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VERTICAL BANDING EVOKED BY ELECTRICAL STIMULATION OF THE BRAIN IN ANAESTHETIZED GREEN SUNFISH, *LEPOMIS CYANELLUS*, AND BLUEGILLS, *LEPOMIS MACROCHIRUS*

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SUMMARY

A pattern of dark vertical bands is a characteristic agonistic display in the green sunfish, *Lepomis cyanellus* and the bluegill, *L. macrochirus*. The rapidity with which the display can appear and disappear indicates that it is neurally controlled. Electrical stimulation of the brain was carried out in anaesthetized green sunfish and bluegills to map those regions from which this colour change can be elicited. Banding was evoked by stimulation of sites near the midline in the preoptic area, ventral thalamic–dorsal hypothalmic transition zone, the midbrain tegmentum (just dorsal to the nucleus prerotundus pars medialis), in and near the torus semicircularis, in the basal midbrain (region of the crossing tectobulbar tracts), and in the rostral basomedial medulla. A 'transition' zone was located basally in the middle medulla, caudal to which only paling was evoked. Areas found to be negative for evoked banding included the telencephalic lobe, the inferior lobe of the hypothalamus, the optic tract, the optic tectum, the body and valvula of the cerebellum and the caudal medulla. It is postulated that the vertical banding pattern is made up of a separate, selectively controlled system of dermal melanophores. The possible neural mechanisms controlling banding are discussed.

INTRODUCTION

Many teleosts are capable of rapid colour change in which colour appears and disappears in a matter of seconds. This has been studied extensively in cichlids because of their highly developed social behaviour and ability to change colour rapidly between interactions (Barlow, 1963; Neil, 1964; Baldacinni, 1973). Among North American centrarchids, several sunfish (*Lepomis*) are also capable of rapid changes in colour and pattern (Miller, 1963; McDonald, 1966; McDonald & Kessel, 1967; Stacey & Chiszar, 1975). The speed of these changes suggests that they are under neural control, a concept which has been substantiated by extensive research in a variety of teleosts by cutting and electrical stimulation of sympathetic fibres supplying the skin (Pye, 1964; Scott, 1965; Healey, 1967).

The external colour and pattern of fish is produced by chromatophores, of which melanophores are the most significant in producing rapid colour change. Concentration of the melanin granules within the melanophores causes lightening and dispersion of the melanin darkening (Bagnara & Hadley, 1973). It has been observed in cichlids that the melanophores may occur in greater density in specific patterns which appear to be under selective neural control as separate systems (Baerends & Baerends-van Roon, 1950), a concept which appears to be applicable to the present study.

Although the anatomy and peripheral control of dermal melanophores have been investigated extensively, relatively little data is available on the central control of rapid colour change. This study was undertaken to investigate central neural control of rapid colour change and to locate specific areas in the brain where the vertical banding pattern can be evoked by electrical stimulation in the green sunfish, *Lepomis cyanellus*, and the bluegill, *L. macrochirus*.

GENERAL METHODS

Animals

Twenty-two green sunfish, *Lepomis cyanellus*, and nine bluegills, *L. macrochirus*, were used in this study. The green sunfish were obtained from spring-fed ponds near Albuquerque, New Mexico, and the bluegills from waters in and near New Orleans, Louisiana. They were held in 80 gal indoor community tanks. Lighting for the green sunfish was controlled on a 16 h photoperiod, and was uncontrolled for bluegills with 12–15 h of illumination. Length, to the fork of the tail, of the green sunfish varied from 15 to 19 cm (averaging 17·2 cm), and for the bluegills ranged from 13 to 16·5 cm (averaging 14·9 cm).

Procedure

A fish was placed in a plastic pail containing (2 l of 3% urethane solution until it lost equilibrium and its respiration rate slowed (approximately 2–5 min). It was then moved to a rectangular plastic pan containing 10 l of 0·3% urethane solution where it was secured in a stainless-steel holding device (Demski & Knigge, 1971; Demski & Gerald, 1974). The fish remained in the holding device during the entire testing sequence which involved exposure of the dorsal surface of the brain, insertion of the electrode in various parts of the brain, electrical stimulation along the electrode tracts, and marking of electrode sites or tracks.

When in the holding device, the mouth was held by a nose clamp over a hollow mouthpiece through which the 0·3% urethane solution was continuously recirculated by an airlift system. A porous 'airstone' was placed in the bath and air was forced through it to continuously oxygenate the urethane solution. The fish was held partially submerged in the holding device with the water surface just below the exposed part of the top of the skull. Its head was stabilized by the nose clamp and two rods which were tightened into place with the opposing points pushing against the bone of the skull just above and rostral to the opercular flap extension. In this manner, the head of the fish was held rigidly against movement while the body and tail could move from side to side.

All test procedures were conducted with the fish anaesthetized and held in the holding device. The skin and musculature over the skull were surgically removed and the

bone was cut away to expose the entire dorsal surface of the brain. Monopolar electrodes consisting of stainless steel insect pins (size 00) were used in all experiments. They were electrolytically sharpened and were insulated with five coats of Epoxylite. The tips were bared for approximately 0·5 mm and each electrode was tested for insulation leaks by passing d.c. current through the electrode in a saline solution and checking for bubbles along the insulated shaft. Electrodes were moved using a micromanipulator calibrated in 0·1 mm. The majority of electrodes were oriented in a dorsal-to-ventral direction and four were inserted obliquely, in a lateral-to-medial direction. Electrical stimulation of the brain was accomplished using biphasic square wave pulse-pairs of 2 msec duration at a frequency of 50 Hz generated by a Nuclear Chicago Constant Current Stimulator (Model 7150). The stimulus waveform was monitored on a Tektronix 502A oscilloscope as the voltage drop across a 1000 Ω resistor in series with the electrode. An indifferent lead was placed in the solution bathing the fish.

Stimulation

The lowest threshold site for evoked vertical banding, or the site of maximum banding evoked at 50 μA, was located on each electrode track. The number of electrode tracks run in a single fish ranged from 1 to 12. Tests were discontinued on a fish if the behaviour evoked by electrical stimulation persisted for more than 1 min after stimulation ceased, or if the condition of the fish had obviously deteriorated. Such deterioration was indicated by very slow gill movement, overall dark colouration, excessive bleeding or obvious damage to the exposed tissue.

Electrode tracks were generally not closer than 1 mm apart, to avoid confusion in identifying sites. At each track the electrode was first moved slowly through the brain with a continuous current of 50 μA. If banding did not occur during the electrode passage at 50 μA, the track was considered negative. In some cases, these negative tracks were marked by a procedure described later.

If banding occurred at 50 μA, the current was turned off and electrode movement was stopped. From this point, either a 'standard' or a 'maximizing' procedure was followed. In the 'standard' procedure the electrode tip was advanced in increments of 0·1 mm, and at each interval a current of 10, 20, 30, 40 and 50 μA was successively applied, each for a period of 10 sec. When the lowest threshold at which banding was evoked was exceeded by two successively higher thresholds, the electrode was returned to the level exhibiting the lowest threshold. This point was then marked for Prussian blue staining (Akert & Welker, 1961) by applying an anodal current (d.c.) of 20 μA for 20 s through the electrode tip. In the 'maximizing' procedure the electrode was moved down and up with the current continuously on at 50 μA and was stopped in the position where the strongest banding response occurred. The lowest threshold at that site was then determined and the site was marked as described above. On three dorsoventral tracks the limits where banding was evoked at 50 μA were marked to bracket the region involved.

Eleven negative electrode tracks (all in green sunfish) were marked in their entirety by slowly raising the electrode while an anodal current (20 μA) was being passed continuously until the electrode left the brain surface. Marking of each such negative

track was followed by eliciting a positive banding response at some other location to ascertain that the fish still responded normally.

The electrically evoked vertical banding pattern is characterized by the relatively rapid (less than 10 s latency) appearance of 7–9 dark brownish-black vertical bands across the sides of the fish, the same as have been observed during displays by normal fish. A strong or moderate response is one in which distinct dark banding occurred, and if the site was stimulated at the next greater current level, it would appear to be of the same intensity. After each 10 s stimulation period, the animal was allowed to return to prestimulation colouration before the next current level was tested (usually less than 30 s). Strong banding was elicited at the majority of marked banding sites. However, four moderate banding sites were also marked where they appeared to be significant, three in green sunfish and one in a bluegill. In addition, four sites were marked in green sunfish where stimulation evoked paling, but no banding.

Histology

After testing, the electrode was removed and the fish was sacrified. The head was removed and fixed in 10% buffered formalin solution. After fixation, brains were removed, dehydrated in xylene and embedded in paraffin. The brains were then serially sectioned in the transverse plane (10 μm thickness). Sections were stained with Prussian blue (Akert & Welker, 1961) to mark the stimulation sites, and neutral red (Humason, 1967) for nuclear groups. The marked stimulation sites were plotted on a series of representative transverse sections of the sunfish brain. Anatomical terminology used in this study was adopted from Demski, Bauer & Gerald (1975).

RESULTS

Green Sunfish

A total of 145 electrode tracts were made in 22 green sunfish. Forty-six sites from which vertical banding was evoked were marked and histologically identified.

Initially, to localize quickly the general regions where the banding response could be evoked, seven positive banding sites were located and marked using the relatively rapid 'maximizing' procedure (Fig. 2). Strong banding was subsequently evoked at 30 additional sites on dorsoventral tracks using the 'standard' technique. These sites lay in a generally medial pathway extending caudally from the ventral thalamic-dorsal hypothalamic transition zone (between the third ventricle and the nucleus prerotundus pars lateralis), through the mid-brain tegmentum above the nucleus prerotundus pars medialis to the level of the oculomotor nuclear complex. It continued through the crossing tectobulbar fibres into the basomedial medulla. In addition, strong banding was evoked at sites in the torus semicircularis and adjacent dorsolateral midbrain tegmentum in a distribution seemingly connected to the more medial positive sites at these levels (Fig. 1). Moderate banding was also evoked at two sites in the preoptic area at currents higher than 50 μA (75 and 100 μA respectively), indicating that this region may also be involved in control of vertical banding (Fig. 1).

Lowest threshold sites for strong banding were also located on four oblique tracks. Their location confirmed the sites identified on dorsoventral tracks in the thalamic-

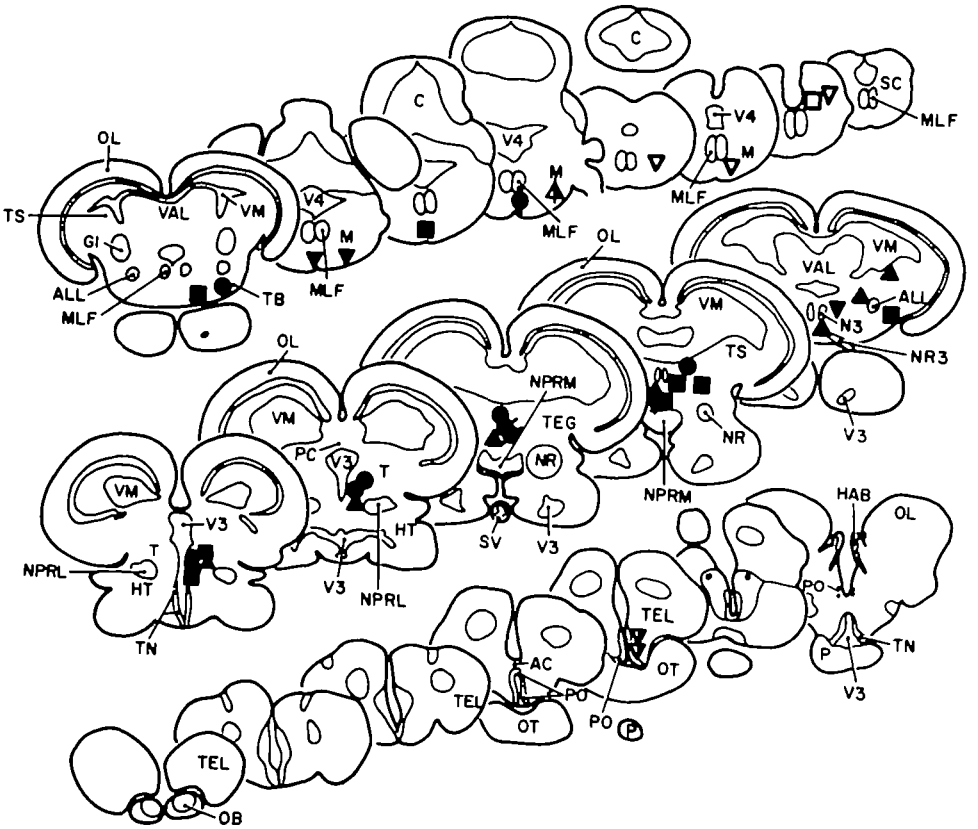

Fig. 1. Transverse sections through the green sunfish brain showing the lowest threshold sites from which banding and/or paling was evoked by electrical stimulation using the 'standard procedure' (see text). Sections are taken from the olfactory bulb (lower left) to the rostral spinal cord (upper right). Symbols represent sites where significant responses were evoked, as follows. Banding: ✐ strong banding at 10 μA; ●, strong banding at 20 μA; ■, strong banding at 30 μA; ▲, strong banding at 40 μA; ▼, strong banding at 50 μA; ▽, moderate banding at 50 μA; Banding and Paling (bimodal): ✐ strong banding at 22 μA and paling at 50 μA; ✐, moderate banding at 40 μA and paling at 50 μA; Paling,: □, paling at 30 μA; ▽, paling at 50 μA.

Abbreviations: AC, anterior commissure; ALL, acoustico-lateral lemniscus; C, cerebellum; GI, ganglion isthmi; HAB, habenula; HT, hypothalamus; M, medulla; MLF, medial longitudinal fasiculus; N_3, nucleus of oculomotor nerve; NPRL, nucleus prerotundus pars lateralis; NPRM, nucleus prerotundus pars medialis; NR, nucleus rotundus; NR_3, oculomotor nerve; OB, olfactory bulb; OL, optic lobe: OT, optic tract; P, pituitary; PC, posterior commissure; PO, preoptic area; SC, spinal cord; SV, saccus vasculosus; T, thalamus; TB, tectobulbar tract in basal midbrain; TEG, midbrain tegmentum; TEL, telencephalon; TN, tuberal nuclei; TS, torus semicircularis; V_3, third ventricle; V_4, fourth ventricle, VAL, valvula of the cerebellum; VM, midbrain ventricle.

hypothalamic transition zone, midbrain tegmentum near the oculomotor nuclear complex and the torus semicircularis (Fig. 2).

Three of the areas strongly implicated in the vertical banding system were given more discrete boundaries by bracketing the portion of the electrode track from which banding could be evoked at currents less than 50 μA. These were the medial thalamic–hypothalamic transition zone, medial midbrain tegmentum above the nucleus prerotundus pars medialis, and the area of the crossing tectobulbar fibres (Fig. 2).

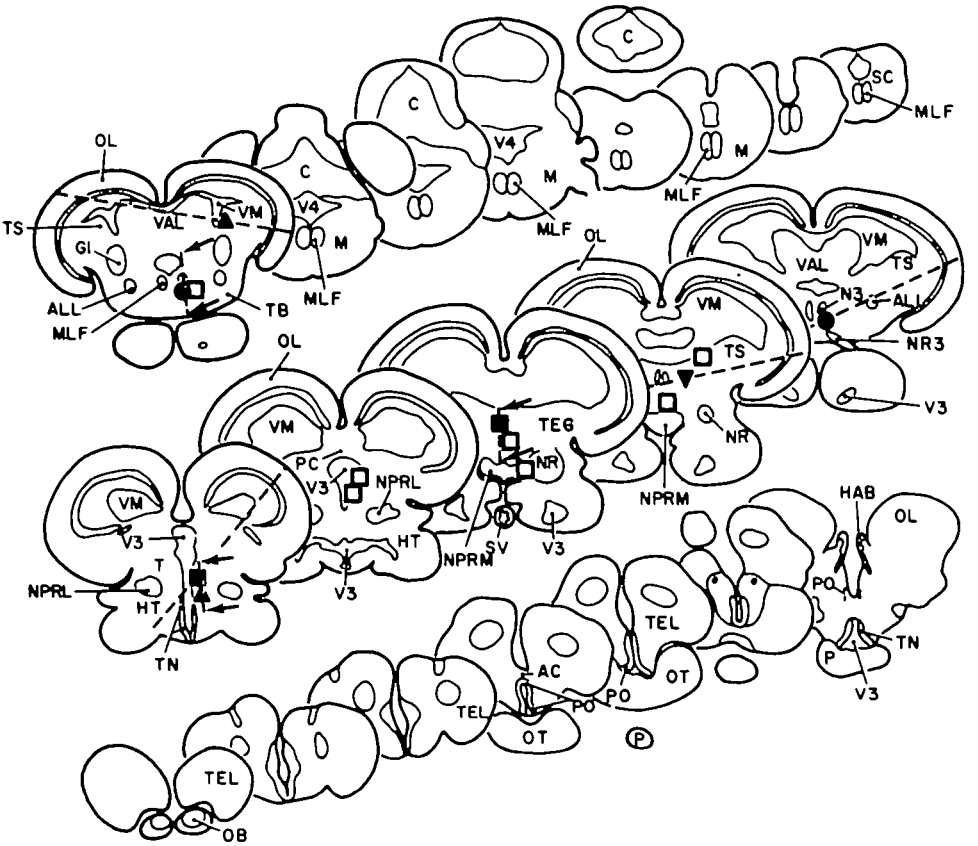

Fig. 2. Transverse sections through the green sunfish brain showing supplementary sites and ranges where banding was evoked by electrical stimulation. Sections are taken from the olfactory bulb (lower left) to the rostral spinal cord (upper right). Inclined dashed lines indicate oblique electrode tracks on which lowest threshold sites are shown: arrows on near-vertical dashed lines indicate limits on tracks within which banding was evoked at 50 μA, and the lowest threshold site in each range is shown. Strong banding was evoked using the 'standard technique' at current levels indicated by the following symbols: ●, 20 μA; ■, 30 μA; ▲, 40 μA; ▼, 50 μA. Moderate banding was evoked at sites marked with open squares at 50 μA or less by use of the 'maximizing technique' (see text). See Figure 1 for abbreviations.

At two points in the mid-basal medulla, banding was evoked at lower and paling at higher thresholds (Fig. 1). At these 'bi-modal' sites the strongest banding occurred at the medial site and moderate banding at the lateral site. Caudal to these 'bi-modal' points strong banding responses were not evoked; however, paling was evoked at four sites which were marked and mapped (Fig. 1).

A total of 75 negative electrode tracks were run while searching for banding sites. Of these, 11 were marked *in toto* for later histological location. The negative regions (in which stimulation consistently failed to evoke banding at 50 μA include the telencephalic lobe, optic tract and optic tectum, inferior lobe of the hypothalamus, body and valvula of the cerebellum, caudal medulla and rostral spinal cord. Many of these negative areas were further identified by negative reactions at 50 μA on the dorsoventral positive tracks above the regions where banding was evoked.

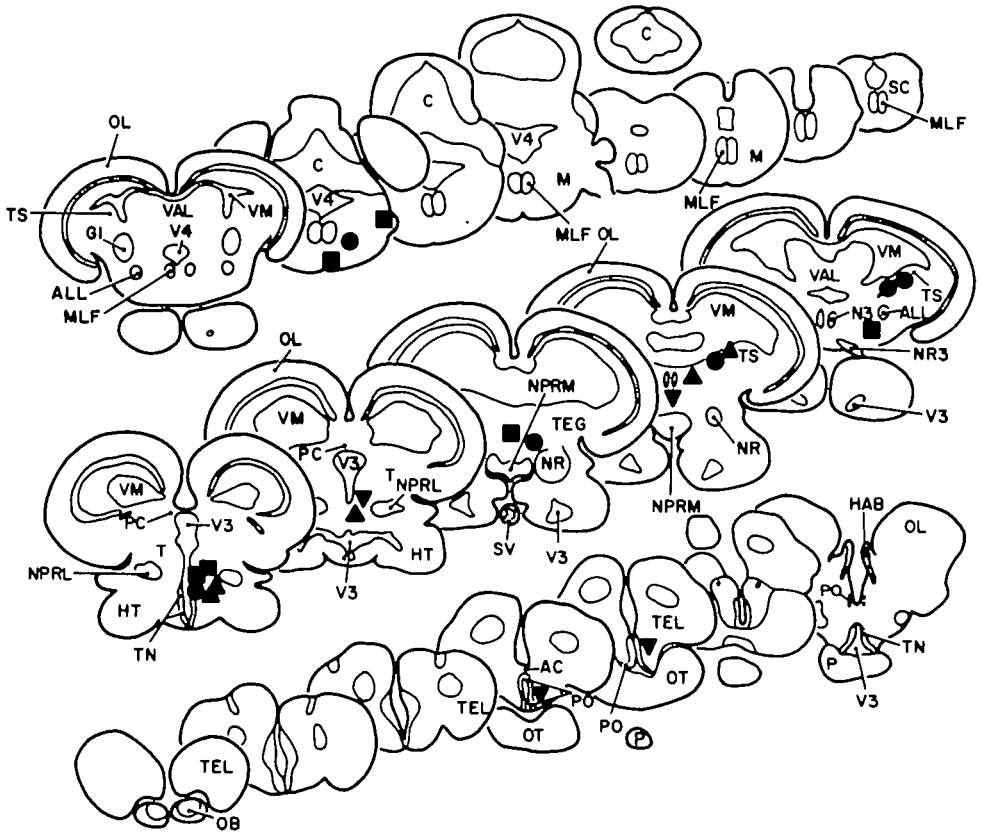

Fig. 3. Transverse sections through the sunfish brain illustrating the lowest threshold sites where vertical banding was evoked by electrical stimulation using the 'standard procedure' in bluegills (see text). Sections are taken from the olfactory bulb (lower left) to the rostral spinal cord (upper right). Strong banding was evoked at the current levels indicated by the following symbols: ✱, 10 μA; ●, 20 μA; ■, 30 μA; ▲, 40 μA; ▼, 50 μA. See Fig. 1 for abbreviations.

Bluegills

A total of 44 electrode tracks were made in nine bluegills. Twenty-one sites from which vertical banding was evoked were marked and histologically located (Fig. 3). Distribution of the sites follows the same pattern as the positive banding sites in the green sunfish. Note that strong banding was evoked at 50 μA in two sites in the preoptic area.

Three bluegills were decapitated to demonstrate the effects upon skin coloration of (*a*) removal of brain control and (*b*) direct stimulation of the spinal cord. One was a mature fish and two were immature. The mature fish darkened immediately after decapitation. It banded at 30 μA and blanched at 100 μA, in response to spinal cord stimulation, and then returned to the darkened state when stimulation was stopped. This test was repeated several times with the same results. The other two immature fish became strongly banded after decapitation, blanched in response to spinal cord stimulation at 100 μA, and returned to the banded coloration after stimulation stopped.

Immature bluegills are typically banded (McDonald, 1966); they may not yet have developed the number of melanophores required to become generally dark. Note that in each case the fish returned to its pre-stimulation colour when stimulation was discontinued.

DISCUSSION

Banding control regions and their interconnexions

The results suggest that the regions from which banding was evoked form a system in which the preoptic area, thalamic–hypothalamic transition zone and the torus semicircularis are control centres, with outputs via fibres passing through the midbrain tegmentum and the area of the tectobulbar tracts to the rostral medulla. This concept is consistent with the existence of known connections between these regions.

The preoptic area has connections with the hypothalamus via fibres in the medial forebrain bundle (Ariëns Kappers, Huber & Crosby, 1936) and the hypothalamus has connexions with the motor centres in the medulla (Aronson, 1963; Schnitzlein, 1964). The torus semicircularis may have connections with the medulla via the reticular formation (see below), and also with the thalamus (Brickner, 1929).

It is probable that fibres from the diencephalon and torus semicircularis pass through and synapse in the reticular formation and that the reticular systems were responsible for activating the banding response observed. In a recent study (concerned mainly with efferent pathways of the optic tectum in the teleosts *Eugerres* and *Holocentrus*), the reticular formation of the mesencephalon, pons, and medulla was well characterized (Ebbesson & Vanegas, 1976). The reticular system is a coordinating apparatus, large elements of which have been found in teleosts just rostral to the nucleus of the oculomotor nerve (Ariëns Kappers *et al.* 1936). In this study two of the lowest threshold sites for banding (10 μA) were found in this region.

Involvement of the diencephalon in colour change of teleosts has been indicated in earlier studies. Von Frisch (1911) evoked darkening in the minnow, *Phoxinus phoxinus*, by unipolar electrical stimulation in the forebrain, both before and after removal of the telencephalon, and concluded that darkening probably resulted from stimulating the diencephalon. Demski (1969) evoked vertical banding in the bluegill, *Lepomis macrochirus*, by electrical stimulation in the diencephalon. The present study confirms the involvement of the teleostean diencephalon in colour change and implicates the preoptic area and thalamic-hypothalamic transition zone as the specific diencephalic areas involved in the banding response of green sunfish and bluegills.

The role of the diencephalon in defensive colour change is compatible with widely held concepts of the functions of this region, which is considered to be a major correlating centre for sensory information (see review by Sarnat & Netsky, 1974). Also, the preoptic and hypothalamic areas have a regulatory effect over autonomic centres, and melanophores in the skin of teleosts are supplied by autonomic fibres (Young, 1931).

The region of the torus semicircularis in teleosts has long been known to receive acoustic input and there is recent evidence that it also responds to visual stimuli. Auditory responses were recorded in the torus semicircularis of the tench, *Tinca tinca* (Grözinger, 1967), and visual and auditory responses were recorded in the torus semicircularis in the goldfish, *Carassius auratus* (Page, 1970; Page & Sutterlin, 1970),

Thus the involvement of the torus semicircularis in control of banding is reasonable since banding has been observed as a response to both visual and vibratory stimuli (Bauer, 1978). Also, the fine structure of the torus semicircularis in certain teleosts indicates that it may have autonomic functions similar to the mesencephalic central gray in tetrapods (Ito, 1974). This is compatible with the concept that melanophores in the banding pattern are controlled via autonomic fibres.

Negative regions

The telencephalon (exclusive of the preoptic area) and the body and valvula of the cerebellum are major 'negative' regions in which electrical stimulation did not evoke banding in the anesthetized fish. Stimulation of the inferior lobe of the hypothalamus also failed to evoke banding in this study; however, Demski (1969) induced banding in several sites of this region using free-swimming bluegills. The difference may be due to reduced sensitivity caused by the anaesthetic. This effect was demonstrated in the spiny boxfish, *Chilomycterus schoepfi*, in which electrical stimulation in the midbrain near the torus semicircularis evoked 'puffing', a defensive response, in unanesthetized fish, but not in fish under anesthesia (Demski, unpublished observation).

Electrical stimulation of the optic tectum consistently failed to evoke banding. As a major recipient of visual input, it may play a role in neural control of banding. However, electrical stimulation of the brain may not be an appropriate technique for determining involvement of the optic tectum because it may not provide the complex pattern of stimulation required to simulate an appropriate retinal output. This might be resolved by lesioning the optic tectum while applying electrical stimulation to areas where banding has been evoked.

Failure to evoke banding in these 'negative' regions does not mean that they may not in some way participate in the control of banding under natural conditions. The regions found positive for banding may be those with relatively strong or direct connections to the motor areas, and other involved regions may be found by additional studies using other methods.

Responses by dermal melanophores

It has been demonstrated conclusively that melanophores respond to excitation by aggregation of melanin granules (paling) and that removal of the stimulation results in dispersion of melanin, or darkening (Scott, 1965; Fujii & Novales, 1969*a*). During this study melanophores in the banding pattern were observed under 40× magnification and appeared to 'expand' during electrical stimulation of a positive banding site in the brain.

Von Frisch (1911) concluded that the dermal melanophores were held in a state of tonal aggregation by a 'paling centre' in the medulla, and that darkening resulted from inhibiting action of an anterior 'darkening centre' upon the 'paling centre' so that the melanophores assumed a resting (dispersed) state. He further concluded that this was accomplished through a single system of aggregating fibres, a view which has been widely supported (Pye, 1964; Scott, 1965; Healey, 1967; Bagnara & Hadley, 1973). It has also been suggested that there may be separate aggregating and dispersing fibres (von Gelei, 1942; Parker, 1948; Fujii & Novales, 1969*b*). However, evidence

supporting 'double innervation' is indirect and inconclusive; therefore, the ensuing discussion is based on the premise of single innervation.

Pye (1964) demonstrated that dermal melanophores of the minnow, *Phoxinus phoxinus*, respond to electrical stimulation at any level in the spinal cord and suggested that the general state of excitation in the sympathetic chain might be raised by such stimulation so that the whole chromatic outflow would be influenced and cause paling. A similar situation was illustrated in this study when paling was evoked by electrical stimulation in the exposed spinal cord in the bodies of three decapitated bluegills, *Lepomis macrochirus*. It appeared that the level of stimulation which caused paling (100 μA) may have been sufficient to spread and excite fibres to melanophores in both the banding pattern and in the superficial system responsible for general body coloration. Conversely, it has been demonstrated by cutting experiments that interruption of the neural pathway below the medulla stops the excitation of the melanophores and results in dispersion of melanin, or darkening (von Frisch, 1911; Pye, 1964; Scott, 1965; Healey, 1967). This was also demonstrated when the bluegills were decapitated during this study.

During these experiments dispersion of melanin in melanophores in the banding pattern was evoked by electrical stimulation of many sites in and above the rostral medulla while stimulation of more caudal sites evoked paling, but not banding. On the premise that darkening results from reduced excitation of melanophores, it follows that electrical stimulation in the brain may cause inhibition to be applied, in the medulla, to those chromatomotor fibres which control the melanophores in the vertical banding pattern. Location of this 'centre' in the medulla was reinforced by the finding of two 'bi-modal' sites. At these sites banding at lower and paling at higher thresholds were both evoked at one electrode site in each of two different fish in an intermediate area in the medulla (Fig. 1). These responses suggest that:

(*a*) The sites were close to the 'centre' where inhibition was applied to outgoing chromatomotor fibres.

(*b*) The lower currents activated the 'incoming' fibres which, in turn, inhibited the 'outgoing' fibres to the melanophores in the banding pattern, causing banding.

(*c*) The higher currents may have spread and directly excited the outgoing chromatomotor fibres to all dermal melanophores, causing paling. Additional detailed electrical stimulation studies in the medulla and rostral spinal cord are needed to better define medullary involvement in neurally controlled colour change.

Peripheral control of melanophore systems

Initial coloration of fishes differed. Some were darker than others, and some had faint visible vertical bands. The vertical banding, or accentuation of banding, evoked in this study may have resulted from either darkening of the banding pattern (no background colour change), or lightening of the background colour (causing bands to stand out), or a combination thereof.

Darkening or lightening of general body colouration (with no banding) was evoked at many sites in the brain. These sites were not histologically located or mapped because the responses occurred as an electrode was being advanced while seeking positive banding sites in anaesthetized fish.

Each change was complete as a unit (i.e. lightening or darkening occurred over the

entire body, or the total banding pattern appeared, disappeared or changed in intensity). This clearly suggests that the melanophores responsible for general body colouration, and those making up the banding pattern occur in two separate, selectively controlled systems. Thus, it appears that the concept of separate melanophore systems proposed by Baerends & Baerends-van Roon (1950) may be applicable to this study of green sunfish and bluegills. These authors described the morphological layering of chromotophores in several cichlid species from a superficial System I of small, evenly distributed, integumentary melanophores, to the deeper layers; System II containing larger melanophores which occur in greater numbers in the vertical bars, and System III containing those melanophores which are concerned with longitudinal bands and 'eye spots'.

Application of the above concept to this study would indicate that a change in overall body colouration is caused by a change in the stimulation level of a system of melanophores distributed over the entire body surface (System I). It also suggests that vertical banding is dependent upon the level of stimulation of another system in which melanophores occur in greater concentration in a banding pattern (System II). It follows then, that aggregation in both Systems I and II would cause a fish to become very light or pale, dispersion in System II would produce or accentuate the vertical banding pattern, and dispersion in System I, or in Systems I and II, would result in darkening. In the latter case banding could be masked by the dark outer layer, System I, and exposed if melanin in System I were to aggregate. More detailed studies are needed which include examination of the skin under magnification to observe possible differential responses by separate melanophore systems to electrical stimulation of the brain in sunfish.

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