The Emergence of Tuning in Newly Generated Tuberous Electroreceptors

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Tuning curves of afferent electroreceptive fibers in the anterior lateral line nerve of the weakly electric fish, *Sternopygus macrurus*, indicate that the tuberous electroreceptors of each individual are well-tuned to its own electric organ discharge (EOD) frequency. In order to study how receptor tuning may develop, new receptor organs were induced to form in regenerating cheek skin, and their tuning properties were compared with those of intact receptors from the same fish. At 3 weeks after the onset of regeneration, new receptors of a given fish were broadly tuned with best frequencies (BFs) lower than that fish's EOD frequency and the BFs of its own intact tuberous receptors. Three weeks later, regenerated receptors of the same fish were indistinguishable from intact receptors in BF, although tuning curves were occasionally slightly broader than normal.

To determine if the presence of an ongoing electric field is necessary for the genesis of proper tuning, receptors were allowed to regenerate in fish deprived of their EODs. At 6 weeks, tuning curves of these receptors also had BFs that were tuned similarly to intact receptors and to each individual's characteristic EOD frequency (determined by recordings of the pacemaker nucleus in the medulla). Thus, as regenerating receptors mature, they gradually become more sharply tuned and tuned to progressively higher frequencies until reaching the correct BF, which matches the EOD frequency; however, tuning to the appropriate EOD frequency occurs without reference to the ongoing electric field.

Weakly electric fish generate low-voltage electric fields around themselves from an electric organ in the posterior body and tail. One function of the electric field is for electrolocation, that is, the detection of objects around the fish by the distortions that they introduce in the fish's electric field (Bullock, 1982; Heiligenberg, 1977; Lissmann and Machin, 1958). Additionally, the electric organ discharge (EOD) waveform of each species is species-specific and is used in social interactions (Hopkins, 1972, 1974, 1981; Hopkins and Bass, 1981; Hopkins and Heiligenberg, 1978).

These fish possess 2 classes of specialized electroreceptor organs in their skin, each with a characteristic morphology and physiology (Suga, 1967; Szabo, 1965). Ampullary receptor organs respond only to DC and low-frequency electric fields (<50

Hz), presumably generated by potential prey, and are not thought to participate in the reception of EODs (but see Bell and Russell, 1978; Hagedorn and Heiligenberg, 1985), while a fish's own EOD, and those of conspecifics, are sensed with tuberous electroreceptors (Szabo, 1974; Zakon, 1986). Afferent fibers innervating both types of receptor organ run in the anterior lateral line nerve.

During both electrolocation and communication it is important to maintain a high signal-to-noise ratio of this sensory channel by filtering out the extraneous EODs of other species. Tuberous receptors of many species have a narrow frequency selectivity with a distinct best frequency (BF), which is closely matched to the peak of the power spectrum of the species-specific EOD (Bastian, 1976, 1977; Hopkins, 1976; Hopkins and Heiligenberg, 1978; Scheich and Bullock, 1974; Scheich et al., 1973; Viancour, 1979a, b; Zakon and Meyer, 1983). This is the case for both orders of electric fish, the Gymnotiformes and Mormyriformes, and is true for species that produce an ongoing nearly sinusoidal discharge of a specific fundamental frequency ("wave" species), as well as for those that produce an irregularly occurring EOD pulse with a broader power spectrum ("pulse" species).

In a number of species the EOD undergoes substantial age- or sex-dependent changes in its waveform and frequency composition (Bass and Hopkins, 1983; Hagedorn and Carr, 1985; Hagedorn and Heiligenberg, 1985; Hopkins, 1972; Westby and Kirschbaum, 1978, 1981, 1982). Certain of these changes can be mimicked in the laboratory by treatment with sex steroids (Bass and Hopkins, 1983, 1984; Meyer, 1983). In the few cases studied to date, including those after hormonal manipulations, receptor tuning has shifted in tandem with the EOD to track and remain tuned to it (Bass and Hopkins, 1984; Meyer and Zakon, 1982; Meyer et al., 1986; Zakon and Meyer, 1983).

How electroreceptors initially become tuned and maintain their tuning, how the close correspondence between EOD power spectrum and receptor frequency sensitivity occurs, and how steroid hormones influence tuning are key questions. The results of such studies are likely to have broad consequences, as the tuning of electroreceptors is thought to be determined by active ion conductances (Bennett, 1967; Hopkins, 1976; Viancour, 1979a; Zakon, 1984b; Zakon and Meyer, 1983). Additionally, the frequency tuning of auditory hair cells of a number of vertebrate species has recently been shown to depend on similar "electrical membrane filters" as electroreceptors (Ashmore and Pitchford, 1985; Crawford and Fettiplace, 1981; Fuchs, 1985; Lewis and Hudspeth, 1983). Yet in no system is the development of tuning of these receptor cells understood. Thus, an appreciation of the factors responsible for electroreceptor tuning coupled with a more detailed knowledge of their membrane biophysics might lead to further understanding of the control of hair cell receptor frequency tuning in particular and, more generally, the regulation of and hormonal influences upon membrane excitability.
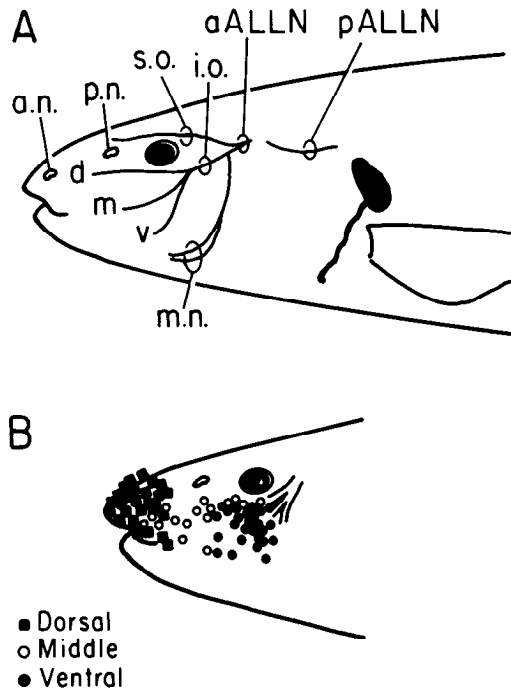
Figure 1. Organization of the aALLN on the head. *A,* Camera lucida drawing of the aALLN of a whole cleared fish (9.0 cm) stained with Sudan black B [method of Filipski and Wilson (1984); material courtesy of H.-Y. Yan]. Supraorbital (*s.o.*), infraorbital (*i.o.*), and mandibulary (*m.n.*) branches supply the top of the head, side of the head, and lower jaw, respectively. The infraorbital component trifurcates into dorsal (*d*), middle (*m*), and ventral (*v*) branchlets. *B,* Location of receptive fields recorded from each of the 3 branchlets of the infraorbital branch of the aALLN. After a unit was encountered, its location on the head was noted by searching for its receptive field with a small dipole electrode. The top of the fish's head, just below eye level, was out of the water so that the nerve was not submerged. Each symbol refers to recordings made from a particular branchlet. Data are pooled from both normal (*n* = 2) and regenerated (*n* = 4) fish. Note that the dorsal branchlet innervates the snout, the ventral branchlet innervates the region below the eye (the cheek), and the middle branchlet innervates the intermediate region, even in the regenerated animals.

One factor that could conceivably influence the development of receptor tuning is the EOD itself. It is important to know, therefore, whether receptors must be exposed to an ongoing EOD to become tuned or whether they can become tuned without it. An ongoing EOD appears unnecessary for mediation of hormone-induced changes of tuning in previously tuned receptors (Keller et al., 1986; but see Bass and Hopkins, 1984). Nevertheless, its role, if any, in the inception of tuning is not known.

Tuberous receptor organs are present in larval fish at the time the EOD commences (Kirschbaum and Denizot, 1975). Yet only one study has been made on the acquisition of tuning in these newly generated receptor cells (Meyer et al., 1986) and this was after their exposure to the EOD. Moreover, the types of experimental manipulations necessary to study the ontogeny of tuning in embryos and larvae are difficult to achieve in such small animals. Gymnotiform fishes, however, possess a remarkable capacity for regeneration (Ellis, 1913; Kirschbaum and Meunier, 1981), and this extends to the peripheral electrosensory system (Denizot and Baillet-Derbin, 1969; Yialamas and Zakon, 1984; H. H. Zakon and D. Y. Sanchez, unpublished observations), providing a convenient preparation for investigating this question.

After skin removal, receptors appear *de novo* in regenerating skin, probably induced from the overlying epithelium by returning nerve fibers (Bailey, 1937; H. H. Zakon and D. Y. San-

chez, unpublished observations). This regeneration of receptors is most likely a manifestation of the naturally occurring lifelong addition of receptor cells (Zakon, 1984a). Newly generated cells, whether during normal cell addition or regeneration, must become tuned so that, rather than being confined to embryonic or larval phases, the tuning process is ongoing, and studies of its ontogeny need not be restricted to larval stages.

In order to study the inception of tuning in newly generated receptors, I have exploited the ability of these fish to generate new receptor organs in regenerating skin and have monitored receptor tuning during the regeneration process in the presence or absence of the fish's endogenous EOD.

Materials and Methods

Animals

Sternopygus macrurus (11–25 cm) used in this study were collected in Caño Mamon, a stream in the Apure drainage basin in Portuguesa, Venezuela. They were housed in the laboratory under a 12L/12D day–night cycle in a temperature-controlled chamber (25.0 ± 0.5°C), kept in tanks with water at 5 kΩ cm resistivity, and fed daily on frozen bloodworms (chironomid larvae).

Anatomy

In preparation for nerve recordings, the branching pattern and course of the anterior lateral line nerve (ALLN) was determined by dissection of 2 formalin-fixed specimens and in 1 additional fish that had been cleared and stained whole with Sudan black B according to the method of Filipski and Wilson (1984). Observations on the course of electroreceptive afferents as they near their termination in the skin were made on pieces of cheek skin silver-stained with the Winkelmann and Schmit (1957) method, prepared for a previous study (Zakon, 1984a).

Surgery

EODs were measured prior to surgery. Fish were placed in a plastic bucket with water from their home tank, and the EOD was amplified with a Grass P-15 amplifier, viewed on an oscilloscope, and an accurate measurement of the frequency made with a counter/timer (Fluke). They were lightly anesthetized with MS-222 (procaine methane sulfonate, Aldrich) and, with the aid of a dissecting microscope, a piece of cheek skin was removed. The skin was carefully lifted from below with forceps, while the thin membranous tissues connecting it to the underlying muscle were cut with iris scissors. During the removal of the skin, care was taken to avoid cutting blood vessels. This procedure left untouched the branchlets of the anterior portion of the anterior lateral line nerve (aALLN), which run deep in or just on the surface of the muscle (Fig. 1A). The piece of skin was generally about 0.5 × 1.0 cm², but varied somewhat with the size of the fish. Fish were returned to their home tanks to recover and were removed for study at various intervals thereafter.

One or two days before skin removal, 5 fish had their electric organs silenced by a high spinal transection. The firing frequency of the electric organ is set by a large midline nucleus of electrotonically coupled cells located in the medulla, termed the pacemaker nucleus (PMN), which then synapses upon neurons in a relay nucleus immediately below it (Bennett et al., 1967). The axons from this latter nucleus form a long descending tract that contacts electromotoneurons along the length of the spinal cord. Transection of these long tracts renders the electric organ inactive, thereby removing the fish's electric field, but leaves the PMN intact and still firing at its normal frequency (Meyer et al., 1984). Thus, the firing frequency of the PMN may be measured even in an electrically silent fish.

In preparation for surgery, fish were anesthetized with MS-222 and were placed in a tray with a tube in the mouth flowing water with MS-222 over their gills. While the fish were respired, the vertebral column immediately posterior to the pectoral fins was exposed, chipped open with a scalpel, and the spinal cord was cut with iris scissors or fine forceps. The muscle masses overlying the vertebral column were pushed back in place, and the wound was sutured shut and then glued with histoacryl glue (Braun-Melsungen, FRG). Fish were returned to separate, electrically isolated compartments of a 20 gallon tank to recover.

Single-unit recording

Details of the stimulation and recording procedures are given in Zakon and Meyer (1983). Briefly, after its EOD was measured, a fish was curarized (0.1–0.3 ml : 1.0 mg/ml tubocurarine, Sigma) and placed in a foam-lined holder, its mouth on a respirator tube providing it with a constant stream of aerated water during the experiment. Curare not only immobilizes the fish, but shuts off its EOD by blocking the neuroelectrocyte junction (Bennett et al., 1967). Before surgery, a swab of cotton soaked in 5% procaine was brushed over the skin. A small superficial incision was made either posterior to the eye to expose the aALLN, which innervates electroreceptors on the head, or just dorsal to the gill cover to gain access to the posterior branch of the anterior lateral line nerve (pALLN), which innervates electroreceptors on the body. The fish was then positioned so that most of its body and the side of its head below the eye were submerged, leaving only the exposed nerve and the dorsalmost part of the head out of water.

The frequency and intensity of sinusoidal electric stimulus fields around the fish could be varied; they were produced by a function generator (Wavetek) and attenuated over a 70 dB range in 1 dB steps (Hewlett-Packard 10 dB and 1 dB calibrated potentiometers in series). Stimulus fields were isolated with a transformer and placed transversely across the fish by means of a carbon rod on each side of the tank, each about 5 cm from the fish. These fields could be monitored with electrodes in the tank for calibration. Throughout this study the reference value of 0 dB equals a field with a strength of 200 mV/cm.

A glass micropipette (3 m NaCl, 20–50 MΩ) was advanced into the nerve with a hydraulic microdrive, and unit activity was evoked by a search stimulus. The search stimulus was a sine wave at 10–20 mV/cm and of a frequency between 100 and 150 Hz, known to be well above threshold for tuberous receptors (Hopkins, 1976; Zakon and Meyer, 1983). BF (the frequency to which the unit is most sensitive), threshold, and Q_{10dB} (BF/bandwidth at 10 dB above threshold) were taken for each unit; then the unit's receptive field was localized (see below). If time permitted, further data points were taken to construct a complete tuning curve. Criterion for threshold was 1:1 firing, that is, one spike per stimulus cycle, which yields a reproducible measure of BF (±3%) and threshold (±1 dB). Although this is well above more sensitive measures of threshold, it can be justified for the following reasons: (1) There is a good correlation between impulse-evoked receptor oscillation frequency and BFs measured in this way (Zakon and Meyer, 1983); (2) a white-noise analysis of tuberous electroreceptors in a related species, Eigenmannia (T. A. Viancour and H. I. Krausz, personal communication), has shown that the receptors behave linearly; (3) determination of tuning curves with other techniques, as a just noticeable increment in firing, is in good agreement with a 1:1 criterion (H. H. Zakon, unpublished observations).

Ampullary receptors were not excited by the search stimulus, but were recognized by the standard criteria of (1) regular spontaneous firing rate (10–20 spikes/sec) and (2) a low-frequency bandpass characteristic: Their spontaneous spiking was strongly modulated by stimuli from 0.5 to 40 Hz (Fig. 7) and uninfluenced or much less influenced by stimuli of 50–60 Hz or higher in frequency (Suga, 1967; Zakon, 1986). They were noted as being ampullary, and their location was confirmed, but no further analysis was carried out.

In order to determine receptive fields, the wide-field stimulus in the tank was switched off, and units were localized with a small dipole electrode constructed of Teflon-coated stainless steel wires and with a tip separation of ~500 μm. The resolution varied depending on the amount of spontaneous activity of the unit and the intensity used to search but was on the order of 1 mm². Once localized, units could easily be scored as regenerated or intact, as there was always a wound margin clearly demarcating new and old skin.

Receptor oscillations

If tuberous receptors that are sharply tuned are stimulated by a brief current pulse, they will produce a receptor potential that oscillates, or "rings," at the BF (Bennett, 1967; Meyer and Zakon, 1982; Watson and Bastian, 1979). Receptor oscillations were recorded in order to gain an additional measure of tuning that would be independent of any possible postsynaptic contributions (synaptic efficacy, refractory period of the afferent).

Responses to current pulses were recorded from single receptor units, or at least from small groups of them in a localized region of skin, by placing the tip of a wide-bore (500 μm–1 mm) fire-polished pipette tightly over them and exciting them with a constant-current pulse (500 μsec–1.0 msec, 100–500 nA) through the bridge circuit of a WPI M707 electrometer. A Teflon-coated stainless steel wire, insulated except at the tip and placed inside the mouth, served as the return electrode. The resulting signals recorded through the pipette were digitized at 20 μsec intervals and averaged with a Nicolet 4094 digital oscilloscope. Oscillation frequency was determined by taking the reciprocal of the intervals between successive peaks as measured directly from the oscilloscope by a cursor (Meyer and Zakon, 1982).

Pacemaker recordings

Since the PMN continues rhythmically firing at the same frequency after spinal cord transection and in the absence of afferent feedback, its firing frequency can be used to determine the presumptive EOD frequency. Field potentials from the PMN were recorded at the termination of all experiments with spinally transected fish. These fish were still under curare—the PMN slows down or shuts off entirely with systemically applied anesthetics (Bullock et al., 1972)—but were topically treated with procaine. The skin was cut and the bone removed along the midline over the cerebellum; then a microelectrode (3 m NaCl) with a tip broken to about 5–10 μm was advanced downward for about 1–3 mm until the large distinctive field potential could be recorded within, or adjacent to, the PMN, and its firing frequency was measured.

Results

Organization of the electrosensory periphery of the head

The main trunk of the aALLN is composed of afferents that innervate all of the electroreceptors of the head, both tuberous and ampullary. Running in this nerve are also afferents to the hair cells of the freestanding neuromasts and, nearby, small fibers of the trigeminal system, all of which can be seen terminating in the skin with silver stains (Szabo, 1965; Zakon, 1984a, and unpublished observations). The main trunk of the nerve is divided into 3 large branches: a supraorbital branch that runs over the eye and innervates receptors on the dorsal aspect of the head, an infraorbital branch that innervates receptors on the side of the head from the snout to the operculum, and a mandibular branch that innervates receptors on the ventral surface of the head (Carr et al., 1982) (Fig. 1A).

Posterior and ventral to the eye, the infraorbital branch divides into 3 smaller rami, which I denote the dorsal, middle, and ventral branchlets due to their respective positions (Fig. 1A). The dorsal branchlet runs ventral to the eye coursing superficially within the underlying muscle, while the middle and ventral branchlets run down into the muscle masses on the side of the head. As they near their termination point in the skin, axons leave the branchlet in which they run by joining small fascicles en route to the skin surface. This can be most readily appreciated in whole-mounts of silver-stained specimens of skin in which small bundles of axons course for a few hundred micrometers just beneath and parallel to the skin and then are cut as they descend into the muscle (Zakon, 1984a). This termination pattern ensures that only receptors in intact skin within a few hundred microns of the wound margin are denervated when skin is removed from the cheek.

The locations of single-unit receptive fields indicate that the dorsal branchlet innervates receptors on the snout and around the upper jaw, the middle branchlet innervates receptors behind that region up to the cheek, and the ventral branchlet innervates receptors on the cheek (Fig. 1B). Recordings made from the trunk of the infraorbital nerve 2–3 mm posterior to the confluence of the 3 branchlets suggest that afferent fibers from each region tend to remain in proximity within the nerve as units recorded in the dorsal portion of the nerve tend to innervate the snout, while those recorded ventrally innervate the cheek region. Additionally, dissection of fixed material indicates that, where fascicles converge onto the nerve branchlets, rostrally originating fascicles always remain more dorsal than caudally originating ones, thereby conferring at least a crude somatotopy.
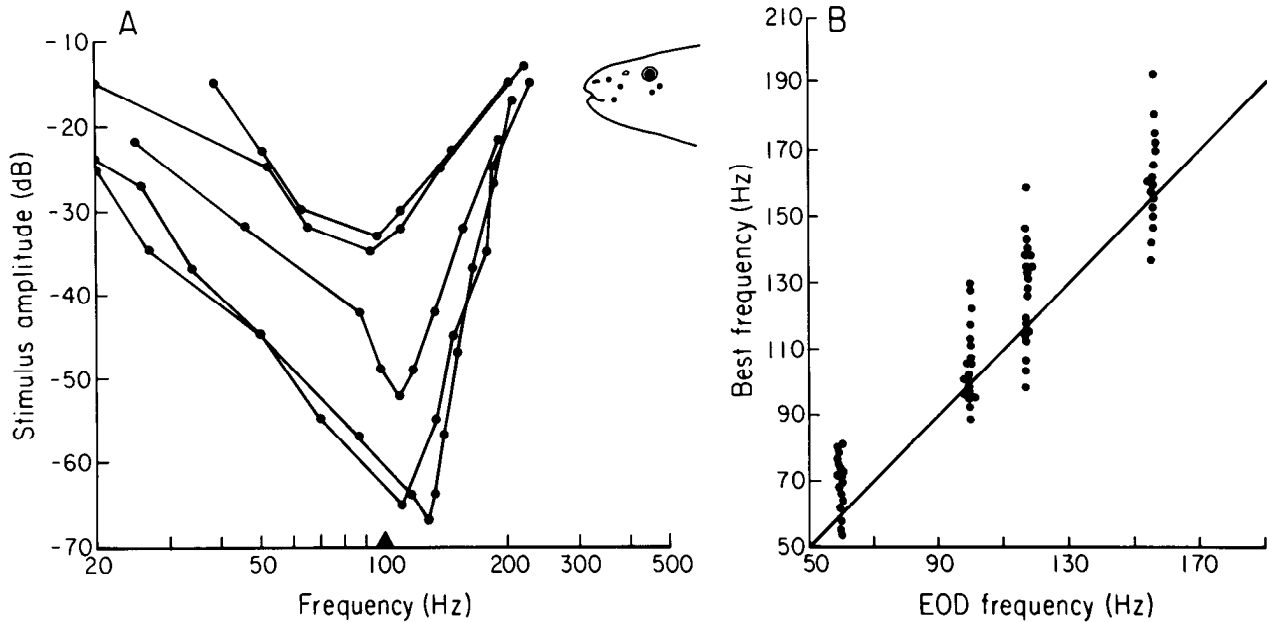
Figure 2. Tuning properties of units recorded from an intact fish. *A,* Tuning curves from a fish with an EOD frequency of 100 Hz (indicated by the *filled arrowhead* on the abscissa). Note that the most sensitive units have BFs at or above the EOD frequency, while the less sensitive units have BFs below. *B,* Distribution of BFs from 4 fish. Each *column of dots* represents all of the BFs recorded from a given fish plotted against its EOD frequency. In this and subsequent figures, the solid 45° line is the line of perfect fit if receptors were tuned 1:1 to the EOD frequency.

Although ampullary and tuberous afferents from the same somatotopic location fasciculate within the periphery, once they enter the CNS, they separate into distinct somatotopically mapped ampullary and tuberous regions (Heiligenberg and Dye, 1982).

Single-unit recordings: baseline observations

Previous recordings of unit tuning curves in this species have only been made from the pALLN, which innervates receptors on the body (Hopkins, 1976; Zakon and Meyer, 1983). To provide baseline data for the regeneration study and in order to compare these data with those collected in other studies, tuning curves were taken from both the aALLN and the pALLN. These recordings were typically made with a wide-field stimulus with no attempt to localize receptors, although receptive fields were determined in a few fish (Fig. 2*A*).

There were no differences in any of the measures taken between units from the aALLN and pALLN (see Table 1), and their tuning properties were as previously reported (Hopkins, 1976; Zakon and Meyer, 1983). Tuning curves of units were "V"-shaped (Fig. 2*A*). The variation in BF recorded for each fish, whether on the head or body, ranged over 30–40 Hz, with the mean BF close to, but usually slightly above, the EOD frequency for that individual (Fig. 2*B*, Table 1). Q_{10dB}, a measure of broadness of tuning, varied from less than 0.5 (broadly tuned) to greater than 3.0 or 4.0 (sharply tuned), with a population

average of 1.66 ($n = 3$ fish, 20 or more receptors recorded from each fish) for the aALLN and 1.55 for the pALLN. As reported previously (Zakon and Meyer, 1983), the most sensitive units (thresholds below 200 μV/cm) were also the most sharply tuned and tended to be tuned about 10–30 Hz higher than the EOD frequency (Fig. 2*A*).

Single-unit recordings: regenerated receptors

Three weeks

Single units with receptive fields within the regenerated skin could be reliably evoked in all fish recorded from 3 weeks after skin removal ($n = 5$). At this time a few were encountered that adapted so rapidly that it was impossible to obtain reliable tuning curves from them. They were discarded from further analysis. Also excluded from further analysis were units encountered in the border region between the regenerated and intact skin that could not be unambiguously localized. (As mentioned above, this also prevented scoring potentially denervated receptors as intact.) By this time, distinctly ampullary and tuberous types of responses could be recorded.

As illustrated in Figure 3, tuning curves with distinct BFs could be taken from many units, and even those with an indistinct BF had a region of maximum sensitivity. For these, BF was taken as the arithmetic midpoint of the region of greatest sensitivity. Two important details emerge from these data. When

Table 1. Mean BF and Q_{10dB} of afferents in the pALLN and aALLN

Fish	EOD (Hz)	Best frequency (Hz) aALLN	n	pALLN	n	Q_{10dB} aALLN	pALLN
1/C	100	105.2 (11.5)	20	101.5 (15.2)	20	1.24 (0.43)	1.44 (0.85)
4A	150	156.3 (21.0)	27	156.3 (10.3)	24	2.39 (1.44)	1.83 (0.71)
3A	156	162.5 (20.0)	19	161.9 (17.6)	20	1.37 (0.45)	1.39 (0.53)

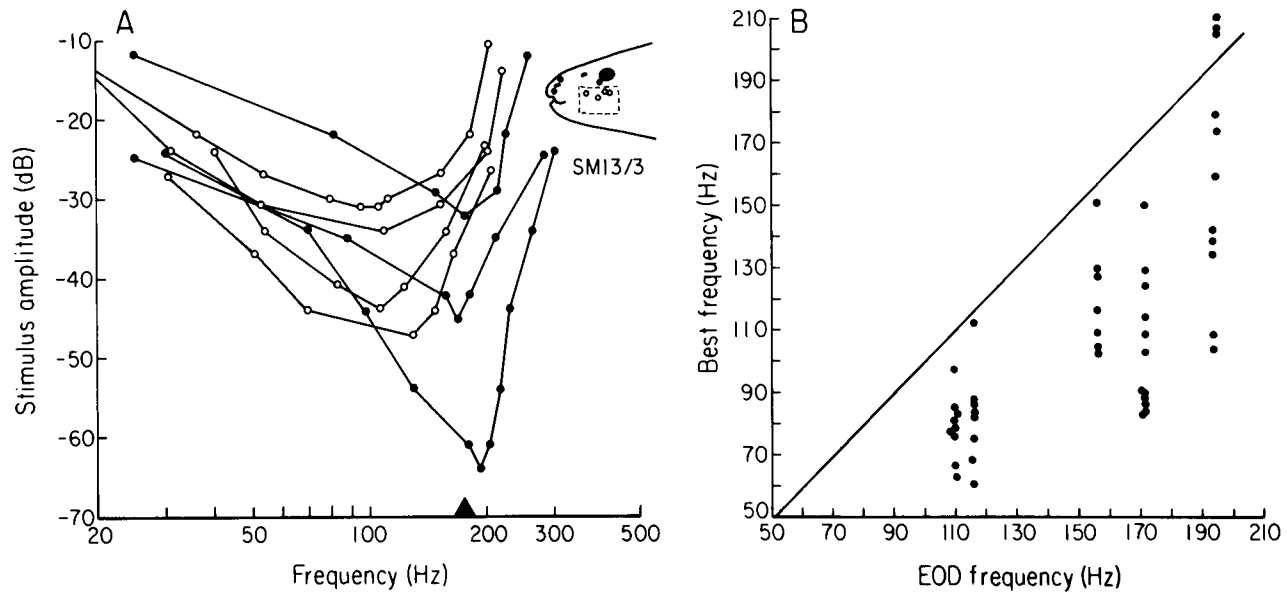SD given in parentheses; n = number of afferents recorded per fish.

Figure 3. Tuning properties of units recorded after 3 weeks of skin regeneration. *A*, Tuning curves of 7 units from a fish with an EOD frequency of 194 Hz (indicated by the *filled arrowhead* on the abscissa). As seen in the fish head inset, 4 units (tuning curves with *open circles*) were localized to the patch of regenerated skin (bordered by the *dotted lines*), while 3 others were from intact skin (*filled circles*). *B*, BFs of receptors in regenerated skin from 5 fish.

regenerated and intact units from the same fish are compared statistically, the mean BF of regenerated units is lower (4 of 4 fish, $p < 0.05$; t test) and the scatter in the distribution of BFs is greater ($p < 0.05$, sums-differences correlation test for unequal standard deviations) than that of units innervating receptors in normal skin (Table 2). Second, the Q_{10dB} of these units is lower than of normal units in the same fish (Table 2). Thus, although units are tuned at this time, they are broadly tuned and tuned well below the EOD frequency (Fig. 3).

When units of both ampullary and tuberous types were localized on the face with the small dipole, each was always found to have a single punctate receptive field within the resolution of the stimulus dipole (about 1 mm²), even within the regenerated patch of skin. Thus, at least with this resolution, it is clear that regenerated afferent fibers do not widely innervate receptors scattered across the regenerated skin. Also, recordings made in the dorsal branch only revealed units with receptive fields on the snout, never in the regenerated skin, and units recorded in the ventral branch only possessed receptive fields in the cheek region (Fig. 1*B*). This indicates that neither sprouting of afferents from adjacent regions into the regenerating patch nor the ingrowth of returning afferents to inappropriate regions of skin occurred.

Six weeks

Six weeks after skin removal the tuning of receptor units appeared nearly normal (Fig. 4). No statistically significant difference existed between the BFs of regenerated and normal units in 6 of 7 fish, or of Q_{10dB} in 5 of 7 fish (Table 2). The one individual that still displayed a mismatch between BFs of intact and regenerated receptors was large and had had a large region of skin removed (Fig. 5*A*), which evidently took longer to regenerate. The scatter of BFs of intact and regenerated units was no different at this time (sums-differences correlation test).

In order to confirm that the mean BF of regenerating receptors actually changed over time, repeated recordings were made from 3 individuals at 3 and 6 weeks (fish 3A, 5, 13). Figure 5 illustrates BFs of units recorded from these fish from the dorsal branchlet, which innervates intact units on the snout, and the ventral branchlet, which innervates the regenerating patch of skin. As

mentioned above, at 3 weeks after skin removal, the regenerating units were tuned significantly below the intact units. Three weeks later, however, regenerated units, recorded from the same nerve branch, were tuned appropriately. In each case, the mean BFs of regenerated receptors from the same fish at 3 and 6 weeks were significantly different ($p < 0.05$, t test, 1-tailed), while no statistically significant difference was observed between receptors from intact skin in the same fish at these times.

Receptor oscillations

It is important to be certain that the low-pass characteristic of the tuning curves taken at 3 weeks postremoval is due to the filter properties of the receptors, rather than the reduced efficacy of the receptor–afferent synapse at higher frequencies of stimulation or an unduly protracted refractory period in the axon. The latter was tested by stimulating regenerated receptors clearly tuned below EOD frequency, with a low frequency (<100 Hz) stimulus; afferents from *Sternopygus* will typically fire 2 or more spikes per cycle if strongly stimulated in this frequency range (Bullock and Chichibu, 1965; Keller et al., 1986). Although this is not a direct measure of refractory period, the interspike interval derived in this manner gives at least a maximum value of refractory period. Minimum interspike intervals ($n = 10$) were generally 1.8–2.0 msec, a value identical to normal fibers, suggesting that afferents ought to be able to fire at frequencies of up to 500 Hz before refractory period limits their frequency response.

A more direct test was applied by recording impulse-induced receptor oscillations (Fig. 6). Oscillations could be recorded from intact regions of skin around the regenerating skin at 3 weeks postremoval. As previously reported (Meyer and Zakon, 1982; Zakon and Meyer, 1983), these were usually 10–30 Hz higher than the EOD frequency, as it is the more sharply tuned receptors that produce the strongest oscillations and the greatest number of cycles, and these receptors are typically tuned higher than the EOD by this extent (Zakon and Meyer, 1983).

When recordings were made from receptors in regenerated skin, on the other hand, the impulse response at this time, if present, was merely a single hump, which is to be expected from a broadly tuned filter (French, 1971) (Fig. 6*A*). When recordings
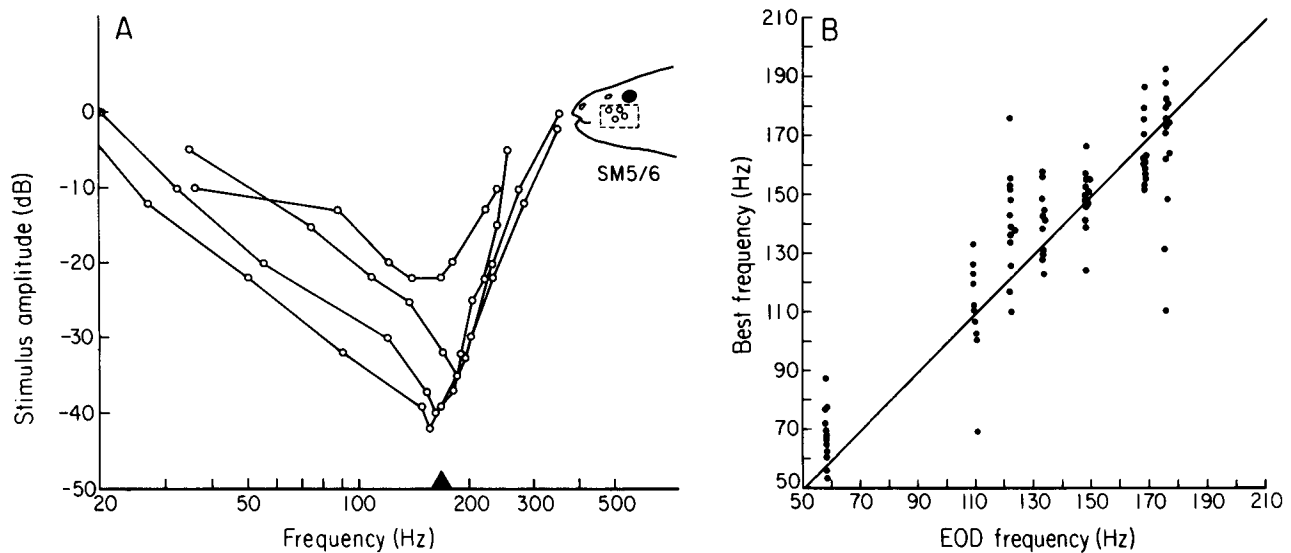
Figure 4. Tuning properties of units recorded after 6 weeks of skin regeneration. *A*, Tuning properties of 4 regenerated units from a fish with an EOD frequency of 168 Hz (*filled arrowhead* on the abscissa). Fish head inset gives the location of the units. *B*, BFs of receptors in regenerated skin from 7 fish.

were made at weekly intervals thereafter, at the time oscillations first occurred within the regenerated patch, they were of a lower frequency (Fig. 6D) and with fewer cycles than oscillations recorded from intact receptors. The oscillation frequency and number of cycles gradually increased until, by 6 weeks, they were almost identical to the oscillations of the intact receptors (Fig. 6B).

Receptor tuning in the absence of an ongoing EOD

Tuned receptors can be identified at 3 weeks postremoval, raising the question: Are the inception of tuning and the eventual matching of units BFs to the EOD frequency a result of, or influenced by, entrainment by the ongoing discharge? The high thresholds of units and the distance of their BFs from EOD frequency would suggest that they would be poorly stimulated by the EOD as they are first becoming tuned. Average values of EOD intensity for this species are 1.0–2.0 mV/cm, which would be well below 1:1 threshold for these newly tuned units. It might be possible, however, for entrainment to occur below this level of threshold. The electric field was removed by high spinal transection and tuning curves taken at 6 weeks in order to investigate this question.

As reported previously (Keller et al., 1986; Meyer et al., 1984), most fish recovered after spinal transection. After lying on their sides for 2–3 weeks, they regained the ability to swim and, if left long enough (>2 months), regained an EOD. When bipolar electrodes are placed near a fish at 2 weeks after spinal transection, occasional bursts of weak nonsynchronized electrical activity can often be recorded directly over the tail in some individuals (Keller et al., 1986). The maximum amplitude of this activity is in the range of 50–100 μV/cm, and the energy is broadly distributed from 50 to 250 Hz. However, with one exception, no such electrical activity, and certainly no EOD, could be detected anywhere in fish at 6 weeks postremoval. (One of the 5 transected fish had a detectable EOD at 6 weeks, so that data from this individual (fish 6A) were included in the 6 week regenerate EOD-intact group instead.)

Both ampullary and tuberous receptors could be recorded. The responses of ampullary receptors appeared normal; they responded to low-frequency stimuli by a modulation of their high spontaneous activity. Figure 7 illustrates the responses of an intact and regenerated ampullary receptor to a 4 Hz sine

wave. The responses are essentially identical.

It has been demonstrated that receptor tuning remains stable for up to 2–4 weeks after the EOD has been eliminated (Keller et al., 1986; Meyer et al., 1984). Similarly, tuning curves of units from the snout appeared normal after 6 weeks of EOD deprivation. Recordings of regenerated units made at this time were similar to units recorded from the same fish in normal skin and indistinguishable in threshold, Q_{10dB}, and tuning curve shape from regenerated units of fish with an EOD (Fig. 8, Table 2).

To determine whether the regenerated receptors of a given fish matched its EOD, the presumptive EOD frequency was measured in these fish by recording the firing frequency of the PMN. The distribution of BFs recorded in the 4 electrically silenced fish, each with a different EOD frequency (determined by PMN recordings) is given in Figure 9A. When the data from 6 week regenerates with and without functional EODs are considered (Fig. 9B), it is clear that the scatter of BFs is no greater than that of regenerated units from fish with an intact EOD (sums-differences correlation test). The correlation coefficients for mean BF versus EOD in both intact and regenerated receptors are identical and indicate a very good relationship ($r = 0.98$). Thus, an ongoing EOD appears to be unnecessary for determining the tuning of newly generated receptors, the extent of the variability of tuning within an individual, and the match between the receptors and the EOD (or in this case the PMN).

Discussion

Development of tuning in newly generated receptors

The key findings of this study are that new tuberous receptors are produced during skin regeneration; that during the inception of tuning they are tuned lower than the EOD; that they appear to gradually increase their BF until they become appropriately tuned to the fish's EOD; and that this process occurs in the absence of an ongoing EOD.

The anatomical details of receptor regeneration will be the subject of another report (H. H. Zakon and D. Y. Sanchez, unpublished observations) but will be briefly mentioned here to provide evidence that receptors are newly generated. During the first 2 weeks after skin removal, cells from the wound margin migrate into the vacant space and form new skin clearly stratified into dermal and epidermal layers. At about this time, nerve
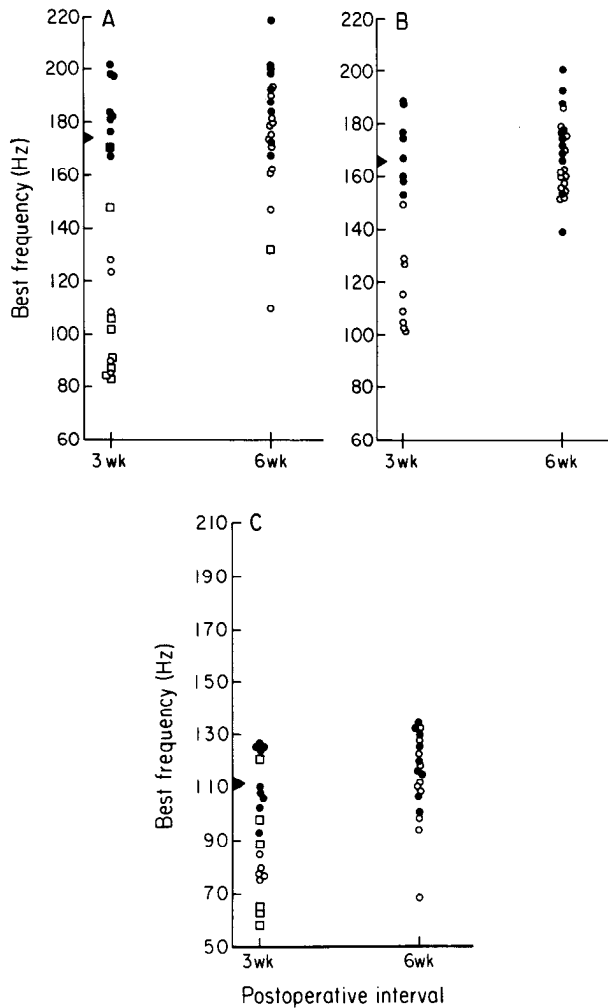
Figure 5. Change in distribution of BF at 3–6 weeks of skin regeneration in 3 individuals with EOD frequencies of 171 (*A*), 168 (*B*), and 116 (*C*) Hz. Each *column of dots* represents BFs from 1 individual recorded at a particular time. *Filled circles* are units that were localized to intact skin; *filled squares* are units that were recorded from the dorsal branch but were lost before they could be localized. *Open circles* are units that were localized to regenerated skin; *open squares* are units that were recorded in the ventral branch but were lost before they could be localized. The *filled arrowhead* on the ordinate indicates EOD frequency recorded at 3 weeks.

Figure 6. Receptor oscillations recorded in a fish during the process of regeneration. Oscillations recorded from the regenerated skin at 3 weeks (*A*) and 6 weeks (*B*), and from the normal skin at 6 weeks (*C*). At 3 weeks, no oscillations, or ones composed of only a small "hump," could be recorded from receptors in the regenerated skin; at 4 weeks, oscillations of more than 1 cycle could be recorded; by 6 weeks, normal-looking oscillations could be recorded from regenerated skin. Bar, 10 msec. *D, Filled circles* represent mean oscillation frequency ($n = 5$ for each point; SD given as bars) from receptors in intact skin; *open circles* are the same from regenerated skin.

fibers are first observed in the new skin (silver-stained whole-mounted material), often contacting small spheres of tightly packed cells, presumably epidermal cells, that have begun to sink into the dermis; these are the presumptive receptor organs. At this time, it is not possible to distinguish between ampullary and tuberous receptor types. Gradually, a tiny lumen appears with a small cell-filled canal leading into it. Over the next week, the 2 types of organs begin to become distinct as diminutive receptor cells accrue and grow, until the receptor organs appear morphologically normal by 3–4 weeks postremoval.

It is not yet known which cell type gives rise to the new receptor organs. They may originate from support cells of older receptor organs that migrate in from beyond the wound margin, as occurs in the regeneration of the amphibian lateral line system (Stone, 1933), or they may be induced from epidermal cells by the returning nerve fibers, as reported for both mechanosensory and electrosensory lateral lines in catfishes (Bailey, 1937). Yet, although the identity of the precursor cells remains unknown, the dramatic sequence of morphological changes is strong evi-
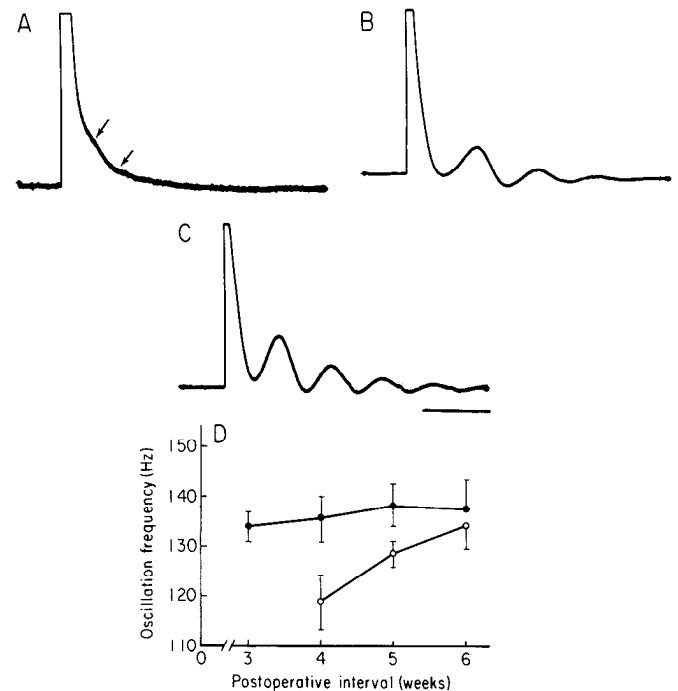
dence for the recent origin of the receptor organs in regenerated skin.

Newly generated electroreceptors, recorded early in the process of regeneration (3 weeks after skin removal), are broadly tuned, with BFs well below the EOD frequency, and ultimately increase in BF and sharpness of tuning until they are tuned to the EOD. Since tuning curves are recorded from the nerve fibers rather than directly from receptor cells, some caution must be used in interpreting these data. Two other factors which might contribute to the low-pass characteristics of the tuning curves of these newly generated receptor units, besides the tuning properties of the electroreceptor membranes, are the input–output function of the synapse between the receptor cell and the afferent, and the refractory period of the afferent.

Refractory period has been shown not to be limiting here as long as stimulus frequencies are below 500 Hz. Newly regenerated synapses may fail at lower frequencies of stimulation than normal synapses (Dennis and Miledi, 1974; Schmidt and Edwards, 1983). I did not measure synaptic efficacy in this study, so that it is not certain what contribution this parameter might make to the tuning curve. However, the receptor oscillation data indicate that the receptor cells are, in fact, broadly tuned and tuned below the EOD frequency. Since each receptor organ is composed of tens of sensory receptor cells, it is not apparent whether all of the receptor cells within an organ are similarly broadly tuned or whether some might be more sharply tuned, each to a different frequency. This remains to be determined by intracellular recordings of receptor cells during various stages of regeneration.

It is not surprising that electroreceptor tuning shows such a developmental shift, as it is known to be labile in a number of
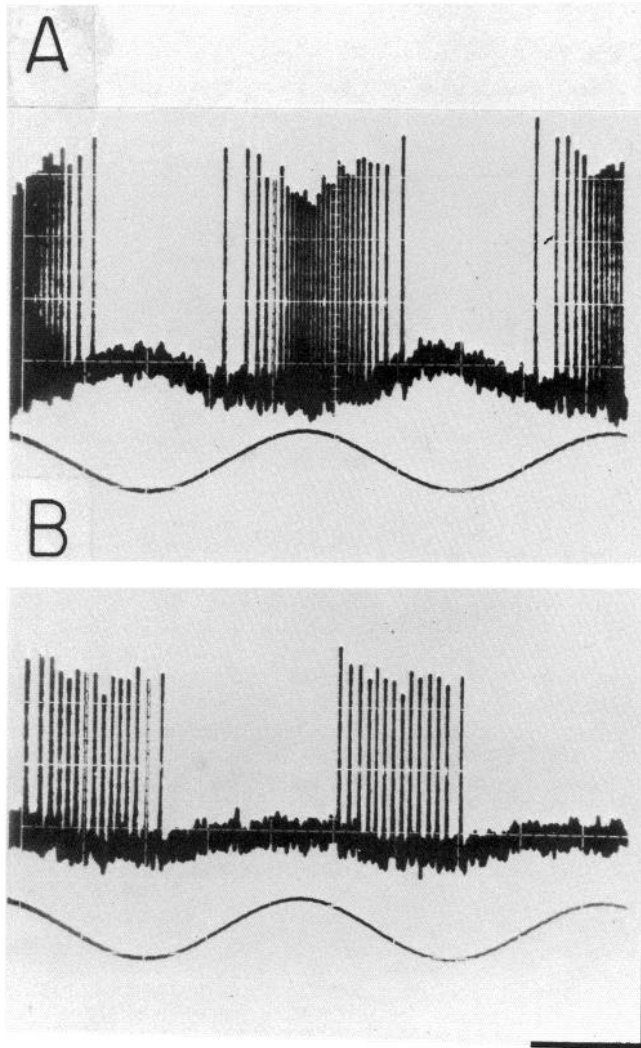
Figure 7. Responses to 4 Hz sinusoidal stimuli of ampullary receptors from fish that had been electrically-silenced for 6 weeks. *A,* Ampullary afferent from intact skin whose firing rate is modulated by stimulus as in a normal ampullary receptor. Stimulus intensity, 20 mV/cm. *B,* Ampullary afferent from regenerated skin; stimulus intensity, 6.5 mV/cm. The intact unit is more sinusoidally modulated by the stimulus than the regenerated unit, but this amount of variation is typical of intact receptors. Bars, 15 msec and 0.3 mV.

species. Larval *Sternopygus* discharge at around 100 Hz, and the EOD of maturing females gradually shifts upward in frequency (adult range, 100–200 Hz) and that of maturing males shifts downward in frequency (adult range, 50–100 Hz) (Hopkins, 1972, 1974; Meyer, 1983). This sex-dependent shift can be mimicked in the laboratory by the administration of sex-steroid hormones: Androgens lower EOD frequency, while estrogens raise it (Meyer, 1983). After systemic treatment with androgens, recordings of oscillatory receptor potentials and mean unit BFs from tuberous electroreceptors in *Sternopygus* decrease in BF by about 20–40 Hz to remain tuned to the EOD (Meyer and Zakon, 1982; Zakon and Meyer, 1983). A similar observation has been made in the mormyrid fish, *Brienomyrus brachyistius triphasic,* in which the juveniles and females possess a pulse-type EOD with peak energy at about 1.2 kHz, while the peak energy of mature males' EOD is around 0.3 kHz (Bass and Hopkins, 1983, 1984). Treatment of females and juveniles with androgens lowers the peak power spectrum of their EOD into

the male range (Bass and Hopkins, 1983). As in *Sternopygus,* after androgen administration, the receptors, which are tuned close to the peak energy in the EOD pulse, shift their BFs to lower values, closer to the new EOD power spectrum (Bass and Hopkins, 1984).

A major question is how the receptor cells "recognize" when they are correctly tuned to the EOD frequency either during hormone-induced shifts of EOD or during ontogeny. One hypothesis we have put forward to explain the ability of the receptor to track the new EOD after it is shifted by androgen administration is that the electric field itself may exert a direct influence upon the receptor cell (Meyer and Zakon, 1982; Meyer et al., 1984; Zakon and Meyer, 1983). This seems not to be the case: After administration of androgens, receptors are capable of *shifting* their BF to a lower value after spinal transection has silenced the EOD (Keller et al., 1986; Meyer et al., 1984). All attempts to shift the tuning of receptors in electrically silenced fish with exogenous electric fields have failed (Keller et al., 1986; J. H. Meyer and H. H. Zakon, unpublished observations). Similarly, in this study, newly generated receptors can *develop* tuning in the absence of an electric field.

Factors other than the EOD itself must be involved in tuning of developing receptor cells. Since the animals in this study were not gonadectomized, and it is well-demonstrated that the administration of androgens influences tuning, it is possible that circulating gonadal steroids play a role in the tuning process during regeneration as well. Initially, it was assumed that steroid levels in these fish would be negligible, as the animals in this study were thought to be juveniles (fish of this species captured in the wild are not reported to be reproductive until they are about 25–30 cm; Hopkins, 1974), and fish were not kept under environmental conditions reported to be conducive to gonadal recrudescence in a related species, *Eigenmannia* (Kirschbaum, 1979). Because of this, neither the state of the gonads nor plasma steroid levels were monitored in these fish. However, recent histological examination of the gonads of another series of fish of similar size range and kept under similar housing conditions in our laboratory has indicated that the testes often contain an abundance of mature sperm and some females may become gravid (H.-Y. Yan, personal communication). These results indicate that some circulating steroids must have been present in the regenerating fish (Fostier et al., 1983). Thus, whether sex hormones are necessary for the inception of tuning must be investigated in an additional series of experiments using gonadectomized fish.

A second possibility is that, in some way, the intact and rhythmically firing pacemaker nucleus itself can influence the tuning of the receptor cells. Direct neural mediation is unlikely, as the only known output from this nucleus is to spinal cord motoneurons (Bennett et al., 1967; Elekes and Szabo, 1981; Ellis and Szabo, 1980), and the electroreceptors receive no efferent contacts (Lissman and Mullinger, 1968; Szabo, 1965, 1974; Szamier and Wachtel, 1970; Wachtel and Szamier, 1966). If the PMN has no direct neural route, perhaps it can influence receptors by the release of a blood-borne factor. Studies of hormone-mediated retuning or the development of tuning of regenerated receptors in fish with lesions of the PMN or in which the firing frequency of the PMN is independently altered will allow a test of this hypothesis.

If these other factors ultimately have no influence on tuning, 2 other possibilities are left: Either the information is transmitted by the afferent fiber or it resides latent in the precursor cells. Abundant evidence indicates that the receptor cells are trophically dependent upon their afferent innervation (Bennett, 1967; Roth and Szabo, 1969; Szamier and Bennett, 1973); various types of electroreceptors degenerate, at the quickest, within 48 hr after denervation (Szamier and Bennett, 1973), or they
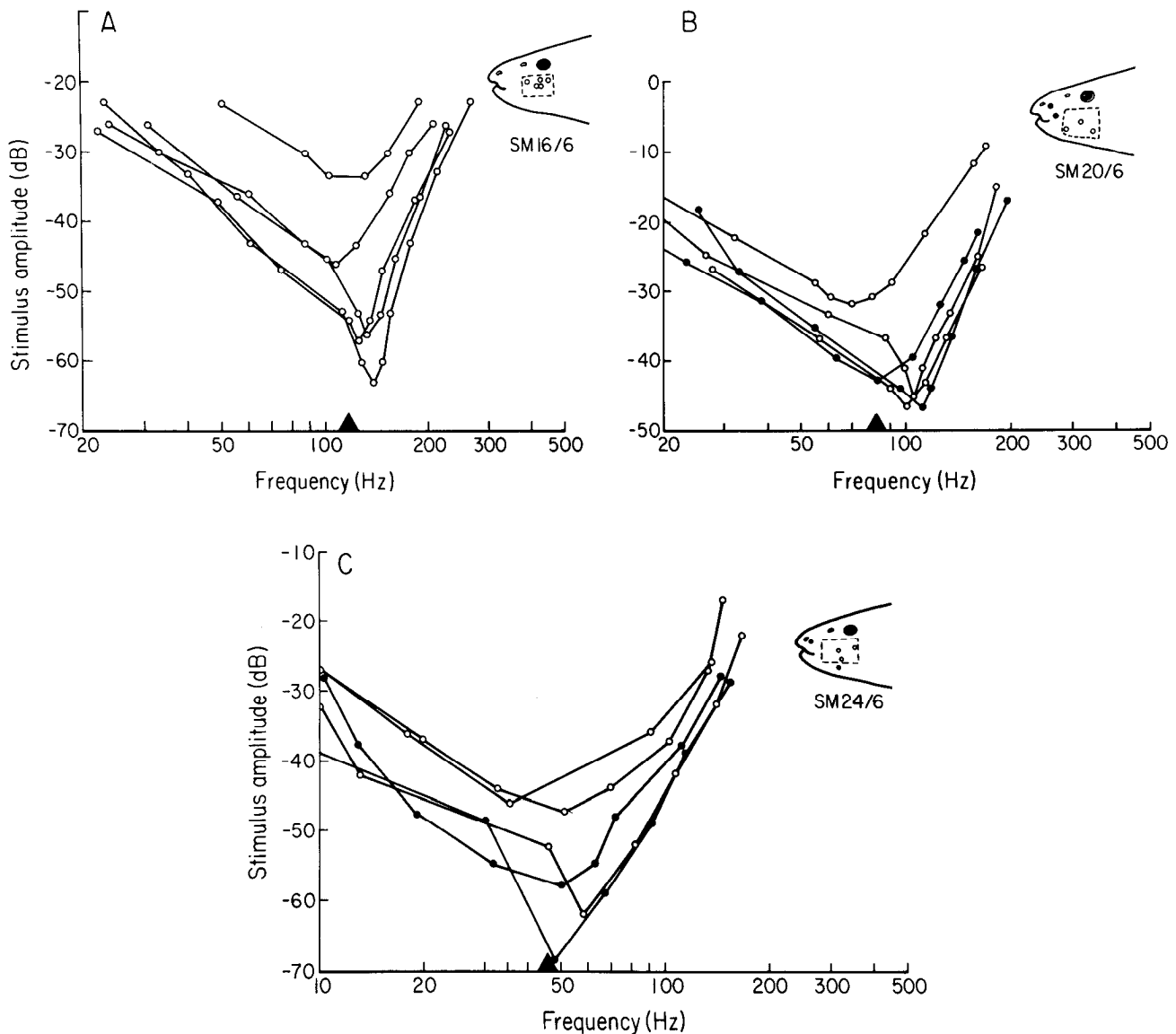
Figure 8. Tuning curves recorded after 6 weeks of skin regeneration from 3 electrically silenced fish. *Filled arrowhead* on the abscissa gives the discharge frequency of the PMN, which indicates the previous EOD frequency of each fish. *A*, PMN frequency of 115 Hz; *B*, PMN frequency of 79 Hz; *C*, PMN frequency of 46 Hz.

may linger for as long as a month but ultimately die (Denizot and Libouban, 1985). Whether the afferent nerves additionally provide instructions for tuning is another matter.

It is also possible that instructions for tuning are latent in the precursor cells. New receptor cells in already-existing organs appear to come from the support or accessory cells forming the base of the receptor epithelium (Denizot and Baillet-Derbin, 1969; Denizot and Libouban, 1985; Roth and Szabo, 1969). During the development of the free-standing neuromasts of the amphibian lateral line in normal ontogeny or during regeneration of the tail, similar stem cells send off progeny that migrate to give rise to new receptor organs (Stone, 1933). On the other hand, experiments on the regeneration of the lateral line hair cells and ampullary electroreceptors in catfish (*Amieurus nebulosus*) suggest that new receptor cells are, instead, induced from epidermal cells by the ingrowing nerve fibers (Bailey, 1937). Perhaps a genetically determined "set point" exists for receptor cell tuning latent in the genome of the precursor cells, which may be moved about by external factors such as hormones.

Maturation of the "electrical filter" and its relevance to development of hair cell tuning

The most likely mechanism to explain the upward shift in BF of new receptors would be the maturation of tuning of the "electrical filter" in the receptor cell membrane (Hopkins, 1976; Meyer and Zakon, 1982; Viancour, 1979a, b; Zakon and Meyer, 1983). Beside their ubiquitous occurrence in electroreceptors, such "electrical filters" have been observed in the hair cells of the sacculus (bullfrog: Lewis and Hudspeth, 1983) and the cochlea (turtle: Crawford and Fettiplace, 1981; chick: Fuchs, 1985; frog, amphibian papilla: Ashmore and Pitchford, 1985). But the inner ear of these vertebrates also possesses mechanical filters in the mechanisms of the otolith or basilar and tectorial membranes. Since mechanical and electrical filters are cascaded, for best sensitivity, the hair cell's electric filter must be tuned within the passband of the preceding mechanical filter stage, as they are in the turtle (Crawford and Fettiplace, 1981). One method of accomplishing this during development would be for the hair
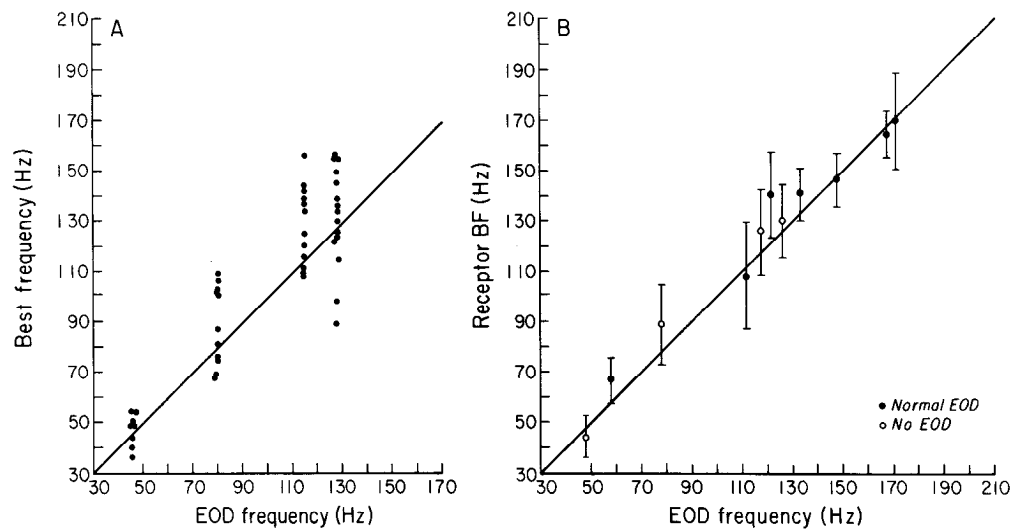
Figure 9. Properties of regenerated tuberous receptors of electrically silenced fish. *A,* Distribution of BFs of 4 fish versus PMN frequency. Note that, even in the absence of the EOD, the BF matches the firing frequency of the PMN. *B,* Distribution of mean and SD of BFs of regenerated receptors for 7 fish with EODs and 4 fish without EODs versus firing frequency of EOD (or PMN in electrically silenced fish).

cell electrical filter to become tuned to a dominant energy component of the mechanically driven local cochlear microphonic potential. However, if the EOD-independent tuning of newly generated electroreceptors bears upon this problem, it suggests that the tuning of hair cells may also be independent of the cochlear microphonic.

Lewis and Hudspeth (1983) have demonstrated that the tuning of saccular hair cells is based on the kinetics of an inward Ca^{2+}- and an outward Ca^{2+}-activated K^+ current. Modeling studies support the idea that the BF of such a tuned receptor would shift with the strength of each ion current (Koch, 1984; Lewis, 1984). Preliminary evidence suggests that similar ion species may underlie the tuning of tuberous electroreceptors

(Zakon, 1984b). The gradual upward shift in tuning of newly generated receptor cells may be due to changes in the passive properties of the receptor cell membrane, as addition of new membrane to the growing receptor cells might change total membrane impedance. Alternatively, this shift might be explained by maturation of active membrane properties by insertion or modification of ion channels in the basal membrane of the receptor cells, until the receptor cell is tuned correctly. A number of studies have demonstrated changes in ionic currents during development and regeneration (Angaut-Petit and Mallart, 1985; Masukawa et al., 1985; Spitzer, 1979).

It will be interesting to observe whether hair cells in the chick or turtle cochlea also undergo a gradual upward shift in BF and

Table 2. Receptor BF and Q_{10dB} as a function of regeneration time

Fish	EOD	BF (mean and ±SD) Intact	n	Regen.	n	Q_{10dB} (mean and ±SD) Intact	Regen.
Regeneration time: 3 weeks							
3A	110	115.3 (12.5)[b]	9	77.2 (11.8)	12	1.62 (0.66)[b]	0.71 (0.20)
12	116	—		81.8 (15.0)	8	—	0.97 (0.37)
5	156	174.5 (13.1)[b]	8	117.3 (17.0)	8	2.08 (0.83)[a]	1.10 (0.50)
13	172	184.3 (12.3)[b]	9	105.1 (21.2)	12	2.24 (0.83)[a]	0.94 (0.39)
31	194	213.6 (17.8)[a]	5	163.0 (39.8)	12	3.12 (2.00)	1.75 (1.17)
Regeneration time: 6 weeks							
6	59	71.5 (7.4)	22	67.0 (9.8)	10	1.27 (0.80)	0.87 (0.28)
3A	112	119.8 (11.7)	9	108.8 (18.9)	10	1.22 (0.15)	1.01 (0.37)
10	122	138.3 (15.5)	13	140.2 (17.0)	17	1.57 (0.42)	1.43 (0.73)
6A	133	147.2 (6.3)	7	140.2 (11.2)	11	1.44 (0.20)	1.29 (0.45)
9	148	146.7 (22.8)	18	146.5 (11.4)	13	1.82 (0.60)	1.98 (1.24)
5	168	174.8 (17.0)	13	163.6 (10.6)	13	2.50 (1.32)[a]	1.59 (0.50)
13	171	191.8 (15.6)[a]	11	169.2 (19.9)	13	2.77 (1.22)[b]	1.49 (0.49)
Silenced fish: 6 weeks							
24	47	44.2 (8.0)	9	46.7 (6.2)	7	0.71 (0.25)	0.91 (0.40)
20	79	88.6 (12.9)	17	89.3 (15.9)	12	1.25 (0.23)	1.11 (0.28)
16	115	135.5 (15.8)	7	128.9 (15.3)	12	1.82 (0.93)	1.71 (0.87)
40	126	134.8 (17.4)	15	128.9 (21.9)	15	1.19 (0.34)	1.10 (0.31)

Data were collected from intact and regenerated (Regen.) patches of skin at 3 and 6 weeks after skin removal in fish with an ongoing EOD and at 6 weeks from skin in fish with the EOD surgically eliminated. n = number of intact or regenerated afferents recorded per fish.

[a] Statistically significant difference between intact and regenerate populations at $p < 0.05$.

[b] Statistical significance at $p < 0.01$. The t test was used throughout for statistical comparison.

increase in sharpness during their early maturation if a similar process underlies tuning in all of these cells.

Significance of regeneration to development of electroreceptors

It is important to ascertain the extent to which regeneration in adult animals parallels ontogeny. In this species, as in a number of other gymnotiform wave fish, growth of the electroreceptive periphery occurs throughout life and new receptor cells are constantly being added to the existing population within a receptor organ (Zakon, 1984a). Furthermore, in *Sternopygus,* when receptor organs reach a particular number of cells, each organ divides into 2 daughter organs (Zakon, 1984a). Each afferent axon innervates only 1 receptor organ in a small fish, while older individuals have either 1 or numerous receptor organs contacting each axon, over 10 in large (>30 cm) individuals. Throughout life, new receptors must be tuned as they are added. Thus, the generation of receptor cells during skin regeneration in postlarval fish must take advantage of this ongoing process.

The only study of the development of tuning in larvae is on the gymnotiform *Apteronotus,* a high-frequency EOD species. Larval *Apteronotus* discharge their electric organ at a frequency of about 300–400 Hz after hatching. As they mature over the next month, the EOD frequency more than doubles to about 700–800 Hz (Kirschbaum, 1983; Meyer et al., 1986). As determined by recording the impulse-evoked receptor oscillations, receptors are initially tuned to the low-frequency EOD and gradually shift upward in BF concurrently with the EOD (Meyer et al., 1986). Steroid hormone levels of these fish are not known, but since no exogenous steroids were given, this may represent a steroid-independent form of receptor plasticity.

Similarly, the EOD waveform undergoes developmental changes in a number of other species of weakly electric fish. The EOD gradually increases in frequency in larval *Eigenmannia,* and changes entirely in the larval mormyrid *Pollimyrus isidori,* from a larval discharge of about 1 msec in duration to an adult discharge of about 100 μsec duration (Westby and Kirschbaum, 1978, 1982). The discharge of larval *Hypopomus* begins as a monophasic pulse and matures into a diphasic pulse of higher peak power frequency (Hagedorn and Carr, 1985, and personal communication). In all of these cases, the peak power frequency of the EOD *increases* during development.

It is not known if the receptors of each species gradually track the EOD during early maturation, as they do during their development in *Apteronotus.* During regeneration of tuberous receptors of *Sternopygus,* the electroreceptors also shift upward in frequency, although in the presence of a constant EOD. Whether the process of a gradual correlated increase in peak power of the EOD and receptor tuning in any way confers an adaptive advantage upon the larvae or, instead, whether it is merely the by-product of necessary developmental processes involved in the genesis of tuning and the maturation of membrane excitability must be the subject of future investigations.

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