Differential Activation of Glutamate Receptor Subtypes on a Single Class of Cells Enables a Neural Oscillator To Produce Distinct Behaviors

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Spiro, John E. Differential activation of glutamate receptor subtypes on a single class of cells enables a neural oscillator to produce distinct behaviors. J. Neurophysiol. 78: 835–847, 1997. Electric fish generate different types of abrupt modulations of their electric organ discharge (EOD) rhythm to convey specific social signals. Intracellular recordings were made from neurons of the medullary pacemaker nucleus, which generates and transmits the rhythm that drives the EOD, to study the neuronal basis of two such modulations of the regular EOD rhythm, sudden accelerations, and abrupt interruptions. Recordings were both in vivo, and in a new in vitro brain preparation of Hypopomus pinnicaudatus (order Gymnotiformes). In vivo recordings during triggered behaviors indicated that abrupt modulations of the EOD rhythm are generated in the medullary pacemaker nucleus at the level of the relay cells, which are the projection cells of the nucleus, and not the pacemaker cells. In the in vitro brain stem preparation, cells of the pacemaker nucleus were spontaneously and rhythmically active as in the intact animal. Distinct modulations of the pacemaker nucleus rhythm that closely resembled those seen during natural behaviors could be triggered by electrical stimulation ofafferent fibers. Modulations of the rhythm also could be triggered by direct pharmacological activation of the relay cells. When non-N-methyl-D-aspartate (NMDA) receptors were activated, relay cells were transiently depolarized and generated bursts of synchronized action potentials. NMDA receptor activation, alternatively, initiated a prolonged depolarization in the relay cells, during which time they failed to relay the regular pacemaker rhythm. The two firing states of the relay cell directly correlate with sudden accelerations and abrupt interruptions of the EOD.

INTRODUCTION

South and Central American electric fish (order Gymnotiformes) generate an electric organ discharge (EOD) with a specialized organ in their tail (see Bullock and Heiligenberg 1986 for general references on electric fish). Two types of neurons in the medullary pacemaker nucleus, pacemaker and relay cells, produce and transmit a rhythmic drive to the spinal motoneurons that innervate the electric organ. Although the fish often produce extremely regular electrical discharges, they also modulate the frequency of their EOD in different behavioral contexts, most robustly during courtship and aggressive encounters (Black-Cleworth 1970; Hagedorn 1986; Hopkins 1974; Kawasaki and Heiligenberg 1989; Westby 1975). These displays include sudden accelerations and sudden interruptions of the otherwise regular rhythm.

Electroreceptors in the fish’s skin transmit information about the generated electric field back to the CNS. Completing the loop, the pacemaker nucleus receives synaptic input from brain regions, the pacemaker nuclei, which participate in the processing of electrosensory, and other sensory, information (e.g., Heiligenberg 1991). Changes in the activity in these areas influence the firing of the pacemaker and relay cells, which in turn change the rate of the EOD. Figure 1 diagrams the organization of the pacemaker nucleus and its inputs, and Fig. 2 shows the cells that make up the nucleus. See Bennett et al. (1967), Bennett (1971), and Dye and Meyer (1986) for further references on electric fish pacemakers.

How do prepacemaker afferents interact with the pacemaker nucleus, which has only two neuronal cell types connected in a feed-forward manner, to produce several distinct output patterns? A partial answer came from studies that showed that different glutamate receptor subtypes were necessary for synaptic transmission between different prepacemaker areas and the pacemaker and relay cells (Dye et al. 1989; Kawasaki and Heiligenberg 1990; Keller et al. 1991; Metzner 1993). For example, Kawasaki and Heiligenberg (1990) introduced glutamate receptor antagonists into the pacemaker nucleus of an intact animal while distinct modulations of the pacemaker rhythm were triggered by stimulating either the sublemniscal prepacemaker nucleus (SPPn) or prepacemaker nucleus, “chirp” subdivision (PPhC) (Fig. 1). 2-Amino-5-phosphonovaleric acid (APV), an N-methyl-D-aspartate (NMDA) receptor antagonist, reversibly eliminated the response to SPPn stimulation (i.e., a sudden interruption of the rhythm), whereas 6-cyano-7-nitroquininaline-2,3-dione (CNQX), a non-NMDA receptor antagonist, had no effect. CNQX did, however, block rapid accelerations of the rhythm triggered by PPhC stimulation, whereas APV did not.

Intracellular recording showed that stimulation of SPPn and the PPhC primarily affected the relay cells (Kawasaki and Heiligenberg 1989) (see also Fig. 3). A subsequent study localized NMDA receptors to the soma of relay cells using a monoclonal antibody (Spiro et al. 1994). Taken together, the results suggested that SPPn fibers triggered sudden interruptions by selectively activating NMDA receptors on relay cells and that PPhC fibers triggered sudden accelerations by selectively activating non-NMDA receptors on the same cells. How activation of the receptors on relay cells generated the changes in relay cell firing, however, remained unclear. This study focuses on the properties of the relay cells that contribute to these responses.

These results were previously presented in abstract form.
FIG. 1. Lateral view of Gymnotiform brain and schematic diagram of medullary pacemaker nucleus and its afferents in *Hypopomus* A: unpaired midline pacemaker nucleus is composed of 2 neuronal cell types, pacemaker and relay cells. In an adult fish, there are $\sim 20$ cells of each type. Pacemaker cells are electrotonically interconnected and make mixed electrotonic and chemical synaptic contacts on relay cells. Relay cells are output cells of nucleus; their axons innervate motoneurons that drive the electric organ. Also shown are approximate locations of 2 regions (each marked (*)) whose projection neurons make synaptic contacts on the relay cells and modulate their firing rate: prepacemaker nucleus, “chirp” subdivision (PPnC), named for effect that its stimulation has on pacemaker rhythm (rapid frequency accelerations that sound like chirps on an audio monitor), and sublemniscal prepacemaker nucleus (SPPn). Activity in these regions triggers qualitatively distinct modulations of otherwise regular pacemaker rhythm (see Fig. 3). Question mark, degree of coupling between relay cells is not known; dashed line, outlines approximate borders of reduced in vitro preparation (METHODS). Additional inputs onto pacemaker cells, which are not involved in abrupt changes of rhythm, are omitted for clarity. B: inputs onto relay cells as shown in A (within dashed box) are illustrated in more detail, including hypothesized cellular loci of synapses and neurotransmitter receptors (see DISCUSSION).

(Spiro 1994; Spiro and Heiligenberg 1992) and in a doctoral thesis (Spiro 1995).

METHODS

Fish

*Hypopomus pinnicaudatus* (order Gymnotiformes) (described by Hopkins 1991) was used in all experiments. Adult fish (10–16 cm) of both sexes were purchased from a commercial dealer or were generously donated by Dr. Philip Stoddard (Florida International University) and were maintained in large aquaria.

Recordings and stimulation in vivo

Fish were paralyzed with an injection of flaxedil, and the EOD, attenuated from the cholinergic block, was monitored with an electrode over the tail as a measure of the pacemaking activity transmitted by the spinal motoneurons. After application of a local anesthetic, a small hole was drilled in the skull to provide access for stimulating and recording electrodes (Kawasaki and Heiligenberg 1989). Electrodes filled with 3 M NaCl (1–5 MΩ) for recording of extracellular potentials or with 3 M KCl (30–100 MΩ) for intracellular recordings, were lowered to the level of the pacemaker nucleus using a Burleigh microdrive. Pacemaker cells were distinguished readily from relay cells based on the waveform of their action potentials: pacemaker cells fired an action potential after a slowly depolarizing phase (pacemaking potential), whereas relay cells fired a rapidly rising spike straight from the baseline voltage. On some occasions, double-barreled electrodes were used to record intracellularly from cells in the pacemaker nucleus while simultaneously introducing $\gamma$-amino-n-butyric acid (GABA; Sigma) into the local extracellular space with iontophoretic current, to stop the pacemaker rhythm. The recording barrel was filled with 3 M KCl, and the other barrel, cut back for a distance between the tips of 10–100 μm, was filled with GABA (0.5 M in water, pH 3.5 with HCl; 30–70 nA positive DC).

Areas presynaptic to the pacemaker nucleus, the midbrain SPPn and the diencephalic PPnC, were stimulated with an iontophoretic application of l-glutamate [Sigma; 0.1 M in water, pH 8, ca. 100 nA (negative DC) with an inner tip diam of the electrode of <10 μm]. These regions were localized by monitoring the tail signal while probing the midbrain or diencephalon with the stimulating electrode. A characteristic change in the EOD indicated that the electrode was in the desired brain region (see RESULTS). Electrodes filled with indium also were used on occasion to stimulate these areas with current pulses or to make small lesions for histological identification of stimulation sites.

In vitro preparation

Fish were anesthetized in ice-cold oxygenated artificial cerebral spinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 0.75 KH₂PO₄, 1.2 MgSO₄, 1.6 CaCl₂, 24 NaHCO₃, and 10 d-glucose, pH 7.4 after saturation with 95% O₂:5% CO₂. This ACSF formula (Turner et al. 1994) reflects minor changes in the composition of standard fish physiological saline. The brain, with part of the spinal cord, was removed from the skull and pinned ventral-side-up while submerged in a Sylgard-lined recording chamber. The entire procedure took <5 min. Early in this study, recordings from the pacemaker nucleus were made in “whole-brain” preparations because attempts also were made to stimulate and record directly from the pacemaker regions in the diencephalon and midbrain. These deeper brain areas, however, were apparently not viable, although their processes were (see RESULTS). A preparation reduced to the
brain stem regions surrounding the pacemaker nucleus (refer to Fig. 1 for approximate borders) therefore was used in later experiments; less tissue in the chamber allowed for more stable recordings and faster washout of pharmacological agents. Results reported here are from the more reduced preparation.

In previous preparations made from pacemaker nuclei of another genus, *Apteronotus*, in which the pacemaker nucleus protrudes from the ventral surface of the brain, the nucleus was excised and placed in an interface chamber of the type commonly used for slice preparations (Dye 1988; Meyer 1984; Schaefer and Zakon 1996). A more intact preparation was required for the *Hypopomus* pacemaker nucleus because, in this genus, the nucleus is located deeper into the ventral surface and cannot be completely isolated without damaging its structure.

The chamber was superfused continuously with oxygenated ACSF at room temperature; the bath level was kept as low as possible. The borders of the midline pacemaker nucleus, including the outline of three to seven relay cells near the ventral surface, could be seen through a dissecting microscope. Small cactus spines were placed at the far rostral and caudal extents of the remaining tissue to stabilize the preparation. Although the meninges often were disturbed during the removal of the brain from the skull, and, on occasion, an occluding superficial midline blood vessel was removed, deliberate attempts to further remove the meninges to improve access to the nucleus were not undertaken. These efforts generally resulted in damage to relay cells, the somata and dendrites of which lie on the ventral surface of the brain. The preparation was allowed to recover for 1 h before intracellular recordings were made. Using extracellular electrodes, however, a pacemaker rhythm could be detected within a few minutes. Action potentials still were recorded on occasion from pacemaker and relay cells after the tissue was in vitro >48 h. More than 100 fish were used.

**Fig. 2.** Photomicrographs of sections through medulla at level of the pacemaker nucleus. A: in an in vitro preparation, a relay cell ( \( \land \) ) was filled intracellularly with biocytin to reveal its structure; tissue then was cut on cross-section (60 \( \mu \)m), and tracer was visualized with an HRP/DAB reaction and counterstained with Nissl stain, neutral red. Axon ( \( \land \) ) left nucleus and extended down spinal cord. Dendrites ( \( * \) ) extended beyond plane of focus. A smaller pacemaker cell soma is marked ( \( \vee \) ). Other somata are filled by dye coupling. Ventral surface of brain is visible at bottom. B: a mid-sagittal section (40- \( \mu \)m-thick) through brain stem, counterstained with neutral red. Pacemaker cells (1 marked with \( b \)) form a subnucleus that is generally dorsal to, and surrounded by, relay cell subnucleus (1 soma marked with \( R \)). Dorsal is up. Scale bars, 100 \( \mu \)m.
FIG. 3. Rapid accelerations and sudden interruptions of electric organ discharge (EOD) followed abrupt changes in activity in relay cells and not pacemaker cells. Intracellular recordings in vivo from a relay cell (A) and a pacemaker cell (B), while pre-pacemaker regions (SPPn or PPnC) were stimulated with iontophoresis of L-glutamate. A simultaneous record of EOD, attenuated by cholinergic block, is shown below intracellular data. SPPn stimulation (A1) caused a prolonged depolarization in relay cell (note 4-s break in record) and a concurrent interruption in regular EOD (?), which resumed after relay cell repolarized. With a similar stimulus, a pacemaker cell (B) continued to fire action potentials regularly, even though rhythmic EOD was interrupted (?). A high-frequency residual signal remained in EOD recording during interruption. Unlike SPPn stimulation, PPnC stimulation (A2) caused a rapid, transient acceleration of relay cell frequency that resulted in a burst in the firing rate of EOD. Degree of acceleration varied considerably from a strong form (left), to a weaker form (right), but additional pulses in EOD always followed additional spikes in relay cell firing 1 for 1. All recordings in A are from same relay cell in same penetration. Iontophoretic stimulus was brief (<1 s). Timing for stimulus is not shown because there was a lag on order of seconds (which varied between stimulus locations) between start of iontophoretic current and effect in cell, presumably reflecting time occupied by spread of L-glutamate in nuclei. As is common in intact fish of this genus, baseline repetition rate of EOD varied somewhat throughout experiment.

for the development of the in vitro preparation. Data from the last 25 preparations are included in this report.

Electrophysiological recordings

The cells of the pacemaker nucleus were spontaneously and rhythmically active in vitro. A field potential recording, which reflects a sampling of a number of cells, was obtained readily using a 3 M NaCl electrode (1–5 MΩ) inserted into the nucleus. Intracellular recordings were obtained using conventional sharp borosilicate electrodes (10–50 MΩ, filled with 3 M KCl) or, in some cases, quartz electrodes pulled on a Sutter laser puller (similar resistances), and Getting 5A, and WPI M707A intracellular amplifiers. Signals were DC amplified and stored on VHS tape using a Vetter PCM device with a minimum bandwidth of 4 kHz and analyzed off-line using software purchased from Run Technologies (Laguna Hills, CA) running on an IBM-compatible 80486 PC. Data were transferred to a Macintosh computer for preparation of figures.

Electrical stimulation in vitro

A concentric bipolar electrode (inner pole 25 μm diam platinum, outer pole 200 μm diam stainless steel, FHC, Brunswick, ME) was used to stimulate the afferents to the pacemaker nucleus electrically (approximately 90-ms train, 5-μA pulses of 1-ms duration at 250 Hz). Stimulation elicited distinct modulations of the pacemaker rhythm whose temporal patterns mimicked changes in the EOD seen in natural behaviors (see RESULTS).

Drug application in vitro

Drugs of interest, diluted into ACSF, were loaded into micropipettes with tips broken to ~10 μm. Using micromanipulators, the tips of the pipettes were brought close to the surface of the brain under visual inspection, until they were directly over the cells of the pacemaker nucleus were spontaneously and rhythmically active in vitro. A field potential recording, which reflects a sampling of a number of cells, was obtained readily using a 3 M NaCl electrode (1–5 MΩ) inserted into the nucleus. Intracellular recordings were obtained using conventional sharp borosilicate electrodes (10–50 MΩ, filled with 3 M KCl) or, in some cases, quartz electrodes pulled on a Sutter laser puller (similar resistances), and Getting 5A, and WPI M707A intracellular amplifiers. Signals were DC amplified and stored on VHS tape using a Vetter PCM device with a minimum bandwidth of 4 kHz and analyzed off-line using software purchased from Run Technologies (Laguna Hills, CA) running on an IBM-compatible 80486 PC. Data were transferred to a Macintosh computer for preparation of figures.

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Labeling of cells intracellularly

Electrode tips were backfilled with a 2% solution of Neurobiotin (Vector laboratories) in 3 M KCl. Electrodes were then topped off with 3 M KCl and had resistances of 20–40 MΩ. In early experiments, Biocytin (Sigma) was used, and electrodes were generally >40 MΩ. Cells were filled by passing depolarizing current pulses (500 ms, 50% duty cycle) of 1–5 nA for 5–30 min. At the end of the experiment, brains were placed in cold 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and left in the same solution for ≤3 days at 4°C. Processing for detection of biocytin or Neurobiotin was modified slightly from earlier protocols (Horikawa and Armstrong 1988; Kita and Armstrong 1991): brains were cut at 40–60 μm on a Vibratome, and...
sections were collected into 0.02 M phosphate saline buffered to pH 7.4 (PBS). They then were transferred to a solution of 0.5% H$_2$O$_2$ at room temperature for 10 min to inhibit endogenous peroxidase activity, rinsed in PBS (3 × 10 min), and transferred to Vector Elite ABC reagent for 2.5 h at room temperature. Sections were washed in 0.1 M (hydroxymethyl)aminomethane (Tris) buffer pH 7.2 (3 × 10 min), and transferred to a solution containing 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.4 mg/ml) and nickel ammonium sulfate (0.06% final concentration) in 0.1 M Tris buffer pH 7.2. H$_2$O$_2$ was added (0.001% final concentration), and the reaction was monitored (5–15 min) and stopped by washing in 0.1 M Tris buffer pH 7.2. Sections then were mounted on subbed slides and dried. They were dehydrated through alcoholic, cleared in Histoclear, and coverslipped with Permout. Some sections were counterstained with the Nissl stain, neutral red. Photomicrographs were made using an Olympus camera system and Kodak T-Max 100 film.

RESULTS

Relay cells, and not pacemaker cells, participate in abrupt modulations of the EOD rhythm: intracellular recordings from pacemaker and relay neurons in vivo

Recordings were made from pacemaker and relay cells in intact, curarized _H. pinnicaudatus_ to determine which cells within the pacemaker nucleus participate in the abrupt changes of the EOD frequency observed during sudden accelerations and abrupt interruptions. The results confirmed previous observations in a closely related species _H. brevisrostris_ (Kawasaki and Heiligenberg 1989), and provided a direct source for comparison with the newly developed in vitro preparation. As shown in Fig. 3, both rhythm modulations originated at the level of the relay cell, because despite the drastic changes in relay cell firing during the triggered behaviors, pacemaker cell firing remained largely undisturbed.

Pacemaker and relay cells were recorded intracellularly while discrete areas in the midbrain and diencephalon, known from tracing experiments to project to the pacemaker nucleus (Kawasaki and Heiligenberg 1989; Kennedy and Heiligenberg 1994; W. Heiligenberg, unpublished observations), were stimulated with focal iontophoretic application of L-glutamate (Fig. 3). Stimulation using current trains was not shown (Kawasaki and Heiligenberg 1989). Similarly, if the pacemaker cell already was depolarized sufficiently when the stimulating electrode was moved from the SPPn to the PPnC. At the latter location, application of glutamate triggered a burst of spikes in the relay cell, each of which was matched with individual pulses in the EOD (Fig. 3A2) (see Kawasaki and Heiligenberg 1989 for a more complete discussion), indicating that the relay cells fired in synchrony during these high-frequency spikes. A strong acceleration, such as that shown in Fig. 3A2, also delayed the next pulse of the baseline rhythm. These brief accelerations of the EOD observed after stimulating PPnC strongly resembled common communicatory signals called ‘chirps’ (Hagedorn 1986; Hopkins 1974; Kawasaki and Heiligenberg 1989; see Black-Cleworth 1970 and Westby 1975 for an alternative nomenclature and discussion of EOD modulations in pulse species). The degree of acceleration of the EOD varied greatly during both natural chirps and stimulated behaviors (Fig. 3A2) (Kawasaki and Heiligenberg 1989).

The recordings in Fig. 3A were from a single relay cell; the stimulating electrode was moved between the two pre-pacemaker areas while an intracellular recording was maintained. This recording demonstrated directly that activity in a given relay cell is correlated with both types of FMs, prolonged interruptions and rapid bursting. Pacemaker cells continued to fire regularly throughout interruptions (Fig. 3B) and brief accelerations (Kawasaki and Heiligenberg 1989) (data not shown for _H. pinnicaudatus_), indicating that the primary effect of stimulation of SPPn and PPnC was on the relay cells. During PPnC stimulation, the pacemaker cells did occasionally fire extra action potentials, however, if the pacemaker cell already was depolarized sufficiently when the relay cell action potential occurred (data not shown) (Kawasaki and Heiligenberg 1989). Similarly, pacemaker cells fired an extra spike during the rapid phase of depolarization of the relay cell membrane potential associated with sudden interruptions (Fig. 3B).

Properties of pacemaker and relay cells in vitro

In vitro, the cells of the pacemaker nucleus were spontaneously active within the same frequency range observed for the EOD of naturally behaving fish (10–40 Hz). Simultaneous intracellular recordings of a pacemaker and relay cell are shown in Fig. 4. The characteristic action potential waveforms and the phase relationship between pacemaker and relay cell firing observed in vivo were maintained in vitro. Similar to in vivo recordings, baseline potentials were between −60 and −90 mV, and spike heights varied between 20 and 50 mV in stable recordings. This variability in spike heights may be attributable to differences in recording locations within the cell. Although not measured routinely, input resistances for relay cells (1–3 MΩ) were in the range of...
published values from studies in vivo in a related genus (*Steatogenys*) (Bennett et al. 1967) and results in vivo presented here. Probably because relay cell somata are larger and located closer to the surface, recordings from relay cells were more common and more stable than those from pacemaker cells.

Biocytin or neurobiotin was iontophoresed into cells to reveal their structure. Extensive processes of a relay cell (Fig. 2A) were observed after a biocytin fill. The subcellular recording site (e.g., soma vs. axon) could not be reliably determined from cell fills with these compounds.

Firing properties of relay cells during intracellular current injection

Penetration of a relay cell either in vivo or in vitro often resulted in high-frequency, small spikes overlying the regular, larger action potentials that followed the pacemaker cell rhythm. Several seconds of hyperpolarizing current abolished these spikes, or they disappeared spontaneously as the recording stabilized. Because the potentials were similar in frequency and size to the spikes seen in relay cells after stimulation of PPnC (Fig. 3A2), these small spikes were investigated more thoroughly in the in vitro preparation to determine if they might constitute the activity seen during rapid accelerations or chirps.

Depolarizing current steps injected into relay cells elicited high-frequency action potentials that were superimposed on the ongoing large action potentials arising from the synchronized input from pacemaker cells (Fig. 5A). Larger current injections resulted in higher frequencies of firing and smaller amplitude spikes. To observe these small spikes in the absence of the large, regular action potentials, the inhibitory transmitter GABA was introduced into the bath in the vicinity of the pacemaker nucleus by ejecting the drug with a pressure pulse from a micropipette (concentration of GABA in the pipette was 100 mM in ACSF). In *Hypopomus*, pacemaker cells, and not relay cells, receive a GABAergic projection from the diencephalon, and stimulation of this projection, or exogenous addition of GABA, specifically inhibits the pacemaker cells (Kawasaki and Heiligenberg 1990; Kennedy and Heiligenberg 1994; J. E. Spiro and W. Heiligenberg, unpublished observations). In the presence of GABA, the frequency of the large spikes in the relay cells, the ones that normally followed pacemaker cell firing, slowed and eventually stopped and the baseline voltage of the relay cell recording did not change; in the absence of pacemaker cell input, relay cells did not fire action potentials spontaneously.

The current-frequency relationship for one relay cell’s action potentials is plotted in Fig. 5, B and C, during 2-s current injections of various amplitudes. Whereas larger current injections produced fast, regular spikes, smaller amplitude currents produced action potentials at a lower, more variable frequency (compare 3 and 1.2 nA in Fig. 5B). This relationship is qualitatively similar to the difference in the action potential frequencies seen in relay cells during strong versus weak chirps (Fig. 3A2). Whereas the firing rate of many kinds of neurons shows spike frequency adaptation during a constant current pulse (Hille 1992), the spike frequency in the relay cells slowly increased during the 2-s period. Similar results were obtained from a total of 11 recordings of relay cells in eight preparations.

The ionic basis of both types of spikes in the relay cell was studied by brief applications of the sodium channel blocker TTX. Concentrations in the range of 20 μM in the pipette gave rapid and reversible results, although the final concentration in the bath and the extent of diffusion could not be determined. After application of TTX, the amplitude of the large spike in the relay cell rapidly decreased to approximately half, then decreased further and eventually disappeared entirely during the course of 1 min (Fig. 6A). Two components of the relay cell action potential were observed during the transition period. The amplitude of the component marked with an arrow did not vary with intracellular current injection, suggesting that this component represents the potential from the pacemaker cells (not yet affected by the TTX) transmitted electrotonically. Figure 6B illustrates a similar experiment where current pulses were delivered to the relay cell before and after focal TTX application. The small, fast spikes caused by current injection persisted for a few minutes longer than the large spikes before eventually also disappearing (not shown). Both action potentials recovered to full amplitude after ~30 min. Because TTX was applied near the surface of the brain in the vicinity of the relay somata and most likely more distant from the dendrites, the differential disappearance of the two types of action potentials suggests that the sites of generation of the large action potential that follows the pacemaker cell rhythm and
PACEMAKER RHYTHM MODULATIONS GENERATED AT THE RELAY CELL

The small fast spike are probably spatially distinct. Similar results were obtained from a total of five cells in three preparations.

The results from current injections and pharmacological manipulations show that the relay cells can generate action potentials at much higher frequencies than the normal pacemaker rhythm, in the absence of input from pacemaker cells. The similarity of these fast spikes generated by the relay cells to those seen in vivo recordings during chirps suggests that they underlie the abrupt acceleration of the EOD characteristic of chirps.

**Triggered modulations of the pacemaker nucleus rhythm in vitro**

**ELECTRICAL STIMULATION OF AFFERENTS.** Whereas DC injection into relay cells could elicit fast action potentials as

![Image](https://via.placeholder.com/150)

**FIG. 5.** Properties of relay cells after intracellular current injection. DC current injected into relay cells caused high-frequency action potentials, which were superimposed on larger ongoing action potentials (*A, *), which followed pacemaker cell rhythm. *A:* a 3-nA current pulse caused a burst of smaller spikes. After introduction of γ-aminobutyric acid (GABA) into bath (below), action potentials that follow pacemaker rhythm slowed and eventually stopped, and only smaller action potentials were observed on depolarization. *B:* plot of instantaneous frequencies of small spikes during 2-s current injections of various amplitudes in a preparation treated with GABA (as in *A*). *C:* plot of mean frequency near end of 2-s step as a function of current amplitude.

![Image](https://via.placeholder.com/150)

**FIG. 6.** Changes in relay cell action potentials after tetrodotoxin (TTX) application. *A:* recording from a relay cell in an in vitro preparation after sodium channel blocker TTX was introduced into vicinity of relay nucleus ~1 min before start of trace. Spike eventually disappeared entirely (not shown). *B:* current pulses were injected into a relay cell before (top) and after (bottom) focal introduction of TTX to ventral surface of pacemaker nucleus (as in *A*). Small spikes disappeared shortly after, and both types of spikes eventually recovered (not shown).
seen during chirps, sustained depolarizations of relay cells, as observed during sudden EOD interruptions, could not be triggered with simple current injections. Sustained depolarizations of the relay cells could be triggered in the in vitro preparation, however, by electrically stimulating pacemaker nucleus afferents. Cells bodies located within the SPPn and PPN complex, including the PPNc and two nearby regions whose output cells make contact on pacemaker cells, send axons to the pacemaker nucleus that run loosely intermingled with each other along the ventral surface of the brain (W. Heiligenberg, unpublished observations). These projection axons were stimulated with brief current pulses delivered through a concentric bipolar electrode while recording intracellularly from a relay cell.

The most common response was a transient smooth acceleration of the pacemaker rhythm, which is also the most common electromotor behavior (a novelty response) seen in this genus of fish. This response probably reflected selective stimulation of fibers that make contact on pacemaker cells, because the rate of firing of pacemaker cells increased smoothly. Another set of responses primarily affected relay cells (Fig. 7) and strongly resembled either changes in relay cell firing seen after direct stimulation of SPPn (sustained depolarizations) or PPNc (rapid accelerations) in vivo. The responses were often blends of different rhythm modulations, an observation consistent with the anatomy, which indicates that the fibers from the different presynaptic regions run intermingled with each other and therefore might be activated with the same stimulus. Small changes in the stimulus parameters (e.g., length of train, amplitude of current, position of electrode) were often sufficient to change the type of response. A consistent map of the parameter space that tended to elicit a particular response was therefore impossible to obtain.

Because prepacemaker cell bodies are absent in the in vitro preparation, these results suggest, as do the following data, that endogenous or network properties of the relay cells are sufficient to generate both sudden accelerations and abrupt interruptions.

**DIRECT APPLICATION OF NEUROTTRANSMITTER TO RELAY CELLS.** Previous work showed that application of glutamate receptor antagonists to the pacemaker nucleus blocked behaviors involving modulations of the EOD frequency (Dye et al. 1989; Kawasaki and Heiligenberg 1990; Keller et al. 1991; Metzner 1993). Here, in the in vitro preparation, glutamate receptor agonists were applied directly to the pacemaker nucleus to determine if activation of the different receptors was sufficient to trigger distinct rhythms.

While recording intracellularly from relay cells, L-glutamate, NMDA, AMPA, or kainate, the principal agonists for the ionotropic glutamate receptor subtypes, were applied locally to the surface of the brain near relay cells using pressure pulses. The results for NMDA and L-glutamate are shown in Fig. 8. Relatively high concentrations of L-glutamate and NMDA (1–2 mM) were loaded into the pipettes. Concentrations were chosen that produced rapid and consistent responses. Also, at these concentrations, the pipette delivering the agonists could be placed close to, but not touching, the surface of the brain, which eliminated movement artifacts.

The distance of the pipette from the surface of the brain largely determined the duration of the pulse needed to elicit a consistent response. The concentrations of the agonists at the relay cell were indeterminate but certainly significantly lower than the pipette concentration due to dilution in the bath.

A short pulse of NMDA produced a large and prolonged depolarizing response in the relay cell (Fig. 8A) that strongly resembled the response in a relay cell after SPPn stimulation in vivo: after an initial slow depolarization, the membrane potential rapidly depolarized and maintained this state for several seconds, after which time it rapidly repolarized. While depolarized, relay cells fired small, high-frequency spikes that increased in amplitude

![Figure 7](https://example.com/figure7.png)

**FIG. 7.** Electrical stimulation of ventral surface of brain in vitro triggered abrupt changes in relay cell rhythm resembling those seen after stimulation of SPPn or PPNc in vivo. Intracellular recordings were made from relay cells while a train of brief current pulses (duration indicated by a line underneath intracellular trace; ~5 μA pulses at 250 Hz) was delivered to ventral surface of brain. Parameters of stimulus train and placement of electrodes were such that relay cell fired either a sustained depolarization (A; note break in time axis) or a burst of action potentials (B), resembling firing patterns following SPPn or PPNc stimulation in vivo, respectively (compare with Fig. 3). Stimulus artifacts are visible [top; an example is marked (f)]. During sustained depolarization, relay cell fired high-frequency action potentials. Spikes after regular pacemaker rhythm (*) became more apparent as cell repolarized. Recordings in A and B were from different preparations.
with increasing repolarization. The regular input from the pacemaker cells (*) was visible overlying the fast spikes. The frequency of the pacemaker cell potential, recorded simultaneously, changed very little after application of NMDA to the relay cell region (Fig. 8A, bottom). This observation was consistent with in vivo results, which showed that after SPPn stimulation, the relay cell can be depolarized abruptly in the absence of a change in the pacemaker cell rhythm (Fig. 3). Shorter pulses of the same concentration of NMDA resulted in depolarizations that were not sustained, followed by a slow repolarizing phase, and control pulses containing only ACSF had no observable effect (not shown). Similar results were obtained when NMDA was applied to a total of 16 relay cells in nine preparations.

Pulses of L-glutamate, presumably the endogenous agonist (Kennedy and Heiligenberg 1994), triggered a different response: bursts of action potentials on top of brief depolarizations in the membrane potential (Fig. 8B). The time course of the response closely resembled that after PPN stimulation in intact preparations. Two general types of responses were observed: in some recordings, brief pulses elicited small, high-frequency action potentials in the relay cells that were superimposed on the ongoing larger action potentials in relay cells that followed the pacemaker rhythm (Fig. 8B1). In other recordings, a single-amplitude action potential was recorded, and the regular pacemaker cell rhythm was not observed during the burst in frequency (Fig. 8B2). It is possible that the different types of spikes observed after a L-glutamate puff reflected different recording locations (e.g., axon vs. soma). From dual recordings of relay cells (see below), it is likely that the different spike sizes also reflected different degrees of synchrony in relay cell firing.

Depolarizing responses with fast spiking also were observed after pulse applications of the specific non-NMDA agonists, AMPA and kainate, using identical methods to those used for L-glutamate and NMDA. The results with these agonists, however, were highly variable between trials and preparations (DISCUSSION).

NMDA was pulsed onto relay cells in the presence of TTX, which blocks voltage-gated Na⁺ currents. While TTX blocked the large action potentials in the relay cell that followed the pacemaker cell rhythm (Fig. 9, Fig. 6), NMDA application was still sufficient to trigger a sustained depolarization of the cells. The TTX block was probably local to the relay cell region because small amplitude potentials were observed in the relay cell during the sustained depolarization. These potentials probably originated far out in the dendrites or were transmitted electrically from other cells. Similar results were obtained from a total of four relay cells in two preparations.
Interactions between relay cells during rhythm modulations

Relay cells normally fire in synchrony in part because they receive synchronous activation from pacemaker cells. During PPhC triggered brief accelerations or chirps, they also fire in synchrony even though they are not necessarily driven by pacemaker cells or by patterned prepacemaker cell firing (Kawasaki and Heiligenberg 1989) (Fig. 3A2, EOD trace). During SPPn-triggered sustained depolarizations, however, fast spikes in relay cells are not necessarily time-locked to each other (Fig. 3A1, and below). Recordings were made from pairs of relay cells in vitro during both electrically and pharmacologically triggered modulations of the pacemaker rhythm to investigate the nature of the synchrony between relay cell firing.

After a current pulse to the surface of the brain, both relay cells of a pair depolarized and maintained a sustained depolarization (Fig. 10A). The initiation of the sustained depolarization as well as its recovery were simultaneous but the timing of the individual spikes was not (compare different action potentials during recovery).

Simultaneous recordings also were made from two relay cells while a pulse of L-glutamate was delivered to the pacemaker nucleus. This type of stimulation contained no patterned input. The two relay cells depolarized and fired a burst of fast, synchronized action potentials, after which time, one cell continued to fire a smaller amplitude action potential, whereas only a small voltage deflection was observed in the other cell. Stable paired recordings were difficult to maintain, and therefore a large sample size was not obtained.

Discussion

General results

With only two neuronal cell types and feed-forward connections (Figs. 1 and 2), the pacemaker nucleus is remarkable in that it is capable of producing not only steady rhythmic firing but also outputs with distinct temporal dynamics.

A major finding of this study is that direct neurotransmitter application to the relay cells is sufficient to generate distinct modulations of the pacemaker rhythm that resemble natural behaviors. Whereas the name “relay” cell suggests a passive role, an endogenous spiking mechanism and a distinct response to NMDA receptor activation are shown to underlie the ability of these cells to participate actively in pacemaker rhythm modulations. As a result, the pacemaker nucleus can produce more output patterns, thereby expanding the behavioral repertoire of the animal. The observations presented here suggest a more segregated role for the two glutamate receptor subtypes on relay cells than has been reported in other neurons.

Relay cells can fire fast spikes in the absence of pacemaker cell input

Although relay cells normally fire action potentials at the frequency of pacemaker cells (10–40 Hz) and they are not spontaneously active in the absence of synaptic input (Fig. 5), they are capable of generating high-frequency (50–250 Hz) spikes during depolarizing current steps (Figs. 5 and 6). These potentials are sensitive to TTX (Fig. 6), and they are fast, suggesting that they are Na+ spikes. The slower time course of disappearance of these spikes after the focal introduction of TTX compared with the sudden disappearance of the action potential that follows the pacemaker cell rhythm suggests that the two potentials are generated at spatially distinct sites on the relay cell (Fig. 6). Alternatively, this difference in the time to block may reflect two classes of spatially overlapping TTX-sensitive Na+ channels on the relay cells with different sensitivities to TTX (e.g., Yoshida 1994 for review).

During strong chirps, the relay cells fire high-frequency, regularly spaced spikes (Fig. 3A2) that strongly resemble the potentials observed during current injections. This correlation suggests that in vivo chirps are generated by PPhC input depolarizing relay cells and activating an endogenous spiking mechanism, rather than, for example, PPhC driving the relay cell firing one for one. Because chirps are blocked with the non-NMDA receptor antagonist CNQX but not the NMDA receptor antagonist APV (Kawasaki and Heiligenberg 1990), the PPhC fibers may selectively activate non-NMDA receptors on relay cells. This possibility was tested by applying a pulse of AMPA, a selective non-NMDA receptor agonist, directly to relay cells in vitro. Such applications did produce bursts of spikes that resembled chirps. Subsequent applications at the same location, however, tended to elicit much smaller responses or no response, possibly due to desensitization of channels caused by a failure to clear AMPA from the tissue. When L-glutamate was applied to the relay cells in a similar manner, it gave consistent brief depolarizations with fast spiking (Fig. 8B) as observed during chirps.

Whereas AMPA is selective for non-NMDA receptors, glutamate, which is likely the endogenous agonist (Kennedy and Heiligenberg 1994), presumably activated both NMDA and non-NMDA receptors. However, whereas the response of a relay cell to a brief application of glutamate was similar to PPhC or AMPA stimulation, glutamate did not trigger the sustained depolarization that characterized the response to...
FIG. 10. Dual recordings from relay cells during triggered modulations of rhythm. Simultaneous intracellular recordings were made from pairs of relay cells while afferent pathway (A) was stimulated with current pulses to produce a sustained depolarization [stimulus duration indicated (-----), artifact marked (⊥)] or during a pulse application of L-glutamate (B) to relay cell region. Both cells in A depolarized and repolarized in synchrony, however, smaller, faster spikes were not synchronous (compare spikes near end of sustained depolarization). In B, cell in top had 2 types of action potentials in addition to large spike that followed pacemaker rhythm. Larger spikes (1 marked with *) were synchronous with spikes in other cell. Smaller spikes (1 marked with f) were correlated only with small voltage deflections in 2nd cell.

SPPn or NMDA stimulation. That glutamate did not also trigger a sustained response was somewhat surprising, especially because NMDA receptors have a higher affinity for L-glutamate compared with non-NMDA receptors (Patneau and Mayer 1990), albeit concentrations used in this report were likely high enough to activate both receptors. Perhaps there are molecular differences between ionotropic glutamate receptors of fish versus mammals that might account for this paradoxical result. In combination with Kawasaki and Heiligenberg’s (1990) data using antagonists, the evidence suggests that activation of non-NMDA receptors alone is sufficient to generate chirps.

Activation of NMDA receptors on relay cells is sufficient to produce sustained depolarizations that interrupt the transmission of the pacemaker cell rhythm

Whereas activation of relay cells through non-NMDA receptor appears functionally equivalent to a current injection, NMDA receptor activation is more complex. Brief NMDA receptor activation in vitro (e.g., 200 ms in Fig. 9) was sufficient to trigger a prolonged depolarized response in relay cells that greatly outlasted (>10 s) the stimulus pulse. How does activation of NMDA receptors on relay cells, either by SPPn stimulation (Fig. 3A1), stimulation of SPPn fibers (Fig. 7A), or direct activation of the receptors (Figs. 8A and 9), trigger a sustained depolarization in relay cells? Because even large current injections do not mimic the effects of NMDA application, the data suggest that some of the more unusual properties of NMDA receptors (Bekkers and Stevens 1990), such as their permeability to Ca2+, may be involved in the specificity of the response.

Characteristics of the sustained depolarizations resemble plateau potentials that have been described in both invertebrate and vertebrate neurons (for review, Kiehn 1991). The features include: a depolarizing response that greatly outlasts the stimulus and stereotyped initiation of and recovery from the depolarization. Similar bistable membrane states have been observed in other neurons, such as mammalian motoneurons (Hochman et al. 1994) and cat neocortical neurons (Flatman et al. 1986) after exposure to NMDA. The possibility that some interneurons, as yet unidentified, participate in the sustained depolarization by providing continued synaptic activation to relay cells, was deemed unlikely because NMDA still was able to initiate a sustained depolarization in the presence of TTX (Fig. 9), although the duration of the depolarization was somewhat shorter. More comprehensive biophysical experiments will be necessary to investigate the currents that underlie the sustained depolarization, but the failure of TTX to block the depolarization also suggests that voltage-gated Na+ currents are not necessary for the process (although see Flatman et al. 1986).

Why do relay cells fail to relay the pacemaker cell rhythm during sustained depolarizations? While depolarized as a result of NMDA receptor activation, relay cells fire high-frequency spikes, which mostly likely result from activating the fast spiking mechanism described above. The functional consequence of the sustained depolarization for the network is that the electric organ no longer fires coherently, and the regular EOD is interrupted (Fig. 3A1). With the current data, we cannot distinguish between a number of possibilities for the failure of the relay cells to transmit the regular pacemaker cell rhythm, which is not affected by the relay cell depolarization (Fig. 3B). It is possible, for example, that the opening of channels causes a decrease in input resistance of the relay cell, and therefore the effect of the pacemaker cell postsynaptic potential is diminished, but more complex network properties also may be involved. Alternatively, the fast spiking in various relay cells may drive the motoneurons or the electric organ asynchronously, thus preventing a coherent discharge.
NMDA and non-NMDA receptors may be segregated on single relay cells

In many neurons, NMDA and non-NMDA receptors are colocalized at synaptic sites as evidenced by the observation, for example, that an excitatory postsynaptic potential (EPSP) can be separated into two components after the application of selective receptor subtype antagonists (e.g., Bekkers and Stevens 1989). The NMDA component shows a voltage dependence and has slower kinetics compared with the faster non-NMDA component. Interactions between the two components account for interesting computational ramifications (e.g., Daw et al. 1993; Nelson and Sur 1992).

In the Hypopomus pacemaker nucleus, the two receptor subtypes may be segregated on single relay cells as illustrated in Fig. 1B, although the evidence is indirect. Anatomic studies, based on placing tracer into SPPn and PPnC and following the anterograde transport to the pacemaker nucleus, show that SPPn fibers surround relay somata, whereas PPnC fibers are more diffusely distributed in the relay subnucleus (Kennedy and Heiligenberg 1994). Considered together with observations that the effect of SPPn stimulation is blocked with the NMDA receptor antagonist APV and PPnC stimulation is blocked with the non-NMDA receptor blocker CNQX (Kawasaki and Heiligenberg 1990), the hypothesis (Fig. 1B) is that NMDA receptors are on the somata, with non-NMDA receptors on the dendrites. Direct evidence from immunohistochemistry confirms that NMDA receptors are localized to the relay somata (Spiro et al. 1994), although similar studies have not yet been completed with probes specific for non-NMDA receptors. It is possible that the proposed spatial segregation of the receptors on an individual cell in part underlies the specific response that the cell shows to different agonists by providing differential access to different intrinsic currents, for example. Results in this report do not directly address this issue, but the generation of the in vitro preparation makes possible studies using new techniques to probe the subcellular loci of different receptor subtypes. For example, Dalva and Katz (1994) have addressed a similar issue of glutamate receptor subtype distribution on single cortical neurons in an in vitro slice preparation. Using a technique in which caged glutamate was focally released at various points along a neuron while recording from the same neuron in the presence of selective receptor antagonists, apical dendrites were found to have a higher percentage of NMDA receptors compared with basal dendrites. This technique has an advantage over immunohistochemistry because it assays functional receptors.

Modulation at the level of relay networks

The conclusion from this report, that the interaction between synaptic and endogenous currents allows relay cells to assume active roles in gating the transmission of the pacemaker rhythm to the electric organ, has some interesting parallels in other systems, suggesting that control at the level of relay networks may be a more general phenomenon in nervous systems. For example, the relay cells in the mammalian lateral geniculate have a low-threshold Ca\(^{2+}\) current (reviewed in Llinás 1988), which underlies their ability to function in distinct relay modes. When nonretinal inputs slightly depolarize the membrane potential from resting potential, the Ca\(^{2+}\) current is activated, and the firing rate of the neurons reflects the visual stimulus rather linearly. Small hyperpolarizing inputs originating from interneurons remove the inactivation of the current, however, and subsequent depolarizations (such as from a retinal EPSP) then can cause a nonlinear bursting response. Interestingly, this burst mode might actually support signal detection better than the tonic mode (Godwin et al. 1996; Guido et al. 1995).

In the electric fish, prepacemaker regions modulate the pacemaker rhythm at both the level of the pacemaker and relay cell. As shown in this report, modulation at the level of the relay cell allows for abrupt changes of the EOD rhythm that would be difficult to bring about at the level of the pacemaker cell. During a sustained depolarization of a relay cell, for example, the pacemaker cells keep firing regularly, even though the EOD is interrupted abruptly. Because the pacemaker cells firing frequency does not change with this interruption, when the relay cell repolarizes the EOD rhythm restarts very close to the frequency before the interruption.

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