

Different classes of glutamate receptors mediate distinct behaviors in a single brainstem nucleus

(electric fish/neuroethology/motor control/neuronal pacemaker)

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ABSTRACT We have taken advantage of the increasing understanding of glutamate neuropharmacology to probe mechanisms of well-defined vertebrate behaviors. Here we report a set of experiments that suggests distinct roles for two major classes of glutamate receptors in a discrete premotor nucleus of the brainstem. The medullary pacemaker nucleus of weakly electric fish is an endogenous oscillator that controls the electric organ discharge (EOD). Its regular frequency of firing is modulated during several distinct behaviors. The pacemaker nucleus continues firing regularly when isolated *in vitro*, and modulatory behaviors can be reproduced by stimulating the descending input pathway. Glutamate agonists applied to the pacemaker *in vitro* produced increases in frequency, while glutamate antagonists selectively blocked stimulus-induced modulations. Experiments with glutamate antagonists in the intact animal resulted in specific effects on two well-characterized behaviors. Our data indicate that these behaviors are separately mediated in the pacemaker by receptors displaying characteristics of the kainate/quisqualate and *N*-methyl-D-aspartate subtypes of glutamate receptor, respectively.

Weakly electric gymnotiform fish generate electric fields by means of electric organs in their tails. These fields are monitored by electroreceptors on the body surface, where local variations in electric organ discharge (EOD) amplitude and phase indicate the presence of nearby objects, allowing these animals to electrolocate in their nocturnal environment. This system is also a principal medium of intra-specific communication. The two species used in these experiments (*Apteronotus leptorhynchus* and *Eigenmannia* sp.) are "wave-type" electric fish, which generate regular, quasi-sinusoidal EODs. The chief communicatory signal is the chirp, a brief and rapid increase in EOD frequency that is naturally evoked during agonistic and courtship encounters (1, 2). Among the slower modulations is one that is not a communication *per se* but a slow frequency shift that preserves electrolocating abilities in the presence of interference from nearby conspecific fish, the jamming avoidance response (JAR) (3, 4). Contamination of the fish's EOD field by a signal at a frequency within a few hertz of the fish's own frequency impairs electrolocation and causes the animal to shift its EOD frequency away from that of the interfering signal. These behaviors can be experimentally induced and quantified during electrophysiological recording.

The electric organ is controlled by the medullary pacemaker nucleus, the sole brain region firing in synchrony with the EOD in the absence of electroreceptive reafference (5, 6). The pacemaker nucleus is an unpaired, midline structure composed of only two neuronal cell types: intrinsic pacemaker cells and projecting relay cells. All synaptic connec-

tions made among neurons within the nucleus are purely electrotonic in *Apteronotus*, while in *Eigenmannia* they are of a morphologically mixed type (7–9). Both cell types alter their firing rate in a manner similar to and slightly in advance of that of the electric organ during modulatory behaviors, and both show underlying depolarizations that induce the acceleration of the chirp (10). The sole source of input fibers to the pacemaker nucleus is the prepacemaker nucleus—a small, diencephalic complex containing separate cell clusters of chirp- and JAR-commanding neurons (11, 12). A single action potential in a "chirp" prepacemaker cell can induce a measurable acceleration in EOD frequency (13), whereas "JAR" cells increase their tonic firing rate during the smooth frequency rises of the JAR (14). The prepacemaker neurons are the source of thin, myelinated axons that form chemical boutons on both pacemaker and relay cells (8).

The pacemaker nucleus of *Apteronotus* continues firing synchronously and regularly when removed from the brainstem and placed in a brain slice chamber (15, 16). Chirp-like responses can be elicited *in vitro* by applying a current pulse through electrodes placed on the pathway taken by descending prepacemaker fibers (17). At a slightly higher threshold, a train of pulses produces a relatively long-term frequency elevation (LTFE) lasting several seconds after the stimulus. The time course of the relaxation to basal frequency after such a stimulus train is similar to that of the JAR and other slow communicatory modulations (18).

METHODS

For the *in vitro* experiments, preparation and recording procedures were as described in ref. 17. Application of agonists and antagonists required rapid delivery to the entire nucleus and rapid clearance. Rapid washout was necessary to prevent agonist-induced uncoupling. Bath application usually had long lasting or irreversible effects because of slow transport through the thickness of the tissue at the air/fluid interface and binding properties of the drugs. This was also deleterious in the antagonist experiments because these compounds also showed some agonistic properties and because reversibility was sought against a background of gradual decline in the effect of afferent stimuli (see Fig. 2). Therefore, agents were delivered by releasing single drops of isotonic saline containing specified concentrations of drug through a micropipette suspended above the tissue during intracellular recording.

Abbreviations: EOD, electric organ discharge; JAR, jamming avoidance response; LTFE, long-term frequency elevation; psp, postsynaptic potential; NMDA, *N*-methyl-D-aspartate; PDA, piperidine dicarboxylic acid; APV, D-(–)-2-amino-5-phosphonovaleric acid; GAMS, γ -D-glutamylaminomethylsulfonic acid.

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Although the precise concentrations of the compounds after their delivery to the tissue could not be determined, a control experiment was performed to estimate the effective, peak concentrations by using the acceleration in pacemaker frequency caused by increased KCl as a bioassay. A micropipette containing concentrated KCl was suspended over the tissue, and a drop was applied in a manner similar to that described above. The resulting frequency increase was then compared to that in response to bath-applied KCl at defined concentrations, the effects of which were more rapid and readily reversible than pharmacological agents. Comparison of the effect of a drop of 3 M KCl to the concentration of KCl in the medium that gave an equivalent frequency increase in the same preparation gave an estimate of the peak concentration in the tissue as more dilute by a factor of approxi-

mately 300 than in the pipette solution. This method took into account the degree of penetration into the tissue as well as dilution in the bath. It did not account for K^+ and glutamate uptake mechanisms or variations in droplet size, making this only an approximation. In the examples of Fig. 1, saline drops contained 50 mM glutamate, 1 mM kainate, 5 mM quisqualate, and 5 mM *N*-methyl-D-aspartate (NMDA), respectively. In Fig. 2, pipette solutions contained 50 mM piperidine dicarboxylic acid (PDA), 25 mM (γ -D-glutamylaminomethylsulfonic acid (GAMS), and 50 mM D(-)-2-amino-5-phosphonovaleric acid (APV). All compounds were from Sigma except APV and GAMS which were from Cambridge Research Biochemicals.

For the *in vivo* experiments, preparation of animals and recording were as described previously (10). In *Eigenman-*

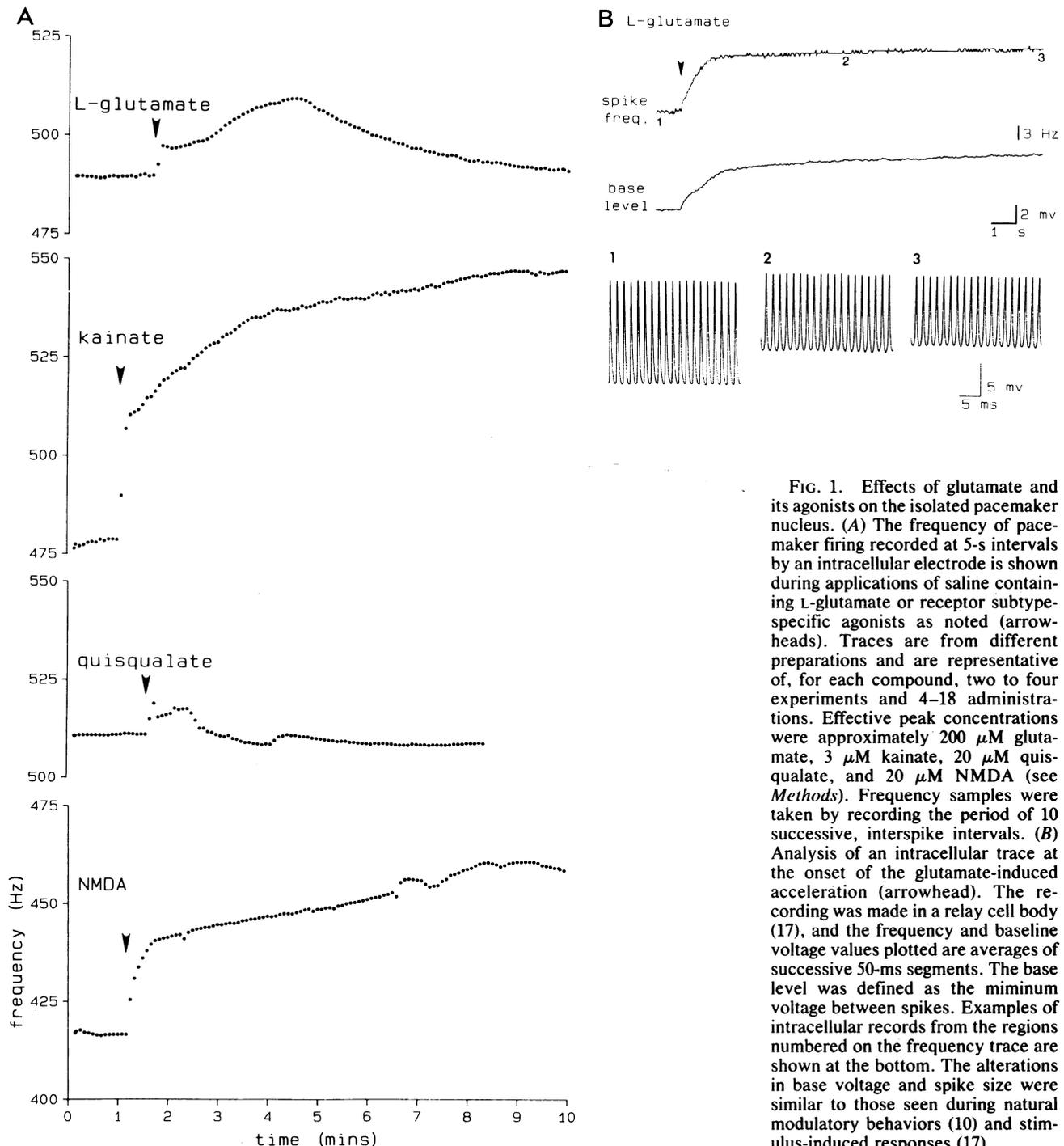


FIG. 1. Effects of glutamate and its agonists on the isolated pacemaker nucleus. (A) The frequency of pacemaker firing recorded at 5-s intervals by an intracellular electrode is shown during applications of saline containing L-glutamate or receptor subtype-specific agonists as noted (arrowheads). Traces are from different preparations and are representative of, for each compound, two to four experiments and 4–18 administrations. Effective peak concentrations were approximately 200 μ M glutamate, 3 μ M kainate, 20 μ M quisqualate, and 20 μ M NMDA (see *Methods*). Frequency samples were taken by recording the period of 10 successive, interspike intervals. (B) Analysis of an intracellular trace at the onset of the glutamate-induced acceleration (arrowhead). The recording was made in a relay cell body (17), and the frequency and baseline voltage values plotted are averages of successive 50-ms segments. The base level was defined as the minimum voltage between spikes. Examples of intracellular records from the regions numbered on the frequency trace are shown at the bottom. The alterations in base voltage and spike size were similar to those seen during natural modulatory behaviors (10) and stimulus-induced responses (17).

nia, curare silences the electric organ, so pacemaker frequency was monitored by recording spinal volleys with an electrode fitted over the tail. Frequency was sampled and averaged for every 10 discharge intervals. Chirps were evoked by electrical stimulation of the prepacemaker chirp areas (12, 13). Spontaneous chirps, which are performed only by *Apteronotus* under these conditions, were blocked by PDA in other experiments with this animal (not shown). Since vigorous chirps can cause transient cessation of the EOD in *Eigenmannia* (1, 2), the sampling scheme in some cases recorded chirps as decreases in instantaneous frequency (see Fig. 3 *Middle*). The JAR was elicited by a sinusoidal electrical field clamped at a frequency alternatively 4 Hz below [frequency difference (Df) < 0] or above (Df > 0) the frequency of the fish's EOD substitute (see Fig. 3 *Bottom*). Drugs were applied in isotonic saline by pressure injection through a triple-barrel microelectrode in the pacer-

maker nucleus. One barrel was filled with 3 M NaCl for recording the pacemaker field potential in order to guide electrode placement. The other two barrels were filled with saline containing the tested compounds for injection. Records in the center column of Fig. 3 were taken within 30 s of drug application, after the transient acceleration accompanying pressure injection had declined. Solutions contained 50 mM PDA, 5 mM GAMS, and 0.5 mM APV, and recovery traces were made 380, 190, and 460 s later, respectively.

RESULTS AND DISCUSSION

In the present experiments, we have begun investigating neurotransmitter pharmacology in the pacemaker nucleus. Our *in vitro* preparation of the pacemaker required nearly simultaneous delivery of tested agents to all or most of the electrically coupled cells in order to reproduce modulatory

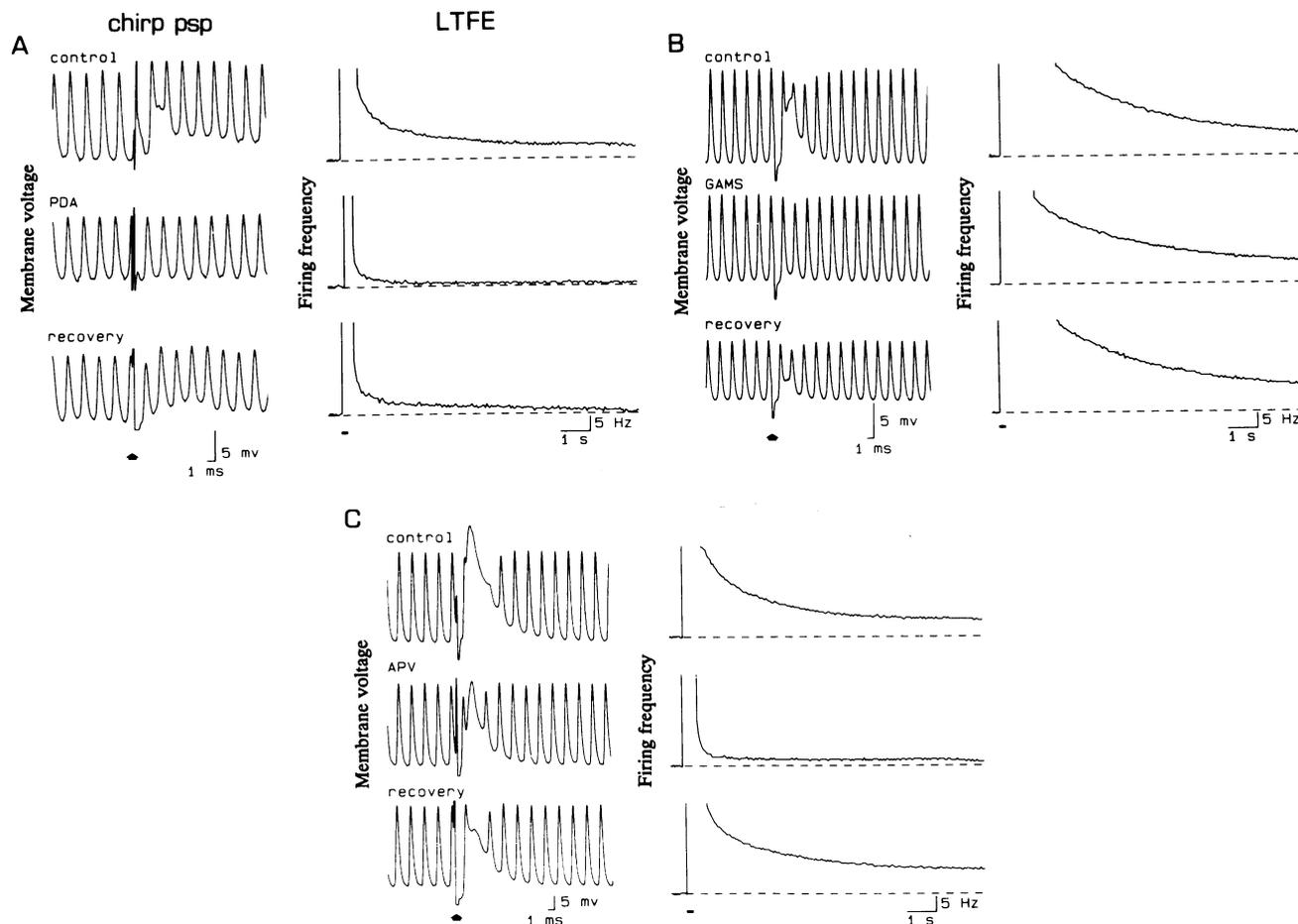


FIG. 2. Effects of application of glutamate receptor antagonists on evoked responses *in vitro*. (A) Single-current pulses shown by arrowhead (*Lower Left*) through electrodes placed on the pathway of prepacemaker fibers induced single chirp-like psp in an intracellular recording from a relay cell (*Left*). In the frequency trace (*Right*), a 200-ms (500-Hz, 200- μ s pulse width) train of stimuli (small bar below traces) caused transient, high-frequency firing followed by a prolonged period of modestly elevated frequency and a slow relaxation to baseline (LTFE) (*Right*, top trace). Three minutes after transient exposure to 170 μ M PDA, the psp and the LTFE were largely eliminated (*Right*, middle trace). Recovery record (*Right*, bottom trace) was taken 68 min later. All psp records were from the same cell, which in part accounts for the diminution of the psp at recovery. In all cases, there was a gradual decline in psp and LTFE amplitude over the course of the experiment, apparently a consequence of cutting the input fibers on removal of the nucleus. The degradation in psp size was accelerated in cells chronically or repeatedly penetrated. In these experiments, application of a concentration of PDA sufficient to eliminate the evoked responses did not permit recovery by more than 50%. (B) Similar treatment with 80 μ M GAMS selectively blocked the chirp psp (*Left*) with relatively little effect upon the LTFE (*Right*). The LTFE trace recommences earlier because of the greatly reduced depolarization during the pulse train. Records of the middle trace were taken 1 min after exposure to GAMS and of the bottom trace were taken 9 min after. (C) Application of 170 μ M APV effectively blocked the LTFE (*Right*) without greatly affecting the chirp psp (*Left*). Records of the middle trace were taken 3 min after APV application and of the bottom trace were taken 72 min after APV application for the chirp psp and 39 min after APV application for the LTFE. In the frequency traces (*Right*), the first point plotted after the stimulus train was the first frequency measured within 30 Hz of the prestimulus frequency. A vertical line is placed between this point and the 30-Hz ceiling. This cut-off level allowed scaling that clearly revealed the LTFE. Summating psp resulting from the stimulus sometimes caused temporary cessation of firing in the nucleus through depolarization block. No data points exist for this period of time.

effects. Therefore, compounds were administered in drops of saline applied to the surface of the tissue. Fig. 1 shows records of the frequency of firing of the pacemaker nucleus during these applications. Administration of either glutamate (Fig. 1A, top trace) or aspartate (not shown) resulted in transient accelerations. Accompanying intracellular recordings revealed depolarizations of baseline voltage and decrements in spike amplitude (Fig. 1B) qualitatively similar to changes in these parameters seen during chirps; repeated applications resulted in gradual desensitization (data not shown). Agonists selective for the three major classes of glutamate receptors, kainate, quisqualate, and NMDA (19, 20) were applied in a similar manner. The amplitudes of the frequency increments and depolarization were greater in response to kainate and NMDA than to glutamate or aspartate, were apparent at lower concentrations, and persisted longer (Fig. 1A). Quisqualate produced little or no acceleration (Fig. 1 shows the greatest quisqualate response).

The control traces in Fig. 2 illustrate the responses *in vitro* to single-pulse (voltage records; Fig. 2 Left) and pulse-train (frequency records; Fig. 2 Right) stimulation of the prepacemaker input pathway. Administration of glutamate receptor blockers to the preparation dramatically reduced the amplitude of the depolarizing postsynaptic potential (psp) underlying the chirp-like response, the LTFE following a train of stimuli, or both responses. The nonspecific glutamate antagonist PDA ($n = 3$) caused a reversible decline in the chirp response and similarly reduced the LTFE (Fig. 2A). A diminution of both responses was obtained by bath administration of PDA (5 mM and 25 mM) but could not be washed out rapidly enough to test recovery (not shown). Bath-applied glutamate (100 μ M and 250 μ M) similarly blocked the response but also caused a variable increase in frequency and uncoupling of pacemaker cells (in preparation). In three of three experiments, application of the kainate/quisqualate receptor blocker GAMS blocked chiefly the short-term, chirp-like responses, leaving the LTFE little affected (Fig.

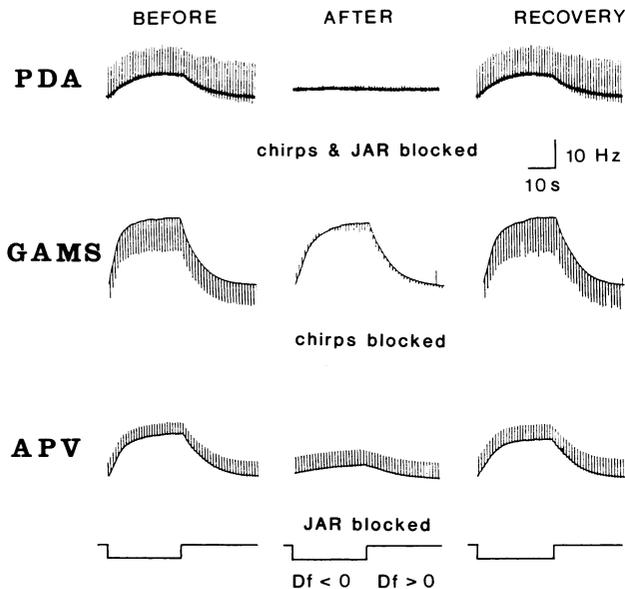


FIG. 3. Effect of glutamate antagonists on electromotor behaviors in *Eigenmannia*. Chirps evoked by a stimulating electrode placed in the prepacemaker nucleus are seen as upwardly or downwardly directed spikes in the EOD frequency record. These overlie the slow frequency shifts of the JAR elicited by an electric field mimicking EODs of a conspecific animal (Bottom). PDA (and kynurenic acid; not shown) effectively blocked both chirps and the JAR (Top). GAMS blocked only the chirps (Middle), and APV blocked only the JAR (Bottom). GAMS and APV examples are from the same animal. Df, frequency difference.

2B). On the other hand, the NMDA-receptor antagonist APV selectively blocked the LTFE (Fig. 2C) in three of four experiments. (In the unsuccessful experiment, both responses were blocked equally.)

Based upon these results from the *in vitro* experiments, we turned to the intact animal to test the effects of the pharmacological agents on natural behaviors. Antagonist effects *in vivo* were more readily reversible than those *in vitro*, and the results were entirely consistent. Fig. 3 illustrates the effects upon two frequency modulations of a behaving *Eigenmannia* after glutamate antagonists were injected into the pacemaker nucleus (*In vivo* experiments on *Apteronotus* gave similar results; *Eigenmannia* is presented because its larger JAR gave a clearer demonstration of the recovery.) In these records, the curarized animal performed the slow upward and downward frequency shifts of the JAR, while overlying chirps were induced at regular intervals by electrically stimulating the prepacemaker chirp area (13). The first row illustrates that PDA reversibly blocked both the JAR and the rapid accelerations of chirping ($n = 3$). When GAMS was injected ($n = 4$), it substantially eliminated chirping, while leaving the JAR unaffected (second row). APV ($n = 7$) had the complementary effect, eliminating the JAR alone (third row).

Parallels in the pharmacological effects between *in vitro* and *in vivo* experiments suggest that the higher-threshold *in vitro* response to trains of stimuli, the LTFE, is largely consequent to excitation of the distinct class of prepacemaker fibers responsible for the JAR. In the light of the characterization of these agents in other systems, our results indicate that the input producing the LTFE is mediated by the NMDA receptor, while that responsible for chirping is mediated by the kainate/quisqualate type. We favor the kainate receptor for the latter response because of the more substantial acceleration produced by kainate (Fig. 1A). Yet we cannot rule out mediation through the quisqualate receptor because (i) chirps have a much faster time course than do the agonist effects illustrated in Fig. 1, and (ii) the quisqualate response may have been affected by uptake processes (21). The development of a kainate- or quisqualate-specific antagonist and an examination of its effect on chirping would resolve this issue. While differences may exist in the pharmacological effects of the compounds used in these experiments between gymnotiform fishes and those, chiefly mammalian, systems in which they have been thoroughly characterized, it remains that these agents perform a selective dissection of distinct behaviors in these fish.

In accord with work on swimming patterns in the lamprey (22) and larval *Xenopus* (23) spinal cords, our results appear to offer another example of the exploitation of the kinetically distinct properties of different glutamate receptors to affect motor patterns. To our knowledge, the present example is unique in illustrating the parcellation of these properties within a compact and identified cell group into separate behaviors. To what degree this is accomplished by fine-scale, anatomical segregation of synapses and differences in receptor subtype localization between or within the cells will be the subject of future studies.

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- Hopkins, C. (1988) *Annu. Rev. Neurosci.* **11**, 497-535.
- Hagedorn, M. & Heiligenberg, W. (1985) *Anim. Behav.* **33**, 254-265.
- Bullock, T. H., Hamstra, H. R. & Scheich, H. (1972) *J. Comp. Physiol. A* **77**, 1-48.
- Heiligenberg, W. (1986) in *Electroreception*, eds. Bullock, T. H. & Heiligenberg, W. (Wiley, New York), pp. 613-649.
- Bennett, M. V. L. (1971) in *Fish Physiology*, eds. Hoar, W. S. & Randall, D. J. (Academic, New York), Vol. 5, pp. 493-574.

6. Dye, J. C. & Meyer, J. H. (1986) in *Electroreception*, eds. Bullock T. H. & Heiligenberg, W. (Wiley, New York), pp. 71–102.
7. Tokunaga, A., Akert, K., Sandri, C. & Bennett, M. V. L. (1980) *J. Comp. Neurol.* **192**, 407–426.
8. Elekes, K. & Szabo, T. (1981) in *Sensory Physiology of Aquatic Lower Vertebrates*, eds. Szabo, T. & Czeh, G. (Pergamon, Budapest), pp. 107–127.
9. Elekes, K. & Szabo, T. (1985) *Exp. Brain Res.* **60**, 509–520.
10. Dye, J. & Heiligenberg, W. (1987) *J. Comp. Physiol. A* **161**, 187–200.
11. Heiligenberg, W., Finger, T., Matsubara, J. & Carr, C. E. (1981) *Brain Res.* **211**, 418–423.
12. Kawasaki, M., Maler, L., Rose, G. J. & Heiligenberg, W. (1988) *J. Comp. Neurol.* **276**, 113–131.
13. Kawasaki, M. & Heiligenberg, W. (1988) *J. Comp. Physiol. A* **162**, 13–21.
14. Rose, G. J., Kawasaki, M. & Heiligenberg, W. (1988) *J. Comp. Physiol. A* **162**, 759–772.
15. Maler, L., Boland, M., Patrick, J. & Ellis, W. (1983) in *The Neurobiology of Zinc, Part A: Physiology, Anatomy, and Techniques*, eds. Frederickson, C. J., Howell, G. A. & Kasarolis, E. J. (Liss, New York), pp. 199–212.
16. Meyer, J. H. (1984) *J. Comp. Physiol. A* **154**, 659–668.
17. Dye, J. (1988) *J. Comp. Physiol. A* **163**, 445–458.
18. Dye, J. (1987) *J. Comp. Physiol. A* **161**, 175–185.
19. Watkins, J. C. & Evans, R. H. (1981) *Annu. Rev. Pharmacol. Toxicol.* **21**, 165–204.
20. Mayer, M. L. & Westbrook, G. L. (1987) *Prog. Neurobiol.* **28**, 197–276.
21. Zaczek, R., Balm, M., Arlis, S., Drucker, H. & Coyle, J. T. (1987) *J. Neurosci. Res.* **18**, 425–431.
22. Grillner, S., Wallen, P., Dale, N., Brodin, L., Buchanan, J. & Hill, R. (1987) *Trends Neurosci.* **10**, 34–41.
23. Roberts, A., Soffe, S. R. & Dale, N. (1986) in *Neurobiology of Vertebrate Locomotion*, eds. Grillner, S., Stein, P. S. G., Stuart, D. G., Forssberg, H. & Herman, R. M. (Macmillan, Basingstoke, U.K.), pp. 279–306.