Mauthner Cell-Initiated Electromotor Behavior Is Mediated via NMDA and Metabotropic Glutamatergic Receptors on Medullary Pacemaker Neurons in a Gymnotid Fish

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Weakly electric fish generate meaningful electromotor behaviors by specific modulations of the discharge of their medullary pacemaker nucleus from which the rhythmic command for each electric organ discharge (EOD) arises. Certain electromotor behaviors seem to involve the activation of specific neurotransmitter receptors on particular target cells within the nucleus, i.e., on pacemaker or on relay cells. This paper deals with the neural basis of the electromotor behavior elicited by activation of Mauthner cells in *Gymnotus carapo*. This behavior consists of an abrupt and prolonged increase in the rate of the EOD. The effects of specific glutamate agonists and antagonists on basal EOD frequency and on EOD accelerations induced by Mauthner cell activation were assessed. Injections of both ionotropic (AMPA, kainate, and NMDA) and metabotropic (*trans*-($\pm$)-1-amino-1,3-cyclopentanedicarboxylic acid) glutamate agonists induced increases in EOD rate that were maxi-

mal when performed close to the soma of pacemaker cells. In contrast, injections in the proximity of relay cells were ineffective. Therefore, pacemaker neurons are probably endowed with diverse glutamate receptor subtypes, whereas relay cells are probably not. The Mauthner cell-evoked electromotor behavior was suppressed by injections of AP-5 and ($\pm$)-amino-4-carboxy-methyl-phenylacetic acid, NMDA receptor and metabotropic glutamate receptor antagonists, respectively. Thus, this electromotor behavior relies on the activation of the NMDA and metabotropic glutamate receptor subtypes of pacemaker cells. Our study gives evidence for the synergistic effects of NMDA and metabotropic receptor activation and shows how a simple circuit can produce specific electromotor outputs.

Key words: glutamate receptors; NMDA; metabotropic; pacemaker; Mauthner cell; electric organ discharge; electric fish; escape response

Gymnotiform fish rhythmically emit electric organ discharges (EOD) for electrolocation and social communication (Lissman, 1958; Black-Cleworth, 1970; Hagedorn, 1986). Each EOD is generated by an electric organ in response to a command discharge of a medullary structure, the pacemaker nucleus (PMn) (Szabo, 1957; Bennett, 1971; Dye and Meyer, 1986; Heiligenberg, 1991), which contains both intrinsic pacemaker cells and projecting relay cells.

The pacemaker command discharge is modulated in the context of different behavioral and experimental circumstances by at least two prepacemaker structures located in the midbrain and diencephalon (Kawasaki et al., 1988; Keller et al., 1991). These modulations represent meaningful electromotor behaviors whose basic mechanisms have been studied in *Hypopomus*, another pulse-emitting electric fish, and in the wave-emitting species *Eigenmania* and *Apteronotus*. Data obtained from *in vitro* and *in vivo* experiments (Dye et al., 1989; Kawasaki and Heiligenberg, 1989, 1990; Spiro, 1997; Juranek and Metzner, 1998) suggest that prepacemaker glutamatergic modulatory drives produce different electromotor outputs according to their cellular targets within the

PMn, i.e., pacemaker or relay cells. Moreover, within the nucleus, there is evidence that the activation of different glutamatergic receptor subtypes located on the same cell types could result in different electromotor behaviors. For example, in *Hypopomus*, glutamatergic innervation initiates slow and sustained EOD frequency rises by activation of NMDA receptors on pacemaker cells, whereas the activation of NMDA receptors on relay cells results in sudden interruption of the EOD. In addition, direct relay cell activation mediated by ionotropic non-NMDA receptors evokes brief accelerations of the EOD frequency, accompanied by a decrease in pulse amplitude (chirps).

In contrast to the wealth of data regarding prepacemaker structures and their control of PMn discharges in other weakly electric fish, prepacemaker structures and their modulatory influences on the PMn are as yet unknown in *Gymnotus carapo*. Several electromotor behaviors have been described in this species (Black-Cleworth, 1970; Westby, 1974; Kramer et al., 1981; Barrio et al., 1991). Falconi et al. (1995) have reported that Mauthner cell (M-cell) activation in this species results in a short latency and large increase in the EOD rate [Mauthner-initiated abrupt increase in rate (M-AIR)], in addition to promoting the motor escape response common to other teleosts (Faber and Korn, 1978; Zottoli et al., 1995). This electromotor behavior characteristically begins abruptly and has a relatively long duration (up to 5 sec). M-cell activation induces short latency and prolonged excitatory synaptic effects that are restricted to the pacemaker cells (Falconi et al., 1997).

In this study, we first explored the sensitivity of the PMn of *Gymnotus carapo* to glutamate agonists to identify the different

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receptor subtypes that might be present. We then applied several glutamate antagonists during testing for the M-AIR to test the hypothesis that this particular electromotor behavior relies on the specific activation of glutamatergic receptor subtypes of pacemaker cells. The evidences obtained suggest that both NMDA and metabotropic receptor subtypes on pacemaker cells are co-activated to produce this prolonged modulation of PMn discharges.

Parts of this paper have been published previously (Falconi et al., 1996).

MATERIALS AND METHODS

In this study, the effects of specific glutamate agonists and antagonists on basal EOD frequency and on EOD accelerations induced by M-cell activation were assessed in 31 specimens of *Gymnotus carapo* (11–22 cm in length). The fish were captured in a lake in southeast Uruguay (Laguna del Sauce, Maldonado) and were kept in fresh water aquaria at a temperature maintained between 20 and 25°C.

Surgical, recording, and stimulation procedures were as described in detail by Falconi et al. (1995, 1997). They are in accordance with the guidelines of the Ministerio de Ganadería, Agricultura y Pesca, División Fauna, Uruguay. Briefly, fish were anesthetized by immersion in iced water. All surgical areas and fixation points were infiltrated with Lidocaine. Paravertebral muscles were removed from one side at a point ~80% down the fish's length, a bipolar stimulating electrode was placed in contact with the vertebral column, and the threshold for inducing the tail flip (characteristic of Mauthner axon activation) was determined. Most of the axons of relay cells that run in the electromotor bulbospinal tract have already left this tract before they reach the level in which the stimulating electrodes were placed (Ellis and Szabo, 1980). Following these procedures, the animals were injected with D-tubocurarine (1–3 µg/gm, i.m.) at doses that produced paralysis but did not completely eliminate the EOD. Electrical stimuli consisted of rectangular current pulses (0.15–0.3 mA, 0.2 msec) that were phase-locked with a delay of 5 msec after the EOD (Falconi et al., 1997). The stimulus strength was adjusted to activate both Mauthner axons and, thus, to obtain maximal EOD accelerations (Falconi et al., 1997).

Micropipettes filled with different solutions of glutamate agonists or antagonists (see below) (Fig. 1) were first used to make extracellular records of PMn field potentials and, subsequently, for pressure ejection of drugs. The tips of these micropipettes were positioned at different locations within the PMn as indicated by the characteristic waveform of the spontaneous pacemaker field potentials (Figs. 1, 2) (Kawasaki and Heiligenberg, 1990).

Drug-containing solutions were applied by pressure (Picospritzer II; General Valve, Fairfield, NJ). Pulses of 20–50 psi and 10–500 msec duration were used. To calibrate drug applications, before each experiment, pressure and pulse duration parameters were adjusted while visualizing the formation of microdroplets under a microscope. The volumes of these microdroplets, calculated from their diameters, were relatively small (between 5 and 15 pl). It was thus supposed that the injection affected a restricted volume of brain tissue. The validity of this supposition was substantiated in a series of control experiments in which the site of maximal effect of an agonist was determined first (see below), and then similar volumes were injected 250 µm lateral to this location (outside the boundaries of the PMn). Injections at this distance from the site of maximum effect did not have noticeable effects on EOD frequency.

Glutamate agonists were injected at four different depths within the PMn to determine the site of maximal effect. Antagonists were usually injected at the site of maximal effects of the corresponding agonist. Control experiments included the injection of similar volumes of the vehicle solution. Two-tailed *t* test was used for the statistical analysis of differences of effects after drug and vehicle injections.

In four animals, the location of micropipette tips within the nucleus was confirmed by extracellular deposit of pontamine sky blue (PSB), which was pressure injected (10–15 pl). In five other animals, double-barreled electrodes were lowered within the nucleus. One barrel contains Glu (5 mM) and was first used to make extracellular records of PMn field potentials and, subsequently, for pressure ejection of Glu. The other barrel contained 2% PSB in 0.5 M NaAc, pH 8.3, which was iontophoresed (1–5 µA, negative DC) to directly mark a given Glu ejection site (Lee et al., 1969). The animals were then deeply anesthetized by cold; the

Figure 1. Diagram of the putative basic neural circuit that mediates M-AIR and the experimental arrangement showing micropipettes for extracellular recording and pressure injection. The M-cell probably innervates an as yet unidentified interneuron (Int), which in turn innervates prepacemaker structures. PMn field potentials were recorded with the agonist-antagonist-filled micropipette. Injections were performed at different depths within the nucleus. The maximal effects of the agonists were obtained at the level of the pacemaker neurons. Glutamate receptor antagonists were injected with an independent micropipette at this level. M-AIR was generated by stimulating M-cell axons at the spinal cord level (Spinal cord stimulation). In five experiments, the two micropipettes were placed near the same location within the PMn. One micropipette was filled with a solution of specific glutamatergic agonist and the other with its respective antagonist. In these experiments, we were able to test the effects of a given glutamatergic antagonist on both M-AIRs and agonist application.

brains were removed and fixed overnight by immersion in formalin (10%). Brainstems were mounted in a Vibraslicer (Campden Instruments, Loughborough, UK) and serially sliced (100 µm) in the transverse plane. Sections were counterstained with Neutral Red.

Electrical recordings from the PMn were obtained with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). A Grass Instruments (Quincy, MA) P15 preamplifier was used to monitor the EOD. The signals were displayed on an oscilloscope and stored on magnetic tape. Data analyses were performed using a Macintosh CI microcomputer (Apple Computers, Cupertino, CA). Superscope software (GW Instruments, Somerville, MA) was used to construct interval versus time and instantaneous frequency versus time plots.

Drugs and solutions. The effects of the substances (dissolved in 154 mM NaCl) were assessed: L-glutamic acid (glutamate) (1–10 mM), its agonists NMDA (500 µM), AMPA (100 µM), 2-carboxy-4-(1-methylethyl)-3-pyrrolidinacetic acid (kainate) (100 µM), and *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (*trans*-ACPD) (5 mM), and its antagonists (±)-2-amino-5-phosphonopentanoic acid (AP-5) (500 µM), 6-cyano-7-nitroquinoxaline-2,3-dione HBC-complex (CNQX) (1–3.9 mM), and (±)-amino-4-carboxy-methyl-phenylacetic acid (MCPG) (1–10 mM) for NMDA, AMPA-kainate, and metabotropic glutamate receptor (mGluR) subtypes, respectively. These substances were purchased from Research Biochemical (Natick, MA). The concentrations expressed above refer to the micropipette filling solutions. During pilot experiments, dose-response relationships were evaluated, and concentrations of drugs were selected to produce maximal effects with smallest volumes.

RESULTS

Most EOD accelerations observed in other species of weakly electric fish appear to involve the activation of several glutamate receptor subtypes in different cellular targets within the PMn (Dye et al., 1989; Kawasaki and Heiligenberg, 1989, 1990; Spiro, 1997; Juranek and Metzner, 1998). However, little is known about the neurotransmitters involved in the modulation of PMn

activity in *Gymnotus carapo*. The sensitivity of the different PMn cell types to glutamatergic agonists for different ionotropic receptor subtypes was first examined. Agonists were pressure injected at different sites within the nucleus while EOD was monitored continuously.

Glutamate-induced increases in EOD frequency

In this series of experiments, we explored the effects of glutamate injected within the PMn close to the cell bodies of either PM cells or to relay cells. This was possible for the following reasons. First, pacemaker and relay cells are spatially segregated. Second, the relative location of the micropipette could be estimated according to the particular waveform of the spontaneous field potential. The somas of the pacemaker cells are located in a dorsal position and give origin to apical and lateral dendrites. Apical dendrites are thin and travel vertically within the medullary raphe, whereas lateral dendrites are thick and ramify profusely near the parent cell body in the dorsal portion of the nucleus. The somas of the relay cells are located in the ventral portion of the nucleus together with their dendritic arborizations (Bennett et al., 1967; Ellis and Szabo, 1980; Trujillo-Cenóz et al., 1993). A similar arrangement of cells and processes was outlined by Kawasaki and Heiligenberg (1990) in *Hypopomus*. These authors observed that the waveform of the spontaneous field potential depended on the different levels of recording within the nucleus. The same pattern of field potentials distribution is found in *Gymnotus carapo* (Fig. 2). The *left column* illustrates the field potentials recorded at different levels within the nucleus. In the *middle traces* of this column, two negative components may be distinguished. These components reach a maximum amplitude at different depths within the nucleus. As indicated by the PSB labeling (Fig. 3), the maximal early negative potential was recorded in the proximity of PM cell somas, whereas the maximal negativity of the late potential was recorded near relay cell somas. In the following descriptions, we refer to the level at which the early negative field potential was maximal as level 0 μm . Glutamate agonists and antagonists were injected at this level or at known distances from it. Dorsal injections were performed either 300, 200, or 100 μm above this level. Likewise, ventral injections were performed 100, 200, or 300 μm below 0. Examples of the effects of glutamate on EOD frequency are illustrated in Figure 2 (*right column*). Dorsal injections did not evoke discernible changes in EOD frequency. However, when glutamate was applied at the level of pacemaker cell somas (0 μm), a large (37.2% increase) and abrupt EOD acceleration was observed. This acceleration lasted 1.5 sec. Injections at $-100 \mu\text{m}$ had much shorter and delayed effects. At more ventral locations ($-200 \mu\text{m}$), glutamate injections did not have effects.

The EOD waveform did not change after glutamate injections. In Figure 3, we illustrate the effects of glutamate injections performed at two levels: 0 and $-300 \mu\text{m}$, which correspond to the location of PM and relay cell somas, respectively. Sites of injection were correlated with the characteristic field potential recorded at these two levels and confirmed by extracellular deposit of PSB at the ventral site (Fig. 3A). Figure 3B shows EOD frequency versus time plots in the *left panel*, raw EOD recordings in the *middle panel*, and two superimposed single EODs recorded before and after the glutamate injections in the *right panel*. Two facts are noteworthy. The first is that, even during the maximum increase in EOD frequency, the EOD waveform and amplitude remained the same. The second is that ventral injection of glutamate, at the level of the relay neuron somas, did not modify either the rate or the shape of the EODs.

Figure 2. Accelerations of the EOD induced by glutamate injections at different depths within the pacemaker nucleus. *Left*, Spontaneous pacemaker field potentials recorded at different depths within the PMn. The numbers indicate the distance from the level at which the first negative peak attains its maximum. This level, labeled 0 μm , corresponds to the location of PM cell somas (see Results). +100 μm and +200 μm indicate positions dorsal to level 0 μm along the same vertical recording microelectrode track, and $-100 \mu\text{m}$ and $-200 \mu\text{m}$ correspond to more ventral positions located within the nucleus. Each trace represents the average of 100 individual field potentials. *Right*, Plots of the EOD frequency versus time after glutamate injection (5 mM, 5 msec, 5 psi) at the levels indicated in *left*. EOD frequency increases were observed when glutamate was injected near PM cells. The moment of injection is indicated by an arrowhead. Dotted lines show the basal EOD frequency (numbers at right).

mate, at the level of the relay neuron somas, did not modify either the rate or the shape of the EODs.

Effect of ionotropic glutamate agonists on EOD rate

The responses to ionotropic glutamate agonist injections within the PMn were investigated. Kainate, AMPA, and NMDA all induced EOD accelerations that were maximal when the injection was made at the level of the PM cell somas (0 μm) (Fig. 4, *top traces*). At more ventral locations within the nucleus ($-300 \mu\text{m}$) (*middle traces*), these agonists were almost ineffective. EOD accelerations induced by these compounds clearly differed in their time course. Both kainate and AMPA injections induced an increase in EOD frequency that was rapid and shorter lasting compared with the response to NMDA. The effect of NMDA

Figure 3. Effects of glutamate on EOD when injected near the somas of pacemaker and relay cells. *A*, Camera lucida drawing of a transversal section of the PMn. The somas of the small pacemaker cells are located in a dorsal position, whereas the somas of the relay cells are located in the ventral portion of the nucleus. Two sites are indicated (0 and $-300\ \mu\text{m}$) from which field potentials were recorded (*Field Potential*; averages of 100 individual recordings) and in which glutamate (5 mM) was injected. The ventral site of injection ($-300\ \mu\text{m}$) was marked with PSB and is represented by the shaded area. *B*, *Left column*, Plots of the EOD frequency versus time after glutamate (5 mM, 5 msec, 10 psi) injection either near PM cells ($0\ \mu\text{m}$) or at the level of relay cells ($-300\ \mu\text{m}$). Dotted lines show basal EOD frequency (15 Hz in this experiment). Raw recordings used to construct these plots are shown in the *middle column*. *Middle column*, Raw head-to-tail recordings of EODs from a partially curarized animal obtained before and after injections of glutamate. *Arrowheads* indicate the moment of injection. *Right column*, The *top trace* shows two superimposed EODs. One was a “control” EOD obtained before the injection, and the other was the third EOD after the injection. This EOD coincides with the peak of the response to glutamate when applied at $0\ \mu\text{m}$. The *bottom trace* illustrates the superposition of a control EOD and the third EOD after the injection of glutamate at $-300\ \mu\text{m}$. Note that the superimposed EODs were identical.

Figure 4. Glutamate ionotropic agonists induce an increase in EOD frequency when injected in the proximity of PM cells. *Top row*, Plots of EOD frequency versus time from an experiment in which kainate ($100\ \mu\text{M}$, 25 msec, 15 psi), AMPA ($100\ \mu\text{M}$, 25 msec, 10 psi), and NMDA ($500\ \mu\text{M}$, 20 msec, 10 psi) were injected near the PM cells. *Middle row*, Similar injections as above but in the proximity of relay cells ($-300\ \mu\text{m}$). *Arrowheads* indicate the moment of injection. *Bottom row*, Pairs of single EOD recordings taken before (*left*) and 500 msec after (*right*) each agonist injection performed at $-300\ \mu\text{m}$. Note the absence of any change of EOD waveform after the injections of these ionotropic glutamate agonists.

consisted of a slower rise in EOD rate, which was followed by a prolonged (5 sec) elevated “plateau” frequency and a slow return to basal EOD frequency. Similar results were obtained in five animals (15 injections). For kainate, AMPA, and NMDA injections respectively, the mean $\pm$ SD amplitudes of increased frequency responses were 9.2 ± 4.4 , 8.7 ± 4.1 , and 9.9 ± 0.9 Hz, and their mean total durations were 9.25 ± 2.2 , 20 ± 5 , and 58.6 ± 14 sec.

Effects of AP-5 and CNQX on the M-AIR

Because PM neurons (but not relay cells) are endowed with several different ionotropic receptor subtypes, the question was

then asked whether M-AIR responses depended on the activation of any particular subtypes of these receptors. We first assessed the effects of specific blockers for NMDA and non-NMDA receptor subtypes (AP-5 and CNQX, respectively) on M-AIR (Fig. 1). Two series of experiments were performed. In the first series (10 animals), AP-5 or CNQX was injected into the PMn while the M-cells were activated antidromically by stimulation at the spinal cord level. These experiments were technically simpler than those described below and allowed us to compare in the same animal the relative suppressor effect of antagonists on M-AIR. The second type of experiments, which were performed successfully in five animals, consisted of positioning the tip of two independent micropipettes in the proximity of PM cells. One micropipette was filled with a solution of a specific glutamate agonist and the other with the respective antagonist. Using this approach, we were able to test the effects of a given glutamate antagonist on both M-AIRs and on agonists application.

An example of the effects of AP-5 on M-AIR and on responses to NMDA is illustrated in Figure 5, *A* and *B*. In this experiment, responses were tested before, and 20 sec and 15 min after AP-5 injections. After the injection of AP-5 (Fig. 5*A*, *middle*), the amplitude of M-AIR was reduced by 75%, and the response was shortened by 40%. After 15 min, this response had almost completely recovered (Fig. 5*A*, *right*). AP-5 similarly blocked the excitatory effects of NMDA applied in the proximity of PM cells by pressure injection (Fig. 5*B*). Again, the effects of AP-5 had almost totally vanished 15 min after its injection. Similar effects of AP-5 on M-AIR were observed in all animals tested ($n = 7$) with mean $\pm$ SD reductions of M-AIR amplitude and duration of 72 ± 6.9 and $62 \pm 4.8\%$, respectively.

It is noteworthy that AP-5 per se did not induce changes in the basal EOD frequency. This suggests that NMDA receptors do not participate in the setting of the pacemaker resting frequency as in other gymnotiform fish, described for instance in *Eigenmannia* (Kawasaki and Heiligenberg, 1990).

Figure 5. Effects of AP-5 on M-AIR and EOD accelerations induced by NMDA and kainate injections. Two independent micropipettes, one filled with a solution of AP-5 (500 μ M) and the other with NMDA (500 μ M), were lowered near the PM cells (see Fig. 1). AP-5 suppressed both M-AIR (*A*) and EOD (*B*) accelerations induced by NMDA. After these records were obtained, the micropipette containing NMDA was replaced by another filled with kainate (100 μ M). AP-5 did not have any effect on EOD accelerations induced by this agonist (*C*). *A*, Plots of M-AIRs obtained before (*left*), 30 sec after (*middle*), and 15 min after (*right*) the injection of AP-5 (200 msec, 35 psi). *B*, Plots of EOD frequency versus time of NMDA effects (35 msec, 25 psi) before and after the injection of AP-5. *C*, Plots of EOD frequency versus time of effects evoked by kainate injections (15 msec, 10 psi) performed before (*left*), 30 sec after (*middle*), and 15 min after (*right*) AP-5 injection. The moment of M-cell activation or of the injections of agonist are indicated by asterisks and arrowheads, respectively. Dotted lines show basal EOD frequency indicated in each trace by the numbers at left.

After the completion of this experiment, the electrode containing NMDA was replaced by another filled with kainate. Kainate responses, as illustrated in Figure 5*C*, were not affected by AP-5 injections, which previously had almost eliminated NMDA responses.

The effect of CNQX on M-AIR was examined in seven animals (25 injections). An example of the most consistent results obtained after the injection of this antagonist is shown in Figure 6. Injections of CNQX, which almost completely blocked kainate-induced responses (Fig. 6*B*), did not affect either M-AIR (Fig. 6*A*) or NMDA (Fig. 6*C*) responses. This was observed in 21 injections. However, in a minority of cases, CNQX had a minimal (6% reduction of M-AIR) and erratic (4 of 25 injections) suppressor effect on M-AIR.

Metabotropic glutamate receptor activation during M-AIR

The prolonged time course of M-AIR and the involvement of glutamatergic innervation in this behavior raises the possibility that glutamate receptor subtypes associated with prolonged postsynaptic effects, i.e., mGluRs, were also activated. To test this possibility, pharmacological evidence supporting the existence of these kind of receptors was investigated by assessing the effects of *trans*-ACPD on EOD rate (eight animals). This substance acts on group I and II mGluRs (Pin and Duvoisin, 1995).

An example of *trans*-ACPD effects is shown in Figure 7. This agonist induced a long-lasting EOD rate increase. The effect was

Figure 6. Effects of CNQX on M-AIR and EOD accelerations induced by kainate and NMDA injections. The effects of CNQX (3.9 mM, 300 msec, 18 psi) on M-AIR (*A*) and on the EOD frequency increase induced by kainate (*B*) (100 μ M, 10 msec, 25 psi) and NMDA (100 μ M, 20 msec, 35 psi) were assessed (see experimental diagram in Fig. 1). *A*, Plots of EOD frequency versus time of M-AIRs obtained before (*left*), 30 sec after (*middle*), and 15 min after (*right*) injection of CNQX. *B*, Plots of EOD frequency versus time of the effects of kainate before and after injection of CNQX. Kainate effects were almost completely blocked by CNQX injection (*Kainate + CNQX*). *C*, Plots of EOD frequency versus time of the effects of NMDA before and after injection of CNQX. As expected, CNQX injection did not modify NMDA effects. The moment of M-cell activation or of injections of agonists are indicated by asterisks and arrowheads, respectively. Dotted lines show basal EOD frequency indicated in each trace by the numbers at left. Recordings in *A* and *C* were obtained from the same animal.

larger and had a shorter latency when *trans*-ACPD was injected at the level of pacemaker cell somas (0 μ m level) (*middle* trace). In most experiments (six of eight animals), two components could be distinguished in the response. First, there was an early and relatively small peak in EOD rate that was followed by a delayed, sustained, and larger component. This second component took ~ 10 sec to reach its maximum (a frequency increase of 5 Hz) and lasted ~ 60 sec. In the remaining two animals (for an example, see Fig. 8*B*), the response to *trans*-ACPD consisted only of the slow component. When *trans*-ACPD was injected at a distance of 200 μ m from level 0, the effects were greatly reduced and, after an injection at 400 μ m, they were undetectable. These data indicates that mGluRs activated by the agonist *trans*-ACPD were most likely restricted to PM cells. Similar slow responses were observed in all animals. Their mean $\pm$ SD amplitudes were 3.4 ± 1.0 Hz, and their mean $\pm$ total durations were 81.3 ± 28.9 sec. Differences with values obtained after vehicle injections were statistically significant ($p < 0.001$).

To investigate the possible role of these receptors in M-AIR, two independent micropipettes were located near PM cell somas, one filled with a solution of *trans*-ACPD and the other with MCPG, a specific metabotropic glutamate receptor antagonist (three animals, eight injections). An example of the effects of MCPG is illustrated in Figure 8. MCPG was injected near PM cells, and M-AIR and *trans*-ACPD effects were tested before (*left*), 20 sec after (*middle*), and 15 min after (*right*) the injection. This glutamate antagonist reduced both the amplitude and dura-

Figure 7. Accelerations of EOD induced by a metabotropic glutamate receptor agonist (*trans*-ACPD) injected at different depths within the PMn. The response to *trans*-ACPD (500 μM, 20 msec, 30 psi) was larger and shorter in latency when injected in the proximity of PM cells (0 μm). This response was slow and long-lasting. The moment of injection is indicated by an arrowhead. Dotted lines indicate basal EOD frequency (20 Hz in this experiment).

tion of M-AIR (Fig. 8A) and concomitantly almost eliminated *trans*-ACPD effects (Fig. 8B). Both responses partially recovered 15 min after MCPG injection. Similar results were obtained in the three animals tested. M-AIR amplitudes were reduced by $22 \pm 4.5\%$, and their duration was reduced by a $32 \pm 7.5\%$. The suppression of both M-AIR and *trans*-ACPD effects were statistically significant ($p < 0.002$).

DISCUSSION

EOD frequency rises induced by glutamate and its agonists

The present study demonstrates that glutamate itself, as well as specific ionotropic and metabotropic agonists of glutamate receptors, induced considerable EOD accelerations when injected into the PMn of *Gymnotus carapo*. The effects of kainate and NMDA were blocked by CNQX and AP-5, respectively. Those of *trans*-ACPD were blocked by MCPG. The maximal actions of glutamate agonists were obtained when pressure injections were placed close to the PM cell somas. Therefore, it is likely that PM cells in *Gymnotus carapo* are endowed with diverse ionotropic and metabotropic glutamate receptors subtypes. The existence of ionotropic glutamate receptors in PM cells in *Hypopomus*, *Eigenmania*, and *Apteronotus* has been suggested previously by Dye et al. (1989) and Kawasaki and Heiligenberg (1990). However, to our knowledge, the evidence obtained in the present work is the

Figure 8. Effects of MCPG on M-AIR and *trans*-ACPD-induced EOD accelerations. In this figure, we illustrate the results of an experiment in which two independent micropipettes, one filled with a solution of MCPG (2.4 mM) and the other with *trans*-ACPD (500 μM), were lowered near the PM cells. The effects of MCPG on both M-AIR (A) and the EOD frequency increase induced by *trans*-ACPD (B) were tested in this experiment. A, Plots of EOD frequency versus time of M-AIRs obtained before (left), 30 sec after (middle), and 15 min after (right) injection of MCPG (300 msec, 30 psi). B, Plots of EOD frequency versus time of the effects of *trans*-ACPD (20 msec, 30 psi) before and after the injection of MCPG. The mGluR antagonist significantly reduced M-AIR amplitude ($\sim 30\%$) and almost completely suppressed the effects of *trans*-ACPD. This suppression was reversible, and both the M-AIR and the effects of *trans*-ACPD partially recovered 15 min after the injection. The moment of M-cell activation or of *trans*-ACPD injection are indicated by asterisks and arrowheads, respectively. Dotted lines show basal EOD frequency indicated in each trace by the numbers at left.

first indication of the existence of metabotropic glutamate receptor subtypes in PM cells of gymnotiform fish.

Injections of glutamate agonists did not modify EOD waveform, nor did they produce “extra” EODs, even when applied in the proximity of relay cells (Figs. 3, 4). These data indicate that relay cells are not directly activated by these compounds and suggest that they lack these glutamatergic receptors. In contrast, in *Hypopomus*, direct glutamatergic relay cell activation through ionotropic non-NMDA and NMDA receptors has been demonstrated. Activation of these receptors mediates chirps and sudden interruptions, respectively (Kawasaki and Heiligenberg, 1990; Spiro, 1997). *Gymnotus carapo*, which displays a more restricted electromotor behavioral repertoire (for example, chirping has not been described) (Black-Cleworth, 1970; Westby, 1974, 1975; Kramer et al., 1981; Barrio et al., 1991), interestingly does not have glutamate receptors in relay cells. This probably denotes important interspecies differences in the organization of even simple electromotor behaviors. Interspecies variability in pacemaker organization is also suggested by the differences between NMDA subunits distribution within the pacemaker nucleus of *Hypopomus*, *Eigenmania*, and *Apteronotus* (Spiro et al., 1994; Bottai et al., 1997, 1998).

NMDA and *trans*-ACPD induced slower responses than AMPA or kainate, which was expected according to the actions of these glutamate agonists in other cell types (Collingridge et al., 1988; Kawasaki and Heiligenberg, 1990; Daw et al., 1993; Pin and Bockaert, 1995; Pin and Duvoisin, 1995). A limiting factor for the occurrence of NMDA-mediated postsynaptic potentials is the channel blockade produced by Mg^{2+} ions, which takes place at hyperpolarizing potentials (-70 mV) (Nowak et al., 1984). PM cells are spontaneously depolarizing neurons that regularly fire action potentials (minimum membrane potential level of -52 mV) (Falconi et al., 1997). Therefore, Mg^{2+} blockade of the NMDA receptor-associated channel is unlikely. Additionally, certain NMDA receptor variants may conduct at near resting mem-

brane potentials, as described in *Apteronotus* electrosensory system (Berman et al., 1997). According to this, for NMDA effects to be manifest, preceding AMPA–kainate mediated depolarizations are not necessary, as in most other glutamatergic synapses (Collingridge et al., 1988; Daw et al., 1993). In addition, a permanent relief of Mg^{2+} blockade will permit the expression of the full time course of the NMDA postsynaptic effect.

Activation of mGluRs results in a variety of long-lasting physiological modifications mediated via specific intracellular second messengers (Pin and Duvoisin, 1995; Sánchez-Prieto et al., 1996). One of the most characteristic effects of mGluR activation is an increase in neuronal excitability, usually associated with a reduction of K^+ currents. However, it would be premature to speculate about the mechanism of the excitatory effect of mGluR activation on PM cells, which should be further investigated.

Glutamate receptors activated during M-AIR

AP-5 injections produced a considerable reduction of both amplitude and duration of M-AIR (Fig. 5*A*). Although to a lesser extent, MCPG also had a highly consistent suppressor effect on this response (Fig. 8*A*). Therefore, we conclude that, during M-AIR, there is a coactivation of NMDA and metabotropic glutamate receptor subtypes. Our study thus provides the first strong evidence of the participation of mGluR in a specific electromotor behavior.

Non-NMDA ionotropic glutamate receptors do not play any apparent role in M-AIR, as suggested by the lack of any significant effect of CNQX on this response (Fig. 6). The functional role of non-NMDA ionotropic receptor subtypes in pacemaker cells remains to be elucidated.

The finding that NMDA and metabotropic glutamate receptors, but not AMPA receptors, are involved in a glutamatergic-mediated pacemaker cell depolarization and behavioral response suggests the segregation of glutamatergic inputs on a single class of cells to produce a specific electromotor output. Segregation of behavior-specific synaptic inputs to the PMn appears to be a common neural design in gymnotiform fish (Fig. 9) (Spiro, 1997; Juranek and Metzner, 1998).

In most central vertebrate glutamatergic synaptic contacts, NMDA and non-NMDA receptors are colocalized, and usually both NMDA and non-NMDA components of glutamatergic synaptic effects could be demonstrated (for review, see Daw et al., 1993). However, our data indicate that prepacemaker glutamatergic fibers could activate NMDA receptors without any non-NMDA component, suggesting that ionotropic glutamatergic receptors at the subsynaptic membrane are only of the NMDA subtype. This notion is supported by studies in other gymnotid fish and young mammals that suggest the existence of glutamatergic synaptic contacts in which NMDA, but not AMPA, receptors are expressed (Liao et al., 1995; Spiro, 1997).

A remarkable characteristic of M-AIR is that a single action potential in an identifiable neuron (the M-cell) triggers a short latency and prolonged electromotor response. To explain this characteristic, we have postulated the existence between Mauthner and pacemaker neurons of a paucisynaptic pathway in which repetitive discharges occur (Falconi et al., 1995). In the present work, we have collected evidence that expands our original view. We suggest that the prepacemaker elements responsible for M-AIR are glutamatergic neurons and that glutamate acts on NMDA and metabotropic receptors of the PM cells. Therefore, it is likely that the long duration of M-AIRs results from a combination of factors that include repetitive discharges of the glutamater-

Figure 9. Different neural strategies underlie the organization of different electromotor behaviors in two pulse type gymnotiform fish. Only glutamatergic inputs to the pacemaker nucleus have been considered. Two neuronal types, pacemaker (PM-cells) and relay (R-cells) cells and different glutamate receptor subtypes are represented. *A* and *B* are based on studies of Kawasaki and Heiligenberg (1990), Spiro et al. (1994), and Spiro (1997) in *Hypopomus*. *C* summarizes our interpretation of the present data in *Gymnotus carapo* (see Discussion). The key for glutamatergic receptors is represented in the inset.

gic prepacemaker structure(s) and the activation of postsynaptic NMDA and metabotropic glutamate receptors of pacemaker neurons, which would facilitate temporal summation of synaptic responses (Collingridge et al., 1988; Daw et al., 1993). In turn, repetitive activation of prepacemaker terminals may provide a significant glutamate concentration rise that has been postulated to be a prerequisite to activate mGluRs (Batchelor et al., 1994).

Neuroethological significance of our data

Distinct neural strategies seem to have evolved in gymnotiform fish to perform different electromotor behaviors. The behavioral repertoire of gymnotiform fish elicited by glutamatergic inputs to the PMn has been explained in terms of differential activation of ionotropic glutamate receptors of specific cellular targets within the PMn (Spiro, 1997; Juranek and Metzner, 1998). In this section, we will illustrate this notion by comparing the proposed neural strategies at the level of the PMn of various electromotor behaviors described in *Hypopomus* (Fig. 9*A,B*) and the strategy suggested in the present report in *Gymnotus carapo* (Fig. 9*C*).

Two possible neural designs have been postulated in *Hypopomus* (Kawasaki and Heiligenberg, 1990; Spiro, 1997). On one hand (Fig. 9*A*), two different electromotor behaviors could result from activation of the same glutamate receptor subtype located in different cellular types of the PMn. Smooth EOD frequency rises, which can be observed during courtship, result from a glutamatergic input acting on NMDA receptors of PM cells. Sudden interruptions, which can be seen in the context of aggressive encounters, have been explained as being attributable to direct relay cell depolarization in response to NMDA receptor activation.

On the other hand (Fig. 9*B*), activation of different glutamate receptor subtypes located in the same cell type may account for different electromotor behaviors. Activation of relay cells as mentioned above, but through non-NMDA ionotropic glutamate receptors, mediates chirping instead of sudden EOD interruptions.

This electromotor behavior could be observed in the context of aggression and courtship.

In this study, we provide evidence that suggests that *Gymnotus carapo* exhibits another strategy used to produce EOD modulations during Mauthner cell-associated motor behaviors (Fig. 9C). In this species, NMDA and metabotropic glutamate receptors on the same cellular type (PM cells) are synergistically activated to produce an abrupt and prolonged modulation of PMn discharges. Interactions between these receptors have not been explored in our study, but the possibility exists of nonlinear summation of effects at the postsynaptic level. In fact, potentiation of NMDA-evoked responses by mGluR agonists has been reported in vertebrate preparations (Kinney and Slater, 1993).

In conclusion, our study complements an ongoing effort to unveil the neural basis of a long-duration electromotor behavior triggered by a single action potential in an identifiable reticulospinal cell (Mauthner cell) in *Gymnotus carapo*. This behavior seems to result from a combination of factors, including repetitive discharges of glutamatergic prepacemaker structure(s) and the synergistic coactivation of postsynaptic NMDA and metabotropic glutamate receptors of pacemaker neurons. This neural strategy seems to be particularly well suited to producing long-lasting outputs in response to brief inputs in simple circuits and may serve to enhance the fish electrolocative sampling of the environment during Mauthner cell-mediated behaviors.

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