The Journal of Neuroscience, October 1994, 14(10): 6289–6299

Immunolocalization of NMDA Receptors in the Central Nervous System of Weakly Electric Fish: Functional Implications for the Modulation of a Neuronal Oscillator

John E. Spiro,[1] Nils Brose,[2] Stephen F. Heinemann,[2] and Walter Heiligenberg[3]

[1]Department of Biology and [3]Group in Neurosciences, University of California at San Diego, La Jolla, California 92093-0202, and [2]Molecular Neurobiology Laboratory, Salk Institute, La Jolla, California 92037

Using a monoclonal antibody raised against the R1 subunit of the rat NMDA receptor, we mapped the distribution of NMDA receptors in the brains of three genera of electric fish. On Western blots, the antibody recognized a glycoprotein of approximately 105 kDa throughout the CNS. On tissue sections, it strongly labeled a number of neuronal somata and dendrites in the medulla, with weaker immunoreactivity in the forebrain and across much of the rest of the nervous system. At the ultrastructural level, reaction product was localized, though not exclusively, to the postsynaptic region of synapses.

To study the role of NMDA receptors in a specific neural circuit, we focused on the medullary pacemaker nucleus. Neurons in this nucleus, which fire action potentials regularly and trigger each electric organ discharge (EOD), receive glutamatergic input from identified premotor areas. Activity in these areas can cause the pacemaker nucleus to produce outputs with distinct temporal dynamics, which are observed in the behaving animal as modulations of the EOD.

The projection cells of the pacemaker nucleus, the relay cells, were heavily labeled with the anti-NMDA R1 antibody in all genera studied. These results are consistent with the previous finding that a particular EOD modulation mediated by the connection from one premotor area of the brain to the relay cells is blocked by application to the pacemaker nucleus of NMDA receptor blockers. Our results complement ongoing efforts to study this nucleus and provide additional evidence for the role of NMDA receptors in diverse neural circuits.

[Key words: NMDA, pacemaker nucleus, amino acid receptor localization, monoclonal antibody, electric fish, ultrastructure]

NMDA receptors have a number of unusual properties compared with other ligand-gated ion channels. They exhibit a voltage dependence based on a Mg^{2+} block and they are permeable to Ca^{2+}. Furthermore, they have relatively slow kinetics and a large single-channel conductance, and they are coactivated by glycine (for recent reviews, see Gasic and Hollman, 1992; Seeburg, 1993). Despite the widespread occurrence in vertebrates, and possibly in invertebrates, and the large amount of attention that these receptors have received in light of their role in long-term potentiation (LTP), activity-dependent sorting during development and excitotoxicity, little is known about their role in normal information processing in nervous systems (see Daw et al., 1993, for recent review).

We focused on the localization and role of NMDA receptors in the medullary pacemaker nucleus of gymnotiform weakly electric fish. These fish have been studied in great detail (for reviews, see Bullock and Heiligenberg, 1986; Heiligenberg, 1991), and the relative simplicity of their nervous systems in comparison with higher vertebrates offers the possibility to link structural and behavioral studies. The pacemaker nucleus provides a useful system to study the function of NMDA receptors in neuronal circuits because this nucleus receives glutamatergic input from premotor areas, its large cells are accessible to electrodes in both *in vivo* and *in vitro* preparations, and, importantly, its roles in various behaviors have been well characterized.

Gymnotiforms live in fresh-water rivers of South and Central America and produce a regular electric organ discharge (EOD) with a specialized organ in their tail. The fish detect the self-generated field with receptors in their skin and extract information in the returning signal, stemming from distortions caused by objects or other animals, for navigation, prey capture, and communication.

The pacemaker nucleus, which sets the rhythm for the EOD, consists of two types of neurons. Pacemaker cells, which are intrinsic to the nucleus and interconnected electrotonically, fire action potentials regularly. Relay cells, which receive input from pacemaker cells and whose firing generally follows pacemaker cell firing, send axons out of the nucleus and make synaptic contacts on the motoneurons that drive the electric organ. Both pacemaker and relay cells receive input from different identified premotor areas in the midbrain and diencephalon that can trigger the production of distinct patterns of output of the pacemaker nucleus (see Fig. 7*A* for a diagram of the pacemaker nucleus and its afferent connections; for general references on electric fish pacemakers, see Bennet et al., 1967; Dye and Meyer,

Received Feb. 2, 1994; revised Apr. 4, 1994; accepted Apr. 21, 1994.

Grace Kennedy provided invaluable assistance with the histology and especially the electron microscopy. We thank John C. Dye, Walter Metzner, Alfred J. Spiro, Calvin J. H. Wong, and two reviewers for careful readings of early versions of the manuscript. This work was supported by NIMH Grant MH 26149-18 to W.H. and NINCDS grants to S.F.H. (NS11549 and NS28709) and the McKnight Foundation (S.F.H.). J.E.S. was supported by NIH Predoctoral Training Grant 5T32 GM01807. N.B. received financial support from the Deutsche Forschungsgemeinschaft.

Correspondence should be addressed to John E. Spiro, Scripps Institution of Oceanography, 9500 Gilman Drive, Department 0202, University of California, San Diego, La Jolla, CA 92093-0202.

1986). These modulations, observed as changes in the frequency of the EOD during natural behaviors, are diverse and are generated in different behavioral contexts; they can involve smooth rises, smooth falls, or rapid and abrupt increases in frequency as well as complete silences.

When applied to the pacemaker nucleus, drugs that block different subtypes of the glutamate receptor complex selectively block different types of modulations of the pacemaker rhythm in every genus of electric fish investigated (Dye et al., 1989; Kawasaki and Heiligenberg, 1989; Kawasaki and Heiligenberg, 1990; Keller et al., 1991; Metzner, 1993). Kennedy and Heiligenberg (1994) have also demonstrated immunohistochemically that some afferent synaptic terminals onto cells of the pacemaker nucleus contain glutamate. Furthermore, in all genera studied, two premotor areas that each cause a distinct EOD modulation (slow and prolonged vs rapid and transient) may make synapses using different glutamate receptor subtypes (NMDA and non-NMDA, respectively) on individual relay cells (refer to SPPn and PPnC inputs as shown in Fig. 7*A*). These results suggest that the pacemaker nucleus is shared by distinct premotor circuits by nature of the types of receptor activated on postsynaptic neurons. An independent determination of where the glutamate receptors are located in the pacemaker nucleus should help us to understand the mechanism of this sharing.

Recently, antibodies raised against subunits of other amino acid receptors have served as useful tools to explore receptor location on large populations of cells with cellular and often subcellular resolution (Rogers et al., 1991; Blackstone et al., 1992a,b; Hampson et al., 1992; Martin et al., 1992; Petralia and Wenthold, 1992; Huntley et al., 1993; Martin et al., 1993a,b; Vickers et al., 1993). The immunohistochemical approach complements and offers some advantages over previous efforts at receptor localization using microelectrodes and pharmacological manipulations, as well as labeled-ligand binding and *in situ* hybridization techniques.

Here we report the results of a study using a new monoclonal antibody raised against the R1 subunit of the rat NMDA receptor, originally cloned by Moriyoshi et al. (1991), in the detection of NMDA receptors in electric fish. The NMDA R1 subunit appears to be a necessary component for functional NMDA receptors. Therefore, an antibody raised against it is a good probe for NMDA receptors in general (see Discussion). We compared the localization of NMDA receptors in three genera of electric fish, *Apteronotus, Eigenmannia,* and *Hypopomus.* These genera have been studied most extensively in the past and we can therefore make comparative studies of functionally related neural circuits at as many levels as possible.

Preliminary data were presented in abstract form (Spiro and Brose, 1993).

Materials and Methods

Fish. A total of 40 animals representing three genera of weakly electric fish (order Gymnotiformes) were used in this study, *Eigenmannia sp., Hypopomus pinnicaudatus,* and *Apteronotus leptorhynchus.* Adult fish (10–20 cm) of both sexes were obtained through commercial dealers or bred in the laboratory. Fish were maintained in large aquaria and fed live worms regularly.

Antibody production and characterization. A monoclonal antibody was generated using standard techniques (Köhler and Milstein, 1975; Jahn et al., 1985) against a fusion protein encoding glutathion-S-transferase in frame with NMDA R1 residues 660-811 [representing the intracellular loop between putative transmembrane regions III and IV; residue numbers as in Moriyoshi et al. (1991)]. A preliminary characterization of the antibody on Western blots of NMDA R1 transfected human embryonic kidney cell line 293 cells was previously published (Sucher et al., 1993).

Expression and immunodetection of NMDA R1 in the human embryonic kidney cell line 293. A full-length NMDA R1 insert, subcloned into a eukaryotic expression vector that had been modified from pBluescript to include the pcDNAI multiple cloning site and human CMV promoter and enhancer, was obtained from J. Sullivan and G. Sharma (Salk Institute, San Diego, CA). For calcium phosphate–mediated transfection (Graham and van der Eb, 1973; Sambrook et al., 1989), 20 μg of DNA was added to a 100 mM culture dish with 293 cells that were approximately 70% confluent. Cells were harvested after 48 hr into sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

For immunohistochemistry, transfected cells were cultured on poly-lysine-coated coverslips. Cells were fixed for 15 min at room temperature in 4% paraformaldehyde, 4% sucrose, 0.1 M sodium phosphate pH 7.4 (0.1 M PB). Cells were then permeabilized for 5 min in 0.25% Triton X-100, 0.1 M PB pH 7.4, washed 3 × 5 min in 0.1 M PB pH 7.4, and blocked for 1 hr at room temperature in 10% goat serum, 0.1 M PB pH 7.4. Cells were then incubated for 30 hr at 4°C with anti-NMDA R1 monoclonal antibody Cl 54.4 diluted 1:500 in 3% goat serum, 0.1 M PB pH 7.4, washed 4 × 8 min in 0.1 M PB pH 7.4, and incubated for 1 hr at room temperature with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:50 in 3% goat serum, 0.1 M PB pH 7.4. Samples were then washed 4 × 8 min in 0.1 M PB pH 7.4 and incubated for 1 hr at room temperature with Vector Elite ABC reagent (Vector Laboratories, Burlingame, CA), diluted according to the manufacturer's specification in 3% goat serum, 0.1 M PB pH 7.4. After washing 4 × 8 min with 0.1 M PB pH 7.4, immunolabeled cells were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB).

Western blots and deglycosylation experiments. Brains for Western blot analysis were removed from fish anesthetized in oxygenated, ice-cold physiological solution (in mM: 124 NaCl, 2 KCl, 1.25 KH_2PO_4, 1.1 $MgSO_4$, 1.1 $CaCl_2$, 16 $NaHCO_3$, 10 glucose, pH 7.4). They were then dissected in the same solution into four pools and frozen in liquid nitrogen: (1) forebrain (2) ventral medulla (3) spinal cord, and (4) remaining neural tissue, including the electrosensory lateral line lobe, mesencephalon, diencephalon, and cerebellum. Brains were divided into these regions because the dissection can be done rapidly and follows clear and reliable boundaries.

Neural tissue, and additionally liver, eyes, and skeletal muscle from each genus were subjected to SDS-PAGE according to the method of Laemmli (1970). Rat synaptic plasma membranes, prepared as described in Brose et al. (1993) were run alongside. All samples contained 4 M urea, and 20 μg of protein was loaded per lane. Transfer of proteins to nitrocellulose membranes was done as described by Towbin et al. (1979), with minor modifications (Rogers et al., 1991). Detection steps were as in Brose et al., (1993) and proteins were visualized in the linear range with enhanced chemoluminescence (Amersham Corp., Arlington Heights, IL).

For the deglycosylation experiments, *Hypopomus* forebrain tissue (obtained as described above) and rat synaptic plasma membranes were digested in 25 mM sodium phosphate pH 6.0, 10 mM EDTA, 0.2% Triton X-100, 0.5% β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride using 20 mU of endoglycosidase H (endo-β-*N*-acetylglucosaminidase H, EC 3.2.1.96; Boehringer–Mannheim, Indianapolis, IN), which is specific for N-linked sugars, or no additions. Incubations were performed for 4 hr at 37°C and terminated by the addition of SDS-PAGE sample buffer followed by boiling.

Tissue preparation for immunohistochemistry. Fish were deeply anesthetized by immersion in a solution of 3-aminobenzoic acid ethyl ester (MS-222) and then perfused through the heart with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. After perfusion for 30 min with the fixative, the brains were removed and post-fixed in the same solution overnight at 4°C.

Brains were cut at 50 μm on a vibratome, and sections were collected into 0.02 M phosphate-buffered saline pH 7.4 (PBS), rinsed in PBS (3 × 10 min), and then blocked in 5% normal horse serum with 0.1% Triton X-100 in the cold for 1 hr. They were then incubated for 24–48 hr at 4°C in primary antibody diluted in PBS with 0.1% Triton X-100 and 1% horse serum. Ascites fluid (clone 54.4) was diluted 1:20,000 for a final concentration of approximately 0.25 μg/ml. An additional clone, 54.1, was tried initially on alternate serial sections, and the staining patterns compared with 54.4 were indistinguishable (data not shown).

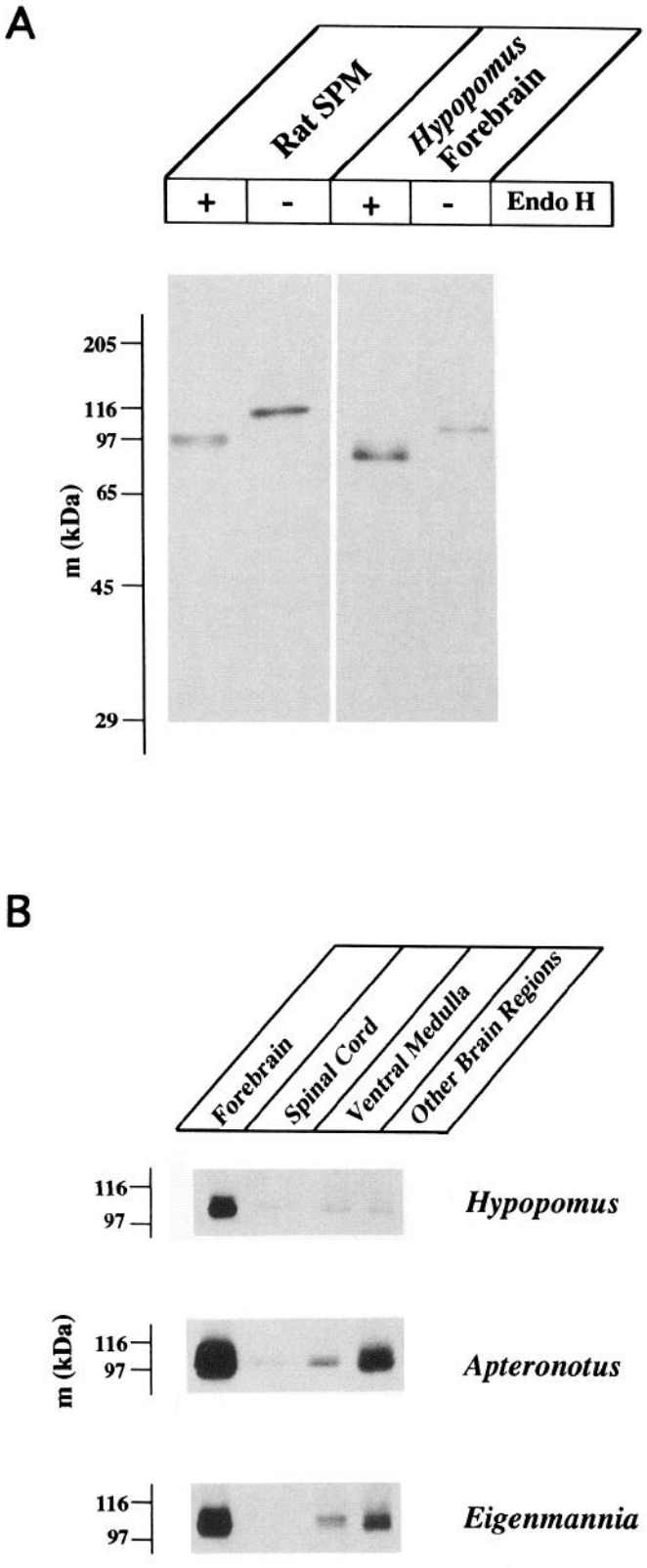


Figure 1. Characterization of the anti-NMDA R1 monoclonal antibody on rat and fish tissue and distribution of NMDA R1 in the fish nervous system. *A*, Western blot analysis comparing rat synaptic plasma membrane (*SPM*) and forebrain tissue from *Hypopomus* (20 μg protein/lane) using a monoclonal antibody generated against the R1 subunit of the NMDA receptor. Some samples were incubated with endoglycosidase H to compare the extent of glycosylation of the proteins. +, sample was incubated with endoglycosidase H; −, no enzyme was added. Positions of molecular weight markers are indicated on the left. *B*, Western blot analysis with the anti-NMDA R1 antibody comparing equal amounts of protein (20 μg/lane) from different brain regions of three genera of weakly electric fish. Lanes are labeled according to the origin of the tissue in the loading sample, and the genus of the fish is indicated on the right. The lane labeled *other brain regions* corresponds to the nervous system tissue remaining after the regions listed in the other lanes were dissected away (see Materials and Methods).

Sections were rinsed in PBS (3 × 10 min) and incubated with biotinylated anti-mouse IgG (Vector Laboratories) at 1:400 in PBS with 0.1% Triton X-100 for 1.5 hr at room temperature, rinsed in PBS (3 × 10 min), and transferred to Vector Elite ABC reagent for 1 hr at room temperature.

Sections were washed in 0.1 M Tris buffer pH 7.2 (3 × 10 min), and transferred to a solution containing DAB (0.4 mg/ml) and nickel ammonium sulfate (0.06% final concentration) in 0.1 M Tris buffer pH 7.2. Hydrogen peroxide was added (0.001% final concentration), and the reaction was monitored (5–10 min) and stopped by washing in 0.1 M Tris buffer pH 7.2.

Sections were then mounted on subbed slides and dried. They were dehydrated through alcohols, cleared in xylene, and coverslipped with Permount. Some sections were counterstained with the Nissl stain neutral red.

In most fish, sections treated as described above, except that the primary antibody was omitted from the incubation, were run alongside the experimental sections to control for any nonspecific signal from the secondary antibody, ABC reagent, or DAB reaction.

As an additional control, alternate sections through one *Hypopomus* pacemaker nucleus were incubated with a preadsorbed primary antibody solution. An excess of NMDA R1 protein (the same protein that was used for immunization but carrying an *N*-terminal His_6 tag instead of glutathione-S-transferase) was added to the primary antibody working solution, and the solution was incubated at 4°C for 48 hr before it was added to tissue sections.

Sections treated identically up to the primary antibody step were also taken for detection with a PAP method and a fluorescein-conjugated secondary antibody. Results from all three methods (biotinylated secondary with ABC reagent, PAP, fluorescent secondary) were qualitatively identical. The ABC detection method was the most sensitive and therefore the method used routinely.

Photomicrographs were made using an Olympus camera system equipped with Differential Interference Contrast (DIC) optics and Kodak T-Max film.

Electron microscopy. Sections were prepared for electron microscopy using a preembedding method. Sections were treated similarly to those prepared for light microscope analysis, except (1) Triton X-100 was omitted from all steps to preserve membrane structure (2) 0.1 M PBS pH 7.4 was used in all steps in lieu of 0.02 M PBS, (3) the primary antibody was used at a dilution of 1:4000 to compensate for the reduced penetration of the antibody. All steps were done at 4°C. The addition of even 0.1% glutaraldehyde led to unacceptable levels of background and, therefore, it was not used. Sections were removed from the DAB, rinsed three times, and treated with 1% osmium tetroxide at room temperature in PBS for 1 hr. They were washed in water, dehydrated through ethanols and *n*-butyl glycol ether, and then embedded in Quetol 651 (EM Sciences, Fort Washington, PA). Sections were cut at 70 nm, placed onto slot grids, stained with lead citrate and 7% uranyl acetate in water, and examined in a Hitachi TEM at 75 kV.

Results

Characterization and specificity of the antibody

Western blot characterization of the antibody on fish and rat tissue revealed that the monoclonal antibody recognizes a single band representing a protein with an apparent molecular weight of 105 kDa in fish, compared with 116 kDa in rat (Fig. 1*A*). Samples from fish and rat ran at reduced apparent molecular weights (81 kDa for fish and 97 kDa for rat SPM) after the tissues were deglycosylated with endoglycosidase H (Fig. 1*A*),

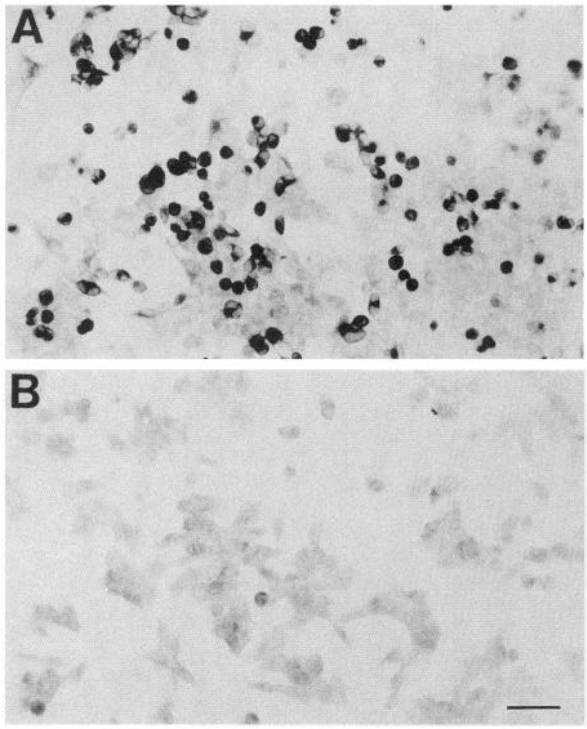


Figure 2. Immunocytochemical detection of NMDA R1 in transfected 293 cells. Human embryonic kidney cells transfected with the full-length NMDA R1 (*A*) and untransfected 293 cells (*B*) were stained with anti-NMDA R1 monoclonal antibody as described in Materials and Methods. Scale bar, 50 μm.

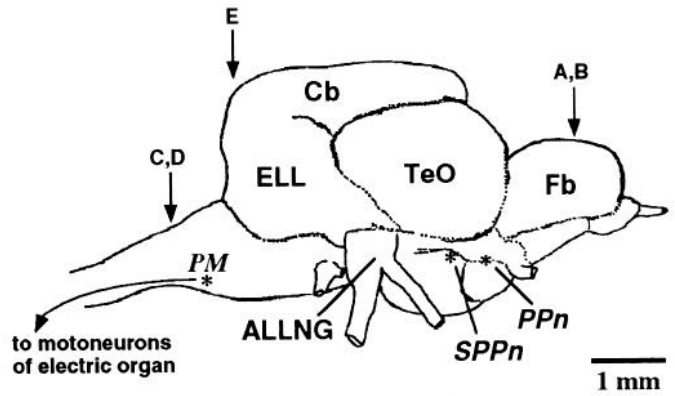


Figure 3. Lateral view of the gymnotiform brain. Some major external structures of the brain are illustrated, and *arrows with letters* mark the positions of transverse sections in Figure 4. *PM, SPPn,* and *PPn* (each marked with an *asterisk*) indicate the approximate positions of the pacemaker nucleus, the sublemniscal prepacemaker nucleus, and the diencephalic prepacemaker nucleus, respectively. *Cb*, cerebellum; *TeO*, optic tectum; *Fb*, forebrain; *ELL*, electrosensory lateral line lobe; *ALLNG*, anterior lateral line nerve ganglion. More caudal cranial nerves are omitted for clarity. Adapted from Keller et al. (1990).

indicating that both proteins are heavily glycosylated with N-linked sugars. This observation is consistent with predictions from the rat primary sequence data indicating that the NMDA R1 transcript has 10 consensus sites for glycosylation (Moriyoshi et al., 1991), and also with data from the characterization of a polyclonal antibody directed against NMDA R1 (Brose et al., 1993).

Although no primary sequence data are currently available for the fish NMDA receptor subunits, we conclude from the similarity in the molecular weight and deglycosylation patterns of the detected antigen in rat and fish brain that the monoclonal antibody used here detects NMDA R1 in fish brain. The high degree of similarity between sequenced NMDA R1 clones (human and rat, 99% identical; Karp et al., 1993) provides additional evidence, although circumstantial, that the protein is evolutionarily highly conserved.

Samples of different brain regions from each genus were also run in separate lanes to examine the distribution of NMDA R1 immunoreactivity across the nervous system (Fig. 1*B*). Signal was strongest in the forebrain in all three species, yet detectable in all brain regions except in *Eigenmannia* spinal cord, where levels were below detection limits (see Discussion). This observation of the widespread distribution of NMDA receptors in electric fish, with an especially high level in forebrain tissue, is consistent with a labeled ligand binding study of *Apteronotus* brain (Maler and Monaghan, 1991). In that study, the authors probed thin sections with ^{3}H-glutamate while blocking non-NMDA binding sites with receptor antagonists. The result is also consistent with reports in mammalian nervous systems, indicating a broad distribution of NMDA R1 mRNA (Moriyoshi et al., 1991) and protein (Brose et al., 1993).

No signal was detected in the lanes with eye or muscle tissue (data not shown). However, a single band close to the apparent molecular weight of the NMDA R1 signal (105 kDa) was detected in the liver. This band was more focused than that seen in brain regions, and it did not run at a different apparent molecular weight when the sample was incubated with endoglycosidase H prior to loading (data not shown), indicating lack of glycosylation. We assume that in liver the antibody recognizes a similar epitope of an unrelated protein.

The antibody was further characterized by transfecting human embryonic kidney cells with NMDA R1, fixing the cells, and incubating them with the antibody as described in Materials and Methods. The antibody was clearly detected on many of the cells transfected with the full-length clone (immunopositive cells are dark; Fig. 2*A*), but not on untransfected cells (Fig. 2*B*).

Summary of immunohistochemistry at the light microscope level throughout the CNS

Vibratome sections from all levels of fish brains were examined for NMDA R1 immunoreactivity. Two types of experiments were run to control for the specificity of the primary antibody and the detection methods. In most trials, some sections were run with the primary antibody omitted from the incubation step. Additionally, a preadsorbtion experiment, in which the monoclonal antibody was preincubated with an excess of fusion protein, was run in one case for *Hypopomus*. Immunoreaction product was absent from sections where the primary antibody had been omitted. In some cases there was a diffuse background staining, presumably from nonspecific secondary binding or endogenous peroxidase activity, but the degree of staining was never as intense as in experimental sections. There was no detectable immunoreaction product on the *Hypopomus* sections that were incubated with preadsorbed antibody (data not shown).

The photomicrographs of Figure 4*A–E* show representative immunoreactivity in the nervous system of *Apteronotus*. The pattern of staining was similar across genera (refer to Fig. 3 for the rostral/caudal position of the sections shown in Fig. 4 and to Maler et al., 1991, for a general atlas of the gymnotiform brain). Somata and dendrites of certain neurons (Fig. 4*A–E*)

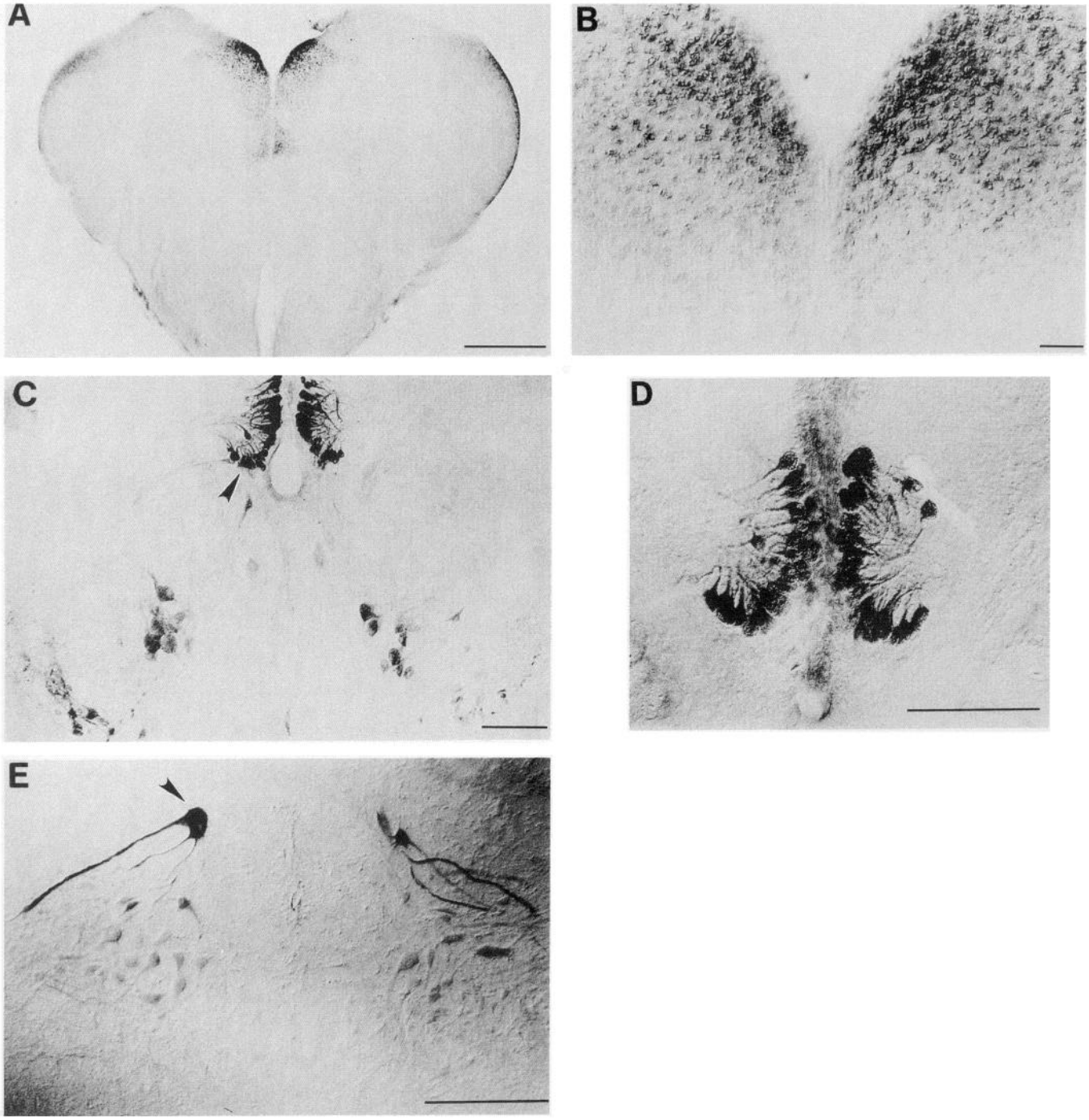


Figure 4. Immunocytochemical detection of NMDA R1 in the CNS of electric fish. *A–E*, Photomicrographs (DIC optics) of 50-μm-thick cross sections at levels of the *Apteronotus* brain containing some of the more intense immunoreactivity. The pattern was similar in the other genera studied (see Results). The approximate position of each section along the rostral–caudal axis of the brain is indicated by the corresponding letter in Figure 3. Dorsal is up in this and following figures. Sections were cut on a vibratome and incubated with the anti-NMDA R1 monoclonal antibody; the antibody was detected using a biotinylated secondary antibody and in the final step a DAB reaction. *A* and *B*, Low- and high-magnification views of sections through the forebrain. Small cells in the dorsomedial region are immunopositive. *C* and *D*, Low- and high-magnification views of sections through the medulla. Cells in the vagal motor nucleus are intensely immunopositive (*arrowhead in C*). *E*, Section through the rostral medulla showing an immunopositive Mauthner cell (*arrowhead*). Scale bars: *A* and *E*, 500 μm; *B–D*, 50 μm.

were strongly stained against a virtually unstained background at this dilution of antibody (diluted 1:20,000, approximately 0.25 μg protein/ml, or up to approximately 1:60,000). Large cells in the reticular formation, Mauthner cells, and cells in the dorsomedial forebrain were labeled particularly strongly. Strong immunoreactivity in these areas was consistent under a variety of fixation conditions and with different primary antibody detection techniques (see Materials and Methods), as well as with another monoclonal antibody clone raised against the same fusion protein (54.1; data not shown).

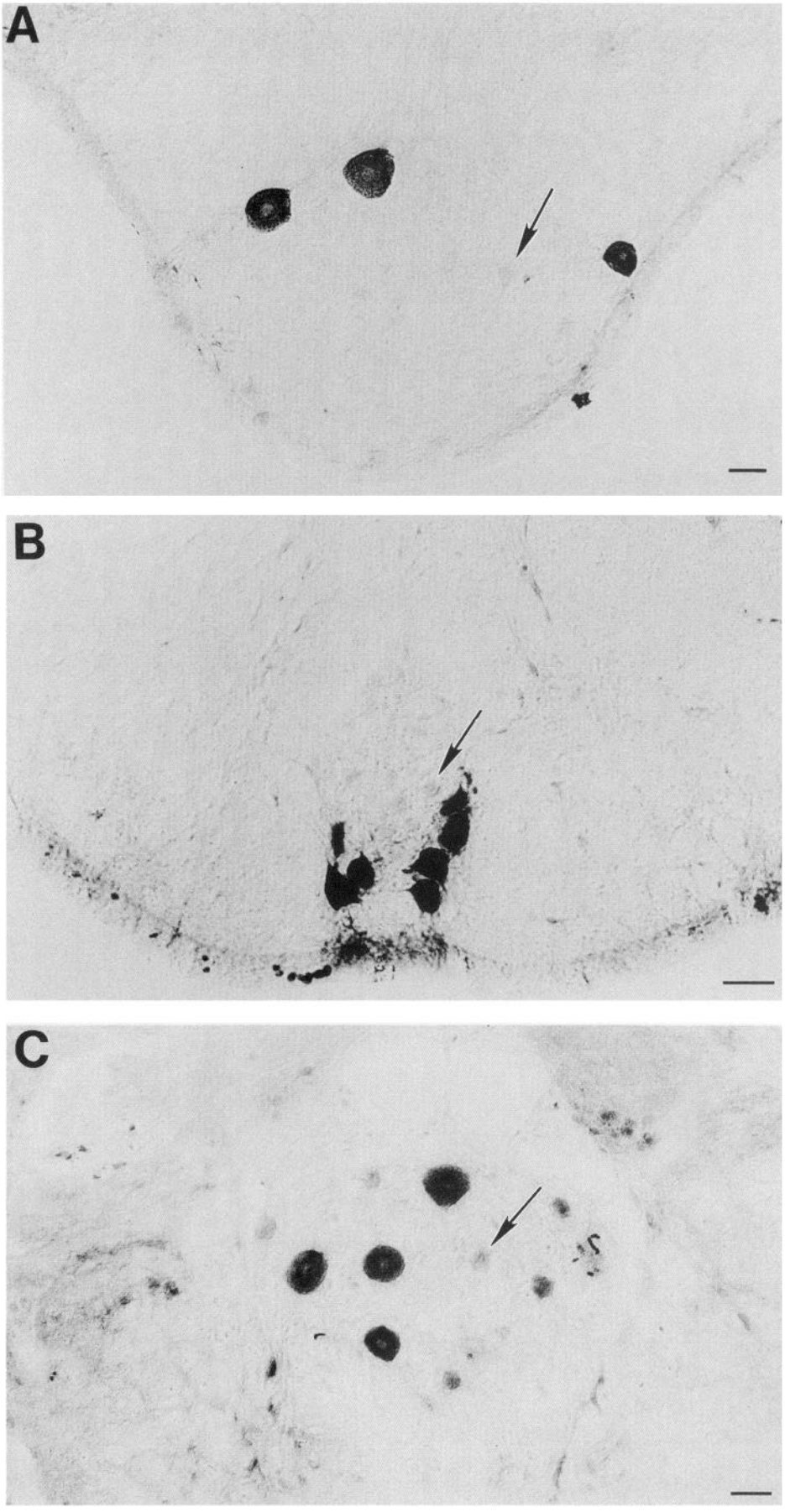


Figure 5. Immunocytochemical detection of NMDA R1 in pacemaker nuclei. Photomicrographs (DIC optics) of 50 μm sections at the level of the medulla show the pacemaker nuclei of *Apteronotus*, (*A*), *Hypopomus* (*B*), and *Eigenmannia* (*C*). Sections were processed for NMDA R1 immunoreactivity as in Figure 4. See Figure 3 for the approximate rostral–caudal level of the pacemaker nucleus. The larger relay cells are intensely immunopositive in all three genera. No signal was detected on pacemaker cells of *Apteronotus* or *Hypopomus*, although pacemaker cells in *Eigenmannia* were weakly immunopositive (one pacemaker soma is marked with *arrow* in each panel). Smaller pacemaker cells are generally restricted to the dorsal pacemaker nucleus in *Hypopomus*, whereas in *Apteronotus* and *Eigenmannia*, the two cell types are distributed throughout the nucleus. Scale bars, 50 μm.

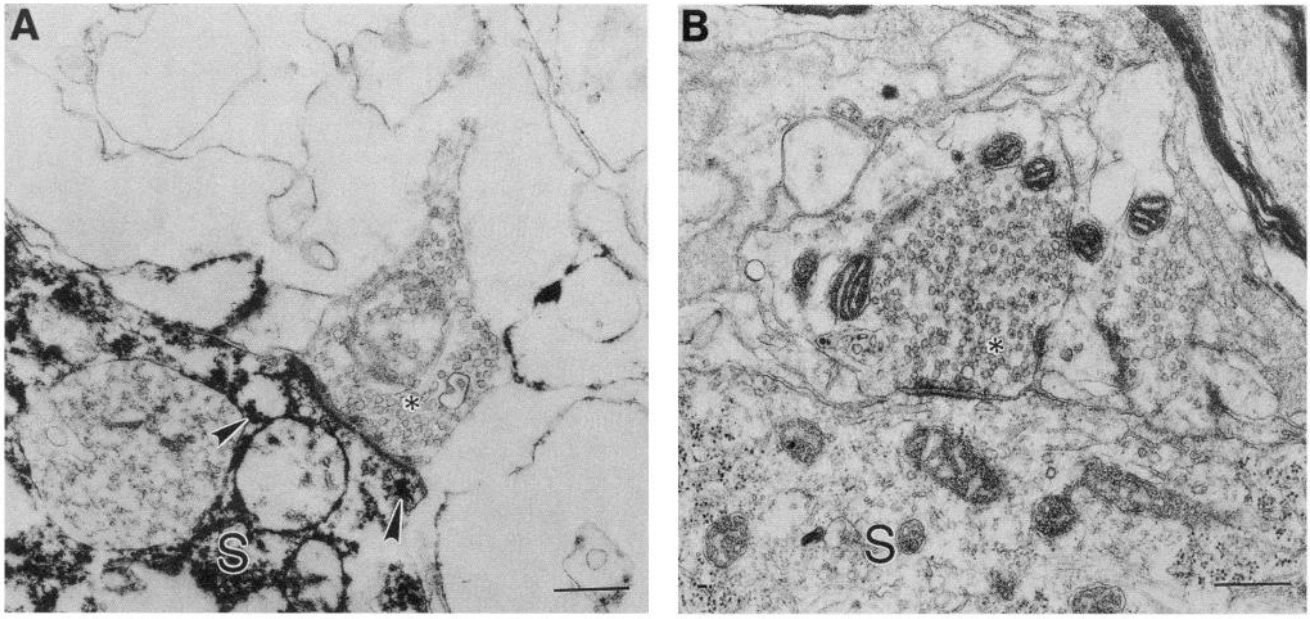


Figure 6. Electron micrograph of anti-NMDA R1 staining on a relay cell. *A,* Section (70 nm) through the pacemaker nucleus of *Hypopomus* processed for the detection of NMDA R1 using a preembedding method. The section is through a relay cell, and a chemical synapse onto the soma (*S*) is visible, most likely from an SPPn fiber (see Discussion). Reaction product is seen around postsynaptic structures (*arrowheads*), and is absent from presynaptic structures (*asterisk*). The morphology suffers from the lack of glutaraldehyde in the fixative (see Discussion). *B,* An unstained section through a relay cell from a fish that was fixed with 3% glutaraldehyde, 1% paraformaldehyde, to illustrate the ultrastructure of well-fixed tissue. Scale bars, 300 nm.

Using more concentrated antibody solutions (~2.5 μg of protein/ml), we could demonstrate consistent and more widespread label in the CNS, including the forebrain. Whereas the strong staining in the medulla was extremely reproducible and readily distinguished from background, the staining in the forebrain was more diffuse, was restricted mostly to somata, and varied more in intensity from case to case. The role of NMDA receptors in forebrain structures is noteworthy given the large amount of information correlating the presence of these receptors in mammals in forebrain structures involved in learning and memory (see Maler and Monaghan, 1991, for a discussion of NMDA receptors in the forebrain of electric fish). In one fish the pituitary was sectioned with the rest of the brain, and was observed to be immunopositive. There is some prior evidence for glutamate receptors (non-NMDA) in pituitaries of mammals (Gasic and Heinemann, 1991).

Immunohistochemistry at the light microscope level in the pacemaker nucleus

The neurons of the pacemaker nucleus, pacemaker and relay cells, are distinguished in tissue sections by the difference in their size: small and large, respectively. In *Hypopomus*, although not in the other genera studied, the two cell types are also spatially segregated in the nucleus: pacemaker cells found more dorsally, and relay cells ventrally. Sections through the pacemaker nuclei of the three genera studied show that relay cells are heavily labeled with the anti-NMDA R1 antibody (Fig. 5*A–C*). All the relay cells of a particular nucleus were normally immunopositive, with reaction product concentrated in the somata and proximal processes. In *Apteronotus* and *Hypopomus* (Fig. 5*A,B*), little if any reaction product was seen on the pacemaker cells, despite intense staining on nearby relay cells. In *Eigenmannia* (Fig. 5*C*), pacemaker cells were also weakly immunopositive.

Immunocytochemistry at the electron microscopic level in the pacemaker nucleus

We examined the anti-NMDA R1 immunoreactivity in the pacemaker nucleus of one specimen of both *Hypopomus* and *Apteronotus* using the electron microscope. At low magnification, DAB reaction product was seen to be concentrated in patches around the perimeter of relay cells, with additional diffuse signal scattered throughout the cytoplasm. At higher magnification, reaction product could be seen postsynaptically at chemical synapses onto relay cell somata (Fig. 6*A*). The reaction product often spread beyond the postsynaptic densities. Signal was absent from presynaptic structures, and was not consistently associated with intracellular organelles. The need to omit glutaraldehyde from the fixation solution compromised the preservation of some ultrastructure and, therefore, limited our ability to characterize the localization more thoroughly. A section through a relay cell from an unstained, glutaraldehyde-fixed specimen is included (Fig. 6*B*) to illustrate the morphology of synapses onto relay cells in well-fixed tissue.

Discussion

Specificity of the antibody for NMDA receptors

We have generated a monoclonal antibody against a fusion protein containing a portion of NMDA R1. The antibody is specific for the NMDA R1 protein in rat brain and, despite the considerable phylogenetic distance, for a similar protein in gymnotiform fish CNS. As NMDA R1 mRNA is expressed in a ubiquitous way in vertebrate brain, and as coexpression of NMDA R1 is necessary for functional expression of other known NMDA receptor subunits, it is likely that NMDA R1 is part of all native NMDA receptors (Brose et al., 1993). Therefore, the antibody used here is a unique tool for the localization of all native NMDA receptors.

NMDA receptor immunoreactivity on tissue sections throughout the CNS, and comparison with Western blot results

The pharmacology of many of the areas tested for NMDA R1 immunoreactivity is not known in the fish used in these experiments (with the notable exception of the pacemaker nucleus, which will be discussed in detail below). However, in many other vertebrate systems, NMDA receptors are involved in motor pattern generation (see Daw et al., 1993, for review), and therefore our results showing intense staining on various motor nuclei may be expected. The presence of immunoreactivity on Mauthner cells, which receive input from multiple sensory sources, may be evidence for the integrative role that NMDA receptors have been postulated to play as a result of their long open time and voltage-dependent Mg^{2+} block.

On Western blots, the strongest signal was found in the forebrain in all three genera, whereas on tissue sections, most consistent cellular label was in the medulla. It is likely, therefore, that the fixation conditions used for the tissue sections sometimes masked the epitope or failed to preserve it in some regions, such as the forebrain. This may also explain why we failed to see intense immunoreactivity on tissue sections in some areas of the brain where physiological responses to NMDA receptor blockers and binding of ^{3}H-glutamate in the presence of non-NMDA receptor blockers were reported previously, such as in the electrosensory lateral line lobe (ELL) (Maler and Monaghan, 1991). Alternatively, we may have failed to resolve a low level of binding with our detection methods. Signal was not detected on Western blots of *Eigenmannia* spinal cord despite the presence of some strongly staining cells on tissue sections; it is possible that the signal from the few strongly immunopositive cells was diluted by a large number of unreactive cells in this tissue and therefore the pooled signal was below detectable limits of the blot. Despite the considerable evidence in mouse and rat to the contrary, we are still unable to exclude the possibility that the antibody only detects one variant of an NMDA R1 transcript, or that NMDA R1 is not present in every NMDA receptor in fish.

NMDA receptor immunoreactivity in the pacemaker nucleus compared with data from physiology and pharmacology experiments

It is difficult to address the question of the functional significance of NMDA receptors in brain circuits due to the complexity of most nervous systems. We have taken the approach that by studying the role of these receptors in particularly simple circuits, such as the pacemaker nucleus, where data about physiology, pharmacology, and behavior can be integrated, we may gain insights that can be generalized to more complex nervous systems.

Previous reports provided evidence for the circuit of the pacemaker nucleus and its afferents as shown in Figure 7*A* with three types of experiments. To establish the general location of areas that make synaptic contact with and modulate the pacemaker rhythm, tracers were injected into the pacemaker nucleus to label afferent nuclei retrogradely. Tracers were also introduced into the afferent nuclei directly to trace their projections to the pacemaker nucleus. To establish the pharmacology of the connections between these areas and the pacemaker nucleus, agonists and antagonists of the glutamate receptor complex were introduced into the pacemaker nucleus while afferent nuclei were stimulated. The results of these experiments suggest that distinct types of modulations of the pacemaker rhythm are mediated via different subtypes of the glutamate receptor complex.

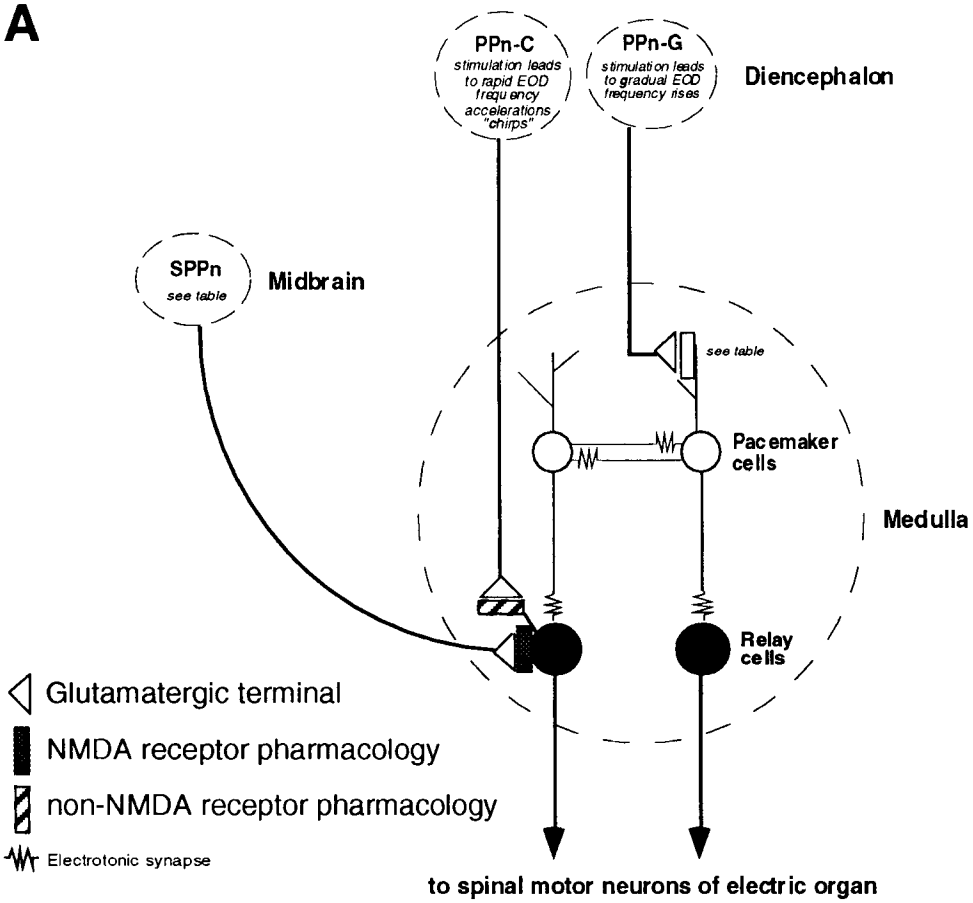
In the present report, we have shown that the relay cells are strongly immunopositive with the anti-NMDA R1 antibody in every genus tested. This observation supports the evidence from previous experiments that in all genera, the effect of stimulation of a midbrain presynaptic area, the sublemniscal pre-pacemaker nucleus (SPPn), on the pacemaker nucleus can be blocked reversibly with the NMDA channel blocker 2-amino-5-phosphonovaleric acid (APV).

Using the electron microscope, reaction product can be localized to the postsynaptic regions of synapses, which provides further support that this antibody recognizes a neurotransmitter receptor. It is likely that the synapses onto relay cells (Fig. 6*A*) are from SPPn fibers given that tracing studies and intracellular recordings have demonstrated that SPPn fibers make synaptic contacts in the vicinity of relay cells (Keller et al., 1991; Kennedy and Heiligenberg, 1994). Reaction product is also present, although more diffusely, throughout the cytoplasm. An intracellular pool of receptors, which may represent those that are in transit to the plasma membrane, was also reported in a recent study using a polyclonal antibody against NMDA R1 (Brose et al., 1993) and in reports of localization of non-NMDA glutamate receptors (Huntley et al., 1993). We expect that a postembedding method that we are now testing, using immunogold particles, will offer better preservation of ultrastructure and higher resolution.

While there was strong labeling of relay cells in all three species studied, pacemaker cell staining was weak in *Eigenmannia*, and essentially absent in *Hypopomus* and *Apteronotus* (see Fig. 7*B* for comparison of the differences across the genera). These results in *Hypopomus* and *Apteronotus* are not consistent with the pharmacology experiments, which indicate that slow rises in EOD frequency are mediated by NMDA receptors on pacemaker cells. We offer three possible explanations for this discrepancy. One possibility, which we have mentioned previously, is that we may have failed to detect all receptors that have NMDA receptor pharmacology; the NMDA R1 subunit in the NMDA receptors on pacemaker cells could differ from other NMDA R1 subunits to the extent that it is not strongly recognized by our antibody, for example. Given that adjacent relay cells were intensely labeled, however, we believe we can eliminate simple arguments such as those based on problems with tissue fixation. Similar to the first explanation, it is possible that the low level of binding that was detected on pacemaker cells still represents an adequate pool of receptors to mediate transmission from the PPnG to the pacemaker cells (see Fig. 7*A*). In this case, the results of the present study would be qualitatively consistent with previous findings.

An alternative third hypothesis regarding the lack of intense signal on pacemaker cells may point to a possible shortcoming in the technique employed to study the pharmacology of the nucleus in previous reports, which involved introducing drugs into the pacemaker nucleus that block glutamate receptor subtypes. Given that there are electrotonic connections between pacemaker and relay cells in these three genera, it is possible that some of the effects of drugs that were thought to be blocking NMDA receptors on pacemaker cells could actually have had primary effects on relay cells. The difficulties in predicting network properties of oscillating cells that are electrotonically coupled were illustrated in a modeling study by Kepler et al. (1990), and in a study in which artificial electrotonic conductances were

A



B

	PPn-G to pacemaker cell glutamate receptor pharmacology	SPPn stimulation leads to:	NMDA R1 Immunoreactivity	
			Relay cells	Pacemaker cells
Apteronotus	NMDA and non-NMDA	EOD frequency rise	+++	-
Eigenmannia	non-NMDA (NMDA)	EOD frequency rise	+++	+
Hypopomus	NMDA (non-NMDA)	Abrupt EOD interruption	+++	-

Figure 7. A, Diagram of the pacemaker nucleus and its afferents. This figure, based on previous reports, summarizes similarities of afferent and internal connections of the pacemaker nucleus for the three genera studied. The glutamate pharmacology of the connections and the effects of stimulation of premotor areas are also indicated. Information about the glutamate receptor pharmacology given in this figure was determined by injecting blockers of the glutamate receptor subtypes (APV or CNQX) into the pacemaker nucleus while stimulating premotor areas. Though not shown, the premotor areas are bilateral, paired structures, whereas the medullary pacemaker nucleus is unpaired. The actual number of pacemaker and relay cells varies in the different genera and with the age of the fish, but is in the range of 20–100 cells of each type. *B,* Summary of differences in the pacemaker nucleus among the electric fish studied. The table includes more detailed information about the differences in the pharmacology and effects of the inputs to the pacemaker nucleus among the different genera, and summarizes the NMDA R1 immunoreactivity in the nucleus (+++, strong label; +, weak label; −, no detectable label). In *Hypopomus*, the abrupt EOD interruption follows a large depolarization of relay cells (see Discussion). Receptor subtypes shown in brackets contribute only weakly to the response.

introduced between actual neurons of the stomatogastric ganglia (Sharp et al., 1992). The authors showed that the effects of coupling neuronal oscillators depend on membrane potentials, intrinsic properties, and coupling strength of the members of an oscillating circuit. All of these factors can strongly influence the network in a complex way, and the types of effects are often not intuitive. If correct, this third hypothesis would imply a more substantial reformulation in our understanding of the organization of the pacemaker nucleus; it would suggest that the PPnG mediates slow frequency changes on the pacemaker nucleus rhythm via synaptic contacts on NMDA receptors on relay cells. The modulatory input on relay cells could then influence pacemaker cell firing through the electrotonic junctions between these two cell types (see Fig. 7*A*).

Further experiments using intracellular recordings in conjunction with pharmacological manipulations should help us to pinpoint the site of action of drugs more precisely and should help to distinguish between the hypotheses raised above.

Possible role of NMDA receptors in the pacemaker nucleus and comparison with other systems

The relay cells of the pacemaker nucleus receive input from a premotor region called the SPPn, and the effects of stimulation of the SPPn in all genera studied are blocked by applying the

NMDA receptor blocker APV to the pacemaker nucleus. In the present study we have shown that relay cells are immunopositive for NMDA R1. What role are NMDA receptors playing in this circuit?

In *Hypopomus*, SPPn stimulation causes a prolonged (few to several seconds) depolarization of relay cell somata; this depolarization leads to a desynchronization of the firing of the relay cell axons, and in turn, the electric organ (Kawasaki and Heiligenberg, 1989). Given that the effect is sustained, it is possible that Ca^{2+} entry through the open NMDA receptor channel may trigger a second messenger cascade that maintains the level of depolarization. We are investigating the possibility of such a mechanism in an *in vitro* preparation of the pacemaker nucleus and its afferents in *Hypopomus*, in which one can record intracellularly from pacemaker and relay cells during pharmacological manipulations.

In *Eigenmannia*, the SPPn provides a tonic excitatory input to the pacemaker nucleus. Inhibition of the SPPn thus causes the pacemaker nucleus to fire at a lower rate, which generates a slow decrease in the fish's EOD frequency similar to the frequency drop observed during a jamming avoidance response (Metzner, 1993). Therefore, the role of the NMDA receptors on relay cells in *Eigenmannia* and *Hypopomus* differs, although perhaps only quantitatively. In *Apteronotus*, the SPPn apparently lacks tonic excitation, and its stimulation causes a rise in pacemaker nucleus frequency. Whereas a rapid component of this rise can be blocked with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a slow and sustained component can be attenuated by APV and 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Heiligenberg and Metzner, unpublished observations). Much as in *Eigenmannia*, the relay cells of *Apteronotus* thus receive sustained activation via NMDA receptors.

In the lamprey spinal cord, where NMDA function has been investigated in detail, specific properties of the NMDA channel, such as its voltage-dependent Mg^{2+} block, are thought to be important for the generation of rhythm (Grillner and Matsushima, 1991, for review). This does not seem to be the case in the electric fish pacemaker nucleus because although APV blocks modulations triggered by stimulation of presynaptic areas, it does not block the ongoing oscillations of the pacemaker nucleus. Consistent with this result, in the present report we show that NMDA receptors are found primarily on the relay cells, and not what are thought to be the cells that generate the rhythm, the pacemaker cells. Similar findings have been reported in recent studies that indicate that despite the presence of NMDA receptors in spinal motor circuits, locomotor and respiratory rhythms are mostly intact in genetically manipulated neonatal mice that lack functional NMDA receptors (Feldman et al., 1993; Smith et al., 1993).

The results of previous experiments summarized in the diagram in Figure 7 also suggest that NMDA and non-NMDA receptors are colocalized in the pacemaker nucleus. On cultured hippocampal and cortical neurons, colocalization has been reported and is likely important for the generation of LTP (Bekkers and Stevens, 1989; Jones and Baughman, 1991). Only a few studies have reported the segregation of these receptor subtypes at synapses (Sillar and Roberts, 1991). In the pacemaker nucleus, NMDA and non-NMDA receptors mediate the input from two distinct premotor areas that make synaptic contact onto relay cells, suggesting that these receptor subtypes may be spatially segregated on relay cells. We are planning to address this issue using a non-NMDA receptor antibody with the same level of resolution as in the present study. A similar synaptic arrangement has been reported in an avian system (Mooney and Konishi, 1991), in which two song nuclei project to single neurons of the robust nucleus of the archistriatum (RA). In a slice preparation, the excitatory postsynaptic potentials (EPSPs) from the two inputs can be selectively blocked with the application of either an NMDA or non-NMDA receptor blocker.

Conclusions

We have generated a monoclonal antibody that is specific for the NMDA R1 protein in rat and fish, and is therefore a useful probe for localizing NMDA receptors at both the light and electron microscope level. By studying the distribution of receptors in the well-studied medullary pacemaker nucleus, we have confirmed some predictions from physiology experiments; namely, that these receptors are involved in the transmission of modulatory information to the nucleus, but not necessarily involved in the generation of the rhythm.

References

Bekkers JM, Stevens CF (1989) NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. Nature 341:230–233.

Bennett MVL, Pappas GD, Gimenez M, Nakajima Y (1967) Physiology and ultrastructure of electrotonic junctions. IV. Medullary electromotor nuclei in gymnotid fish. J Neurophysiol 30:236–300.

Blackstone CD, Levey AI, Martin LJ, Price DL, Huganir RL (1992a) Immunological detection of glutamate receptor subtypes in human central nervous system. Ann Neurol 31:680–683.

Blackstone CD, Moss SJ, Martin LJ, Levey AI, Price DL, Huganir RL (1992b) Biochemical characterization and localization of a non-*N*-methyl-D-aspartate glutamate receptor in rat brain. J Neurochem 58: 1118–1126.

Brose N, Gasic GP, Vetter DE, Sullivan JM, Heinemann SF (1993) Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1. J Biol Chem 268: 22663–22671.

Bullock TH, Heiligenberg W, eds (1986) Electroreception. New York: Wiley.

Daw NW, Stein PS, Fox K (1993) The role of NMDA receptors in information processing. Annu Rev Neurosci 16:207–222.

Dye JC, Meyer JH (1986) Central control of the electric organ discharge in weakly electric fish. In: Electroreception (Bullock TH, Heiligenberg W, eds), pp 71–102. New York: Wiley.

Dye J, Heiligenberg W, Keller CH, Kawasaki M (1989) Different classes of glutamate receptors mediate distinct behaviors in a single brainstem nucleus. Proc Natl Acad Sci USA 86:8993–8997.

Feldman JL, Li Y, Funk GD, Smith JC, Dong X-W, Johnson SM, Lai J, Hsu S, Tonegawa S (1993) Neonatal mice lacking functional NMDA receptors generate respiratory oscillations *in vivo* and *in vitro*. Soc Neurosci Abstr 19:925.

Gasic GP, Heinemann S (1991) Receptors coupled to ionic channels: the glutamate receptor family. Curr Opin Neurobiol 1:20-26.

Gasic GP, Hollmann M (1992) Molecular neurobiology of glutamate receptors. Annu Rev Physiol 54:507–536.

Graham FL, van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.

Grillner S, Matsushima T (1991) The neural network underlying locomotion in lamprey-synaptic and cellular mechanisms. Neuron 7:1–15.

Hampson DR, Huang XP, Oberdorfer MD, Goh JW, Auyeung A, Wenthold RJ (1992) Localization of AMPA receptors in the hippocampus and cerebellum of the rat using an anti-receptor monoclonal antibody. Neuroscience 50:11–22.

Heiligenberg W (1991) Neural nets in electric fish. Cambridge, MA: MIT Press.

Huntley GW, Rogers SW, Moran T, Janssen W, Archin N, Vickers JC, Cauley K, Heinemann SF, Morrison JH (1993) Selective distribution of kainate receptor subunit immunoreactivity in monkey neo-

cortex revealed by a monoclonal antibody that recognizes glutamate receptor subunits GluR5/6/7. J Neurosci 13:2965–2981.

Jahn R, Schiebler W, Ouimet C, Greengard P (1985) A 38,000-dalton membrane protein (p38) present in synaptic vesicles. Proc Natl Acad Sci USA 82:4137–4141.

Jones KA, Baughman RW (1991) Both NMDA and non-NMDA subtypes of glutamate receptors are concentrated at synapses on cerebral cortical neurons in culture. Neuron 7:593–603.

Karp SJ, Masu M, Eki T, Ozawa K, Nakanishi S (1993) Molecular cloning and chromosomal localization of the key subunit of the human *N*-methyl-D-aspartate receptor. J Biol Chem 268:3728–3733.

Kawasaki M, Heiligenberg W (1989) Distinct mechanisms of modulation in a neuronal oscillator generate different social signals in the electric fish *Hypopomus*. J Comp Physiol [A] 165:731–741.

Kawasaki M, Heiligenberg W (1990) Different classes of glutamate receptors and GABA mediate distinct modulations of a neuronal oscillator, the medullary pacemaker of a gymnotiform electric fish. J Neurosci 10:3896–3904.

Keller CH, Maler L, Heiligenberg W (1990) Structural and functional organization of a diencephalic sensory-motor interface in the gymnotiform fish, *Eigenmannia*. J Comp Neurol 293:347–376.

Keller CH, Kawasaki M, Heiligenberg W (1991) The control of pacemaker modulations for social communication in the weakly electric fish *Sternopygus*. J Comp Physiol [A] 169:441–450.

Kennedy G, Heiligenberg W (1994) Ultrastructural evidence of GABAergic inhibition and glutamatergic excitation in the pacemaker nucleus of the gymnotiform electric fish, *Hypopomus*. J Comp Physiol [A] 174:267–280.

Kepler TB, Marder E, Abbott LF (1990) The effect of electrical coupling on the frequency of model neuronal oscillators. Science 248: 83–85.

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Maler L, Monaghan D (1991) The distribution of excitatory amino acid binding sites in the brain of an electric fish, *Apteronotus leptorhynchus*. J Chem Neuroanat 4:39–61.

Maler L, Sas E, Johnston S, Ellis W (1991) An atlas of the brain of the electric fish *Apteronotus leptorhynchus*. J Chem Neuroanat 4:1–38.

Martin LJ, Blackstone CD, Huganir RL, Price DL (1992) Cellular localization of a metabotropic glutamate receptor in rat brain. Neuron 9:259–270.

Martin LJ, Blackstone CD, Levey AI, Huganir RL, Price DL (1993a) AMPA glutamate receptor subunits are differentially distributed in rat brain. Neuroscience 53:327–358.

Martin LJ, Blackstone CD, Levey AI, Huganir RL, Price DL (1993b) Cellular localizations of AMPA glutamate receptors within the basal forebrain magnocellular complex of rat and monkey. J Neurosci 13: 2249–2263.

Metzner W (1993) The jamming avoidance response in *Eigenmannia* is controlled by two separate motor pathways. J Neurosci 13:1862–1878.

Mooney R, Konishi M (1991) Two distinct inputs to an avian song nucleus activate different glutamate receptor subtypes on individual neurons. Proc Natl Acad Sci USA 88:4075–4079.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31–37.

Petralia RS, Wenthold RJ (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. J Comp Neurol 318:329–354.

Rogers SW, Hughes TE, Hollmann M, Gasic GP, Deneris ES, Heinemann S (1991) The characterization and localization of the glutamate receptor subunit GluR1 in the rat brain. J Neurosci 11:2713–2724.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Seeburg PH (1993) The molecular biology of mammalian glutamate receptor channels. Trends Neurosci 16:359–365.

Sharp AA, Abbott LF, Marder E (1992) Artificial electrical synapses in oscillatory networks. J Neurophysiol 67:1691–1694.

Sillar KT, Roberts A (1991) Segregation of NMDA and non-NMDA receptors at separate synaptic contacts: evidence from spontaneous EPSPs in *Xenopus* embryo spinal neurons. Brain Res 545:24–32.

Smith JC, Li Y, Funk GD, Johnson SM, Dong X-W, Lai J, Hsu S, Feldman JL, Tonegawa S (1993) Functional networks for locomotion in spinal cord of neonatal mice lacking NMDA receptors. Soc Neurosci Abstr 19:720.

Spiro JE, Brose N (1993) Localization of NMDA receptors in the CNS of weakly electric fish using a monoclonal antibody. Soc Neurosci Abstr 19:376.

Sucher NJ, Brose N, Deitcher DL, Awobuluyi M, Gasic GP, Bading H, Cepko CL, Greenberg ME, Jahn R, Heinemann SF, Lipton SA (1993) Expression of endogenous NMDAR1 transcripts without receptor protein suggests post-transcriptional control in PC12 cells. J Biol Chem 268:22299–22304.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Vickers JC, Huntley GW, Edwards AM, Moran T, Rogers SW, Heinemann SF, Morrison JH (1993) Quantitative localization of AMPA/kainate and kainate glutamate receptor subunit immunoreactivity in neurochemically identified subpopulations of neurons in the prefrontal cortex of the macaque monkey. J Neurosci 13:2982–2992.