Activation of Ionotropic Glutamate Receptors on Peripheral Axons of Primary Motoneurons Mediates Transmitter Release at the Zebrafish NMJ

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Todd, Keith J., Carrie A. B. Slatter, and Declan W. Ali. Activation of ionotropic glutamate receptors on peripheral axons of primary motoneurons mediates transmitter release at the zebrafish NMJ. J Neurophysiol 91: 828–840, 2004; 10.1152/jn.00599.2003. The development and function of the vertebrate neuromuscular junction (NMJ) is continually being redefined. Previous studies have indicated that glutamate may play a role in the development or function of the NMJ by associating with presynaptic receptors. We have used larval zebrafish (Danio rerio) to investigate the presence of presynaptic ionotropic glutamate receptors (iGluRs) at the NMJ in vivo. In whole-mount zebrafish larvae, antibody staining directed to NR2A subunits colocalized with specific staining of motoneuron axon tracts. Whole cell voltage-clamp recordings of miniature end-plate currents (mEPCs) from axial white muscle were performed during application of iGluR agonists and antagonists. Local perfusion of the NMJ with iGluR agonists resulted in significant increases in the frequency of spontaneous acetylcholine (ACh) release. These increases were blocked by the N-methyl-D-aspartate (NMDA) receptor antagonist d-(-)-2-amino-5-phosphonopentanoic acid (50 μM) and by the non-NMDA receptor antagonist 6-cyano-7-nitroquinazoline-2,3-dione (50 μM). Further pharmacological investigation revealed no effect of the kainate receptor-specific antagonist 2S-4R)-4-methylglutamate (10 μM) on kainate-induced rises in the frequency of spontaneous ACh release. However, these were blocked with the AMPA receptor-specific antagonist 1-[(4-aminophenyl)-4-methyl-7,8-methylenedioxy]-5H-2,3-benzodiazepine (50 μM). Application of glutamate (1 mM) in the presence of the glutamate uptake inhibitor d-threo-β-benzyloxyaspartate (200 μM) resulted in a significant increase in the frequency of mEPCs. These results suggest the presence of AMPA and NMDA receptors in association with motoneuron axons of larval zebrafish.

INTRODUCTION

Neuromuscular transmission occurs through the release of acetylcholine (ACh) into the neuromuscular junction (NMJ) where it binds to postsynaptic nicotinic acetylcholine receptors (nAChRs). Spinal motoneurons projecting from the ventral root of the spinal cord synapse with muscle fibers, forming end-plate junctions where nAChRs are clustered and excitatory transmission occurs (Dale et al. 1936; Katz 1971). Since this classical description of the NMJ, neurotransmitters other than ACh have been located in motoneurons and at the NMJ (Malomouzh et al. 2003; Pinard et al. 2002; Waerhaug and Ottersen 1993). One neurotransmitter that may be released from the motoneuron terminals along with ACh is glutamate (Barthelemy-Requin et al. 2000; Berger et al. 1995; Malomouzh et al. 2003; Waerhaug and Ottersen 1993). Despite the lack of knowledge surrounding the physiological actions of glutamate at the NMJ, there is a growing body of literature that suggests its presence there (Chen et al. 1998; Fu et al. 1995; Grozdanovic and Gossrau 1998; Liou et al. 1996; Pinard et al. 2002).

Glutamate is the predominant excitatory neurotransmitter in the vertebrate CNS. Glutamate-mediated transmission is regulated by ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors (iGluRs) consist of three main groups: N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid receptors (AMPARs), and kainic acid receptors (KARs). NMDARs require glycine as a coagonist, are Mg2+ sensitive and are permeable to K+, Na+, and Ca2+. They are composed of a combination of two NMDAR subunits 1 (NR1) and two NMDAR subunits 2A-D (NR2A-D) (Hollmann and Heinemann 1994), and in some cases, may also contain NR3 subunits (Nishi et al. 2001; Perez-Otano et al. 2001). KARs and AMPARs are often grouped together as one receptor type (non-NMDARs) due to their similar pharmacological and kinetic properties. The majority of AMPA and KA receptors are monovalent cation channels that conduct Na+ and K+, although, some subunit compositions of KA and AMPA receptors are also permeable to Ca2+ (Hollmann and Heinemann 1994). All three receptor types can be found pre- or postsynaptically in the CNS (MacDermott et al. 1999), where they have been reported to be involved in synaptic plasticity phenomena such as long-term potentiation or depression (LTP and LTD) as well as a number of other cellular mechanisms (for review, see Grant and O'Dell 2001; Luscher et al. 2000).

The zebrafish (Danio rerio) has recently gained increasing popularity for developmental studies due to the ease of genetic manipulation, the transparent nature of embryos and larvae, prolific reproduction, and rapid development. Although some developmental changes continue to occur into early adulthood (van Raamsdonk et al. 1983), the motor network is in place by ~30 h postfertilization (hpf) (Drapeau et al. 2002). The body musculature of embryos and young larvae consists of superficial slow red fibers and medial fast white fibers (van Raamsdonk et al. 1978; Waterman 1969). A single layer of red fibers runs parallel to the body while multiple layers of white fibers lie underneath the red fibers at an oblique angle, thereby allowing for visual distinction between the two muscle types (van Raamsdonk et al. 1982). There are two classes of motoneurons within the spinal cord (primary and secondary) that have different functional properties (Liu and Westerfield 1988; Westerfield et al. 1986) and different patterns of innervation (de Graaf et al. 1990; van Raamsdonk et al. 1983). In each body hemi-segment, there are three primary motoneurons and...
20–25 secondary motoneurons (Myers et al. 1986; Westerfield et al. 1986). White muscle fibers are electrically coupled (Buss and Drapeau 2000) and polyinnervated by one primary and two to three secondary motoneurons, while red fibers are only innervated by secondary motoneurons (de Graaf et al. 1990; Liu and Westerfield 1988).

Like other vertebrates, neuromuscular transmission in zebrafish occurs through the action of Ach interacting with nAChRs (Liu and Westerfield 1992; Nguyen et al. 1999). The presence of glutamate at vertebrate NMJs has been well established (Berger et al. 1995; Chen et al. 1998; Fu et al. 1995; Grozdanovic and Gossrau 1998; Liou et al. 1996; Malomouzh et al. 2003; Pinard et al. 2002; Waerhaug and Ottersen 1993); however, there is some controversy over whether iGluRs are located on the postsynaptic or on the presynaptic membrane. It has also been suggested that a developmental switch from expression of iGluRs to metabotropic glutamate receptors occurs (Liou et al. 1996). Because the zebrafish is an excellent model to investigate neuromuscular transmission in vivo, we thought its use might help to resolve some of the controversies surrounding the presence of presynaptic iGluRs.

We investigated the presence of presynaptic iGluRs through immunohistochemistry, electrophysiological, and pharmacological manipulation of the NMJ. Immunohistochemistry suggests the presence of NR2A subunits in association with the axons of primary motoneurons, whereas electrophysiology suggests a role for glutamate receptors in facilitation of Ach release during early development.

Methods

Animals

All use of animals adhered to guidelines established by the University of Alberta and the Canadian Council of Animal Care. Adult zebrafish (Carolina Biological, Burlington, NC) were spawned daily and fertilized eggs were collected and kept at 28.5°C. Prior to all protocols larval zebrafish were acclimated in 0.02% MS-222.

Immunohistochemistry

Whole zebrafish larvae (4–6 days postfertilization, dpf) were fixed for 2 h in 2% paraformaldehyde and permeabilized with 4% Triton X-100 for 30 min. Permeabilized animals were incubated in primary antibody (z-1 mouse anti-zebrafish 1:50, University of Oregon) to identify primary motoneurons, and anti-NR2A (Calbiochem, EMD Biosciences, San Diego, CA) for 48 h at 4°C on a shaker. Fish were washed in phosphate-buffered saline for 3 h every 15 min, then mounted in glycerol for viewing. Imaging was done using a Hamamatsu C4742–95 CCD camera mounted on a Leica DMLFA epifluorescence microscope with water-immersion x20 and x40 objectives. Image capture was done using Openlab software (Improvision, Lexington, MA).

Immunoprecipitation and immunoblotting

Adult zebrafish brains were rapidly dissected in ice-cold physiological saline, placed in sucrose buffer [containing (in mM) 320 sucrose, 0.01 EDTA, and 10 Tris-HCl, pH 7.4] containing protease inhibitors (3 mM PMSF, 40 μM leupeptin, 4 μM pepstatin A, and 0.4 mg/ml aprotinin) and immediately placed on dry ice. Whole brains were homogenized by hand with an ependorf mortar and pestle and centrifuged at 1,000 g for 15 min. The supernatant was removed and the pellet was resuspended in sample buffer containing 2% SDS and protease inhibitors and boiled for 10 min to solubilize membrane proteins. Protein quantification was performed using the Lowry Protein Assay (Bio-Rad, Hercules, CA). For immunoprecipitation, the NR2A antibody (8 μg/ml; Calbiochem, EMD Biosciences, San Diego, CA) was added to the resuspended pellet (500 μg protein) and incubated overnight at 4°C, followed by 30 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. Immunoprecipitates were washed, eluted, subjected to SDS-PAGE (8%), according to the method of Laemmli (1970), and transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked in blocking buffer (5% skim milk powder, 0.3% Tween-20 in TBS (TBS-T)) for 1 h at room temperature and incubated in anti-NR2A primary antibody (1:800) overnight at 4°C. Membranes were washed several times in TBS-T and incubated in HRP secondary antibody (1:200,000 goat anti rabbit IgG, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature on a shaker. Signals were detected with enhanced chemiluminescence (SuperSignal West Femto, Pierce, Rockford, IL) and developed on X-ray film.

Electrophysiology

Anesthetized 4- to 6-day-old zebrafish larvae were pinned through the notochord to silicon elastomer (Sylgard)-lined recording dishes and perfused with extracellular saline containing (in mM) 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 HEPES, 10 glucose, and 0.001 tetrodotoxin (TTX, Tocris, Avonmouth, UK). A region of skin was removed to allow access to the axial muscles. After removal of the skin, red muscle fibers were gently removed with a micropipette. Red fibers run parallel to the body, whereas white fibers lie at an oblique angle, allowing for visual distinction between the two muscle types (van Raamsdonk et al. 1982). Whole cell patch-clamp recordings were performed on axial white fibers in voltage-clamp mode (Hamill et al. 1981). For investigation of (NMDA receptor properties, Mg²⁺-free extracellular solution was used. The Mg²⁺-free recording solution consisted of equimolar NaCl replacing MgCl₂. The pipette solution contained (in mM) 130 CsCl, 2 MgCl₂, 10 HEPES, 10 EGTA, 2 CaCl₂, 4 Na₂ATP, and 0.4 LiGTP. Polished pipettes were pulled from thin-walled glass (World Precision Instruments, Sarasota, FL) and had tip resistances of 1–2.5 MΩ, resulting in whole cell series resistance values of 1.5–5 MΩ. Series resistances were monitored carefully during recordings and abandoned if a change of >15% occurred. All series resistances were compensated by 90–95%, and fibers were clamped at ~65 mV throughout all recordings. Whole cell recordings were performed with an Axopatch 200B and captured with pClamp 8.1 software. Data were sampled at 100 kHz and low-pass filtered at 10 kHz. Recording analysis was performed using Axograph software.

After rupture of the membrane, recordings were allowed to stabilize prior to initiation of recording. After stabilization, the next 2 min of each recording was used as a measure of the baseline frequency of spontaneous release. Miniature endplate current (mEPC) frequency in the presence of agonists was determined at the visually identified peak frequency or after 6–6.5 min of agonist perfusion. Recordings of mEPCs were analyzed for any kinetic changes associated with agonist perfusion, including rise time, decay time, peak amplitude, proportions of small (<400 pA) and large (>400 pA) events (as determined through amplitude distribution analysis), and frequency of spontaneous release. Kainic acid, AMPA, NMDA, and glutamate (Sigma) were used at these concentrations. Vehicle controls were completed for 0.1% DMSO. Because of the high stringency of the recording conditions, we were unable to maintain high-quality recordings for only 12–15 min, and it was difficult to achieve washout of the agonists in most of our experiments. However, in the few recordings that were held for ~20
min, we were able to washout the agonists, and in these cases, we observed a reduction in the mEPC frequency back to approximately baseline levels.

Agonist specificity was investigated through antagonist application. Antagonists were bath applied for 2 min prior to initiation of agonist perfusion. The competitive antagonist d-(-)-2-amino-5-phosphonopentanoic acid (AP-5; 50 μM; Tocris, Avonmouth, UK) was used to block NMDA receptor activation (Ali et al. 2000). Nonspecific [6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), 50 μM; Sigma] and specific non-NMDAR antagonists were used to investigate the contribution of AMPARs and KARs. (2S,4R)-4-methylglutamate (Sym 2081, Sym; 10 μM; Sigma, St Louis, MO) acts specifically on KARs to desensitize them and prevent their activation (Donevan et al. 1998; Zhou et al. 1997). 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466, 50 μM, Sigma) was used as a specific antagonist for AMPARs (Cossart et al. 2002; Donevan and Rogawski 1993). To investigate potential effects of glutamate, the general glutamate transport blocker d-threo-beta-benzylxoxaspartate (d-TBOA; 200 μM; Tocris, Avonmouth, UK) was used (Jabaudon et al. 1999; Waagepetersen et al. 2001). d-tubocurarine (10 μM; Sigma) was used to block nAChRs.

Local perfusion was used to directly apply agonist onto the NMJ. To detect the perfusate, domestic food coloring (5%) was added to the agonist solution. To ensure that the perfusate was removed quickly over the spinal cord was ever visible. To detect the perfusate, domestic food coloring (5%) was added to the perfusate was clearly visible with bath perfusion pipette was placed at the horizontal midline of the larvae flowing ventrally away from the midline.

### Results

#### Immunohistochemistry

Whole-mount preparations of 4–6 dpf zebrafish reveal peripheral staining in the axial musculature with NR2A antibodies (Fig. 1, A1 and B1; n = 6). To identify which peripheral axons were expressing the NMDAR subunits, we performed double-labeling experiments using the NR2A antibody and the zn-1 antibody that specifically targets primary motoneurons in larval zebrafish (Myers et al. 1986). The NR2A staining appears to be associated with motoneurons, as shown by colocalization with zn-1 antibody staining (Fig. 1, A and B). Staining with anti-NR2A reveals what appears to be one common, ventral exit point from the spinal cord, after which axons project to the dorsal, ventral, and mid musculatures (Fig. 1, A1 and B1). The NR2A staining correlates well with previously published images of primary motoneuron projections (Behra et al. 2002; Eisen and Melançon 2001; Myers et al. 1986; Westerfield et al. 1986). The three primary motoneurons, caudal (CaP), middle (MiP), and rostral (RoP), project to the ventral, dorsal, and mid axial musculature, respectively (Myers et al. 1986). NR2A staining colocalizes with zn-1 staining in dorsal, ventral and mid musculature, (Fig. 1, A3 and B3), suggesting that NMDARs containing the NR2A subunit are associated with the axons of primary motoneurons.

Because glial cells are known to express glutamate receptors...
(Porter and McCarthy 1996; Zhou and Kimelberg 2001), it was possible that the observed staining was associated with Schwann cells along motoneurons. We therefore performed immunostains using anti-NR2A antibodies on animals at an age at which myelination of peripheral axons has not yet occurred (Brosamle and Halpern 2002). NR2A staining in 26 hpf embryos (Fig. 1C) reveals similar staining of projections as seen in older animals, indicating that NR2A subunits are associated with the motoneurons and not Schwann cells.

Negative controls were performed by excluding primary antibody from tissue incubations. Incubation in secondary antibodies resulted in dull homogenous staining throughout (Fig. 1D). These controls support the specificity of the primary antibodies used. Antibody staining for other NR2 subunits was inconclusive. In addition we tested antibodies targeted to some AMPA and kainate receptor subunits (GluR1, 2/3, GluR5, 6, 7, and KA2), but the results were also inconclusive.

Immunoprecipitation and immunoblotting

To determine the specificity of the NR2A antibodies in zebrafish, we immunoprecipitated membrane proteins from adult zebrafish brain to first concentrate the protein and then immunoblotted with anti-NR2A. We used adult brains to obtain tissue with high levels of NMDARs. Results clearly indicate a single protein band of 165–170 kDa (NR2A) that is detected by the anti-NR2A antibody (Fig. 1E; n = 7), confirming the specificity of the antibodies. Negative controls were performed in which the primary antibody (anti-NR2A) was omitted from the immunoprecipitation step. NR2A subunits were not detected in these control experiments. Positive controls were performed by running rat brain homogenate (Santa Cruz, Santa Cruz, CA) on SDS-PAGE. Taken together, these results suggest that anti-NR2A detects the NR2A subunits that appear to be associated with the axons of developing primary motoneurons.

Electrophysiology

LOCAL PERFUSION. We next decided to use electrophysiology to investigate the presence of axonal or presynaptic NMDARs at the NMJ and to confirm the immunohistochemical results. We hypothesized that activation of these peripheral NMDARs will likely depolarize the axons and will influence the release of ACh at the endplate. We therefore attempted to record mEPSCs from muscle fibers in the presence of glutamate receptor agonists. This was done using a relatively high bath flowrate (~3 ml/min), flowing from dorsal to ventral over the preparation. The agonist perfusion pipette was placed at or ventral to the horizontal midline of the fish, also perfusing from dorsal to ventral. Whole cell patch-clamp recordings were performed on white muscle at the zebrafish NMJ. We recorded mEPSCs with a high gain (Saha et al. 1994), and we therefore used a relatively high concentration of NMDA to ensure activation of axonal or presynaptic receptors. Separate control experiments were performed whereby we perfused saline or 0.1% DMSO (vehicle) over the preparations for the duration of the recording. We recorded solely from white muscle because the immunohistochemistry suggested that NMDARs were associated with primary and not secondary motoneurons. Application of 500 μM NMDA caused a significant (P < 0.05) rise in the frequency of spontaneous ACh release that was 346.7 ± 145.2-fold (n = 4). This increase was blocked by bath application of 50 μM AP-5 (Fig. 2A; n = 4). Because NMDARs appeared to be present at the NMJ, we tested for the possible presence of AMPARs and KA receptors. Perfusion with KA (250 μM) induced responses that were significantly (P < 0.05) greater than saline controls by 165.5 ± 34.9-fold (n = 4) and that were prevented with the non-NMDAR antagonist CNQX (50 μM; Fig. 2B; n = 4). Graphical representations of raw traces (Fig. 2, C and D) illustrate the significant increase in mEPSC frequency in the presence of KA and NMDA (P < 0.05). There was no increase in mEPSC frequency in any of the control experiments (P > 0.05). The observed increase in mEPSC frequency during local application of agonists directly onto the trunk musculature suggests that glutamate receptors are likely associated with the axons of motoneurons in the peripheral nervous system.

BATH PERFUSION. During local perfusion, multiple factors alter agonist concentration at the endplate, such as perfusate flow rate, bath flow rate, and distance of the perfusion pipette from the endplate. These properties vary from experiment to experiment and make accurate calculations of agonist concentrations at the NMJ very difficult. For this reason, further, more detailed kinetic and pharmacological analyses were performed on recordings taken with the more uniform bath application. Whole cell voltage-clamp recordings from white axial muscle were performed in the presence of NMDA and the specific NMDA receptor antagonist AP-5 (50 μM; Fig. 3; n = 6–8). Representative traces from these recordings illustrate a large increase in frequency of spontaneous ACh release in the presence of NMDA compared with vehicle control experiments (Fig. 3A). The specificity of the bath applied agonists was investigated through application of specific NMDA and non-NMDA receptor antagonists. Bath application of the competitive antagonist to NMDA receptors, AP-5 (50 μM) prevented the NMDA-induced (100 μM) increases in frequency of spontaneous release (Fig. 3A). Application of 10, 50, and 100 μM NMDA allowed for production of a dose-response relationship (Fig. 3B). Bath application of 50 and 100 μM NMDA induced significant increases in the frequency of spontaneous ACh release (P < 0.05). However, the response at 50 μM was variable, therefore 100 μM NMDA was used for further pharmacological studies. Averaged values for individual recordings reveal a significant (P < 0.05) increase in frequency of spontaneous ACh release in the presence of 100 μM NMDA (Fig. 3C). This increase of 77.0 ± 41.5-fold was prevented from occurring in the presence of 50 μM AP-5. We observed no change in mEPSC frequency when AP-5 was applied alone (P > 0.05).

We also investigated the potential presence of AMPA and KA receptors at the NMJ. Representative traces from recordings performed in the presence of each pharmacological application illustrate the AMPA-induced increase in spontaneous ACh release (Fig. 4A). Application of 10, 50, and 100 μM AMPA revealed significant (P < 0.05) effects at concentrations of 50 and 100 μM (Fig. 4B; n = 4–7). For these applications, 0.1% DMSO was used as a vehicle control be-
cause DMSO was used to solubilize AMPA. Averages of agonist and antagonist applications reveal a significant increase of 114.7 ± 37.1-fold (n = 6) in the frequency of spontaneous ACh release in the presence of 50 μM AMPA compared with vehicle control experiments in which there was no change in mEPC frequency over the course of the experiment (Fig. 4C).

The noncompetitive AMPA receptor-specific antagonist GYKI 52466 (50 μM) prevented the effect of AMPA (50 μM) on spontaneous release (P < 0.05). The frequency of spontaneous ACh release was unaltered in the presence of GYKI 52466 alone (P > 0.05).

Because we had determined that AMPARs are present at the larval zebrafish NMJ, we wanted to investigate the contribution of KARs to this response. To do this, we utilized the specific KAR antagonist Sym 2081 (Sym), which desensitizes KARs at low concentrations (Cossart et al. 2002; Zhou et al. 1997). Representative recordings illustrate the KA-induced increase in spontaneous ACh release compared with controls (Fig. 5A). Concentration of 10, 50, and 100 μM KA were tested. No significant response to 10 μM KA was observed; however, in the presence of 50 and 100 μM KA, significant increases (P < 0.05) in mEPC frequency were observed (Fig. 5B; n = 4–6). The more moderate increase (150.7 ± 56.9-fold; n = 5) in the presence of 50 μM KA was chosen for complete pharmacological investigation. We found that Sym 2081 (10 μM) had no effect on the KA-induced increase in mEPC frequency (Fig. 5, A and C; n = 5). In addition, Sym 2081 (10 μM) alone had no effect on the frequency of spontaneous release (P > 0.05; n = 4). To investigate whether KA was nonspecifically activating AMPAR receptors, GYKI 52466 (50 μM) was applied in the presence of KA (50 μM). GYKI 52466 prevented the KA-induced increase in frequency of spontaneous ACh release (P < 0.05), suggesting that KA acts nonspecifically to activate peripheral AMPARs.

Although synthetic iGluR agonists allow for pharmacological investigation of individual receptor types, the endogenous neurotransmitter is glutamate, and we therefore tested whether glutamate was capable of affecting the mEPC frequency in the same manner as the other iGluR agonists.

Representative traces are shown from recordings done in the presence of 1 mM glutamate, saline controls, and uptake inhibitors (Fig. 6A). In the presence of 1 mM glutamate, no significant (P > 0.05) change in frequency of spontaneous release was observed (Fig. 6; n = 5). However, in the presence of the general glutamate uptake inhibitor d-TBOA (200 μM) with 1 mM glutamate, significant (P < 0.05) increases in
FIG. 3. Frequency of spontaneous ACh release is increased during bath application of 100 μM NMDA. Representative 30-s traces of whole cell voltage-clamp recordings from axial white muscle (A). Traces are for bath application of Mg$^{2+}$-free saline vehicle controls, 100 μM NMDA, 100 μM NMDA + 50 μM AP-5, and 50 μM AP-5. The NMDA-induced increase in spontaneous ACh release is blocked by co-application of 50 μM AP-5. Bath application of 10, 50, and 100 μM NMDA resulted in a partial dose-response curve (B). Pooled data for spontaneous ACh release (seen in A) with averages and SE plotted as the normalized frequency (C). No significant difference in frequency of spontaneous ACh release is apparent in the presence of 100 μM NMDA + 50 μM AP-5 or 50 μM AP-5 alone when compared with Mg$^{2+}$-free saline vehicle controls. In the presence of 100 μM NMDA, a significant increase in spontaneous ACh release is observed. Significance indicated by *, $P < 0.05$; $n = 6–8$.

FIG. 4. Frequency of spontaneous ACh release is increased during application of 50 μM AMPA. Representative 30-s traces of whole cell voltage-clamp recordings from axial white muscle (A). Traces are for bath application of 0.1% DMSO vehicle controls, 50 μM AMPA, 50 μM AMPA + 50 μM 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-2,3-benzodiazepine (GYKI 52466), and 50 μM GYKI 52466. The AMPA-induced increase in spontaneous ACh release is blocked by co-application of 50 μM GYKI 52466. Bath application of 10, 50, and 100 μM AMPA resulted in a partial dose-response curve (B). Results show large and significant increases in frequency of spontaneous ACh release at both 50 and 100 μM AMPA. Pooled data for spontaneous ACh release (seen in A) with averages and standard error plotted as the normalized frequency (C). No significant difference in frequency of spontaneous ACh release is apparent in the presence of 50 μM AMPA + 50 μM GYKI 52466 or 50 μM GYKI 52466 alone, when compared with 0.1% DMSO vehicle controls. In the presence of 50 μM AMPA, a significant increase in spontaneous ACh release is observed (*, $P < 0.05$; $n = 4–7$).
MEPC frequency were observed (Fig. 6; n = 6). During application of 200 μM TBOA alone, no change in MEPC frequency was observed (Fig. 6; n = 4).

Last, to ensure that the MEPCs observed during whole cell muscle recordings were due strictly to AChR activation, we used curare to block the iGluR agonist-induced rise in MEPC frequency. Curare (10 μM) blocked all MEPCs occurring in the presence of NMDA (100 μM) and KA (50 μM), confirming that the observed MEPCs are due to ACh release and nAChR activation (Fig. 7; n = 3). Taken together, the agonist and antagonist data for local and bath perfusion suggest the presence of NMDARs and AMPARs in the peripheral nervous system, most probably associated without the motoneuron axons and possibly even presynaptically at the NMJ.

MEPC KINETICS. Bath application of glutamate receptor agonists provided a consistent method of investigating the electrophysiological influence of glutamate receptors on spontaneous ACh release. The present results strongly suggest that the NMDA and AMPARs are located along motoneuron axons or presynaptically at the NMJ or both. However, we did not know if activation of iGluRs also altered the properties of AChR kinetics in addition to affecting transmitter release. We therefore investigated MEPC kinetics in the presence and absence of iGluR agonists.

Analysis of recordings revealed the presence of multiple types of MEPCs (Fig. 8A). Of these pools of events, it is likely that large amplitude events with fast rise times are MEPCs from mature synapses on the muscle being recorded from. A second type of event is of a smaller amplitude (400 pA) and displays relatively long rise times (Fig. 8A). It is probable that these events occur on cells other than the ones being recorded from and are highly filtered due to electrical coupling (Buss and Drapeau 2000; Nguyen et al. 1999). The third type of MEPCs have small amplitudes and fast rise times and may result from activation of immature synapses or simply from synapses with fewer numbers of nAChRs. It has previously been reported that two distinct pools of MEPCs can be recorded at the zebrafish NMJ (Nguyen et al. 1999). Nguyen et al. (1999) also found slow and fast rise time events with small amplitudes, although these were not reported as separate pools.
Amplitude distributions clearly illustrate a bimodal distribution of mEPCs (Fig. 8, B and C) that is not altered in the presence of NMDA or AMPA. It is evident that there is a pool of events that has a mean amplitude of ~200 pA with the division between event pools ~400 pA (Fig. 8, B and C). The large (~400 pA)-amplitude events have a normal distribution with a mean value of 1,422 ± 110 pA (n = 7 experiments) in the
presence of saline that is almost identical to the average amplitude of mEPSCs in the presence of NMDA (1,433 ± 139 pA; n = 8 experiments). The average amplitude of large events is 1,455 ± 34 and 1,340 ± 140 pA in the presence of 0.1% DMSO and AMPA respectively (n = 6 experiments). Neither of these pairs of values are significantly different. Rise times versus amplitude plots clearly illustrate the different pools of mEPSCs (Fig. 8, D and E). Small-amplitude events range in rise times from <0.1 to ~0.4 ms, whereas larger-amplitude events have fast rise times between 0.05 and 0.09 ms.

Although there was no apparent change in mEPSC amplitude of the large events in the presence of glutamate receptor agonists, it was possible that the pharmacological treatments were altering the ACh receptor kinetics. We analyzed the average of all events in the 0- to 2-min baseline period by first dividing them into small and large event pools with a 400-pA division, for each experiment, and obtained values for the rise time, decay time (τ), peak amplitude, and proportion of all events that were large (>400 pA). These values were then compared with the average of all events occurring during agonist application, and the comparisons for large events are shown as ratios in Fig. 9, whereas the comparisons for small events are shown as ratios in Fig. 10. Analysis of agonist application data revealed no significant difference in the data for rise time, decay time, peak amplitude, or proportion of large (>400 pA)-amplitude events in the presence of NMDA, AMPA, KA, or glutamate (Fig. 9). The lack of difference in any of the above properties indicates that glutamate receptor agonists do not act postynaptically but rather on receptors that are located at some point presynaptic to the NMJ. This may be either at the level of the motoneuron axons or at the presynaptic terminal or both. These results also suggest that because the proportion of large events does not change in the presence of the iGluR agonists, then neither does the proportion of small events because these are reciprocal values.

DISCUSSION

We have combined immunohistochemistry and electrophysiology to demonstrate the presence of iGluRs at the developing zebrafish NMJ. This is the first study to show a specific NR2 subunit associated with axons of motoneurons in the periphery. Finally, our results suggest that the NMDARs may be localized to primary but not secondary motoneurons in the zebrafish. Although this is the first report of this occurring in zebrafish, iGluRs have been suggested to be present at the NMJ of Xenopus and rat by a number of different groups (Barthelemy-Requin et al. 2000; Fu et al. 1995; Grozdanovic and Gossrau 1998; Liou et al. 1996; Malomouzh et al. 2003; Waerhaug and Ottersen 1993). Many of these reports have focused primarily on NMDARs; however, we also suggest the presence of non-NMDARs at the NMJ. Our use of specific non-NMDAR antagonists provides support for the presence of AMPARs but not KARs. When taken together, our results strongly suggest the presence of iGluRs in association with motoneuron axons outside the CNS. Furthermore, the abolishment of all mEPSCs in the presence of curare suggests that iGluRs are not associated with muscle fibers.

iGluRs at the NMJ

Data presented here suggest the presence of NMDARs in association with the axons of motoneurons and possibly also with presynaptic structures. The precise location of the iGluRs has yet to be thoroughly mapped out. Staining in 26 hpf embryos was similar to what was present at 4–6 dpf, indicating that NR2A subunits are associated with motoneuron axons and not glia. Although glial cell precursors are present in embryonic zebrafish (Gilmour et al. 2002), and may be associated with axon pathfinding and motoneuron differentiation (Gilmour et al. 2002; Park et al. 2002), it has been shown that myelination does not occur until ~4 dpf (Brosamle and Halp-
NMDARs are the only Ca$^{2+}$ which has been shown to be functionally important when Mg$^{2+}$ block (Hollmann and Heinemann 1994; Kirson et al. 1999). Other differences in NR2 subunits include responses to both glycine (Regalado et al. 2001) and glutamate (Chen et al. 2001) and mechanisms of modulation (Krupp et al. 1996). It has also been suggested that variations in surface expression pattern occur between subunits. Barria and Malinow (2002) suggest that NR2A subunits require ligand binding to be inserted into the membrane, whereas NR2B subunits do not. This may provide a mechanism that allows for selective surface expression of axonal NMDARs. NR2A subunits also have shorter decay times (Cathala et al. 2000), which may be important for faster on and off responses to changes in ligand availability. Also, NR2A are more permeable to Ca$^{2+}$, which has been shown to be functionally important when NMDARs are the only Ca$^{2+}$-permeable receptor at a synapse (Lei and McBain 2002). Any of the preceding subunit-specific characteristics could be important for the selective expression of NR2A subunits at the NMJ.

Recently, the presence of glutamate at the NMJ has been suggested in mammalian preparations (Barthelemy-Requin et al. 2000; Waerhaug and Ottersen 1993). There is also evidence that glutamate and ACh can be coreleased from vertebrate synaptosomes (Vyas and Bradford 1987). It does, however, seem most likely that glial cells surrounding the NMJ are involved in glutamate uptake and release. Although many types of glutamate transporters are probably present at the NMJ, previously published literature suggests that glial transporters are primarily responsible for glutamate clearance (Bergles and Jahr 1998; Danbolt 2001). The lack of effect in the presence of glutamate alone suggests that glutamate is either being taken up or being metabolized (Danbolt 2001; Matthews et al. 2000). This strongly suggests that glutamate is being removed from the site of action at a rate that prevents activation of iGluRs. The lack of effect of TBOA in the absence of applied glutamate suggests that little glutamate is released spontaneously. It is possible that endogenously released glutamate is capable of activating the receptors under normal swimming conditions when motoneurons are firing at frequencies $\leq 30$ Hz. Rapid removal of glutamate reflects the tight regulation of this neurotransmitter at the synaptic cleft to prevent excitotoxicity and diffusion (Asztely et al. 1997).

It is possible that glutamate is released from glial cells surrounding the NMJ. Although this has not been reported to occur at the NMJ, glial cells of the CNS are thought to have vesicular machinery (Calegari et al. 1999) and are able to release glutamate in a nonpathological, vesicular manner (Araque et al. 2000; Pasti et al. 2001). This release could be mediated through a feedback mechanism involving signaling molecules like NO or perhaps through muscarinic AChRs, which are known to be present on glial cells surrounding the NMJ (Robitaille et al. 1997).

Investigation of non-NMDARs suggests the presence of AMPARs, but not KARs, at the NMJ. This is suggested through the use of specific AMPAR and KAR antagonists (GYKI 52466 and Sym 2801 respectively). Because EC$_{50}$ values for activation of AMPARs by KA have been reported to be between 56 and 150 $\mu$M (Jayaraman 1998; Lambolez et al.
The concentrations of KA that we used could most likely activate both AMPA and kainate receptors. Prevention of the KA-induced increase in mEPC frequency by GYKI 52466 supports indiscriminant non-NMDAR activation by KA.

Although antibody staining suggests that only primary motoneurons express NMDARs, both small and large mEPCs increase in frequency during iGluR agonist application. One reason for this may be that both large and small mEPCs are due to spontaneous release from primary motoneurons. Another is that small mEPCs may be due to the presence of immature synapses along different branches of primary motoneurons. It is also possible that secondary motoneurons express NR2 subunits other than NR2A, and therefore were not detected. Whatever the cause, both small and large mEPCs increase in frequency during iGluR agonist applications, with the relative proportion of each remaining the same.

Role of iGluRs at the NMJ

Glutamate is the major excitatory neurotransmitter in the vertebrate CNS. During development, glutamate and its receptors have many important roles in axonal outgrowth (Metzger et al. 1998), learning and memory, (Atwood and Wojtowicz 1999) and synaptic strengthening (Gu et al. 1996). Previously reported work on glutamate receptors at the NMJ has suggested that Ca$^{2+}$ may play roles in developmental changes in the presynaptic terminal (Fu et al. 1995). Aside from this, iGluRs may also alter muscle contraction (Koyuncuoglu et al. 1998) or in strengthening postsynaptic components of the NMJ. It is known that pruning of motoneurons is activity dependent and that postsynaptic AChR clustering is also dependent on the presence of the presynaptic motoneuron (Luo et al. 2002; Sanes and Lichtman 1999). If activation of iGluRs increases ACh release and subsequent activity at the developing NMJ, then they could play a role in both pre- and postsynaptic modifications.

Ionotropic glutamate receptors at the NMJ may be replaced by mGluRs at a later developmental stage (Lio et al. 1996). This may reflect a developmental change in requirements for glutamate at the NMJ. Initial excitatory actions of glutamate at the NMJ. Initial excitatory actions of glutamate may play a role in neuronal and muscular development in the zebralshad embryo. Nat Neurosci 5: 111–118, 2002.


GLUTAMATE RECEPTORS AT THE ZEBRAFISH NMJ


