

Developmental Characteristics of AMPA Receptors in Chick Lumbar Motoneurons

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ABSTRACT: Ca^{2+} fluxes through ionotropic glutamate receptors regulate a variety of developmental processes, including neurite outgrowth and naturally occurring cell death. In the CNS, NMDA receptors were originally thought to be the sole source of Ca^{2+} influx through glutamate receptors; however, AMPA receptors also allow a significant influx of Ca^{2+} ions. The Ca^{2+} permeability of AMPA receptors is regulated by the insertion of one or more edited GluR2 subunits. In this study, we tested the possibility that changes in GluR2 expression regulate the Ca^{2+} permeability of AMPA receptors during a critical period of neuronal development in chick lumbar motoneurons. GluR2 expression is absent between embryonic day (E) 5 and E7, but increases significantly by E8 in the chick ventral spinal cord. Increased GluR2 protein expression is correlated with parallel changes in GluR2 mRNA in the motoneuron

pool. Electrophysiological recordings of kainate-evoked currents indicate a significant reduction in the Ca^{2+} -permeability of AMPA receptors between E6 and E11. Kainate-evoked currents were sensitive to the AMPA receptor blocker GYKI 52466. Application of AMPA or kainate generates a significant increase in the intracellular Ca^{2+} concentration in E6 spinal motoneurons, but generates a small response in older neurons. Changes in the Ca^{2+} -permeability of AMPA receptors are not mediated by age-dependent changes in the editing pattern of GluR2 subunits. These findings raise the possibility that Ca^{2+} influx through Ca^{2+} -permeable AMPA receptors plays an important role during early embryonic development in chick spinal motoneurons. © 2007 Wiley Periodicals, Inc. *Develop Neurobiol* 67: 1419–1432, 2007

Keywords: motoneuron; AMPA receptors; GluR2 expression; development

INTRODUCTION

In the central nervous system, excitatory synaptic transmission is mainly generated by activation of ionotropic glutamate receptors. Based on their pharmacological and molecular properties, ionotropic glutamate receptors can be broadly divided into NMDA, AMPA, and kainate receptors (reviewed by Nakanishi, 1992). AMPA and kainate receptors, com-

monly known as non-NMDA receptors, mediate fast synaptic transmission in central synapses. Because of their intrinsic Ca^{2+} permeability, NMDA receptors were originally thought to be the sole source of Ca^{2+} influx through glutamate receptors; however, non-NMDA receptors also allow a significant influx of Ca^{2+} ions. The Ca^{2+} permeability of AMPA receptors, in particular, is determined by the insertion of one or more edited GluR2 subunits, which significantly reduces the permeability of Ca^{2+} through the channel (Burnashev et al., 1992; Jonas et al., 1994; Brorson et al., 1999). Editing of GluR2 subunits involves a posttranscriptional modification in the pore-forming membrane domain (or M2 domain), which results in the substitution of the amino acid glutamine (Q) for arginine (R) (Lomeli et al., 1994; Carlson et al., 2000). The presence of a positively

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charged arginine in the pore-forming domain of GluR2 reduces the Ca^{2+} permeability of AMPA receptors. GluR2 editing does not appear to be developmentally regulated, and nearly 100% of GluR2 receptor subunits present are fully edited at any given developmental stage (Longone et al., 1998; Carlson et al., 2000). Activation of embryonic GluR2-lacking AMPA receptors provides a significant influx of Ca^{2+} ions that could potentially regulate various aspects of neuronal development, including cell migration, dendritic outgrowth, and cell death (Metzger et al., 1998; Fryer et al., 1999; Catsicas et al., 2001; Poluch et al., 2001).

Previous work has shown that chick lumbar motoneurons (LMNs) express functional non-NMDA receptors at early stages of development. AMPA and kainate activate a common receptor in motoneurons isolated between embryonic (E) day 5 and 6 (O'Brien and Fischbach, 1986; Zorumski and Yang, 1988; Temkin et al., 1997). Moreover, kainate application to a spinal cord preparation induces bursting activity as early as E4 (Milner and Landmesser, 1999). It is possible that kainate stimulates bursting activity by acting directly on spinal motoneurons or activating other spinal cord neurons. Interestingly, activation of ionotropic glutamate receptors is not required for the generation of early network activity in the chick spinal cord (between E4 and E6), which is primarily dependent on cholinergic and GABAergic synaptic neurotransmission (Milner and Landmesser, 1999; Hanson and Landmesser, 2003). However, glutamate activation of AMPA/kainate receptors seems to play a minor role in regulating the shape of spontaneous bursts at early stages of network activity (Milner and Landmesser, 1999). Although glutamatergic neurotransmission does not drive early spinal cord activity in chick embryos, activation of both NMDA and AMPA receptors is critical for the generation of spontaneous activity by E10 (Chub and O'Donovan, 1998). This developmental switch in the nature of the excitatory neurotransmitter driving spontaneous network activity appears to occur around E8 in chick embryos (Milner and Landmesser, 1999). The early appearance of AMPA/kainate receptors in the chick spinal cord suggests that they could play an important role in regulating other aspects of neuronal differentiation. Whether the functional role of AMPA receptors in chick LMNs is related to changes in the Ca^{2+} permeability of these receptors remains to be determined. However, a significant developmental switch in the Ca^{2+} permeability of AMPA receptors has been shown in other developing neurons (Gleason and Spitzer, 1998; Eybalin et al., 2004).

The aim of the present study was to investigate the presence of Ca^{2+} permeable AMPA receptors in

developing chick LMNs and to assess changes in GluR2 expression in the chick spinal cord by combining whole-cell patch-clamp recording, fluorescence calcium imaging, immunoblot, and real-time PCR analysis. Our present results support the conclusion that chick LMNs express Ca^{2+} -permeable AMPA receptors at early stages of motoneuron development (between E5 and E7). Ca^{2+} -impermeable AMPA receptors mediate excitatory responses to glutamate by E11. Changes in the Ca^{2+} permeability of AMPA receptors is correlated with an increase in the expression level of GluR2 mRNA and protein in the motoneuron pool.

METHODS

Motoneuron Labeling, Dissociation, and Cell Culture

Labeling, dissociation and culture of chick LMNs were performed as previously described by Martin-Caraballo and Dryer (2002a,b). Briefly, chick LMNs were retrogradely labeled *in ovo* with DiI (1 mg/mL in 20% ethanol and 80% saline). Dye injection into muscles of the thigh and foreleg was performed 24 h (E6, E8) or 4 days (E11) before spinal cord isolation. For an enriched motoneuron culture, only the ventral sections of the chick spinal cord were excised into a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free solution, mildly trypsinized (E5–E6, 0.05% for 20 min; E8, 0.05% for 30 min; E11, 0.2% for 40 min), dissociated by trituration, and plated onto poly-D-lysine-coated glass coverslips. Basal culture medium consisted of Eagle's minimal essential medium (BioWhittaker, Walkersville, MA) supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, and 10 ng/mL glial derived neurotrophic factor (GDNF). Recording of Ca^{2+} signals or whole-cell currents was performed 3–4 h after spinal cord dissociation or following overnight culture.

Western Blot Analysis

Immunoblot analysis of GluR2 expression was conducted in acutely isolated ventral halves of chick spinal cords at various developmental stages using a specific antibody against the GluR2 subunit (Chemicon, Temecula, CA). No further purification of motoneurons by gradient centrifugation was pursued, since such purification of motoneurons in older embryos does not result in viable cells, especially from embryos older than E7 (our own unpublished results, Mettling et al., 1995). For immunoblot analysis of GluR2 protein expression, ventral spinal cords were isolated in ice-cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free saline and lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO). After determining the protein concentration with a Bradford protein kit (BioRad, Hercules, CA), lysates were combined with 2 \times Laemmli sample buffer and boiled

for 5 min at 95°C. Samples were separated by SDS-PAGE on 8% gels. Proteins were transferred to nitrocellulose membranes, which were previously blocked in Tris-buffered saline, containing 0.1% Tween-20 and 2% nonfat dried milk, before overnight incubation with mouse anti-GluR2 (1:500 dilution). Blots were analyzed using anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase and a chemiluminescent substrate (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). To control for equal loading of protein in each sample, membranes were stripped in a 0.2 M glycine solution (pH 2.8) for 30 min at room temperature and re probed with a β -actin specific antibody (at 1:20,000 dilution, Sigma) followed by incubation with the corresponding secondary antibody and immunodetection.

Laser Capture Microdissection

DiI-labeled spinal cords were isolated at E6, E8, and E11, placed in tissue freezing medium (Richard-Allan Scientific, Kalamazoo, MI), and stored at -80°C . Cryostat sections (20 μm) were attached to RNase-free PEN membrane-covered slides (PALM, Microlaser Technologies AG, Bernried, Germany). Microdissection of the motoneuron pool was performed on a PALM microbeam-equipped Zeiss microscope. The DiI-labeled motoneuron pool was excised from 25–40 spinal cord sections. The microdissected material was catapulted into a tube (PALM Adhesive caps) containing 30 μL of lysis buffer, provided with the ArrayPure Nano-scale RNA purification kit (Epicenter Technologies, Madison, WI).

RNA Isolation and Real Time PCR

RNA from laser-captured samples was isolated with the ArrayPure Nano-scale RNA purification kit according to the manufacturer's instructions. Isolated RNA was used for cDNA synthesis by reverse transcription with Omniscript reverse transcriptase system (Qiagen). For quantitative analysis, cDNAs of GluR2 and β -actin (used as a normalizer) were amplified in separate samples using their corresponding primers (supplied by Sigma Genosys), whose amplification efficiencies have been matched. Quantification of cDNA was performed by quantitative real-time PCR using 5'-Fam3 and 3'-Black Hole Quencher (BHQ) probe on an Applied Biosystems PRISM 7500 sequence detection system. Primers and TaqMan probe designs were based on the *G. gallus* GluR2 mRNA published sequence (X89508) and consisted of the following sequences: forward primer (5'GCGGCAAGGATGCGATATT-3'), reverse primer (5'TGTAGGATGAGATTATGATGAGGGTAA-3'), and TaqMan probe (5'CCAAGATCCCTGTCTGGGCGCA-3'). PCR reactions consisted of one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and one cycle of 60°C for 1 min. At the completion of the PCR reaction, the amount of target message in each sample was estimated from a threshold cycle number (C_T), which is inversely correlated with the abundance of its initial

mRNA. Values of GluR2 and β -actin transcripts in each sample were obtained by interpolating C_T values on a standard curve. The standard curve was derived from serial dilutions of known quantities of the target message. All PCR reactions for the standard curve and the experimental samples were run simultaneously in duplicates. Each reaction also included a control containing no reverse transcriptase enzyme to test for DNA contamination. GluR2 mRNA expression was normalized to β -actin to correct for differences in RNA concentration according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Immunohistochemistry

Spinal cords were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, incubated overnight in 30% sucrose/PBS, and embedded in tissue freezing medium before cryostat sectioning. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min. Slides containing spinal cord sections were blocked in PBS with 2% Tween, 5% bovine serum albumin, and 5% goat serum for 1 h at room temperature. Slides were incubated with a GluR2 specific primary antibody (at 1:600 dilution, Chemicon) in blocking solution at 4°C overnight. After three washes with PBS, slides were exposed to a biotinylated mouse secondary antibody conjugated to HRP for 1 h at room temperature. The peroxidase reaction with diaminobenzidine was performed using the ABC kit from Vector Laboratories (Burlingame, CA). Controls for the specificity of immunostaining were assessed by omission of the primary antibody.

Electrophysiology

Dissociated motoneurons were identified during patch-clamp recordings using an Olympus 71 \times inverted microscope equipped with Hoffman optics and rhodamine filters. Recordings were performed at room temperature (22–24°C). Recording electrodes were made from thin wall borosilicate glass (3–4 M Ω) and filled with a solution consisting of 120 mM CsCl, 2 mM MgCl_2 , 10 mM HEPES, 10 mM EGTA, 1 mM ATP, and 0.1 mM GTP (pH 7.4 with CsOH). Where indicated, spermine (50–500 μM) was added to the pipette solution to prevent washout of inward rectification (Koh et al., 1995). To study channel permeability, cell cultures were bathed in two different solutions: standard chick saline and a 10 mM $\text{Ca}^{2+}/\text{Na}^+$ -free solution. The standard chick saline contained 145 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl_2 , 5.4 mM CaCl_2 , 5 mM glucose, and 13 mM HEPES (pH 7.4 with NaOH). Standard chick solution was also supplemented with 600 nM TTX and 200 μM CoCl_2 ions to block voltage-gated Na^+ and Ca^{2+} channels, respectively. To investigate the Ca^{2+} permeability of AMPA receptors, cell cultures were perfused with an external solution in which NaCl was replaced with the impermeant cation *N*-methylglucamine (NMG), and 10 mM CaCl_2 . The composition of the 10 mM $\text{Ca}^{2+}/\text{Na}^+$ -free extracellular solution was 135 mM NMG, 10 mM CaCl_2 , 5 mM glucose, and 10 mM

HEPES (pH 7.4 with HCl). Under these recording conditions, kainate currents are mediated by the flow of Ca^{2+} and Cs^+ ions. The permeability ratio ($P_{\text{Ca}}/P_{\text{Cs}}$) in the 10 mM $\text{Ca}^{2+}/\text{Na}^+$ -free solution was calculated from the reversal potential (E_r), according to the extended GHK constant field equation using estimated ion activities (Mayer and Westbrook, 1987): $P_{\text{Ca}}/P_{\text{Cs}} = 0.25 \times (a_{\text{Cs}}/a_{\text{Ca}}) \times \exp(E_r F/RT) \times [\exp(E_r F/RT) + 1]$, where $a_{\text{Cs}} = \text{Cs}^+$ activity (activity coefficient = 0.75), $a_{\text{Ca}} = \text{Ca}^{2+}$ activity (activity coefficient = 0.55), and F , R , and T have their usual meaning. All E_r values were adjusted for an estimated junction potential of 10.2 mV (in 10 mM $\text{Ca}^{2+}/\text{Na}^+$ -free solution) and 4.7 mV (in normal chick solution). Drugs were applied using a gravity-fed perfusion system (Bioscience Tools, San Diego, CA). Voltage commands and data acquisition and analysis were performed with a MULTICLAMP 700A amplifier and PCLAMP software (Axon Instruments, Foster City, CA). Pipette offset and whole-cell capacitance were compensated automatically with the MultiClamp 700B Commander. Throughout, all data values are presented as mean \pm SEM. Statistical analyses consisted of Student's unpaired t -test when single comparisons were made, and one-way ANOVA followed by *post hoc* analysis using Tukey's honest significant difference test for unequal n for the more typical experimental designs that entailed comparisons between multiple groups (STATISTICA software, Tulsa, OK). Throughout, $p \leq 0.05$ was regarded as significant. In every experiment, data were collected from a minimum of two platings (i.e. from multiple cultures).

Intracellular Free Ca^{2+} Measurements

Changes in intracellular $[\text{Ca}^{2+}]$ resulting from activation of Ca^{2+} permeable AMPA receptors were detected with the ratiometric dye Fura-2. Cell cultures were incubated for 30 min with Fura-2 AM (5 μM , Molecular Probes, OR) and 0.2% pluronic acid in the dark. Cultures were washed and incubated for an additional 30 min in the dark to complete de-esterification of the dye. Cells were viewed with a Nikon microscope equipped with xenon epifluorescence optics and a 40 \times water immersion objective. Cells were illuminated with 340 and 380 nm light from a 75-W xenon source, and the emitted fluorescence was collected at 510 nm with a Hamamatsu CCD camera. Image collection and analysis were performed with the computer software Simple PCI (Compix). Recorded Ca^{2+} signals were corrected for background fluorescence and presented as the ratio of the fluorescent peak signals generated at 340 and 380 (F_{340}/F_{380}). This ratio represents relative changes in intracellular $[\text{Ca}^{2+}]$ without conversion to absolute values of intracellular-free Ca^{2+} . There is minimal overlap in the fluorescent profile of Fura-2 measured at peak values (Abs = 340/380 nm, $E_m = 510$ nm) and the retrograde label DiI (Abs = 550 nm, $E_m = 560$ nm) used to identify LMNs. Drugs were applied for 30–60 s with a ValveLink 8 perfusion system (AutoMate Scientific, San Francisco, CA). Initially, control Ca^{2+} signals generated by activation of voltage-gated Ca^{2+} channels were induced with 30 mM extracellular K^+ .

AMPA-generated Ca^{2+} signals were recorded in standard chick saline supplemented with 600 nM TTX and 200 μM CoCl_2 to block voltage-gated Na^+ and Ca^{2+} channels, respectively.

Analysis of GluR2 Editing

The extent of GluR2 mRNA editing at the Q/R site was determined as previously described by Greig et al. (2000). Specific primers consisting of (forward) CCTCAGAAGTC-CAAGCCAGGAGTG and (reverse) CAGGAAGGCAGC-TAAGTTAGCCG were used to amplify the GluR2 region containing the Q/R editing site. The PCR product was digested with the restriction endonuclease BbvI for 3 h at 37°C, and the digested products were separated on a 5% nondenaturing polyacrylamide gel. The amplified PCR product (331 bp) is cleaved into two fragments in the edited state (311 and 20 bp), whereas it generates three fragments in the unedited state (248, 63, and 20 bp). The extent of RNA editing was quantified by calculating the ratio of cleaved to uncleaved PCR products. PCR bands were sequenced to confirm the identity of the PCR products.

Chemicals and Drugs

Phanthotoxin, poly-D-lysine, tetrodotoxin, and trypsin were from Sigma. BbvI was obtained from New England Biolabs (Beverly, MA). GDNF was obtained from R&D Systems (Minneapolis, MN). (*RS*)- α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, GYKI 52466, and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) were provided by Tocris Cookson (Ellisville, MS). Fura-2AM and pluronic acid were purchased from Molecular Probes (Carlsbad, CA). Culture medium and supplements, including serum, were from BioWhittaker (Walkersville, MA).

RESULTS

Developmental changes in the functional properties of AMPA receptor-generated currents were assessed by whole-cell voltage-clamp recordings from LMNs isolated between E5 and E11. These responses were generated shortly after motoneuron isolation and, therefore, were less likely to be modified by the cell culture conditions. We should point out that previous recordings of AMPA and kainate responses in chick spinal neurons were made in long-term cultures (between 3 and 14 days in culture), supplemented with a variety of trophic factors that may not be a necessary component of the normal milieu (O'Brien and Fischbach, 1986; Zorumski and Yang, 1988). Functional expression of AMPA/kainate receptors in chick LMNs was examined by puffing 500 μM of

AMPA or kainate onto isolated neurons during a series of voltage steps.

Electrophysiological recordings of kainate responses in a Na^+ -containing extracellular solution indicate that chick LMNs generate significant inward currents at negative potentials at early stages of development. Using our standard chick saline, stimulation of E6 or E11 motoneurons with kainate generated nondesensitizing currents with a linear current–voltage (IV) relationship over the range of -90 to $+50$ mV and a reversal potential near 0 mV [Fig. 1(A–D)]. At E6, the corrected reversal potential of kainate-evoked currents was -6.9 ± 3.3 mV ($n = 15$), whereas at E11 it was -6.2 ± 2.5 mV ($n = 14$). Since kainate activates both kainate and AMPA receptors, we examined the effect of GYKI 52466, a noncompetitive receptor blocker selective for AMPA receptors (Paternain et al., 1995). At all ages tested, kainate-generated currents were sensitive to bath application of GYKI 52466 in a dose-dependent manner. In E6 LMNs, kainate-generated currents were reduced by over 60% in the presence of $50 \mu\text{M}$ GYKI 52466, whereas near 80% of kainate currents were blocked in E11 LMNs [Fig. 1(E,F)]. At all ages tested, incubation of motoneurons with $100 \mu\text{M}$ GYKI 52466 resulted in a near elimination of kainate-generated currents as shown for other cell populations (Pemberton et al., 1998; Morkve et al., 2002; Vitten et al., 2004). After washout of GYKI 52466, kainate-generated currents partially recovered [Fig. 1(E)]. These results suggest that the majority of kainate-generated currents were due to activation of AMPA receptors.

Previous findings in mammalian neurons (including rat spinal motoneurons) have shown strong inward rectification of kainate-generated currents at positive membrane potentials (Koh et al., 1995; Greig et al., 2000). It appears that inward rectification is mediated by channel blocking with polyamine-like compounds present in the intracellular milieu. Washout of endogenous polyamines by the pipette solution can be prevented by application of spermine to the recording pipette solution. Interestingly, in chick LMNs, AMPA-generated currents show a linear IV relationship over the range of recorded voltages, indicating lack of inward rectification characteristic of highly Ca^{2+} -permeable AMPA receptors in mammalian neurons. As demonstrated in Figure 1(C,D), insertion of $50 \mu\text{M}$ spermine in the pipette solution generated linear IV relationships at all ages recorded. No inward rectification was observed in the presence of $500 \mu\text{M}$ spermine (not shown). To quantify possible changes in the rectification of kainate-generated currents, we compared the rectification index of E6 and E11 LMNs. The rectification index was calculated as the ratio of the

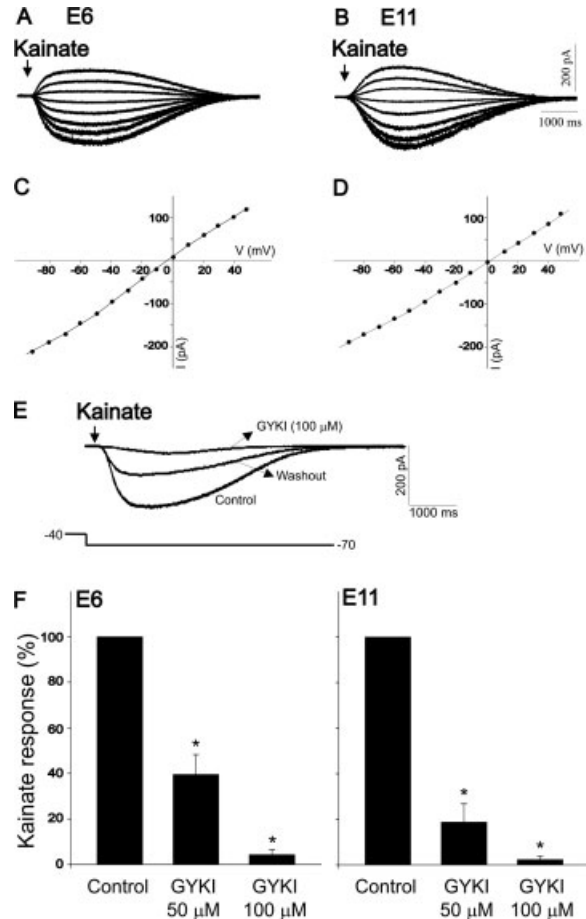


Figure 1 Kainate-induced currents in isolated chick LMNs. (A,B) Typical responses of E6 and E11 LMNs to kainate in standard chick solution. Whole-cell currents were evoked by puffing kainate ($500 \mu\text{M}$) onto isolated spinal cord neurons at holding potentials ranging from -90 to $+50$ mV. Voltage commands were applied in 10-mV steps, but only a few traces are shown for clarity. Currents were recorded in normal chick saline (Na^+ -based) using a CsCl-pipette solution containing $50 \mu\text{M}$ spermine. (C,D) Current–voltage (IV) relationship of kainate-evoked responses for the cells shown in (A) and (B). (E) Effect of GYKI 52466 on the kainate-evoked currents. Notice significant decrease in kainate current amplitude after bath perfusion with $100 \mu\text{M}$ GYKI 52466. Kainate-evoked current was recorded at a holding potential of -70 mV. (F) The inhibitory effect of GYKI 52466 on kainate-evoked currents was dose-dependent ($n = 6$, $p \leq 0.05$ vs. control).

kainate-evoked current slope between $+10$ and $+30$ mV to the slope between -50 and -30 mV. The rectification index in the presence of $50 \mu\text{M}$ spermine was 1.2 ± 0.2 ($n = 18$) for E6 neurons and 1.0 ± 0.2 ($n = 14$, $p > 0.5$) for E11 motoneurons. Since ATP present in the pipette solution is a potential chelator of intracellular polyamines (Bowie and Mayer,

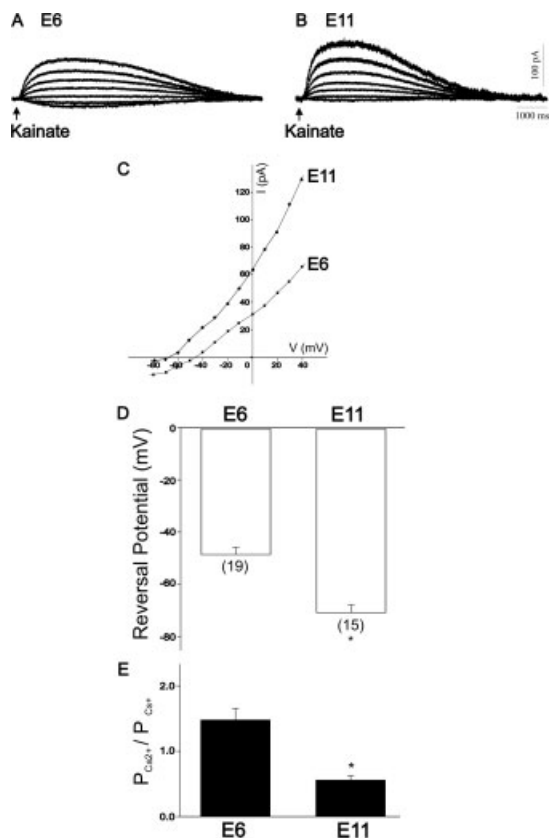


Figure 2 Developmental change in the Ca^{2+} -permeability of AMPA receptors in chick LMNs. (A,B) Typical whole-cell currents in E6 and E11 LMNs evoked by kainate application in 10 mM Ca^{2+}/Na^{+} -free external solution, at holding potentials ranging from -80 to $+40$ mV. (C) Current–voltage relationship of kainate-evoked responses for the cells shown in (A) and (B). (D) Plot showing the value of the reversal potential at E6 and E11. (E) Plot showing the value of the relative permeability of Ca^{2+} to that of Cs^{+} ($P_{Ca^{2+}}/P_{Cs^{+}}$) at E6 and E11. $P_{Ca^{2+}}/P_{Cs^{+}}$ was calculated according to the extended GHK constant field equation (see Methods). In this and subsequent figures, the number of cells recorded is given above each bar and the asterisk denotes $p \leq 0.05$ vs. E6.

1995), we also tested whether a higher concentration of spermine could still generate inward rectification under our recording conditions. No significant differences in the rectification index were observed when 500 μM spermine was added to the pipette solution (rectification index at E6, 1.0 ± 0.1 , $n = 6$; E11, 1.0 ± 0.1 , $n = 7$, $p > 0.5$). Comparison of the rectification index in E6 and E11 LMNs suggests that, in avian spinal neurons, polyamine blockage with spermine does not affect channel permeability at positive membrane potentials.

To determine the relative Ca^{2+} permeability of AMPA/kainate receptors, a Na^{+} -free, high Ca^{2+}

extracellular solution was used (see Methods). An example of kainate-generated currents in E6 and E11 LMNs is presented in Figure 2(A,B). Under our recording conditions, IV curves were slightly outwardly rectifying. Age-dependent changes in the $P_{Ca^{2+}}/P_{Cs^{+}}$ were assessed using the constant field equation (see Methods) based on the reversal potential of kainate-generated currents in the 10 mM Ca^{2+}/Na^{+} -free extracellular solution [Fig. 2(C)]. The reversal potentials of E6 LMNs were significantly more positive than those in E11 motoneurons [E6 -48 ± 2.7 mV,

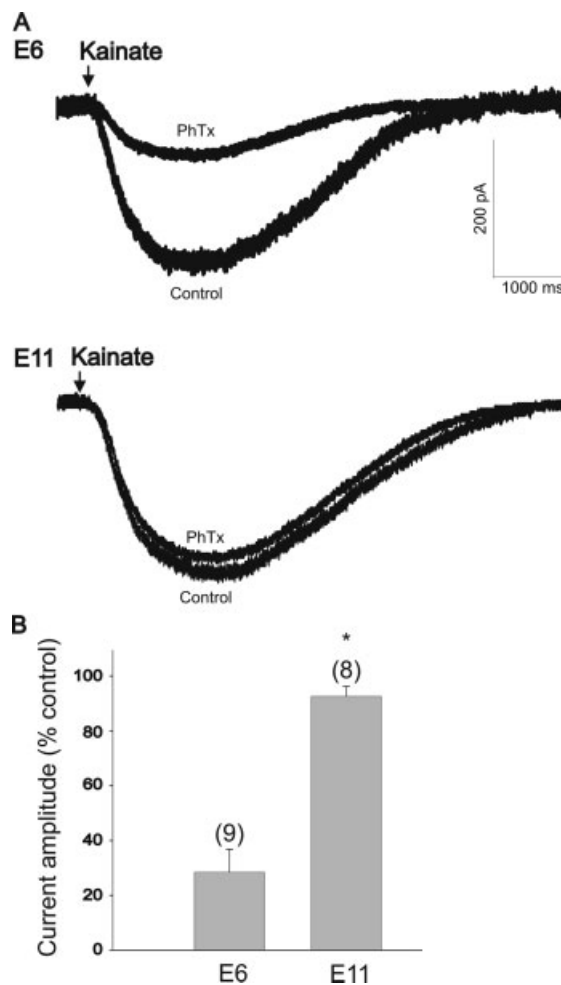


Figure 3 Effect of philanthotoxin (PhTx) on kainate-evoked currents in E6 and E11 motoneurons. (A) Example of kainate-evoked currents in E6 and E11 motoneurons before (control) and after a 5-min bath application of philanthotoxin (1 μM). Kainate-evoked currents were recorded at a holding potential of -70 mV. (B) Summary of philanthotoxin blockade of kainate-evoked currents in chick spinal motoneurons at two stages of development (E6 and E11). Philanthotoxin blockade was expressed as a percentage of control currents. Notice significant block of kainate-evoked currents in E6 neurons, but little effect of philanthotoxin application in E11 motoneurons ($p \leq 0.05$ vs. E6).

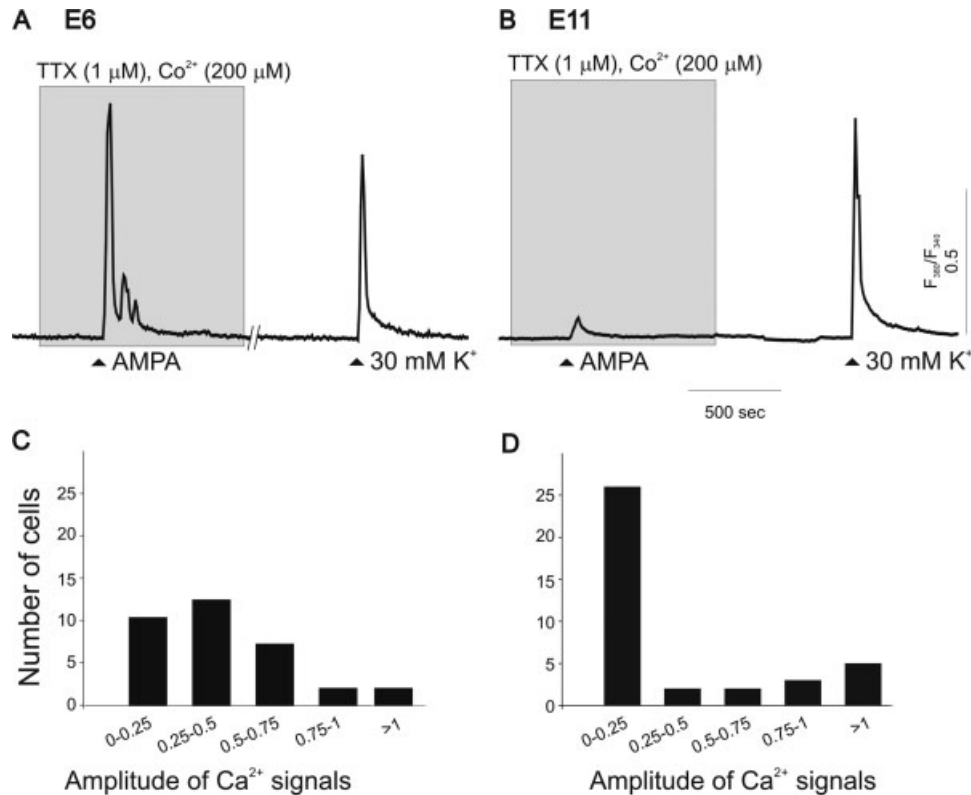


Figure 4 Developmental changes in the Ca²⁺ response of acutely isolated LMNs following AMPA receptor stimulation between E6 and E11. (A,B) Typical Ca²⁺ responses recorded at E6 and E11. (C,D) Graphic representation showing the range of Ca²⁺ signals in DiI-labeled motoneurons. After stimulation with 50 μ M AMPA, the majority of E6, but not E11, motoneurons responded with large Ca²⁺ signals. Acutely isolated cells were incubated with the ratiometric Ca²⁺ indicator Fura-2 for 30 min and stimulated with 50 μ M AMPA for 30 s. Stimulation with 30 mM K⁺ served as a control. AMPA-mediated responses were recorded in the presence of 600 nM TTX and 200 μ M Co²⁺ ions to block voltage-gated Na⁺ and Ca²⁺ channels, respectively.

$n = 19$; E11 -70.2 ± 2.9 mV, $n = 15$; Fig. 2(D)]. The calculated P_{Ca}/P_{Cs} revealed that E6 LMNs have higher Ca²⁺ permeability than do E11 motoneurons [E6 1.5 ± 0.2 , $n = 19$; E11 0.6 ± 0.1 , $n = 15$; Fig. 2(E)]. Age-dependent changes in the Ca²⁺ permeability of AMPA receptors in chick spinal motoneurons were further studied using philanthotoxin, a polyamine toxin that targets Ca²⁺-permeable AMPA receptors (Toth and McBain, 1998). Extracellular application of philanthotoxin (1 μ M) caused a significant reduction in the kainate-evoked currents in E6, but not in E11 motoneurons (Fig. 3). The blocking effect of philanthotoxin on kainate-evoked currents was significant at early stages of development (E6) when compared with older motoneurons [E11, Fig. 3(B)].

To investigate whether changes in the Ca²⁺ permeability of AMPA/kainate receptors generate significant changes in intracellular Ca²⁺ concentration

([Ca²⁺]_i), acutely isolated LMNs were loaded with the ratiometric Ca²⁺ indicator Fura-2 AM. Ca²⁺ signals were measured in response to 30-s application of AMPA (50 μ M) in the presence of TTX (500 nM) and CoCl₂ (200 μ M) in order to block voltage-gated Na⁺ and Ca²⁺ channels, respectively. Stimulation with 30 mM extracellular K⁺ was used as control. Typical Ca²⁺ signals in response to 50 μ M AMPA at E6 and E11 are shown in Figure 4(A,B). In the majority of E6 LMNs, AMPA receptor stimulation generated large Ca²⁺ signals that were comparable in amplitude to those generated with 30 mM extracellular K⁺ [Fig. 4(A)]. In E11 LMNs, however, AMPA receptor stimulation generated a small Ca²⁺ signal (indicated by fluorescent ratios between 0 and 0.25) in most motoneurons [$\sim 70\%$ of all recorded neurons, Fig. 4(B)]. However, a small portion of E11 motoneurons still generated a significant Ca²⁺ signal in response to AMPA receptor activation, suggesting

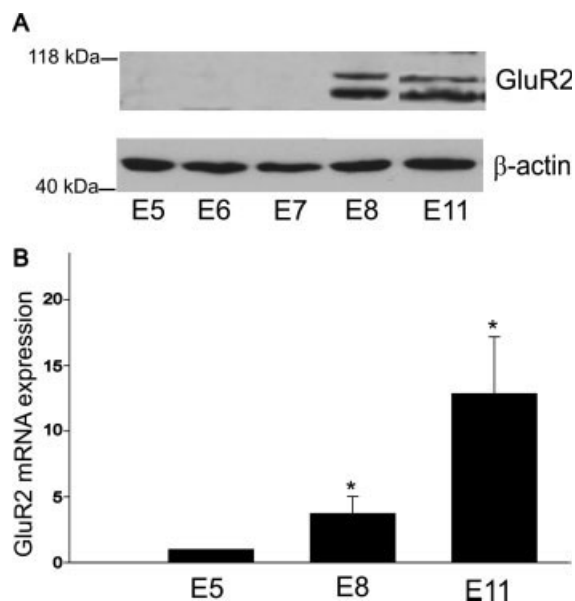


Figure 5 Developmental changes in GluR2 immunoreactivity and mRNA expression in chick ventral spinal cord. (A) Representative example of Western blot data collected from chick ventral spinal cords at various developmental stages (E5, E6, E7, E8, and E11). Each well was loaded with equal amounts of protein (30 μ g), as determined by the Bradford assay. To confirm equal loading, membranes were stripped following GluR2 immunoblot and reprobed for β -actin. (B) Relative abundance of GluR2 mRNA in laser-microdissected motoneurons as detected by real time PCR ($n = 5$, $*p \leq 0.05$ vs. E5).

that in a few older motoneurons there is a significant presence of Ca^{2+} -permeable AMPA receptors. Age-dependent changes in the Ca^{2+} permeability of

AMPA/kainate receptors resulted in a leftward shift in the distribution of peak Ca^{2+} signals between E6 and E11 neurons [Fig. 4(C,D)]. The AMPA-generated increase in intracellular Ca^{2+} was specific and can be eliminated by GYKI 52466 or the broad AMPA/kainate receptor blocker CNQX (not shown).

Are changes in the Ca^{2+} permeability of AMPA receptors correlated with changes in the level of expression of GluR2 in the chick spinal cord? To assess the level of GluR2 expression in the chick spinal cord, we used a mouse monoclonal antibody specific to the GluR2 subunit (Chemicon). Immunoblot analysis indicated that GluR2 subunit expression was absent between E5 and E7 in the chick ventral spinal cord [Fig. 5(A)]. By E8, the anti-GluR2 antibody detected two light bands that have a relative molecular weight of ~ 100 kDa [Fig. 5(A)]. These two bands are likely the result of different glycosylation states of GluR2 subunits (Hall et al., 1997). To determine whether changes in GluR2 protein expression are correlated with changes in GluR2 mRNA, we performed a real-time PCR analysis from isolated LMNs. LMNs were retrogradely labeled with DiI and captured using laser microdissection (see Methods). Quantitative PCR analysis indicates a significant change in GluR2 mRNA expression between E6 and E11 LMNs. GluR2 mRNA expression was significantly lower at E6 and increased over 10-fold by E11 [Fig. 5(B)].

Peroxidase immunostaining of spinal cord sections was performed to investigate the pattern of GluR2 expression throughout the spinal cord (Fig. 6). At E6, staining with the GluR2 antibody did not reveal any significant levels of expression throughout the spinal

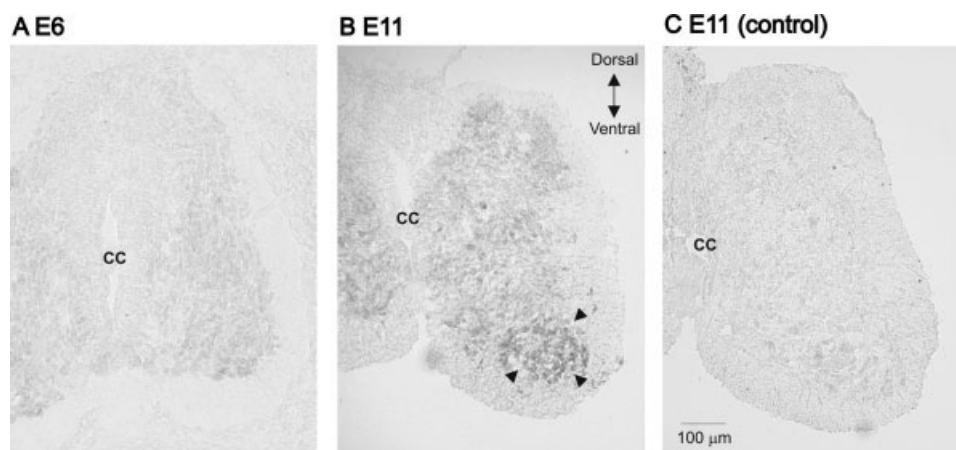


Figure 6 Developmental expression of GluR2 in chick spinal cord. Immunostaining of spinal cord sections for GluR2 expression at E6 (A) and E11 (B). Control (C) underwent the same immunolabeling procedures as sections in (A) and (B), except for the treatment with the GluR2 primary antibody. Note the low level of GluR2 expression at E6 but significant staining at E11 especially in the motoneuron pool (arrows). CC, central canal. Scale bar = 100 μ m.

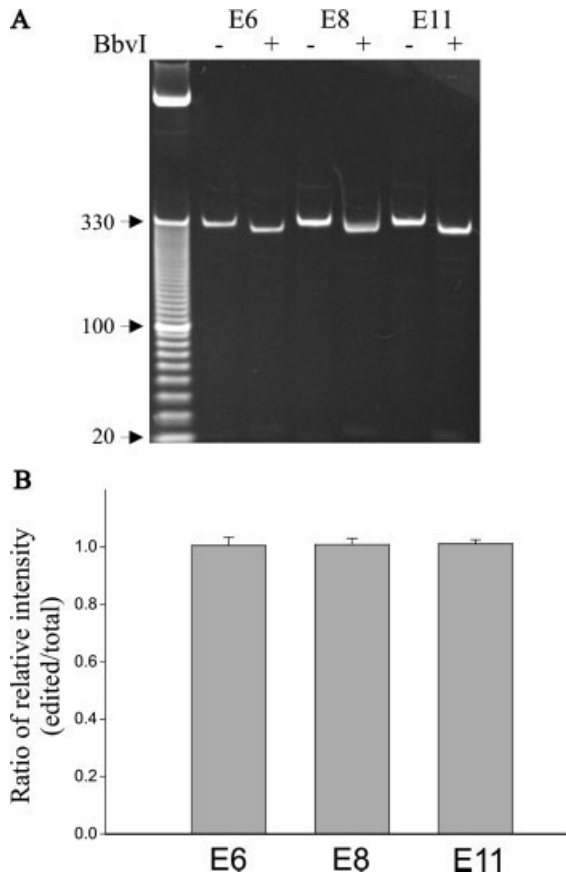


Figure 7 Extent of Q/R editing in chick ventral spinal cord between E6 and E11. GluR2 mRNA was amplified by PCR, and the product containing the Q/R editing site was digested with the restriction enzyme BbvI. Digested products were separated on a 5% polyacrylamide gel (A). The amplified PCR products (331 bp) were cleaved into two fragments (311 and 20 bp). (B) The extent of Q/R editing was quantified by plotting the ratio of band intensities at 311 and 331, which represents the level of cleaved and uncleaved PCR products, respectively.

cord [Fig. 6(A)]. Significant levels of immunostaining were observed in E11 spinal cord sections [Fig. 6(B)]. At E11, GluR2 staining of the spinal cord can be found throughout the gray matter, but not in the white matter. GluR2 staining was especially high in the motor neuron pool [Fig. 6(B), arrows]. Spinal cord sections not exposed to the GluR2 antibody lacked any significant labeling.

The presence of unedited GluR2 subunits in the Q/R site can also generate calcium-permeable AMPA receptors. To investigate whether a low level of GluR2 editing at early stages of motoneuron development can account for higher Ca^{2+} -permeability, we analyzed the level of Q/R editing between E6 and E11 in the ventral spinal cord. mRNA from ventral spinal cords was reverse-transcribed, and the 331 bp-

PCR product containing the GluR2 editing site was cut with the restriction enzyme BbvI. Analysis of the PCR products following digestion with BbvI indicates the presence of only two bands at 311 and 20 bp [Fig. 7(A)]. At all ages tested, the ratio of cleaved to uncleaved PCR products was near 1, suggesting that GluR2 editing is nearly complete between E6 and E11 [Fig. 7(B)].

DISCUSSION

The period spanning E5–E11 is critical for the development of the neuromuscular system in the chick. During this period, chick LMNs innervate and form functional synapses with target muscles in the hindlimb (Dahm and Landmesser, 1991). Failure to form appropriate synaptic connections with target muscles results in a ~50% reduction in the number of motoneurons and a significant reorganization of the motor pool (Chu-Wang and Oppenheim, 1978; Tang and Landmesser, 1993). The present study demonstrates that chick LMNs express functional AMPA receptors as early as E5, which undergo considerable changes in their Ca^{2+} permeability during a critical period of motoneuron development. Between E6 and E11, there is a 2.5-fold decrease in the Ca^{2+} permeability of AMPA receptors in chick LMNs. Changes in the Ca^{2+} -permeability of AMPA receptors are not mediated by age-dependent changes in the editing pattern of GluR2 subunits. Rather, changes in the Ca^{2+} -permeability of AMPA receptors are mediated by increased expression of GluR2 mRNA and protein in the motoneuron pool.

Age-Dependent Changes in GluR2 Expression and Ca^{2+} Permeability of AMPA Receptors

Whole-cell recordings and ratiometric measurements of Ca^{2+} signals following AMPA receptor stimulation indicate that chick LMNs express functional Ca^{2+} -permeable AMPA receptors at early stages of development. Thus, based on measurements of the reversal potential with a 10 mM $Ca^{2+}/0 Na^{+}$ external solution, the Ca^{2+} -permeability of AMPA receptors at E6 is 2.5-fold higher than at E11. Blockage of AMPA-mediated currents by philanthotoxin at E6 but not at E11 is also consistent with the presence of Ca^{2+} -permeable AMPA receptors in younger motoneurons. Expression of functional Ca^{2+} -permeable AMPA receptors results in a significant accumulation of intracellular Ca^{2+} in E6 motoneurons, although a few

older motoneurons also show a considerable Ca^{2+} signal. This observation could be explained by coexpression of Ca^{2+} -permeable and -impermeable receptors in a subpopulation of older motoneurons that may result in a significant accumulation of intracellular Ca^{2+} following AMPA receptor stimulation in a small number of E11 motoneurons. Indeed, it has been shown that clusters of Ca^{2+} -permeable and -impermeable AMPA receptors can coexist in the same motoneuron (Vandenberghe et al., 2001). Alternatively, the presence of Ca^{2+} -permeable AMPA receptors in a few E11 motoneurons could represent a subpopulation of motoneurons undergoing late differentiation. When interpreting the present results, we should also take into consideration that recordings were performed within 24 h after dissociation. Thus, we cannot exclude the possibility that the culture environment could alter some of our findings. However, it is difficult to explain why our dissociation and culture conditions would favor the expression of Ca^{2+} -permeable AMPA receptors at E6 but not at E11. One also has to consider that whole-cell recordings were performed in isolated motoneurons, which results in the elimination of most dendrites during the dissociation procedure. Accordingly, there is the possibility that we have underestimated the contribution of dendritic Ca^{2+} -permeable AMPA receptors if they were present in E11 motoneurons. We should note, however, that space-clamp problems could severely hinder whole-cell recordings from intact motoneurons because of their large size and extensive dendritic tree. Taken together, these changes in the Ca^{2+} permeability of AMPA receptors reflect a general pattern of development also found in other developing neurons including *Xenopus* spinal and mammalian auditory neurons (Otis et al., 1995; Gleason and Spitzer, 1998; Ravindranathan et al., 2000; Eybalin et al., 2004).

Our present results also indicate that changes in the Ca^{2+} permeability of AMPA receptors are most likely mediated by changes in the expression pattern of GluR2. Thus, our immunoblot data show that GluR2 expression is absent from the ventral spinal cord region between E5 and E7 and increases to detectable levels by E8. Immunolabeling of chick spinal cord tissue also reveals little staining at E6, but significant labeling of the motoneuron pool in the ventral horn of older embryos. Although it is possible that immunodetection of GluR2 expression may not be sensitive enough to detect GluR2 protein expression prior to E8, our real-time PCR data also show an incremental pattern of GluR2 mRNA in the motoneuron pool between E6 and E11, suggesting that the changes in GluR2 expression in the chick ventral

spinal cord require *de novo* protein synthesis. According to previous reports, insertion of one or more GluR2 subunits determines the Ca^{2+} permeability of AMPA receptors (Jonas et al., 1994). Therefore, age-dependent changes in GluR2 expression most likely explain the presence of Ca^{2+} -permeable AMPA receptors at early stages of motoneuron development in chick spinal cord motoneurons.

Although insertion of unedited GluR2 subunits could also explain the presence of Ca^{2+} -permeable AMPA receptors, our present results indicate that already by E6 nearly 100% of GluR2 mRNA is edited in the chick spinal cord. Thus, digestion of a GluR2 amplification product with the restriction enzyme BbvI only resulted in two bands, which would be expected if GluR2 subunits were fully edited. The extent of GluR2 editing in the chick spinal cord is similar to that in rat spinal motoneurons at comparable developmental stages (Greig et al., 2000). Although we are unable to predict whether editing levels are higher at earlier stages of spinal cord development (i.e., prior to E5), previous work has demonstrated a small, but nonetheless significant amount of unedited GluR2 in whole chick embryos between E2 and E5 (Lee et al., 1998).

A surprising result of the present study was the lack of inward rectification in Ca^{2+} -permeable AMPA receptors in E6 spinal neurons. These results contrast with previous findings, showing a strong correlation between Ca^{2+} -permeable receptors lacking GluR2 transcripts and inward rectification (Jonas et al., 1994; Kumar et al., 2002). Inward rectification in Ca^{2+} -permeable AMPA receptors is caused by endogenous polyamines that block outward currents (Koh et al., 1995; Shin et al., 2005). Our results do not support a similar effect of polyamines in chick spinal motoneurons, since concentrations of spermine as high as 500 μM did not produce any effect under our experimental conditions. Thus, our present results suggest that high Ca^{2+} -permeability and inward rectification of AMPA receptors are not intrinsically linked in chick spinal motoneurons, which could be explained by species differences or differences in the channel structure (see below). Although inward rectification has been associated primarily with Ca^{2+} -permeable, GluR2-lacking AMPA receptors, there appear to be some exceptions to this rule. For example, AMPA-mediated currents with high Ca^{2+} -permeability and linear IV relationship have been described in rat amacrine cells, even in the presence of spermine (Morkve et al., 2002). Moreover, there is evidence that sensitivity to polyamines and Ca^{2+} influx are not regulated by the same molecular factor (Burnashev et al., 1992; Dingledine et al., 1992;

Washburn et al., 1997). First, the rectification properties and Ca^{2+} permeability of AMPA receptors can be separated by genetic manipulations of the amino acid group in the pore-forming domain of the channel, also known as the Q/R site (Dingledine et al., 1992). Thus, experiments using a heterologous expression system have revealed that inward rectification of AMPA receptors can be altered by the size of the amino acid side chain found at the Q/R site. For example, substitution of glutamine by asparagine in the Q/R editing site of GluR3 subunits generates kainate currents with a linear IV relationship and very high permeability to divalent cations, rather than the inwardly rectifying IV generated by native GluR3 receptors. On the other hand, permeability to divalent cations can be modified by changes in the positively charged amino acid found in the Q/R site, including changes in the size of the side chain or the electron cloud in the guanidine head group (Dingledine et al., 1992). Second, it appears that the Ca^{2+} permeability of AMPA receptors rather than inward rectification and internal polyamine block is more sensitive to the ratio of GluR2 to non-GluR2 subunits (Washburn et al., 1997). Thus, it is possible that cell-specific modifications in the amino acid structure of GluR2 subunits underlie the high Ca^{2+} -permeability and linear IV relationship in E6 chick motoneurons. Extracellular block of AMPA receptors by polyamine toxins (such as *Joro* spider toxin, argiotoxin, and philanthotoxin) is also regulated by the relative abundance of GluR2 subunits (Blaschke et al., 1993; Herlitze et al., 1993; Meucci and Miller, 1998). Previous studies have shown that polyamine toxins bind to a glutamine residue in the M2 domain in order to block kainate-evoked currents (Blaschke et al., 1993; Herlitze et al., 1993). Accordingly, AMPA receptors lacking edited GluR2 should be more sensitive to inhibition by polyamine toxins. However, variations in the GluR2 content of AMPA receptors generate polyamine toxin-insensitive receptors that can still produce significant Ca^{2+} influx (Meucci and Miller, 1998). This situation resembles our present results in E11 motoneurons, where over 90% of AMPA-mediated currents were philanthotoxin-insensitive, although the Ca^{2+} -permeability of the AMPA receptors was not completely eliminated, and in a few neurons, we observed a significant intracellular Ca^{2+} signal following receptor activation. These results further support the notion that the Ca^{2+} permeability and philanthotoxin block of AMPA receptors in E11 motoneurons may arise from some variability in the GluR2 content of the receptors.

Functional Role of Ca^{2+} -Permeable AMPA Receptors

Changes in the Ca^{2+} permeability of AMPA receptors may have important functional implications in neuronal development (Gleason and Spitzer, 1998; Kumar et al., 2002). Previous work has shown that chick LMNs express functional non-NMDA glutamatergic receptors at early stages of spinal cord development (Temkin et al., 1997; Milner and Landmesser, 1999). Moreover, kainate application to a spinal cord preparation induces bursting activity as early as E4, either by acting directly on spinal motoneurons or by other cellular components of the spinal cord circuitry (Milner and Landmesser, 1999). Previous studies have shown that activation of AMPA/kainate receptors is not required for the generation of early network activity in the chick spinal cord (between E4 and E6), although they may have some effect in regulating the shape of spontaneous bursts (Milner and Landmesser, 1999). At later stages of development, however, AMPA receptor activation is required for the generation of spontaneous network activity (Chub and O'Donovan, 1998).

The early appearance of AMPA/kainate receptors in the chick spinal cord raises the possibility that they play an important role in the regulation of other developmental processes. Our present results indicate that changes in GluR2 expression and the Ca^{2+} permeability of AMPA receptors coincide with an important period of functional development in the chick spinal cord. Changes in GluR2 expression and the Ca^{2+} permeability of AMPA receptors could affect neuronal survival. For example, in chick brainstem auditory neurons, inhibition of Ca^{2+} -permeable AMPA receptors prevents normally occurring cell death during a critical period of development (Solum et al., 1997). In the chick spinal cord, approximately half of all LMNs die as a result of programmed cell death by an apoptotic mechanism between E6 and E10 (Chu-Wang and Oppenheim, 1978; Caldero et al., 1998). However, there have been conflicting reports regarding the effect of AMPA receptor activation on motoneuron survival. Activation of Ca^{2+} -permeable AMPA receptors results in a significant loss of rat spinal motoneurons *in vitro*, but not of chick motoneurons (Metzger et al., 1998; Fryer et al., 1999, respectively). Moreover, daily treatment of chick embryos *in ovo* with the kainate antagonist NBQX does not alter the number of LMNs (Caldero et al., 1997; Llado et al., 1999). Thus, it appears that activation of Ca^{2+} -permeable AMPA receptors plays no significant role in regulating neuronal survival in chick spinal motoneurons between E5 and E10.

Nonetheless, it is important to point out that expression of Ca²⁺-impermeable AMPA receptors coincides with a period of increased network activity driven by glutamate neurotransmission in the chick spinal cord (O'Donovan and Landmesser, 1987; Chub and O'Donovan, 1998). We speculate that expression of Ca²⁺-impermeable AMPA receptors in older neurons may limit Ca²⁺ entry through multiple sources during a period when NMDA receptors (which are Ca²⁺-permeable) also become functional in order to generate spontaneous network activity in the chick spinal cord (Chub and O'Donovan, 1998).

Another possibility to consider is that changes in intracellular [Ca²⁺] caused by GluR2 expression may regulate dendritic outgrowth during motoneuron development. Increased intracellular [Ca²⁺] acts as a stop signal for growing dendrites to form synapses (Lohmann et al., 2005). Activation of Ca²⁺-permeable AMPA receptors also downregulates dendritic outgrowth (but not axonal growth) of rat spinal motoneurons in culture (Metzger et al., 1998). The inhibitory effect of AMPA receptors on dendritic outgrowth is specific and can be reversed by the AMPA/kainate blockers CNQX, GYKI 52466, and *Joro* spider toxin, but not by the NMDA blocker AP5 (Metzger et al., 1998; Catsicas et al., 2001). Increased GluR2 expression will limit Ca²⁺ influx and could facilitate dendritic outgrowth and navigation to establish appropriate synaptic connections with sensory inputs. In the chick spinal cord, sensory afferents initiate the first contacts with the dendrites of motoneurons at E7.5 (Lee et al., 1988; Mendelson and Frank, 1991). Interestingly, early monosynaptic potentials between sensory afferents and motoneurons persist in the presence of NMDA receptor blockers, raising the possibility that they are mediated by AMPA receptor activation (Lee et al., 1988). Studies with AMPA receptor antagonists are currently underway to assess whether changes in GluR2 expression and Ca²⁺ permeability of AMPA receptors can affect motoneuron development *in vivo*.

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