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Developmental Regulation and Function of AMPA Receptor Subunits in Chicken Lumbar Motoneurons

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DEVELOPMENTAL REGULATION AND FUNCTION OF AMPA
RECEPTOR SUBUNITS IN CHICKEN LUMBAR MOTONEURONS

A Dissertation Presented

by

Xianglian Ni

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Biology

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Accepted by the Faculty of the Graduate College, the University of Vermont, in partial fulfillment of the requirement for the degree of Doctor of Philosophy specializing in Biology.

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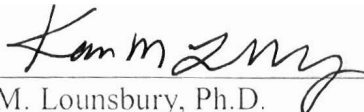


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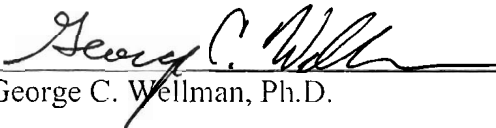
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ABSTRACT

Ca^{2+} influx through ionotropic glutamate receptors regulates 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 into the receptors. Although Ca^{2+} -permeable AMPA receptors are a familiar feature in developing neurons, the developmental function of these receptors during the formation of the nervous system has yet to be established. This study was designed to investigate the expression and functional role of Ca^{2+} -permeable AMPA receptors in developing chicken spinal motoneurons.

Our results demonstrate that chicken lumbar motoneurons express functional AMPA receptors as early as embryonic day (E) 5. Electrophysiological recordings of kainate-evoked currents indicate a significant reduction in the Ca^{2+} permeability of AMPA receptors between E6 and E11. During this developmental period, the Ca^{2+} permeability of AMPA receptors decreases three-fold. Reduction in the Ca^{2+} permeability of AMPA receptors is accompanied by increased expression of GluR2 mRNA in the spinal motoneuron pool. Changes in GluR2 mRNA expression occur in parallel to changes in GluR2 protein expression in the chicken ventral spinal cord. Changes in the Ca^{2+} -permeability of AMPA receptors are not mediated by age-dependent changes in the editing pattern of GluR2 subunits.

At early stages of development, functional AMPA receptors were composed of a combination of GluR3 and GluR4 subunits. mRNA analysis indicates that GluR4 is the most abundant subunit in the chicken ventral spinal cord between E6 and E11. Immunohistochemistry analysis of spinal cord sections also demonstrated that both GluR3 and GluR4 proteins are expressed at E6 and E11.

Expression of Ca^{2+} -permeable AMPA receptors regulates the maturation of dendritic outgrowth in developing spinal motoneurons. Measurements of dendritic length and branching pattern demonstrate significant changes in the dendritic morphology of spinal motoneurons between E6 and E11. Blockade of AMPA receptor activation with CNQX between E5 and E8 causes a significant increase in dendritic outgrowth in lumbar motoneurons, when compared with vehicle-treated embryos. Treatment of chicken embryos with CNQX between E8 and E11, when AMPA receptors become Ca^{2+} -impermeable, has no effect on dendritic morphology. However, blockade of NMDA receptor activation with MK-801 causes a significant reduction in dendritic outgrowth of lumbar motoneurons by E11. These findings indicate that AMPA receptor activation between E5 and E8 limits dendritic outgrowth in developing motoneurons, whereas NMDA receptor activation is involved in dendritic remodeling after the establishment of synaptic contacts with sensory afferents.

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CHAPTER 1: INTRODUCTION

1. Glutamate receptors: a general overview

Glutamate is the most common excitatory neurotransmitter in the brain. It can activate both ionotropic and metabotropic glutamate receptors (GluRs). Based on the action of selective pharmacological agonists, ionotropic GluRs are divided into three categories: N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and kainate receptors (Grossman et al., 2000). The AMPA and kainate receptors mediate fast ionic currents, while NMDA receptors allow slow currents (Niedzielski and Wenthold, 1995). AMPA receptors are either heteromeric or homomeric assemblies of four subunits known as GluR1-4. Each of these subunits, within the macromolecular receptor complex, consists of distinct functional regions. Individual subunits are composed of a carboxyl terminal domain (CTD), three transmembrane domains (M1, M3 and M4), a re-entrant loop (M2), a ligand-binding domain (S1 and S2), and an extracellular amino terminal domain (ATD) (Fig1.1) (Gouaux, 2004). The CTD consists of various structural motifs, which interact with scaffolding proteins and are involved in numerous signal transduction processes (Kim and Sheng, 2004). The M2 region is a pore-forming domain that controls ion permeability and selectivity (Wollmuth and Sobolevsky, 2004). The S1 region is located at the N-terminus of the M1 transmembrane spanning domain and S2 is found between regions M3 and M4 (Mayer and Armstrong, 2004). A study of the ligand-binding

structure of AMPA receptors in GluR2 subunits revealed that the receptor fragment resembled a clamshell and that agonists bind to the cavity between each shell (Armstrong et al., 1998). Direct link and water-mediated interdomain hydrogen bonds are established between the agonist and residues of the S1 and S2 domains (Armstrong and Gouaux, 2000). The ATD is the site of action of various molecules, which can regulate glutamate receptor function (Herin and Aizenman, 2004). This region is also believed to be involved in the assembly of different AMPA receptor subunits into a functional receptor complex (Ayalon and Stern-Bach, 2001).

AMPA subunits can be grouped into two different categories based on the sequence of the C-terminal cytoplasmic domain (Fig 1.1). GluR2 and GluR3 are in the same group because both have short cytoplasmic tails, which contain 50 amino acids. This short tail interacts with specific intracellular proteins such as glutamate receptor-interacting protein (GRIP), AMPAR binding protein (ABP), and protein interacting with C-kinase-1 (PICK-1). These proteins are PDZ-containing proteins capable of interacting with the PDZ binding domains in the C-terminus of GluR2 and GluR3 subunits (Gardner et al., 2005). GluR1 and GluR4 have longer cytoplasmic tails, which connect to a different set of proteins other than GRIP/ABP and PICK-1. GluR1 binds to SAP97 while little is known about GluR4 interaction (Sheng and Lee, 2001). Each AMPA receptor is formed by different subunit combinations, often containing two homo-dimers (Sun et al., 2005). Channels formed with various combinations of AMPA subunits will form receptors with different channel properties (Bettler et al., 1990). A key factor that determines the functional properties of AMPA receptors is the synaptic receptor heterogeneity and therefore it is necessary to know the composition of receptor subunits

in a specific neuronal type in order to understand the cellular function of the receptors within a particular cellular context (Grunder et al., 2000).

Modification of particular GluR subunits has important implications for AMPA receptor function. For example, phosphorylation of the GluR1 subunit at Ser845 by PKA could result in changes in unitary current amplitude, relative proportions of subconductance levels, number of active channels, peak probability, and receptor desensitization (Banke et al., 2000). In addition to modulating GluR1, cAMP-dependent PKA is also involved in interacting with the GluR3 subunit, thus influencing AMPA receptor function (Keller et al., 1992). The carboxyl-terminal domain of GluR3 has been found to contain several motifs, which interact with various scaffolding and signal transduction proteins. These interactions regulate the traffic and localization of AMPA receptors in the membrane (Song and Huganir, 2002; Kim and Sheng, 2004).

Phosphorylation of the GluR4 AMPA receptor subunit by PKA, PKC, and a calcium/calmodulin-dependent protein kinase at Ser-482 is involved in synaptic delivery (Carvalho et al., 1999). GluR4-enriched AMPA receptors are found in several regions of the CNS where they mediate high rate synaptic transmission (Mosbacher et al., 1994). At early developmental stages of the rat hippocampus, GluR4 subunits are involved in synaptic delivery of AMPA receptors to the membrane (Zhu et al., 2000). However, neurons expressing AMPA receptors with a high content of GluR4 subunits are vulnerable to AMPA-induced excitotoxicity due to increased activity in the activator protein-1 (AP-1) DNA-binding protein. AP-1 is a transcription factor, which promotes cell death (Santos et al., 2006).

GluR2 is responsible for many AMPA receptor transmission properties, including Ca^{2+} permeability, receptor kinetics, and rectification. In particular, GluR2 plays a critical role in modulating mammalian AMPA function by being the most highly regulated among all of the AMPA receptor subunits. Regulatory modifications are made at many steps of its production, including gene expression, RNA editing, trafficking, and receptor assembly (Sommer et al., 1991; Hartmann et al., 2004). GluR2 interacts with various proteins, which help the subunit-specific trafficking of AMPA receptors to the synapse. N-ethylamide-sensitive fusion protein (NSF), glutamate receptor interacting protein (GRIP), and AMPAR binding protein (ABP) are only some of the interactive proteins that affect GluR2 expression and AMPA receptor function. Interaction of GRIP and ABP with GluR2 is mediated by phosphorylation of the C terminus of GluR2 at serine 880 (Chung et al., 2000).

Expression of AMPA receptor subunits in various combinations could have important implications for cell function by regulating ion channel conduction and synaptic activity. Several different techniques have been used to detect the changing composition of AMPA receptors in different neuronal populations, including real-time PCR, Western blot and immunochemistry. Different combinations of AMPA receptor subunits can be found in different populations of neurons. In cortical and cerebellar primary cell cultures, all four AMPA subunits are present (Janssens and Lesage, 2001). GluR1 was not found in cochlear and vestibular ganglion cells, while the other three subunits were detected (Niedzielski and Wenthold, 1995). By using quantitative RT-PCR and laser capture microdissection, it was found that GluR2 is expressed at greater levels in most rat brain areas than other AMPA receptor subunits. GluR1 and GluR3 are highly

expressed in rat motor neurons (Sun et al., 2005). Sometimes AMPA subunits were found in specific layers of the retina or the cortex. For instance, GluR1 is present in the ganglion cell layer (GCL) of the retina and inner part of the neuroblast layer (NBL) during early stages of development. However, GluR1 subunits are not found in other layers, like the outer part of the NBL.

Expression of AMPA receptor subunits can be regulated in a time-dependent manner. For example, in the mammalian basal ganglia of the rat, GluR1 and GluR2 expression is high in neonates, but decreases significantly in the adult ganglia. By contrast, both GluR3 and GluR4 are the predominant subunits in the adult ganglia. These differences confer the establishment of proper synaptic connections within the basal ganglia during development (Jakowec et al., 1998). In cultured rat spinal cord neurons, both GluR1 and GluR2/3 were expressed at the earliest sites of postsynaptic receptor clustering. GluR4 was not present until several days after the onset of synaptogenesis (O'Brien et al., 1997). In hippocampal neurons, AMPA receptors containing GluR1 subunits are found throughout all developmental stages (Rao et al., 1998). Unlike GluR1, GluR2/3 is not detected in the inner plexiform layer (IPL) of the rat retina in the first postnatal week, whereas GluR4 begins to be expressed in the IPL on postnatal day (P) 14. GluR4 can also be found broadly distributed throughout the granular cell layer at all developmental stages (Grunder et al., 2000). To study the neural circuitry underlying jaw movements, AMPA receptor subunit expression in trigeminal motoneurons (Mo5) and mesencephalic trigeminal neurons (Me5) was investigated. GluR4 and GluR2/3/4c were found in rostral Me5 throughout the time frame (postnatal day P1, P3, P5, P10, P19-21, P32-35, and P60). GluR1 started to show up at P3, whereas GluR2 was detected between

P10 and P32-35 (Turman et al., 2000). In cochlear and vestibular ganglion cells, AMPA receptors consist of GluR2, GluR3, and GluR4, but not GluR1. This composition confers a critical sensory transduction in cochlear and vestibular ganglia (Niedzielski and Wenthold, 1995).

2. Posttranscriptional modifications of AMPA receptors

AMPA receptor subunits can undergo multiple modifications that have profound effects on the properties of the receptors. GluR2 mRNA editing at the Q/R site, in which genomically encoded information is enzymatically modified, is one important way AMPA receptor properties are regulated (O'Connell, 1997). In this case, a single nucleotide exchange within the first glutamine codon of the GluR2 sequence (CAG to CGG) results in glutamine (Q) being substituted with arginine (R), modifying the site of a selectivity filter in the pore forming M2 region (Fig 1.1) (Sommer et al., 1991). There are three structurally related RNA-editing enzymes that have been identified in mammalian tissues. The double-strand RNA editase ADAR2 is the most efficient enzyme involved in RNA editing of GluR2 subunits. This editing of GluR2 alters the gating and permeability properties of AMPA receptors, causing them to be less permeable to calcium (Rueter et al., 1995). The level of editing of the Q/R site is developmentally regulated. Nearly 100% of GluR2 subunits in rats are edited at the Q/R site after birth. A small percent (1%) of unedited GluR2 is found at early prenatal stages (Burnashev et al., 1992). The level of editing appears to vary among different components of the nervous system. For example,

it was determined that the ratio of Q/R editing in the substantia nigra region of human brain is less than 72% (Nutt et al., 1994).

In addition to mRNA editing of the Q/R site, GluR subunits may also undergo editing at the R/G site, which is located in the loop between the M3 and M4 regions. The first adenosine of the arginine codon (AGA) is replaced with guanosine to form a glycine (G) codon (GGA). R/G editing is found in all four GluR subunits, and is responsible for altering the desensitization kinetics of AMPA receptors (Ammori et al., 2007). The edited form confers faster recovery rates of the AMPA receptor from desensitization. The level of editing of the GluR2 subunit at the R/G site can vary greatly. In the adult rat brain, the ratio of R/G editing is around 80-90% (Lomeli et al., 1994).

Besides RNA editing, each GluR receptor subunit has two different splice variants, called flip and flop (Fig 1.1). These isoforms are produced by alternative splicing of a 114 bp region in the extracellular N-terminal region of the fourth transmembrane domain next to the R/G editing site (Sommar et al., 1990). One of two functionally critical cassettes of 38 amino acids is introduced into the extracellular loop of the GluR subunit (Tanaka et al., 2000). Flip and flop have different functional properties. The flip isoform mediates the steady-state current evoked by AMPA or L-glutamate. By contrast, the flop isoform generates a fast desensitizing component in glutamate-evoked currents (Sommer et al., 1990). Flip and flop isoforms are developmentally regulated in mammalian neurons. Only GluR flip isoforms are found during early embryonic development. Flop isoforms start to be expressed at later developmental stages (Monyer et al., 1991). There are also several splice variants of the C-terminus in GluR2 and GluR4 subunits at early stages of development (Kohler et al.,

1994). Splicing at the flip/flop and editing at the R/G site are responsible for receptor desensitization and mediate the kinetics of synaptic transmission (Lambolea et al., 1992).

3. Role of GluR2 subunit in regulating AMPA receptor properties

Except for GluR2, all the other AMPA receptor subunits have a glutamine (Q) group at the pore forming domain (Greger et al., 2003). Unlike the other subunits, the GluR2 subunit undergoes pre-mRNA editing at the Q/R site, resulting in a positively charged arginine replacing the genetically encoded neutral glutamine group in the pore-forming domain of the GluR2 subunit. This alteration makes an AMPA receptor containing the GluR2 subunit Ca^{2+} impermeable (Fig 1.2) (Van Damme et al., 2002). The glutamine-replaced arginine is the result of the original Q/R site codon CAG going through adenosine deamination and creating the codon CIG (Feldmeyer et al., 1999). Normally, the editing efficiency at the Q/R site is almost 100% in the adult nervous system. But incomplete editing is observed in motor neuron disorders, such as amyotrophic lateral sclerosis (ALS) (Van Damme et al., 2005). Non-editing of GluR2 subunits in pathological states like ALS can result in cell death due to the increased permeability of AMPA receptors to Ca^{2+} and the subsequent activation of various Ca^{2+} -dependent cell death mechanisms. The insertion of at least one edited GluR2 subunit into a functional AMPA receptor determines its Ca^{2+} permeability (Van Damme et al., 2002). AMPA receptors without a single edited GluR2 subunit or containing one or more unedited GluR2 subunits are permeable to Ca^{2+} (Fig 1.2). Compared to AMPA receptors lacking a GluR2 subunit, receptors with one edited GluR2 subunit are relatively

impermeable to Ca^{2+} . Upon binding to their agonists, AMPA receptors allow Ca^{2+} to enter a cell, even at hyperpolarized potentials. Edited GluR2-containing AMPA receptors have relatively lower single-channel conductance than GluR2-lacking AMPA receptors (Brill and Huguenard, 2008; Swanson et al., 1997).

In addition to allowing Ca^{2+} influx into cells, Ca^{2+} -permeable AMPA receptors have two other characteristics: inward rectification and sensitivity to polyamine blockade. Polyamines are a large group of molecules that preferentially block Ca^{2+} -permeable AMPA receptors. For example, kainate-evoked currents generated by activation of Ca^{2+} -permeable AMPA receptors are inhibited by application of 1-naphthyl acetyl-spermine (NAS) in a dose-dependent manner (Van Damme et al., 2002). Similarly, the polyamine toxin Joro spider toxin (JSTX) can produce a rapid blockade of kainate-evoked current generated by Ca^{2+} -permeable AMPA receptors (Greig et al., 2000). Polyamine toxins are non-competitive blockers of AMPA receptors. The antagonism of AMPA receptors is use- and voltage-dependent. GluR2-containing AMPA receptors are not sensitive to polyamine toxins (Stromgaard and Mellor, 2004).

Inward rectification is another characteristic of Ca^{2+} -permeable AMPA receptors. The phenomenon known as inward rectification describes a channel showing an increased conductance with hyperpolarization and a decreased conductance with depolarization. Inward rectification can be detected by measuring the current-voltage (I-V) relationship generated by activation of AMPA receptors. Inward rectification originates when Ca^{2+} -permeable AMPA receptors are capable of allowing significant inward current but very little outward current. This is different from what is found in GluR2-containing AMPA receptors, where the receptors allow the flow of both inward

and outward currents resulting in a linear I-V relationship. Inward rectification may originate from the blocking effect of endogenous polyamines that bind to the pore of Ca^{2+} -permeable AMPA receptors at depolarizing potentials. Binding of endogenous polyamines to the pore of Ca^{2+} -permeable AMPA receptors can affect the inward rectification properties of the receptors. Following whole cell recordings of cells with Ca^{2+} -permeable AMPA receptors, it was observed that the I-V relationship switched from inward to linear after agonist application for 5-10 minutes. This switch in the inward rectification led to the idea that an intracellular factor was lost during whole-cell recordings. Spermine is most likely the intracellular factor as it is a normal cellular constituent, which exists widely in CNS white matter. Further studies have demonstrated that the washout of spermine from the intracellular milieu could be prevented by addition of spermine to the patch-pipette solution, which restored inward rectification (Kamboj et al., 1995). Another factor that can contribute to inward rectification is the presence of Mg^{2+} ions in the intracellular space. For example, block by intracellular Mg^{2+} generates inward rectifying currents in some families of K^+ channels or neuronal nicotinic receptors. Ca^{2+} -permeable AMPA receptors are unaffected by $[\text{Mg}^{2+}]_i$. Thus, spermine and spermidine can fully explain the inward rectification property of AMPA receptors in unedited form (Bowie and Mayer 1995). The mechanisms for polyamine block are suggested as electrostatic interactions between multivalent cations (such as Ca^{2+} and polyamines) and the positively charged arginine located at the Q/R site of the pore-forming domain of GluR subunits (Koh et al., 1995). The levels of ATP in a cell can alter the blocking effect of polyamines on Ca^{2+} -permeable AMPA receptors. Several groups have demonstrated that ATP could chelate cations present in the intracellular milieu, thus

abolishing inward rectification in neuronal nicotinic receptors or certain K^+ channels (Sands and Barish 1992; Fakler et al., 1995). Voltage-dependent blockage of the Ca^{2+} -permeable AMPA receptors by polyamines modulate Ca^{2+} inward current. It has also been suggested that the polyamine block of Ca^{2+} influx at the resting potential prevents the cell from damage caused by excessive Ca^{2+} during cell activation (Bowie and Mayer 1995). In spite of these findings, it has also been observed that there is no inward rectification in Ca^{2+} -permeable AMPA receptors from interneurons of the rat neocortex or from chicken colchlear neurons. Only linear or outward rectifying I-V relationships are obtained (Jonas et al., 1994; Otis et al., 1995). Species differences or channel structure variances could account for the absence of inward rectification in Ca^{2+} -permeable AMPA receptors. Changes in the size of the amino acid side chain at the Q/R site can alter the properties of inward rectification of AMPA receptors. Replacement of glutamine with asparagine at the Q/R editing site of GluR3 subunits results in kainate currents with a linear IV relationship and high permeability to divalent cations (Dingledine et al., 1992).

4. AMPA receptor function

AMPA receptors mediate fast synaptic transmission in response to presynaptic neurotransmitter release. Postsynaptic depolarization through activation of AMPA receptors can initiate neuronal firing. Several findings have demonstrated that AMPA receptors are involved in learning and memory through regulation of long-term potentiation (LTP) and long-term depression (LTD). LTP is caused by receptor insertion or modification while LTD is the result of receptor internalization (Luscher and Frerking

2001). Compared with NMDA receptors, which are relatively stable in the synaptic site, AMPA receptors can be inserted into or removed from the postsynaptic membrane at a relatively high rate. This may explain the phenomenon of learning and memory. The concept of the “silent synapse” was proposed in the 1990’s to describe the existence of NMDA receptors, not AMPA receptors. Upon strong synaptic stimulation, functional AMPA receptors will appear in previously silent synapses, which potentiate synaptic transmission and result in LTP. Internalization of AMPA receptors from the neuronal surface can depress synaptic transmission, which induces LTD (Sheng and Lee, 2001).

Ca^{2+} -permeable AMPA receptors are believed to be involved in a variety of cell functions including regulation of neurite outgrowth, neuronal survival and neuronal plasticity. Activation of Ca^{2+} -permeable AMPA receptors reduced neurite outgrowth in dissociated embryonic chick retinal cells (Catsicas et al., 2001). A reduction in dendrite growth was also observed after activation of Ca^{2+} -permeable AMPA receptors in dissociated rat motoneurons (Metzger et al., 1998). With respect to neuronal survival, the activation of Ca^{2+} -permeable AMPA receptors, and the resulting accumulation of intracellular Ca^{2+} , can cause neurodegeneration under certain pathological conditions, such as amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (Lu et al., 1996). Global ischemia, causing delayed onset of neurological deficits following cardiac arrest, is characterized by an increased expression of GluR2-lacking, Ca^{2+} -permeable AMPA receptors in post-ischemic CA1 neurons, which results in increased influx of toxic Ca^{2+} and Zn^{2+} (Tanaka et al., 2000). Similarly, Ca^{2+} -permeable AMPA receptors mediate the delayed neuronal death associated with status epilepticus (Friedman and Veliskova 1998). In rat auditory brainstem neurons, Ca^{2+} -permeable AMPA receptors are responsible for

forming proper neuronal function once hearing is initiated (Caicedo et al., 1998). This is consistent with the idea that Ca^{2+} -permeable AMPA receptors play a role in the morphological maturation of neuronal circuits (Sanes et al., 1992).

Ca^{2+} -permeable AMPA receptors affect synaptic efficacy in two ways. First, Ca^{2+} influx mediates subunit switches in the AMPA receptor, which changes the synaptic performance by altering channel permeability, kinetics and electrical rectification. Second, Ca^{2+} is involved in regulating intracellular signaling cascades, such as AMPA receptor trafficking, local translation, and gene transcription. The number of Ca^{2+} -permeable AMPA receptors can be adjusted through self-regulating mechanisms (Liu and Zukin, 2007).

5. Developmental regulation of AMPA receptors

AMPA receptors play a major role in determining postsynaptic excitatory neurotransmission and must be carefully regulated. Regulation of postsynaptic receptors can occur at both the gene expression level and at synaptic sites by means of differential targeting. Changes in the receptor subunits themselves are related to developmental maturation of neural circuitry and innervation. There is a critical point at which AMPA receptors undergo a kinetic switch in the neurons of the avian cochlear nucleus magnocellularis (nMag). This switch occurs after innervation and lasts for more than one month in low-density culture. The rate of desensitization of AMPA receptors increased soon after innervation (Lawrence and Trussell 2000). AMPA receptors may also be regulated in an age-dependent manner. There is sufficient evidence that the complex

composition of AMPA receptor subunits varies in vertebrate neurons. In rat primary auditory neurons, GluR1, GluR3 and GluR4 are found before postnatal day (PND) 4, but GluR2 is not expressed until PND10 (Eybalin et al., 2004). A study of rat CA1 pyramidal neurons found that the proportion of GluR1 in AMPA receptor complexes is transiently decreased at PND 18. In addition, there are more GluR1/GluR2 and GluR2/GluR3 heteromeric complexes than homomeric GluR1 between postnatal days 30 to 40 (Seifert et al., 2000).

AMPA receptors may be present at very early developmental stages but do not show any known function. For example, there were no functional AMPA receptors found in CA1 pyramidal neurons until P1-2. However AMPA receptor subunits were detected before P2 in rat CA1 pyramidal cell bodies using immunochemical methods (Durand et al., 1996; Martin et al., 1998). In developing neurons derived from a P19 mouse cell line, AMPA receptors were found to be expressed at 5 days in vitro, by which time neurogenesis is only 60% complete (Lee et al., 2003). It has been observed that AMPA receptors are expressed soon after initiation of neurite outgrowth in *Xenopus* spinal neurons (Gleason and Spitzer 1998). Therefore, it is of great interest to identify the early expression of AMPA receptors in a specific region and cell processes they may mediate.

A lot of work has focused on studying the developmental regulation of postsynaptic AMPA receptors in vertebrates or mammals but AMPA receptors are also expressed at presynaptic sites. By staining AMPA receptor subunits in dorsal root ganglion cells, it was observed that the variations in subunit expression in the presynapse may be involved in transmitter release and important to the characteristic physiological profile of different classes of primary afferents (Lu et al., 2002). A similar result was

found with AMPA receptor subunits located on the axon terminals of corticostriatal and thalamostriatal afferents, implying that glutamate release is adjusted through the presynaptic AMPA receptors (Fujiyama et al. 2004). In Ia muscle spindle afferents, there is less GluR1 subunit expression than in other AMPA receptor subunits (Ragnarson et al., 2003). In vertebrate and human spinal cords, spatial and temporal expression of AMPA receptor subunits has been established. In the ventral horn of the developing rat spinal cord, there is significant down-regulation of GluR1 while expression of GluR3 does not change much. GluR2 and GluR4 show modest down-regulation in the postnatal life of the spinal cord. Overall, AMPA receptor subunits are most highly expressed in developing spinal cord gray matter (Jakowec et al., 1995). In the human spinal cord, it has been discovered that there are more AMPA receptors in the substantia gelatinosa region of spinal cord grey matter than in other regions. Besides spatial regulation, it has also been determined that AMPA receptors are regulated in a temporal manner in the human spinal cord. Glutamate receptors were lost from the late fetal spinal cord (Kalb and Fox 1997). In the rat hippocampus, except for GluR1, proportions of all the other three AMPA receptor subunits changed from postnatal day 1 to 35, showing increased expression at postnatal day 7 or 18 (Ritter et al., 2002). As little is known about the avian spinal cord with regard to the developmental regulation of AMPA receptor subunits, we investigated the subject and the accompanying changes in receptor function and pharmacological roles.

AMPA receptors may undergo a functional switch caused by an alteration in the subunit composition. There is a decrease in the Ca^{2+} permeability of AMPA receptors during development of rat neocortical pyramidal neurons. The changes in the Ca^{2+}

permeability of AMPA receptors are caused by the incorporation of GluR2 subunits into the receptors or changing the stoichiometry to enhance the copy number of GluR2 (Kumar et al., 2002). Similarly, when O-2A progenitor cells from rat brain differentiate into oligodendrocytes and type II astrocytes, AMPA receptors on these cells become less sensitive to Joro spider toxin (JSTx), a blocker of Ca²⁺-permeable AMPA receptors, implying that there is a developmental change in the AMPA receptor subunit composition and more GluR2 subunits are incorporated into the receptors (Meucci et al., 1996). In hippocampal neurons, most AMPA receptors in early developmental stages lack the GluR2 subunit. However, GluR2 is detected in almost every AMPA receptor-positive synapse (Pickard et al., 2000).

6. Regulation of AMPA receptor function by electrical activity

In response to the level of excitatory synaptic transmission, neurons are able to maintain a homeostatic state. It has been suggested that there is an increased glutamate chemosensitivity of the postsynaptic spinal neurons in response to the blockade of excitatory synaptic transmission (O'Brien and Fischbach 1986). To determine the mechanism that accounts for the changes in miniature excitatory postsynaptic current (mEPSC) amplitudes in response to the changes in excitatory synaptic activity, several studies were conducted. It was reported that changes in mEPSC amplitudes are due to altered accumulation of postsynaptic AMPA receptors. It was also observed that there are no changes in excitatory synapses. The changes in AMPA receptor accumulation at excitatory synapses suggest that there is a homeostatic feedback loop which allows

neuronal output adjustment in order to regain the original threshold for plasticity of the postsynaptic neurons (O'Brien et al., 1998). It appears that there is not only a change in the number of AMPA receptors in response to neuronal activity, but also in the subunit composition of AMPA receptors. Continuous activation of Ca^{2+} -permeable AMPA receptors leads to the incorporation of GluR2-containing receptors, which can reduce Ca^{2+} permeability in a self-regulating manner (Liu and Cull-Candy 2000).

AMPA receptors are expressed and distributed diffusely without the appropriate input. Upon activation of postsynaptic excitatory neurotransmitter receptors, AMPA receptor subunits undergo clustering in rat spinal cord neurons. There is developmental clustering of GluR1 subunits, which is induced by postsynaptic AMPA receptor activation. Like GluR1, GluR2/3 is also expressed and clusters at early sites of postsynaptic receptors (O'Brien et al., 1997). The activity has been suggested to have a great impact on AMPA receptor subunit composition. In addition, AMPA receptors are differentially distributed according to the type of synaptic input. It was shown that there is an increase in GluR2 expression parallel to the increase in electrical activity with maturation (Hollmann et al., 1991). GluR4 subunits are specifically targeted to dendrites and somata that are connected to auditory nerve fibers in mammalian cochlear nuclei. However, other AMPA receptor subunits are present at both auditory nerve fibers and parallel fibers of granule cells. This may explain the kinetics difference of the AMPA receptors in cochlear nuclear neurons (Hunter et al., 1993; Gardner et al., 1999).

Some molecules are considered to play important roles in regulating neurotransmitter receptors at synapses. It is of interest to identify these molecules, as it will help to better understand synaptic plasticity and neuronal development. Neuronal

activity-regulated pentraxin (Narp) is highly expressed in both the developing brain and the adult brain. At the same time, it increases significantly in neurons which respond to patterned synaptic activity (Tsui et al., 1996). Narp is able to interact with AMPA receptor subunits, leading to clustering of AMPA receptors. It was suggested that Narp may mediate the development and plasticity of excitatory synapses consistent with clustering of AMPA receptors (O'Brien et al., 1999). A novel protein named glutamate receptor interacting protein (GRIP) is believed to be involved in AMPA receptor clustering because it can interact with the carboxyl termini of the GluR2 and GluR3 subunits (O'Brien et al., 1998). There are also mechanisms that mediate the removal of AMPA receptors from synapses. The internalization of AMPA receptors can be triggered by glutamate, AMPA or insulin. Activation of NMDA receptors is involved in AMPA receptor endocytosis, which is called NMDA-receptor-dependent LTD. Calcium influx and activation of calcium-dependent protein phosphatase calcineurin are required for the AMPA receptor endocytosis (Beattie et al., 2000).

7. Regulation of AMPA receptor function by trophic factors

It is believed that neurotrophins can regulate neuronal physiology in an acute or long-term manner (Thoenen, 1995). Because of their ability to modulate synaptic function and affect synaptic plasticity, they are important for the development of the vertebrate nervous system. Neurotrophins include brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT-4/5), and nerve growth factor (NGF) (Chan et al., 2003). NT-3 was found to increase synaptic responses in neonatal rat

spinal motoneurons. By modulating motoneuron NMDA receptors, NT-3 can facilitate the fast AMPA/kainate receptor-mediated dorsal root evoked synaptic responses. The evoked fast excitatory postsynaptic potentials (EPSP) last for more than 3 hours after washout of NT-3. Long-lasting changes in synaptic transmission elicited by NT-3 resemble LTP (Arvanov et al., 2000). It was also found that neurotrophins are able to affect neurotransmission by modulating the phosphorylation of NR2B components of NMDA receptors, which are activated by calcium/calmodulin-dependent protein kinase II. This results in initiation of the signaling cascade, characterized by an enhanced response by AMPA or kainate receptors (Strack and Colbran, 1998).

BDNF is involved in synaptic plasticity, especially in LTP. AMPA receptors are also involved in directing synaptic plasticity. Thus, it is important to determine the relationship between BDNF and AMPA receptors. In hippocampal neurons cultured with BDNF, GluR1 and GluR2 protein levels increase, a result of activation of the tropomyocin receptor kinase (Trk) receptor by BDNF. In addition, by activating PKC and CaMKII, BDNF phosphorylated Ser-831 on GluR1 and GluR1- or GluR2-containing AMPA receptors are delivered to the synapse in the CA1 region of the hippocampus. It has also been determined that BDNF increases the number of GluR1 subunits in the plasma membrane by increasing the translation activity (Calderia et al., 2007). Similarly, it is suggested that basic fibroblast growth factor (bFGF) enhanced protein levels of the AMPA receptor GluR1 subunit in a concentration-dependent pattern. The rest of the AMPA receptor subunits are not affected. These studies indicate that neurotrophins may be involved in regulating the expression of AMPA receptor subunits, and thus modulating neuronal plasticity (Cheng et al., 1995). Among the four subunits of the

AMPA receptor, GluR2 is of extreme importance because it is able to regulate neuron vulnerability, neuronal survival, and neurite outgrowth. Upregulation of GluR2 levels plays a protective role in neurons by reducing the Ca^{2+} permeability of the AMPA receptors. The effect of neurotrophins on GluR2 subunit expression and the mechanism that would underlie such regulation are not well known. It is suggested that GluR2 expression may be regulated at the gene transcription level. A neuron-restrictive silencer element (NRSE), a gene sequence present within the GluR2 gene promoter, may be involved in the GluR2 expression that is regulated by neurotrophic factors such as BDNF and GDNF (Brene et al., 2000).

8. The avian spinal cord has many advantages for cellular studies of neuronal development

Several developmental processes such as programmed cell death, spontaneous motor activity, and the role of cell-cell interactions in shaping neuronal differentiation were first established in chicken embryos (Hamburger & Balaban, 1963; Hamburger, 1975; Dryer, 1998). An important advantage of the chicken embryo as a model for developmental studies is the easy accessibility of the embryo to *in vivo* manipulations throughout all stages of development (Coleman, 2008). The chick embryo can also be utilized to screen drugs in the pharmaceutical industry (Vargas et al., 2007). With the development of new technologies, such as *in vivo* electroporation and the sequencing of the chick genome, the chick has become one of the most useful experimental systems available (Stern, 2005; Wallis et al., 2004). Another advantage of using the chicken

spinal cord as a developmental model is the wealth of information about the general development of chicken lumbar motoneurons.

The vertebrate central nervous system originates from the ectoderm germ layer. The neural plate is derived from the ectoderm germ layer and develops into a cylindrical structure termed the neural tube (Schoenwolf, 1982). Initially, the neural tube consists of a single layer of multipotent cells in the ventricular zone of mammals and birds. It gains another two layers with the development of the spinal cord, which includes the mantle (or intermediate) and marginal zones. The ventricular zone eventually gives rise to both neurons and glia. Motor neurons are generated at early developmental stages and they are restricted to the ventral region of the spinal cord. Motor neurons in the bird are born between stages 17-23 or days 3-4 in a 21 day incubation period (Landmesser, 1980). Spinal motor neurons originate from the ventricular zone of the developing spinal cord, then migrate and settle in their particular pools (Guthrie, 2002). The developing spinal cord regionalizes along the dorsal-ventral (D-V) and anterior-posterior (A-P) axes. D-V polarity in the developing spinal cord is characterized by the action of extracellular signals originating from organizing centers. Signals that specify the ventral neural tube emanate from the notochord and the floor plate later on (Ericson et al., 1997; Goulding et al., 1993). The notochord is below the neural tube and it is the rod of axial mesodermal cells. The floor plate is very close to motoneurons and it consists of a group of specialized glial cells occupying the ventral midline of the neural tube (Placzek et al., 1991). The patterning of ventral fates is mediated through the action of the Sonic hedgehog (Shh) protein (Roelink et al., 1995). The expression of Shh is found at both the notochord and floor plate (Price and Briscoe, 2004). In addition to Shh, it has also been

suggested that some other signaling molecules are involved in the patterning of the ventral spinal cord. BMPs which are members of the transforming growth factor beta (TGF- β) are believed to play a critical role in dorsal ventral patterning. In addition, they appear to have a potent role in regulating the pattern of ventral cell types (Poh et al., 2002).

Motor neurons are characterized as a well-studied neuronal class in the vertebrate central nervous system. Motor neurons in the developing spinal cord are divided into important functional units termed motor pools. Motor pools are defined as clusters of functionally related motor neurons settling within each of the sub-columns of the limb muscles (LMC) (Hollyday, 1980; Landmesser, 2001). All of the neurons within the same motor pool are electrically coupled and receive the same proprioceptive input from neurons located in the dorsal root ganglion. The cell bodies of motor neurons in the same motor pool innervate a single muscle in the periphery (Brenowitz et al., 1983). By studying limb-moving segments of the avian spinal cord, a lot of knowledge of motoneuron development has been obtained. Lumbosacral motoneurons in the bird originate from the ventricular zone of the neural tube between stages 17-23 (Wenger 1950; Hollyday and Hamburger 1997). The generation and differentiation of a majority of motoneurons correlated to limb-moving segments is controlled by intrinsic factors and extrinsic factors (Wenger 1951). The motoneurons innervate their specific muscles and are gathered in a coherent and elongated manner in chick spinal cord (Landmesser 1978).

Skeletal muscles are innervated by a group of motoneurons. The motoneuron pool and the corresponding muscle are connected into a functional unit. The development of motor innervation was investigated in the hind limb of the chick embryo in order to

determine how the spinal nerve is correlated with the hind limb. It seems that there is a craniocaudal pattern of innervation of limb muscles: the more cranial spinal nerves innervate the dorsal muscle first. In contrast, caudal spinal nerves innervate the ventral muscle mass at a later time (Landmesser and Morris 1975). Axons reach the limb bud at stage 27 and form functional connections at the same time. The process of axon initiation happens in a relative short time; all eight spinal nerves have arrived at the base of the limb by stage 23-24. The crural and ischiadic plexuses innervating the hind limb are being formed at the same time. Crural muscle nerves are formed by axons of motoneurons from lumbar segments L1 to L3 while motoneurons of the ischiadic plexus are found throughout lumbar segments L4 to L8 (Landmesser and Morris 1975; Lance-Jones and Landmesser 1981). Motoneurons do not innervate the limb in a random manner. Instead, they match each other in a high myotypic specification (Cruce 1974). The position of a motoneuron cell body is not directly related to its function, nor is it related to the topographical position of its mature muscle. In fact, it is related to its axonal termination site in the embryonic limb bud. It was found that lateral motoneurons innervate the dorsal muscle mass while motoneurons located within the medial region project to the ventral muscle mass. The medial and lateral motoneurons at different segmental levels are able to arrive at the base of the limb at the midpoint of the dorso-ventral limb axis (Landmesser, 1978). All spinal nerves reach the base of the limb at almost the same time because axon outgrowth is comparatively synchronous (Fouvet, 1973). Although there are many different motoneuron pools, errors are rarely found in axon pathfinding. Axons from different motoneuron pools are mixed with each other within proximal spinal nerves. But once they reach the base of the limb, they

defasciculate and regroup into muscle-specific fascicles (Lance-Jones and Landmesser, 1981).

How motoneuron pools reach their specific muscles has been broadly investigated. Some studies have shown that motoneurons have pool-specific identities when they first extend their outgrowths and that they have cell surface molecule differences so that they can fasciculate with similar axons at the base of the limb and reach their corresponding muscle by recognizing limb derived guidance cues (Landmesser, 2001). Guidance cues could be contact-mediated attractive or repulsive signals. Motoneuron growth cones are able to respond to guidance cues along dorsal-ventral axis (Ferguson 1983). The expression of certain genes was used to identify motoneuron subtypes. For example, motoneurons at stages 17-18 express *islet-1* and never express *LIM-1*. These motoneurons settle medially and innervate ventral muscles (Tsuchida et al., 1994). Gene expression patterns are important to determine the precise development time of motoneuron subtypes. Transcription factors including the LIM and ETS families play important roles as they are differentially expressed in motoneurons which project to distinct muscles (Wenner et al., 2000). In addition to exhibiting a pool-specific pattern, chick motoneurons can also be subdivided into fast/slow and flexor/extensor cells depending on whether they innervate fast or slow muscle fibers. How motoneurons develop and find their proper targets are topics that require further investigation. Electrophysiology, gene techniques and tracing methods are useful tools to acquire more knowledge.

In addition to motoneurons, circuits of interneurons regulate the motor output of the spinal cord. These interneurons are characterized as R-interneurons in the developing

chick spinal cord (Wenner and O'Donovan, 1999). Interneurons expressing engrailed-1 in the embryonic chick spinal cord are regulated by γ -aminobutyric acid (GABA) which is the main inhibitory neurotransmitter. They are connected with motoneurons by projecting their axons into the motor column (Wenner et al., 2000). R-interneurons are involved in developing an inhibitory interneuronal circuit in the embryonic spinal cords. The R-interneuron circuit goes through a reorganization process between embryonic day 8 and 15. R-interneurons receive monosynaptic nicotinic input from motoneurons and project back to motoneurons (Xu et al., 2007).

Motoneuron activation is controlled by both inputs from supraspinal neurons and sensory inputs from somatosensory neurons that mediate spinal cord reflexes. It has been shown that activity of lumbar motoneurons is initiated by electrical stimulation of the brain stem at E6 (Sholomenko and O'Donovan, 1995). In addition to the effects of the descending supraspinal pathways on lumbosacral motor activity, sensory neurons can make synaptic connections with a particular group of motoneurons (Fig 1.3). Sensory neurons and motor neurons form precise monosynaptic connections even in the absence of correlated neural activity (Bruce and Eric, 1991). It has been shown that hindlimb muscle afferents make first synaptic contacts with lumbosacral motoneurons between the 8th and 9th days of development (Lee et al., 1988; Lee and O'Donovan, 1991). Muscle afferents form synaptic connections with motoneurons supplying their own or synergistic muscles. It is important for the muscle spindle afferents to innervate the proper groups of motoneurons so that the feedback loop can function precisely in order to generate a spinal reflex (Eric and Bruce, 1990). A particular group of sensory neurons project axons to muscles and react to changes in muscle length. At the same time, these sensory neurons

also innervate specific groups of motoneurons in the spinal cord. These peripheral and central connections form a negative feedback system which underlies the stretch reflex, thus protecting animals from unexpected changes in muscle length (Simon and Eric, 1997).

9. Generation of spontaneous electrical activity is an early feature of spinal cord development

Spinal somatic motor neurons are one of the few neuronal types that project axons out of the CNS and are therefore easily recognized. Their activity can be identified by recording muscle contraction. Embryonic motility is recorded as episodes or bouts separated by quiescent periods. This pattern of motility is called spontaneous activity. Spontaneous activity is a characteristic feature of developing neuronal networks. It is generated by the interaction of the developing network's high excitability and the presence of the activity-induced transient depression of network excitability. Spontaneous activity is divided into two distinct types: that which occurs before the development of chemical synaptic networks, and that which occurs after. Before the formation of chemical synaptic networks, neurons generate spontaneous activity that is characterized by the generation of calcium transients. After chemical synaptic networks arise, another type of spontaneous activity called network-driven activity appears (O'Donovan, 1999). Network-driven spontaneous activity results in an increase of free intracellular calcium in spinal neurons (O'Donovan et al., 1994). Spontaneous activity can be driven either by glutamatergic/cholinergic or GABAergic/glycinergic spinal networks (O'Donovan 1999).

A balance is maintained between excitatory and inhibitory neuronal circuits so that a state of homeostasis is reached (Palla et al., 2006).

In the chick embryo, spontaneous activity is first observed at E3-E4 and it continues until the chick hatches at E21 (Hamburger and Balaban 1963). The spontaneous activity emerges in isolated lumbar cords, independent of descending and afferent input. Originally, it was believed that rhythmic bursting activity was only involved in refinement of neural projections in developing neural circuits such as those in chick or mouse spinal cords. However, it was recently revealed that early spontaneous activity is also important for early pathfinding decisions (Hanson et al., 2008). Chick lumbosacral motoneurons make two major pathfinding decisions: a binary dorsal-ventral choice and a pool-specific pathfinding choice (also called anteroposterior pathfinding). Moderate decreases in burst frequency resulted in dorsal-ventral pathfinding errors. On the other hand, moderate increases of spontaneous bursting episodes greatly disrupted the anteroposterior pathfinding process so that motoneurons failed to fasciculate into pool-specific fascicles and could not grow to the appropriate muscle targets (Hanson and Landmesser 2006).

10. Role of cell death in spinal cord development

There is a phenomenon of natural occurring cell death in the developing nervous system. It accounts for a 20-80% loss of neurons in a given population. In the chick embryo spinal cord, programmed cell death is observed in the lateral motor column from E5 until E10. It was first considered as a mechanism to correct error, by which aberrant

synaptic connections are eliminated. But this notion was challenged by Landmesser who conducted a study and confirmed that there is no change of the position of motoneuron pools before and after the period of major cell death (Landmesser 1978). Further results have confirmed that prevention of cell death from embryonic day 5 to embryonic day 10 does not change the innervation of motoneuron pools to specific hindlimb muscles. Many motoneurons that are rescued from cell death are able to project axons to their appropriate synaptic targets (Oppenheim 1981).

There are several mechanisms which may explain this phenomenon. It has been demonstrated that cell death is mainly regulated by interactions between motoneurons and their targets (Hollyday and Hamburger 1976). It was revealed that synaptic transmission at the neuromuscular junction is also involved in cell death (Pittman and Oppenheim 1978). After treating chick embryos with a neuromuscular competitive blocker such as curare, it was found that blockade of neuromuscular activity results in an increased number of acetylcholine receptors (AChRs) followed by increased innervation and increased motoneuron survival (Oppenheim et al., 1989). Neuromuscular development was examined in an avian genetic mutant called “crook neck dwarf” in which spontaneous, neurally mediated movements are absent. It was found that there is increased branching of motoneuron axons and a greater number of synaptic contacts in the mutant muscle. The rate of motoneuron survival in the lumbar spinal cord by the end of programmed cell death is significantly higher in mutant embryos than in wild type embryos (Oppenheim et al., 1997). It was proposed that reduced muscle activity would result in increased production or availability of muscle-derived neurotrophic factors, which prevent motoneuron cell death. Afferent input was also examined to see if it plays

a role in the regulation of cell death. Removing supraspinal, propriospinal and primary sensory afferents from the lumbar spinal cord caused a significant loss of motoneurons by E10 (Okado and Oppenheim 1984). Cell death induced by afferent input removal can be decreased by adding CNS-derived neurotrophic factors (Qin-Wei et al., 1994).

In addition to neurotrophic factors, neurotransmitters are suspected to play a role in motoneuron survival. Treating chick embryos in ovo from E6 to E9 with CPP, a competitive NMDA receptor antagonist, results in a significant reduction in motoneuron death (Caldero et al., 1997). Excitatory amino acids could have either a trophic or toxic effect on chick embryo spinal cord motoneurons by modulating intracellular calcium. Early activation of NMDA receptors in chick motoneurons results in a survival-promoting effect. This result is caused by downregulation of the NMDA receptor (Llado et al., 1999). In addition to the NMDA receptor, it was found that periodic blockade of AMPA/kainate receptors is also involved in protecting neurons from naturally occurring cell death and deafferentation-induced neuronal death. AMPA/kainate receptor antagonist CNQX was applied to chick embryos in ovo and increased cell survival in the nucleus laminaris from E8 to E10. It is possible that CNQX may act through calcium-permeable non-NMDA receptors such as the AMPA receptor (Solum et al., 1997).

11. Morphological maturation of spinal motoneurons

The process of neurite outgrowth is a commonly observed phenomenon in developing neurons. The development of dendritic morphology is a process associated with a lot of growth and remodeling (Threadgill et al., 1997). Neurite outgrowth is

affected by many factors including neurotrophic factors, the extracellular matrix, electrical activity, and neurotransmitters. Glutamate is one of the neurotransmitters that has been studied with respect to its regulation of neurite outgrowth. Glutamate can either promote or inhibit dendrite outgrowth depending on the developmental stage or condition. In addition to playing a role in regulating cell survival, ionotropic AMPA receptors are also considered to be responsible for regulating dendrite outgrowth through excitatory neurotransmission. By activating AMPA/kainate receptors on motoneurons obtained from 15-day old rat embryos, glutamate is able to inhibit dendrite but not axon outgrowth. Inhibition of dendritic outgrowth is closely related to Ca^{2+} influx through AMPA receptors. Blockage of Ca^{2+} -permeable AMPA receptors by joro spider toxin (JSTX-3) results in increased dendrite growth in embryonic rat motoneurons. In addition, the effect is dose-dependent and reversible (Metzger et al. 1998). Neurite retraction is observed in tangentially migrating neurons in the intermediate zone of the embryonic rat neocortex after activation of AMPA alone for 6 hours or exposure to both AMPA and desensitization blocker cyclothiazide (CTZ) for 3 hours. This signaling cascade was explored and calcium is proposed as the most probable intracellular mediator (Poluch et al., 2001). The neurite outgrowth regulated by Ca^{2+} -permeable AMPA receptors was investigated in chick retinal cells. It was shown that Ca^{2+} influx through AMPA receptors is responsible for the reduction of neurite outgrowth upon AMPA receptor activation. This effect can be inhibited by applying AMPA/kainate receptor antagonist CNQX. The inhibition of neurite outgrowth by AMPA receptor activation is limited to dendrites and does not include axons (Catsicas et al., 2001).

As mentioned above, glutamate can either enhance or decrease neurite outgrowth, effectively regulating neuronal plasticity and neuropathology. It was revealed that a transient increase in Ca^{2+} influx during the first hour of glutamate activation results in increased dendrite outgrowth while a sustained increase in Ca^{2+} influx leads to dendrite retraction. It was suggested that glutamate-induced dendrite retraction may be accompanied by calpain-mediated decreases in the dendritic microtubule levels. Glutamate-induced dendrite outgrowth is possibly related to calmodulin regulation of cytoskeletal dynamics (Wilson et al., 2000). Similar results were found in immature mouse cortical neurons. By applying kainate on E18 mouse cortical neurons, it was shown that both primary dendrite growth and length are increased. This result indicates that kainate receptor activation plays a role in promoting neurite growth in early developmental stages (Monnerie and Le Roux 2006). There is evidence showing that afferent innervation has a great effect on the development of dendritic branches and spines in hippocampal neurons. Afferents are able to induce the formation of dendritic branches in an activity-independent manner. In contrast, formation of dendritic branches is affected by blockers of glutamate receptors or by blockade of spontaneous activity (Kossel et al., 1997). Among all of the previous experiments conducted, Ca^{2+} is believed to be the key factor in determining neuronal differentiation and morphology. While many experiments have been conducted to study the role of AMPA/kainate receptors in vitro, the role of AMPA receptors on developmental neurons in vivo has yet to be determined. The goal of our study is to determine the role of Ca^{2+} -permeable AMPA receptors in the early developmental stages of chick spinal cord motoneuron morphology.

12. Rationale for studying the developmental regulation of AMPA receptors in spinal motoneurons

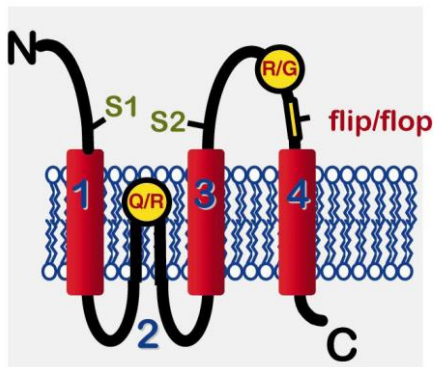
Functional non-NMDA receptors are expressed at very early stages of neurodevelopment. Expression of these receptors may have important implications in the overall formation of neuronal circuits that have yet to be fully explored. In the chicken embryo, functional non-NMDA receptors are expressed as early as E5. These receptors respond to activation by kainate and AMPA (O'Brien and Fischbach, 1986). Little is known regarding the subunit composition of AMPA receptors at early stages of development. However, the functional characteristics of AMPA receptors vary greatly based on their subunit composition, RNA editing, and alternative splicing. Synaptic receptor heterogeneity is a leading factor in determining the various functional properties of AMPA receptors in neurons (Grunder et al., 2000). Changes in GluR2 expression have important implications for AMPA receptor function because GluR2 insertion into functional channels regulates the Ca^{2+} permeability of the receptors (Geiger et al., 1995). RNA editing mediates the permeability and receptor kinetics of glutamate receptors (Lowe et al., 1997). GluR2 editing at a site in the pore forming domain affects rectification and ionic permeability (Verdoorn et al., 1991; Sommer et al., 1991). Editing is developmentally regulated in adult animals. Unedited forms of the AMPA receptor are more common at early stages of development, while edited forms are expressed more at later developmental stages (Lomeli et al., 1994).

The early-expressed AMPA receptors may play a functional role during the maturation of the nervous system. Glutamate and GABA synaptic transmission contribute

to network activity at later stages of development (>E8) (Chub & O'Donovan, 1998). The neuronal circuits that produce spontaneous activity at early stages of development (between E4-E6) rely solely on cholinergic and GABAergic neurotransmission (Milner & Landmesser, 1999; Bekoff, 1976). E8 appears to be a critical point during the development of the chick spinal cord. Although glutamate receptors are not involved in the generation of early spontaneous activity in the spinal cord, they may play a critical role in regulating the morphological differentiation of motoneurons. In *Xenopus*, spinal cord neurons express functional AMPA and NMDA receptors shortly after the initiation of neurite outgrowth (Gleason & Spitzer, 1998). Synaptic activity generated by activation of glutamate receptors also contributes to dendritic remodeling by regulating intracellular Ca^{2+} (Ramoia et al., 1988). Increased intracellular Ca^{2+} prevents growing dendrites from forming synapses (Lohmann et al., 2005). We are using chick lumbar motoneurons as a model to study the developmental expression of AMPA receptors and their role in regulating neuronal differentiation. Our hypotheses include 1) Chick LMNs express lower levels of GluR2 mRNA at E6 than at E11 in individual motoneurons. 2) There are different patterns of AMPA receptor subunit expression in developing chick LMNs and the changes in the Ca^{2+} -permeability of AMPA receptors are solely related to the variations in the expression of the GluR2 subunit. 3) Elevated intracellular Ca^{2+} caused by downregulation of the GluR2 subunit plays a role in regulating dendritic outgrowth in chick LMNs.

In summary, the AMPA receptor is involved in morphological alterations in neurons and has neuroprotective functions (Mattson et al., 1988; Bambrick et al., 1995). Excessive activation of AMPA receptors could result in neurotoxicity and pathological

changes in the nervous system (Meldrum, 1994). Ca^{2+} -permeable AMPA receptors are implicated in some neurological disorders and injuries, such as global ischemia, seizures, and spinal cord injury. Downregulated GluR2 mRNA and protein expression are observed in these diseases. Excessive Ca^{2+} influx through GluR2-lacking AMPA receptors induces subsequent cell death (Liu and Zukin, 2007). Motoneurons are found to be more vulnerable to AMPA receptor activation than other spinal neurons (Vandenberghé et al., 2000). The increased excitation of motoneurons can result in either death of some motoneurons or rapid elimination of nerve-muscle contacts (Tyc and Vrbova, 2003).



RNA editing of GluR subunits

	Q/R SITE		R/G SITE	
	Unedited (Q)	Edited (R)	Unedited (R)	Edited (G)
GluR1	100 %	0 %	100 %	0 %
GluR2	<0.01 %	>99.99 %	10-20 %	80-90 %
GluR3	100 %	0 %	10-20 %	80-90 %
GluR4	100 %	0 %	~50 %	~50 %

Figure 1.1: Structure of the GluR subunits. This secondary structure of the GluR subunits includes an extracellular N-terminus domain, three transmembrane domains (TM1, TM3 and TM4), a hydrophobic segment which lines the ion channel (M2), two binding domains (S1, S2), and an intracellular C-terminus domain. Q/R and R/G are two alternative editing sites. Normally, almost 100% of the GluR2 mRNA is edited at the Q/R site. (Copyright 2000 Elsevier Science B.V.)

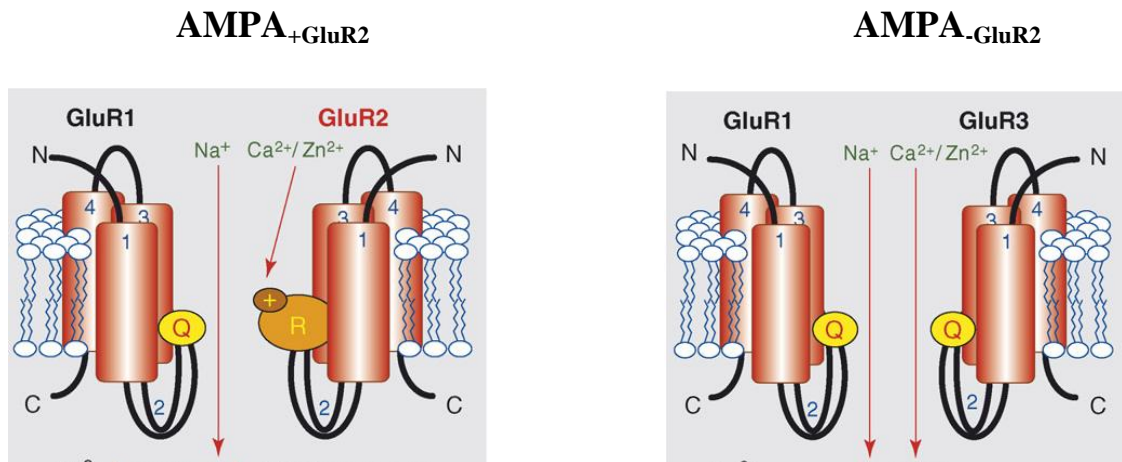


Figure 1.2: The Ca^{2+} permeability of AMPA receptors depends on the subunit composition of the receptor. The presence of at least one edited GluR2 subunit determines the Ca^{2+} permeability of AMPA receptors. GluR2-containing AMPA receptors are impermeable to Ca^{2+} , whereas GluR2-lacking receptors are highly permeable to Ca^{2+} . (Copyright 2007 Elsevier Ltd)

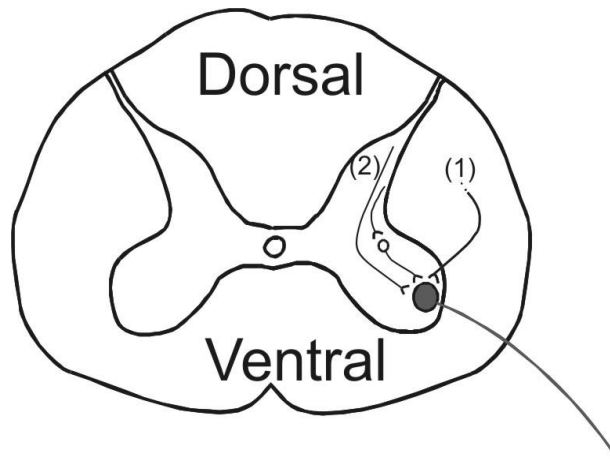


Figure 1.3: Ventral spinal motoneurons receive input from descending supraspinal terminals (1) and sensory inputs (2). Descending supraspinal inputs are critical in mediating purpose locomotion in the adult spinal cord, whereas sensory inputs mediate spinal cord reflexes.

REFERENCES

- Ammori JB, Zhang W, Newman EA, Mulholland MW (2007) Glutamate-induced calcium transients in rat neurons of the dorsal motor nucleus of the vagus. *Journal of Gastrointestinal Surgery* 11(8):1016-1024.
- Armstrong N, Gouaux E (2000) Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structure of the GluR2 ligand binding core. *Neuron* 28:165-181.
- Armstrong N, Sun Y, Chen G-Q, Gouaux E (1998) Structure of a glutamate-receptor ligand-binding core in complex with kainite. *Nature* 395:913-917.
- Arvanov VL, Seebach BS, Mendell LM (2000) NT-3 evokes an LTP-like facilitation of AMPA/kainite receptor-mediated synaptic transmission in the neonatal rat spinal cord. *Journal of Neurophysiology* 84(2):752-758.
- Ayalon G, Stern-Bach Y (2001) Functional assembly of AMPA and kainite receptors is mediated by several discrete protein-protein interactions. *Neuron* 31:103-113.
- Banke TG, Bowie D, Lee HK, Huganir RL, Schousboe A, Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *Journal of Neuroscience* 20(1):89-102.
- Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, Zastrow MV, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nature Neuroscience* 3(12):1291-1300.
- Bekoff A (1976) Ontogeny of leg motor output in the chick embryo: a neural analysis. *Brain Res* 106:271-291.
- Bettler B, Boulter J, Hermans-Borgmeyer I, O'Shea-Greenfield A, Deneris ES, Moll C, Borgmeyer U, Hollmann M, Heinemann S (1990) Cloning of a novel glutamate receptor subunit, GluR5-expression in the nervous-system during development. *Neuron* 5(5):583-595.
- Bochet P, Audinat E, Lambolez B, Crepel F, Rossier J, Iino M, Tsuzuki K, Ozawa S (1994) Subunit composition at the single-level explains functional properties of a glutamate-gated channel. *Neuron* 12:383-388.
- Bowie D, Mayer ML (1995) Inward rectification of both AMPA and Kainite subtype Glutamate receptors generated by polyamine-mediated ion-channel block. *Neuron* 15(2):453-462.

- Brene S, Messer C, Okado H, Hartley M, Heinemann SF, Nestler EJ (2000) Regulation of GluR2 promoter activity by neurotrophic factors via a neuron-restriction silence element. *European Journal of Neuroscience* 12(5):1525-1533.
- Brill J, Huguenard JR (2008) Sequential changes in AMPA receptors targeting in the developing neocortical excitatory circuit. *The Journal of Neuroscience* 28(51):13918-13928.
- Bruce M, Eric F (1991) Specific monosynaptic sensory-motor connections form in the absence of patterned neural activity and motoneuronal cell death. *Journal of Neuroscience* 11(5):1390-1403.
- Burnashev N., Monyer H., Seeburg P. H. and Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8(1):189-198
- Caicedo A, Kungel M, Pujol R, Friauf E (1998) Glutamate-induced Co^{2+} uptake in rat auditory brainstem neurons reveals developmental changes in Ca^{2+} permeability of glutamate receptors. *European Journal of Neuroscience* 10(3):941-954.
- Calderia MV, Melo CV, Pereira DB, Carvalho R, Correia SS, Backos DS, Carvalho AL, Esteban JA, Duarte CB (2007) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of α -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. *Journal of Biological Chemistry* 282(17):12619-12628.
- Caldero J, Ciutat D, Llado J, Castan E, Oppenheim RW, Esquerda JE (1997) Effects of excitatory amino acids on neuromuscular development in the chick embryo. *Journal of Comparative Neurology* 387(1):73-95.
- Carvalho AL, Kameyama K, Huganir RL (1999) Characterization of phosphorylation sites on the glutamate receptor 4 subunit of the AMPA receptors. *Journal of Neuroscience* 19(12):4748-4754.
- Catsicas M, Allcorn S, Mobbs P (2001) Early activation of Ca^{2+} -permeable AMPA receptors reduce neurite outgrowth in embryonic chick retinal neurons. *Journal of Neurobiology* 49(3):200-211.
- Chan YS, Chen LW, Lai CH, Shum DKY, Yung KKL, Zhang FX (2003) Receptors of glutamate and neurotrophin in vestibular neuronal functions. *Journal of Biomedical Science* 10(6):577-587.
- Cheng B, Furukawa K, O'Keefe JA, Goodman Y, Kihiko M, Fabian T, Mattson MP (1995) Basic fibroblast growth-factor selectively increases AMPA-receptor subunit GluR1 protein level and differentially modulates Ca^{2+} response to AMPA and MNDA in hippocampal-neurons. *Journal of Neurochemistry*

65(6):2525-2536.

- Chub N, O'Donovan (1998) Blockade and recovery of spontaneous rhythmic activity after application of neurotransmitter antagonists to spinal networks of the chick embryo. *The Journal of Neuroscience* 18(1):294-306.
- Chung HJ, Xia J, Scannevin RH, Zhang X, Haganir RL (2000) Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *Journal of Neuroscience* 20(19):7258-7267.
- Cruce WLR (1974) The anatomical organization of hind limb motoneurons in the lumbar spinal cord of the frog *Rana-Catesbeiana*. *Journal of Comparative Neurology* 153(1):59-76.
- Dingledine R, Hume RI, Heinemann SF (1992) Structural determinants of barium permeation and rectification in non-NMDA glutamate receptor channels. *Journal of Neuroscience* 12:4080-4087.
- Durand GM, Kovalchuk Y, Konnerth A (1996) Long-term potentiation and function synapse induction in developing hippocampus. *Nature* 381(6577):71-75.
- Eric F, Bruce M (1990) Specification of synaptic connections between sensory and motor neurons in the developing spinal cord. *Journal of Neurobiology* 21(1):33-50.
- Eybalin M, Caicedo A, Renard N, Ruel J, Puel JL (2004) Transient Ca²⁺-permeable AMPA receptors in postnatal rat primary auditory neurons. *European Journal of Neuroscience* 20(11):2981-2989.
- Fakler B, Brandle U, Glowatzki E, Weidemann S, Zenner HP, Ruppersberg JP (1995) Strong voltage-dependent inward rectification of inward rectifier K⁺ channels is caused by intracellular spermine. *Cell* 80(1):149-154.
- Feldmeyer D, Kask K, Brusa R, Kornau HC, Kolhekar R, Rozov A, Burnashev N, Jensen V, Hvalby O, Sprengel R, Seeburg PH (1999) Neurological dysfunctions in mice expression different levels of the Q/R site-unedited AMPAR subunit GluR-B. *Nature Neuroscience* 2(1):57-64.
- Ferguson BA (1983) Development of motor innervation of the chick following dorsal-ventral limb bud rotations. *Journal of Neuroscience* 3(9):1760-1772.
- Fouvet PB (1973) Innervation et morphogenese de la patte chez l'embryon de poulet. I. Mise en place de l'innervation normale. *Arch. Anat Microsc. Morphol. Exp* 62:269-280.
- Friedman LK, Veliskova J (1998) GluR2 hippocampal knockdown reveals developmental

regulation of epileptogenicity and neurodegeneration. *Molecular brain research* 61(1-2):224-231.

Fujiyanma F, Kuramoto E, Okamoto K, Hioki H, Furuta T, Zhou L, Nomura S, Kaneko T (2004) Presynaptic localization of an AMPA-type glutamate receptor in corticostriatal and thalamostriatal axon terminals. *European Journal of Neuroscience* 20(12):3322-3330.

Gardner SM, Trussell LO, Oertel D (1999) Time course and permeation of synaptic AMPA receptor in cochlear nuclear neurons correlate with input. *Journal of Neuroscience* 19(20):8721-8729.

Gardner SM, Takamiya K, Xia Jun, Suh J, Johnson R, Yu S, Huganir RL (2005) Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF. *Neuron* 45:903-915.

Geiger JPR, Melcher T, Koh D-S, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca²⁺ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron* 15:193-204.

Gleason EL, Spitzer NC (1998) AMPA and NMDA receptor expressed by differentiating *Xenopus* spinal neurons. *Journal of Neurophysiology* 79(6):2986-2998.

Gouaux E (2004) Structure and function of AMPA receptors. *Journal of Physiology* 554:249-253.

Greger IH, Khatri L, Kong X, Ziff EB (2003) AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* 40(4):763-774.

Greig A, Donevan SD, Mujtaba TJ, Parks TN, Rao MS (2000) Characterization of the AMPA-activated receptors present on motoneurons. *Journal of Neurochemistry* 74(1):179-191.

Grunder T, Kohler K, Guenther E (2000) Distribution and developmental regulation of AMPA receptor subunit proteins in rat retina. *Investigative Ophthalmology & Visual Science* 41(11):3600-3606.

Guthrie S (2002) Neuronal development: sorting out motor neurons. *Current Biology* 12:R488-R490.

Hamburger V, Balaban M (1963) Observations and experiments on spontaneous rhythmic behavior in the chick embryo. *Dev Biol* 7:533-545.

Hamburger V (1975) Cell death in the development of the lateral motor column of the

- chick embryo. *J. Comp. Neurol* 160:535-546.
- Hanson MG, Landmesser LT (2006) Increasing the frequency of spontaneous rhythmic activity disrupts pool-specific axon fasciculation and pathfinding of embryonic spinal motoneurons. *Journal of Neuroscience* 26(49):12769-12780.
- Hanson MG, Milner LD, Landmesser LT (2008) Spontaneous rhythmic activity in early chick spinal cord influences distinct motor axon pathfinding decision. *Brain Research reviews* 57:77-85.
- Hartmann B, Ahmadi S, Heppenstall PA, Lewin GR, Schott C, Borchardt T, Seeburg PH, Zeihofer HU, Sprengel R, Kuner R (2004) The AMPA receptor subunits GluR-A and GluR-B reciprocally modulate spinal synaptic plasticity and inflammatory pain. *Neuron* 44(4):637-650.
- Herin GA, Aizenman E (2004) Amino terminal domain regulation of NMDA receptor function. *Eur J Pharmacol* 500:101-111.
- Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} permeability of Ka -AMPA gated glutamate receptor channels depends on subunit composition. *Science* 252(5007):851-853.
- Hollmann M, Heinemann S (1994) Cloned glutamate receptors. *Annual Review of Neuroscience* 7: 31-108.
- Hollyday M, Hamburger V (1976) Reduction of naturally-occurring motor neuron loss by enlargement of the periphery. *J. Comp. Neuro* 170:311-320.
- Hollyday M, Hamburger V (1997) An autoradiographic study of the formation of the lateral motor column in the chick embryo. *Brain Research* 132:197-208.
- Hunter C, Petralia RS, Vu T, Wenthold RJ (1993) Expression of AMPA-selective glutamate receptor subunits in Morphologically defined neurons of the mammalian cochlear nucleus. *Journal of Neuroscience* 13(5):1932-1946.
- Jakowec MW, Jackson-Lewis V, Chen X, Langston JW, Przedborski S (1998) The postnatal development of AMPA receptor subunits in the basal ganglia of the rat. *Developmental Neuroscience* 29(1):19-33.
- Jakowec MW, Yen L, Kalb RG (1995) In-Situ hybridization analysis of AMPA receptor subunits gene-expression in the developing rat spinal-cord. *Neuroscience* 67(4):909-920.
- Janssens N, Lesage ASJ (2001) Glutamate receptor subunit expression in primary neuronal and secondary glial cultures. *Journal of Neurochemistry* 77(6):1457-

1474.

- Jona P, Racca C, Sakmann B, Seeburg PH, Monyer H (1994) Differences in Ca^{2+} permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* 12(6):1281-1289.
- Kalb RG, Fox AJ (1997) Synchronized overproduction of AMPA, kainite, and NMDA glutamate receptors during human spinal cord development. *Journal of Comparative Neurology* 384(2):200-210.
- Kamboj SK, Swanson GT, Cull-Candy SG (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainite receptors. *Journal of Physiology-London* 486(2):297-303.
- Keller BU, Hollmann M, Heinemann S, Konnerth A (1992) Calcium influx through subunits GluR1 GluR3 of Kainate AMPA receptor channels is regulated by cAMP dependent protein-kinase. *Embo Journal* 11(3):891-896.
- Kim EJ, Sheng M (2004) PDZ domain proteins of synapse. *Nature Reviews Neuroscience* 5(10):771-781.
- Koh DS, Burnashev N, Jonas P (1995) Block of native Ca^{2+} -permeable AMPA receptors in rat-brain by intracellular polyamines generates double rectification. *Journal of Physiology* 486(2):305-312.
- Kohler M., Kornau H.-C. and Seeburg P. H. (1994) The organization of the gene for the functionally dominant α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor subunit GluR-B. *J. Biol. Chem.* 269: 17367–17370
- Kossel AH, Williams CV, Schweizer M, Kater SB (1997) Afferent innervation influences the development of dendritic branches and spines via both activity-dependent and non-activity-dependent mechanisms. *The Journal of Neuroscience* 17(16):6314-6324.
- Kumar SS, Bacci A, Kharazia V, Huguenard JR (2002) A development switch of AMPA receptor subunits in neocortical pyramidal neurons. *Journal of Neuroscience* 22(8):3005-3015.
- Lambolez B, Audinat E, Bochet P, Crepel F, Rossier J (1992) AMPA receptor subunits expressed by single Purkinje-cells. *Neuron* 9(2):247-258.
- Lance-Jones C, Landmesser L (1981) Pathway selection by chick lumbo sacral moto neurons during normal development. *Proceedings of the Royal Society of London Series B Biological Biological Sciences* 214(1194):1-18.
- Landmesser L (1978) The development of motor projection patterns in the chick hind

- limb. *Journal of Physiology (Cambridge)* 284:391-414.
- Landmesser L (1978) The distribution of moto neurons supplying chick hind limb muscles. *Journal of Physiology (Cambridge)* 284:371-390.
- Landmesser L, Morris DG (1975) The development of function innervation in the hind limb of the chick embryo. *Journal of Physiology (Cambridge)* 249(2):301-326.
- Landmesser LT (1980) The generation of neuromuscular specificity. *Ann. Rev. Neurosci.* 3:279-302.
- Landmesser LT (2001) The acquisition of motoneuron subtype identity and motor circuit formation. *International Journal of Development Neuroscience* 19(2):175-182.
- Lawrence JJ, Trussell LO (2000) Long-term specification of AMPA receptor properties after synapse formation. *Journal of Neuroscience* 20(13):4864-4870.
- Lee MT, Koebbe MJ, O'Donovan MJ (1988) The development of sensorimotor synaptic connections in the lumbosacral spinal cord of the chick embryo. *Journal of Neuroscience* 8:2530-2543.
- Lee MT, O'Donovan MJ (1991) Organization of hindlimb muscle afferent projections to lumbosacral motoneurons in the chick embryo. *Journal of Neuroscience* 11(8):2564-2573.
- Lee YH, Lin CH, Hsu LW, Hu SY, Hsiao WT, Ho YS (2003) Roles of ionotropic glutamate receptors in early developing neurons derived from the P19 mouse cell line. *Journal of Biomedical Science* 10:199-207.
- Liu SQJ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. *Nature* 405(6785):454-458.
- Liu SQJ, Zukin RS (2007) Ca²⁺-permeable AMPA receptors in synaptic plasticity and neuronal death. *TRENDS in Neurosciences* 30(3):126-134.
- Llado J, Caldero J, Ribera J, Tarabal O, Oppenheim RW, Esquerda JE (1999) Opposing effects of excitatory amino acids on chick embryo spinal cord motoneurons: Excitotoxic degeneration or prevention of programmed cell death. *Journal of Neuroscience* 19(24):10803-10812.
- Lomeli H, Mosbacher J, Melcher T, Hoyer T, Geiger JRP, Kuner T, Monyer H, Higuchi M, Bach A, Seeburg PH (1994) Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266(5191):1709-1713.
- Lowe DL, Jahn K, Smith DO (1997) Glutamate receptor editing in the mammalian

- hippocampus and avian neurons. *Molecular Brain Research* 48:37-44.
- Lu CR, Hwang J, Phend KD, Rustioni A, Valtschanoff JG (2002) Primary afferent terminals in spinal cord express presynaptic AMPA receptors. *Journal of Neuroscience* 22(21):9522-9529.
- Lu YM, Yin HZ, Chiang J, Weiss JH (1996) Ca²⁺-permeable AMPA/kainite and NMDA channels: High rate of Ca²⁺ influx underlies potent induction of injury. *Journal of Neuroscience* 16(17):5457-5465.
- Luscher C, Frerking M (2001) Restless AMPA receptors: implications for synaptic transmission and plasticity. *Trends in Neurosciences* 24(11):665-670.
- Martin LJ, Furuta A, Blackstone CD (1998) AMPA receptor protein in developing rat brain: Glutamate receptor-1 expression and localization change at regional, cellular, and subcellular levels with maturation. *Neuroscience* 83(3):917-928.
- Mattson MP, Dou P, Kater SB (1998) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *Journal of Neuroscience* 8:2087-2100.
- Meldrum BS (1994) The role of glutamate in epilepsy and other CNS disorders. *Neurology* 44:S14-S23.
- Mayer ML, Armstrong N (2004) Structure and function of glutamate receptor ion channels. *Annu. Rev. Physiol* 66:161-181.
- McDonald JW, Johnston MV (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Research Review* 15:41-70.
- Metzger F, Wiese S, Sendtner M (1998) Effect of glutamate on dendritic growth in embryonic rat motoneurons. *Journal of Neuroscience* 18(5):1735-1742.
- Meucci O, Fatatis A, Holzwarth JA, Miller RJ (1996) Development regulation of the toxin sensitivity of Ca²⁺-permeable AMPA receptors in cortical glia. *Journal of Neuroscience* 16(2):519-530.
- Monnerie H, Le Roux PD (2006) Glutamate receptor agonist kainite enhances primary dendrite number and length from immature cortical neurons in vitro. *Journal of Neuroscience Research* 83(6):944-956.
- Monyer H, Seeburg PH, Wisden W (1991) Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. *Neuron* 6:799-810.

- Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppertsberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. *Science* 266(5187):1059-1062.
- Ni X, Sullivan GJ, Martin-Caraballo M (2007) Developmental characteristics of AMPA receptors in chick lumbar motoneurons. *Developmental Neurobiology* 67(11):1419-1432.
- Niedzielski AS, Wenthold RJ (1995) Expression of AMPA, kainite, and NMDA receptor subunits in cochlear and vestibular ganglia. *Journal of Neuroscience* 15(3):2338-2353.
- Nutt SL, Kamboj RK (1994) Differential RNA editing efficiency of AMPA receptor subunit GluR-2 in human brain. *Neuroreport* 5:1679-1683.
- O'Brien RJ, Fischbach GD (1986) Modulation of embryonic chick motoneuron glutamate sensitivity by interneurons and agonists. *Journal of Neuroscience* 11:3290-3296.
- O'Brien RJ, Kamboj S, Ehlers MD, Rosen KG, Fischbach GD, Huganir RL (1998) Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21(5):1067-1078.
- O'Brien RJ, Lau LF, Huganir RL (1998) Molecular mechanisms of glutamate receptor clustering at excitatory synapses. *Current Opinion in Neurobiology* 8:364-369.
- O'Brien RJ, Mammen AL, Blackshaw S, Ehlers MD, Rothstein JD, Huganir RL (1997) The development of excitatory synapses in cultured spinal neurons. *The Journal of Neuroscience* 17(19):7339-7350.
- O'Brien RJ, Xu DS, Petralia RS, Steward O, Huganir RL, Worley P (1999) Synaptic clustering of AMPA receptors by the extracellular immediate-early gene product *Narp*. *Neuron* 23(2):309-323.
- O'Connell MA (1997) RNA editing: rewriting receptors. *Curr. Biol* 7:R437-R439.
- O'Donovan M, Ho S, Yee W (1994) Calcium imaging of rhythmic network activity in the developing spinal cord of the chick embryo. *Journal of Neuroscience* 14(11):6354-6369.
- O'Donovan MJ (1999) The origin of spontaneous activity in developing networks of the vertebrate nervous system. *Current Opinion in Neurobiology* 9:94-104.
- O'Brien RJ, Mammen AL, Blackshaw S, Ehlers MD, Rothstein JD, Huganir RL (1997) The development of excitatory synapses in cultured spinal neurons. *Journal of Neuroscience* 17(19):7339-7350.

- Okado N, Oppenheim RW (1984) Cell-death of motoneurons in the chick-embryo-spinal-cord .9. the loss of motoneurons following removal of afferent inputs. *Journal of Neuroscience* 4(6):1639-1652.
- Oppenheim RW(1981) Cell death of motoneurons in the chick embryo spinal cord V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connections. *Journal of Neuroscience* 1(2):141-151.
- Oppenheim RW, Bursztajn S et al. (1989) Cell-death of motoneurons in the chick-embryo-spinal-cord.11. Acetylcholine-receptors and synaptogenesis in skeletal-muscle following the reduction of motoneuron death by neuromuscular blockade. *Development* 107(2):331-341.
- Oppenheim RW, Prevette D, Houenou LJ, Pincon-Raymond M, Dimitriadou V, Donevan A, O'Donovan M, Wenner O, Mckemy DD, Allen PD (1997) Neuromuscular development in the avian paralytic mutant crooked neck dwarf (cn/cn): Further evidence for the role of neuromuscular activity in motoneuron survival. *Journal of Comparative Neurology* 381(3):353-372.
- Otis TS, Raman IM, Trussell LO (1995) AMPA receptors with high Ca^{2+} permeability mediate synaptic transmission in the avian auditory pathway. *Journal of Physiology-London* 482(2):309-315.
- Palla SL, Wenner P, Gonzalez-Islas C, Fagiolini M, Razak KA, Kim G, Sanes D, Roerig B (2006) Developmental plasticity of inhibitory circuitry. *Journal of Neuroscience* 26(41):10358-10361.
- Pickard L, Noel J, Henley JM, Collingridge GL, Molnar E (2000) Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA receptor subunit composition in living hippocampal neurons. *Journal of Neuroscience* 20(21):7922-7931.
- Pittman R, Oppenheim RW (1978) Neuromuscular blockade increases motoneuron survival during normal cell death in the chick embryo. *Nature* 271:364-366.
- Placzek M, Yamada T, Tessier-lavigne M, Jessell T, Dodd J (1991) Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development supplement* 2:105-122.
- Poh A, Karunaratne A, Kolle G, Huang N, Smith E, Starkey J, Wen D, Wilson I, Yamada T, Hargrave M (2002) Patterning of the vertebrate ventral spinal cord. *Int. J. Dev. Biol.* 46:597-608.
- Poluch S, Drian MJ, Durand M, Astier C, Benyamin Y, Konig N (2001) AMPA receptor

activation leads to neurite retraction in tangentially migrating neurons in the intermediate zone of the embryonic rat neocortex. *Journal of Neuroscience Research* 63(1):35-44.

Ragnarson B, Ornung G, Grant G, Ottersen OP, Ulfhake B (2003) Glutamate and AMPA receptor immunoreactivity in la synapse with motoneurons and neurons of the central cervical nucleus. *Experimental Brain Research* 149(4):447-457.

Rao A, Kim E, Sheng M, Craig AM (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *Journal of Neuroscience* 18(4):1217-1229.

Ritter LM, Vazquez DM, Meador-Woodruff JH (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. *Development Brain Research* 139(2):227-236.

Rueter SM, Burns CM, Coode SA, Mookherjee P, Emeson RB (1995) Glutamate receptor RNA editing in vitro by enzymatic conversion of adenosine to inosine. *Science* 267:1491-1494.

Sands SB, Barish ME (1992) Neuronal Nicotinic Acetylcholine-receptor currents in pheochromocytoma (Pc12) cells-dual mechanisms of rectification. *Journal of Physiology-London* 447:467-487.

Sanes DH, Song J, Tyson J (1992) Refinement of dendritic arbors along the tonotopic axis of the gerbil lateral superior olive. *Developmental Brain Research* 67(1):47-55.

Santos AE, Duarte CB, Iizuka M, Barsoumian EL, Ham J, Lopes MC, Carvalho AP, Carvalho AL (2006) Excitotoxicity mediated by Ca²⁺-permeable GluR4-containing AMPA receptors involves the AP-1 transcription factor. *Cell Death and Differentiation* 13(4):652-660.

Seifert G, Zhou M, Dietrich D, Schumacher TB, Dybek A, Weiser T, Wienrich M, Wilhelm D, Steinhauser C (2000) Development regulation of AMPA-receptor properties in CA1 pyramidal neurons of rat hippocampus. *Neuropharmacology* 39(6):931-942.

Sheng M, Lee SH (2001) AMPA receptor trafficking and the control of synaptic transmission. *Cell* 105(7):825-828.

Sholomenko GN, O'Donovan MJ (1995) Development and characterization of pathways descending to the spinal cord in the embryonic chick. *Journal of Neurophysiology* 73(3):1223-1233.

- Simon CM, Eric F (1997) Formation of specific monosynaptic connections between muscle spindle afferents and motoneurons in the mouse. *Journal of Neuroscience* 17(9):3128-3135.
- Solum D, Hughes D, Major MS, Parks TN (1997) Prevention of normally occurring and deafferentation-induced neuronal death in chick brainstem auditory neurons by periodic blockade of AMPA/kainite receptors. *Journal of Neuroscience* 17(12):4744-4751.
- Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Kohler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* 249(4976):1580–1585.
- Sommer B., Kohler M., Sprengel R. and Seeburg P. H. (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channel. *Cell* 67(1):11–19
- Song I, Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. *Trends in Neuroscience* 25(11):578-588.
- Strack S, Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl-D-aspartate receptor. *Journal of Biological Chemistry* 273(33):20689-20692.
- Stromgaard K, Mellor I (2004) AMPA receptor ligands: synthetic and pharmacological studies of polyamines and polyamine toxins. *Medicinal research reviews* 24(5):589-620.
- Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S (2005) Expression profile of AMPA receptor subunit mRNA in single adult rat brain and spinal cord neurons in situ. *Neuroscience Research* 52(3):228-234.
- Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. *The Journal of Neuroscience* 17(1):58-69.
- Tanaka H, Grooms SY, Bennett MVL, Zukin RS (2000) The AMPAR subunit GluR2: still front and center-stage. *Brain Research* 886:190-207.
- Thoenen H (1995) Neurotrophins and neuronal plasticity. *Science* 270(5236):593-598.
- Threadgill R, Bobb K, Ghosh A (1997) Regulation of dendritic growth and remodeling by Rho, Rac, Cdc42. *Neuron* 19:625-634.
- Tsuchida T, Ensini M, Morton SB, Baldassare M, Edlund T, Jessell TM, Pfaff SL (1994)

- Topographic organization of embryonic motor-neurons defined by expression of lim homeobox genes. *Cell* 79(6):957-970.
- Tsui CC, Copeland NG, Gilbert DJ, Jenkins NA, Barnes C, Worley PF (1996) Narp, a novel member of the pentraxin family, promotes neurite outgrowth and is dynamically regulated by neuronal activity. *Journal of Neuroscience* 16(8):2463-2478.
- Turman JE, Macdonald AS, Pawl KE, Bringas P, Chandler SH (2000) AMPA receptor subunit expression in trigeminal neurons during postnatal development. *Journal of comparative neurology* 427:109-123.
- Van Damme P, Bracken D, Callewaert G, Robberecht W, Van Den Bosch L (2005) GluR2 deficiency accelerates motor neuron degeneration in a mouse model of amyotrophic lateral sclerosis. *Journal of Neuropathology and Experimental Neurology* 64(7):605-612.
- Van Damme P, Van den Bosch L, Van Houtte E, Callewaert G, Robberecht W (2002) GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. *Journal of Neurophysiology* 88(3):1279-1287.
- Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 252:1751-1718.
- Wenger BS (1995) Determination of structural patterns in the spinal cord of the chick embryo studied by transplantations between brachial and adjacent levels. *J of Exp Zool* 116:123-163.
- Wenger EL (1950) An experimental analysis of relations between parts of the brachial spinal cord of the embryonic chick. *J of Exp Zool* 114:51-86.
- Wenner P, O'Donovan MJ (1999) Identification of an interneuronal population that mediates recurrent inhibition of motoneurons in the developing chick spinal cord. *Journal of Neuroscience* 19(17):7557-7567.
- Wenner P, O'Donovan MJ, Matisse MP (2000) Topographical and physiological characterization of interneurons that express engrailed-1 in the embryonic chick spinal cord. *Journal of Neurophysiology* 84(5):2651-2657.
- Wilson MT, Kisaalita WS, Keith CH (2000) Glutamate-induced changes in the pattern of hippocampal dendrite outgrowth: a role for calcium-dependent pathways and the microtubule cytoskeleton. *Journal of Neurobiology* 43(2):159-172.

- Wollmuth LP, Sobolevsky AI (2004) Structure and gating of the glutamate receptor ion channel. *Trends Neurosci* 27:321-328.
- Xu HY, Clement A, Wright TM, Wenner P (2007) Developmental reorganization of the output of a GABAergic interneuronal circuit. *Journal of Neurophysiology* 97(4):2769-2779.
- Yin QW, Johnson J, Prevette D, Oppenheim RW (1994) Cell death of spinal motoneurons in the chick embryo following deafferentation: rescue effects of tissue extracts, soluble proteins, and neurotrophic agents. *Journal of Neuroscience* 14(12):7629-7640.
- Zhu JJ, Esteban JA, Hayashi Y, Malinow R (2000) Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nature* 3(11):1098-1106.

Chapter 2

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.

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, commonly 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 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 & Fischbach, 1986; Zorumski & Yang, 1988, Temkin et al., 1997). Moreover, kainate application to a spinal cord preparation induces bursting activity as early as E4 (Milner & 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 & Landmesser, 1999; Hanson & 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 & 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 & 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 & 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 & 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 E5-7). 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 lumbar motoneurons were performed as previously described by Martin-Caraballo and Dryer (2002a, b). Briefly, chick lumbar motoneurons 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 hr (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 (E5-6, 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 (EMEM, 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 hr 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). After determining the protein concentration with a Bradford protein kit (BioRad, Hercules, CA), lysates were combined with 2X 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 to 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 reprobed 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 μ l 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, forty cycles of 95°C for 15 sec, 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 & Schmittgen, 2001).

Immunohistochemistry: Spinal cords were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hr, 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 hr 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 hr at room temperature. The peroxidase reaction with diaminobenzidine (DAB) was performed using the ABC kit from Vector Laboratories (Burlingame, CA). Controls for the specificity of immunostaining was assessed by omission of the primary antibody.

Electrophysiology: Dissociated motoneurons were identified during patch-clamp recordings using an Olympus X71 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 (in mM): 120 CsCl, 2 MgCl₂, 10 HEPES, 10 EGTA, 1 ATP, and 0.1 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 Ca²⁺/ Na⁺-free solution. The standard chick saline contained (in mM) 145 NaCl, 5.4 KCl, 0.8 MgCl₂, 5.4 CaCl₂, 5 glucose, and 13 HEPES (pH 7.4 with NaOH). Standard chick solution was also supplemented with 600 nM TTX and 200 μM CoCl₂ ions to block voltage-gated Na⁺ and Ca²⁺ channels, respectively. To investigate the Ca²⁺ 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 CaCl₂. The composition of the 10 mM Ca²⁺/ Na⁺-free extracellular solution was (in mM): 135 NMG, 10 CaCl₂, 5 glucose, and 10 HEPES (pH 7.4 with HCl). Under these recording conditions, kainate currents are mediated by the flow of Ca²⁺ and Cs⁺ ions. The permeability ratio (P_{Ca}/P_{Cs}) in the 10 mM Ca²⁺/ Na⁺-free solution was calculated from the reversal potential (E_r) according to the extended GHK constant field equation using estimated ion activities (Mayer & Westbrook, 1987): $P_{Ca}/P_{Cs}=0.25 \times (a_{Cs}/a_{Ca}) \times \exp(E_r F/RT) \times [\exp(E_r F/RT) + 1]$, where a_{Cs} = Cs⁺ activity (activity coefficient =0.75), a_{Ca} = Ca²⁺ 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 Ca²⁺/ 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 Ca²⁺ measurements: Changes in intracellular [Ca²⁺] resulting from activation of Ca²⁺ permeable AMPA receptors was detected with the ratiometric dye Fura-2. Cell cultures were incubated for 30 min with Fura-2 AM (5 μ M, 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 40x 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 Inc). Recorded Ca²⁺ signals were corrected for background fluorescence and presented as the ratio of the fluorescent peak signals generated at 340 and 380 (F₃₄₀/F₃₈₀). This ratio represents relative changes in intracellular [Ca²⁺] without conversion to absolute values of intracellular-free Ca²⁺. There is minimal overlap in the fluorescent profile of Fura-2 measured at peak values (Abs=340/380 nm, Em=510 nm)

and the retrograde label DiI (Abs=550 nm, Em=560 nm) used to identify LMNs. Drugs were applied for 30-60 sec with a ValveLink 8 perfusion system (AutoMate Scientific Inc, San Fransisco, 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 μ M $CoCl_2$ 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) CCTCAGAAGTCCAAGCCAGGAGTG and (reverse) CAGGAAGGCAGCTAAGTTAGCCG 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 hr 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 bp 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: Philanthotoxin, poly-D-lysine, tetrodotoxin, and trypsin were from Sigma (St. Louis, MO). 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). Fura2-AM 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 3-14 days in culture), supplemented with a variety of trophic factors that may not be a necessary component of the normal milieu (O'Brien & Fischbach, 1986; Zorumski & Yang, 1988). Functional expression of AMPA/kainate receptors in chick LMNs was examined by puffing 500 μ M 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 non-desensitizing currents with a linear current-voltage relationship over the range of -90 to +50 mV and a reversal potential near 0 mV (Fig. 2.1A, B, C, 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 non-competitive 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 μM GYKI 52466, whereas near 80% of kainate currents were blocked in E11 LMNs (Fig. 2.1E, F). At all ages tested, incubation of motoneurons with 100 μM 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. 2.1E). 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 Fig. 2.1C&D, insertion of 50 μM spermine in the pipette solution generated linear IV relationships at all ages recorded. No inward rectification was observed in the presence of 500 μM 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 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 μM 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 & Mayer, 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 μ M 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 lumbar motoneurons is presented in Fig. 2.2A&B. Under our recording conditions, IV curves were slightly outwardly rectifying. Age-dependent changes in the $P_{\text{Ca}}/P_{\text{Cs}}$ were assessed using the constant field equation (see Methods) based on the reversal potential of kainate-generated currents in the 10 mM $\text{Ca}^{2+}/\text{Na}^+$ -free extracellular solution (Fig. 2.2C). The reversal potentials of E6 LMNs were significantly more positive than those in E11 motoneurons (E6 -48 ± 2.7 mV, $n=19$; E11 -70.2 ± 2.9 mV, $n=15$; Fig. 2.2D). The calculated $P_{\text{Ca}}/P_{\text{Cs}}$ revealed that E6 LMNs have higher Ca^{2+} permeability than E11 motoneurons (E6 1.5 ± 0.2 , $n=19$; E11 0.6 ± 0.1 , $n=15$; Fig. 2.2E). Age-dependent changes in the Ca^{2+} permeability of AMPA receptors in chick spinal motoneurons were further studied using philanthotoxin, a polyamine toxin that targets Ca^{2+} permeable AMPA receptors (Toth & McBain, 1998). Extracellular application of philanthotoxin (1 μ M) caused a significant reduction in the kainate-evoked currents in E6 but not E11 motoneurons (Fig. 2.3). The blocking effect of philanthotoxin

on kainate-evoked currents was significant at early stages of development (E6) when compared with older motoneurons (E11, Fig. 2.3B).

To investigate whether changes in the Ca^{2+} permeability of AMPA/kainate receptors generate significant changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), acutely isolated LMNs were loaded with the ratiometric Ca^{2+} indicator Fura-2 AM. Ca^{2+} signals were measured in response to 30 s application of AMPA (50 μM) in the presence of TTX (500 nM) and CoCl_2 (200 μM) in order to block voltage-gated Na^+ and Ca^{2+} channels, respectively. Stimulation with 30 mM extracellular K^+ was used as control. Typical Ca^{2+} signals in response to 50 μM AMPA at E6 and E11 are shown in Fig. 2.4A and B. In the majority of E6 LMNs, AMPA receptor stimulation generated large Ca^{2+} signals that were comparable in amplitude to those generated with 30 mM extracellular K^+ (Fig. 2.4A). In E11 LMNs, however, AMPA receptor stimulation generated a small Ca^{2+} signal (indicated by fluorescent ratios between 0 and 0.25) in most motoneurons (~70% of all recorded neurons, Fig. 2.4B). However, a small portion of E11 motoneurons still generated a significant Ca^{2+} signal in response to AMPA receptor activation suggesting 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. 2.4C, 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. 2.5A). By E8, the anti-GluR2 antibody detected two light bands that have a relative molecular weight of ~100 kD (Fig. 2.5A). 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 real time PCR analysis from isolated lumbar motoneurons. Lumbar motoneurons 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 lumbar motoneurons. GluR2 mRNA expression was significantly lower at E6 and increased over ten-fold by E11 (Fig. 2.5B).

Peroxidase immunostaining of spinal cord sections was performed to investigate the pattern of GluR2 expression throughout the spinal cord (Fig. 2.6). At E6, staining with the GluR2 antibody did not reveal any significant levels of expression throughout the spinal cord (Fig. 2.6A). Significant levels of immunostaining were observed in E11 spinal cord sections (Fig. 2.6B). 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. 2.6B, 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. 2.7A). 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. 2.7B).

DISCUSSION

The period spanning E5 to 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 & 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 & Oppenheim, 1978; Tang & 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 lumbar motoneurons 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 co-exist 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 hr 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 & 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 & 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 & 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 & 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 & 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 other cellular components of the spinal cord circuitry (Milner &

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 & Landmesser, 1999). At later stages of development, however, AMPA receptor activation is required for the generation of spontaneous network activity (Chub & 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 & 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 (Fryer et al., 1999; Metzger et al., 1998, respectively). Moreover, daily treatment of chick embryos *in ovo* with the kainate antagonist NBQX does not alter the number of lumbar motoneurons (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 Ca^{2+} -impermeable AMPA receptors coincides with a period of increased network activity driven by glutamate neurotransmission in the chick spinal cord (O'Donovan & Landmesser, 1987; Chub & O'Donovan, 1998). We speculate that expression of Ca^{2+} -impermeable AMPA receptors in older neurons may limit Ca^{2+} entry through multiple sources during a period when NMDA receptors (which are Ca^{2+} -permeable) also become functional in order to generate spontaneous network activity in the chick spinal cord (Chub & O'Donovan, 1998).

Another possibility to consider is that changes in intracellular $[\text{Ca}^{2+}]$ caused by GluR2 expression may regulate dendritic outgrowth during motoneuron development. Increased intracellular $[\text{Ca}^{2+}]$ acts as a stop signal for growing dendrites to form synapses (Lohmann et al., 2005). Activation of Ca^{2+} -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 Ca^{2+} 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 & 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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REFERENCES

- Blaschke M, Keller BU, Rivosecchi R, Hollmann M, Heinemann S, Konnerth A. 1993. Single amino acid determines the subunit-specific spider toxin block of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor channels. *Proc. Natl. Acad. Sci. USA* 90(14):6528-6532.
- Bowie D, Mayer ML. 1995. Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* 15(2):453-462.
- Brorson JR, Zhang Z, Vandenberghe W. 1999. Ca(2+) permeation of AMPA receptors in cerebellar neurons expressing glu receptor 2. *J Neurosci.* 19(21): 9149-9159.
- Burnashev N, Monyer H, Seeburg PH, Sakmann B. 1992. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8(1):189-198.
- Caldero J, Prevette D, Mei X, Oakley RA, Li L, Milligan C, Houenou L, Burek M, Oppenheim RW. 1998. Peripheral target regulation of the development and survival of spinal sensory and motor neurons in the chick embryo. *J Neurosci.* 18(1):356-370.
- Caldero J, Ciutat D, Llado J, Castan E, Oppenheim RW, Esquerda JE. 1997. Effects of excitatory amino acids on neuromuscular development in the chick embryo. *J Comp Neurol.* 387(1):73-95.
- Carlson NG, Howard J, Gahring LC, Rogers SW. 2000. RNA editing (Q/R site) and flop/flip splicing of AMPA receptor transcripts in young and old brains. *Neurobiol Aging* 21(4): 599-606.
- Catsicas M, Allcorn S, Mobbs P. 2001. Early activation of Ca(2+)-permeable AMPA receptors reduces neurite outgrowth in embryonic chick retinal neurons. *J Neurobiol.* 49(3): 200-211.
- Chub N, O'Donovan MJ. 1998. Blockade and recovery of spontaneous rhythmic activity after application of neurotransmitter antagonists to spinal networks of the chick embryo. *J Neurosci.* 18(1):294-306.
- Chu-Wang I-W, Oppenheim RW. 1978. Cell death of motoneurons in the chick embryo spinal cord. I. A light and electron microscopy study of naturally occurring and induced cell loss during development. *J Comp Neurol.* 177: 33-58.

- Dahm LM, Landmesser LT. 1991. The regulation of synaptogenesis during normal development and following activity blockade. *J Neurosci.* 11(1):238-255.
- Dingledine R, Hume RI, Heinemann SF. 1992. Structural determinants of barium permeation and rectification in non-NMDA glutamate receptor channels. *J Neurosci.* 12(10):4080-4087.
- Eybalin M, Caicedo A, Renard N, Ruel J, Puel JL. 2004. Transient Ca²⁺-permeable AMPA receptors in postnatal rat primary auditory neurons. *Eur. J Neurosci.* 20(11):2981-2989.
- Fryer HJ, Knox RJ, Strittmatter SM, Kalb RG. 1999. Excitotoxic death of a subset of embryonic rat motor neurons in vitro. *J Neurochem.* 72(2): 500-513.
- Gleason EL, Spitzer NC. 1998. AMPA and NMDA receptors expressed by differentiating Xenopus spinal neurons. *J Neurophysiol.* 79(6):2986-2998.
- Greig A, Donevan SD, Mujtaba TJ, Parks TN, Rao MS. 2000. Characterization of the AMPA-activated receptors present on motoneurons. *J. Neurochem.* 74: 179-191.
- Hall RA, Hansen A, Andersen PH, Soderling TR. 1997. Surface expression of the AMPA receptor subunits GluR1, GluR2, and GluR4 in stably transfected baby hamster kidney cells. *J Neurochem.* 68(2): 625-630.
- Hanson MG, Landmesser LT. 2003. Characterization of the circuits that generate spontaneous episodes of activity in the early embryonic mouse spinal cord. *J Neurosci.* 23(2): 587-600.
- Herlitze S, Raditsch M, Ruppersberg JP, Jahn W, Monyer H, Schoepfer R, Witzemann V. 1993. Argitoxin detects molecular differences in AMPA receptor channels. *Neuron* 10(6):1131-1140.
- Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H. 1994. Differences in Ca²⁺ permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* 12(6): 1281-1289.
- Koh DS, Burnashev N, Jonas P. 1995. Block of native Ca(2+)-permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. *J Physiol.* 486 (Pt 2):305-312.
- Kumar SS, Bacci A, Kharazia V, Huguenard JR. 2002. A developmental switch of

- AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci.* 22(8):3005-3015.
- Llado J, Caldero J, Ribera J, Tarabal O, Oppenheim RW, Esquerda JE. 1999. Opposing effects of excitatory amino acids on chick embryo spinal cord motoneurons: excitotoxic degeneration or prevention of programmed cell death. *J Neurosci.* 19(24):10803-10812.
- Lee JC, Greig A, Ravindranathan A, Parks TN, Rao MS. 1998. Molecular analysis of AMPA-specific receptors: subunit composition, editing, and calcium influx determination in small amounts of tissue. *Brain Res Brain Res Protoc.* 3(2):142-154.
- Lee MT, Koebbe MJ, O'Donovan MJ. 1988. The development of sensorimotor synaptic connections in the lumbosacral cord of the chick embryo. *J Neurosci.* 8(7): 2530-2543.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4): 402-408.
- Lohmann C, Finski A, Bonhoeffer T. 2005. Local calcium transients regulate the spontaneous motility of dendritic filopodia. *Nat Neurosci.* 8(3): 305-312.
- Lomeli H, Mosbacher J, Melcher T, Hoyer T, Geiger JR, Kuner T, Monyer H, Higuchi M, Bach A, Seeburg PH. 1994. Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266(5191): 1709-1713.
- Longone P, Impagnatiello F, Mienville JM, Costa E, Guidotti A. 1998. Changes in AMPA receptor-spliced variant expression and shift in AMPA receptor spontaneous desensitization pharmacology during cerebellar granule cell maturation in vitro. *J Mol Neurosci.* 11(1): 23-41.
- Martin-Caraballo M, Dryer, SE. 2002a. Activity- and target-dependent regulation of large-conductance Ca^{2+} -activated K^{+} channels in developing chick lumbar motoneurons. *J Neurosci.* 22(1): 73-81.
- Martin-Caraballo M, Dryer, SE. 2002b. Glial-cell line-derived neurotrophic factor and target-dependent regulation of large-conductance K_{Ca} channels in developing chick lumbar motoneurons. *J Neurosci.* 22(23): 10201-10208.
- Mayer ML, Westbrook GL. 1987. Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. *J Physiol.* 394:501-527.

- Mendelson B, Frank E. 1991. Specific monosynaptic sensory-motor connections form in the absence of patterned neural activity and motoneuronal cell death. *J Neurosci.* 11(5): 1390-1403.
- Mettling C, Gouin A, Robinson M, el M'Hamdi H, Camu W, Bloch-Gallego E, Buisson B, Tanaka H, Davies AM, Henderson CE. 1995. Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support. *J Neurosci.* 15(4):3128-3137.
- Meucci O, Miller RJ. 1998. Dissociation between the Joro spider toxin sensitivity of recombinant alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and their ability to increase intracellular calcium. *Neuropharmacol.* 37(10-11):1431-1443.
- Metzger F, Wiese S, Sendtner M. 1998. Effect of glutamate on dendritic growth in embryonic rat motoneurons. *J Neurosci.* 18(5): 1735-1742.
- Milner LD, Landmesser LT. 1999. Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J Neurosci.* 19(8): 3007-3022.
- Morkve SH, Veruki ML, Hartveit E. 2002. Functional characteristics of non-NMDA-type ionotropic glutamate receptor channels in AII amacrine cells in rat retina. *J Physiol.* 542(Pt 1):147-165.
- Nakanishi S. 1992. Molecular diversity of glutamate receptors and implications for brain function. *Science* 258(5082): 597-603.
- O'Brien RJ, Fischbach GD. 1986. Characterization of excitatory amino acid receptors expressed by embryonic chick motoneurons in vitro. *J Neurosci.* 6(11):3275-3283.
- O'Donovan MJ, Landmesser L. 1987. The development of hindlimb motor activity studied in the isolated spinal cord of the chick embryo. *J Neurosci.* 7(10):3256-3264.
- Otis TS, Raman IM, Trussell LO. 1995. AMPA receptors with high Ca^{2+} permeability mediate synaptic transmission in the avian auditory pathway. *J Physiol.* 482 (Pt 2):309-315.
- Paternain AV, Morales M, Lerma J. 1995. Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron* 14(1):185-189.

- Pemberton KE, Belcher SM, Ripellino JA, Howe JR. 1998. High-affinity kainate-type ion channels in rat cerebellar granule cells. *J Physiol.* 510 (Pt 2):401-420.
- Poluch S, Drian MJ, Durand M, Astier C, Benyamin Y, Konig N. 2001. AMPA receptor activation leads to neurite retraction in tangentially migrating neurons in the intermediate zone of the embryonic rat neocortex. *J Neurosci Res.* 63(1):35-44.
- Ravindranathan A, Donevan SD, Sugden SG, Greig A, Rao MS, Parks TN. 2000. Contrasting molecular composition and channel properties of AMPA receptors on chick auditory and brainstem motor neurons. *J Physiol.* 523 Pt 3:667-684.
- Shin J, Shen F, Huguenard JR. 2005. Polyamines modulate AMPA receptor-dependent synaptic responses in immature layer V pyramidal neurons. *J Neurophysiol.* 93(5):2634-2643.
- Solum D, Hughes D, Major MS, Parks TN. 1997. Prevention of normally occurring and deafferentation-induced neuronal death in chick brainstem auditory neurons by periodic blockade of AMPA/kainate receptors. *J Neurosci.* 17(12):4744-4751.
- Tang J, Landmesser LT. 1993. Reduction of intramuscular nerve branching and synaptogenesis is correlated with decreased motoneuron survival. *J Neurosci.* 13: 3095-3103.
- Temkin R, Lowe D, Jensen P, Hatt H, Smith DO. 1997. Expression of glutamate receptor subunits in alpha-motoneurons. *Brain Res Mol Brain Res.* 52(1): 38-45.
- Toth K, McBain CJ. 1998. Afferent-specific innervation of two distinct AMPA receptor subtypes on single hippocampal interneurons. *Nat Neurosci.* 1(7):572-578.
- Vandenberghe W, Bindokas VP, Miller RJ, Robberecht W, Brorson JR. 2001. Subcellular localization of calcium-permeable AMPA receptors in spinal motoneurons. *Eur J Neurosci.* 14(2):305-314.
- Vitten H, Reusch M, Friauf E, Lohrke S. 2004. Expression of functional kainate and AMPA receptors in developing lateral superior olive neurons of the rat. *J Neurobiol.* 59(3):272-288.
- Washburn MS, Numberger M, Zhang S, Dingledine R. 1997. Differential dependence on GluR2 expression of three characteristic features of AMPA receptors. *J Neurosci.* 17(24):9393-9406.

Zorumski CF, Yang J. 1988. AMPA, kainate, and quisqualate activate a common receptor-channel complex on embryonic chick motoneurons. *J Neurosci.* 8(11):4277-4286.

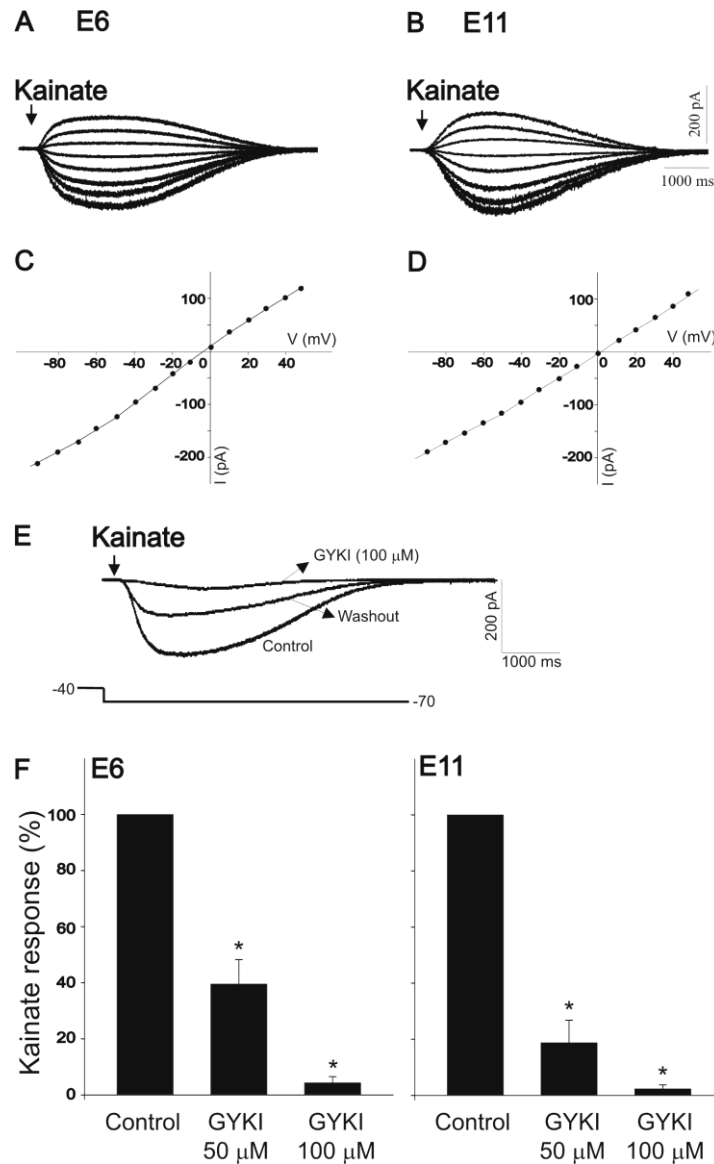


Figure 2.1. Kainate-induced currents in isolated chick LMNs. **A) & B)** Typical responses of E6 and E11 LMNs to kainate in standard chick solution. Whole cell currents were evoked by kainate (500 μ M) puffing onto isolated spinal cord neurons at holding potentials ranging from -90 to $+50$ mV. Holding potential was varied in 10 mV steps. 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 μM spermine. **C) & D)** Current-voltage (IV) relationship of kainate-evoked responses for the cells shown in A & B. **E)** Effect of GYKI 52466 on the kainate-evoked currents. Notice significant decrease in kainate current amplitude after bath perfusion with 100 μM 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).

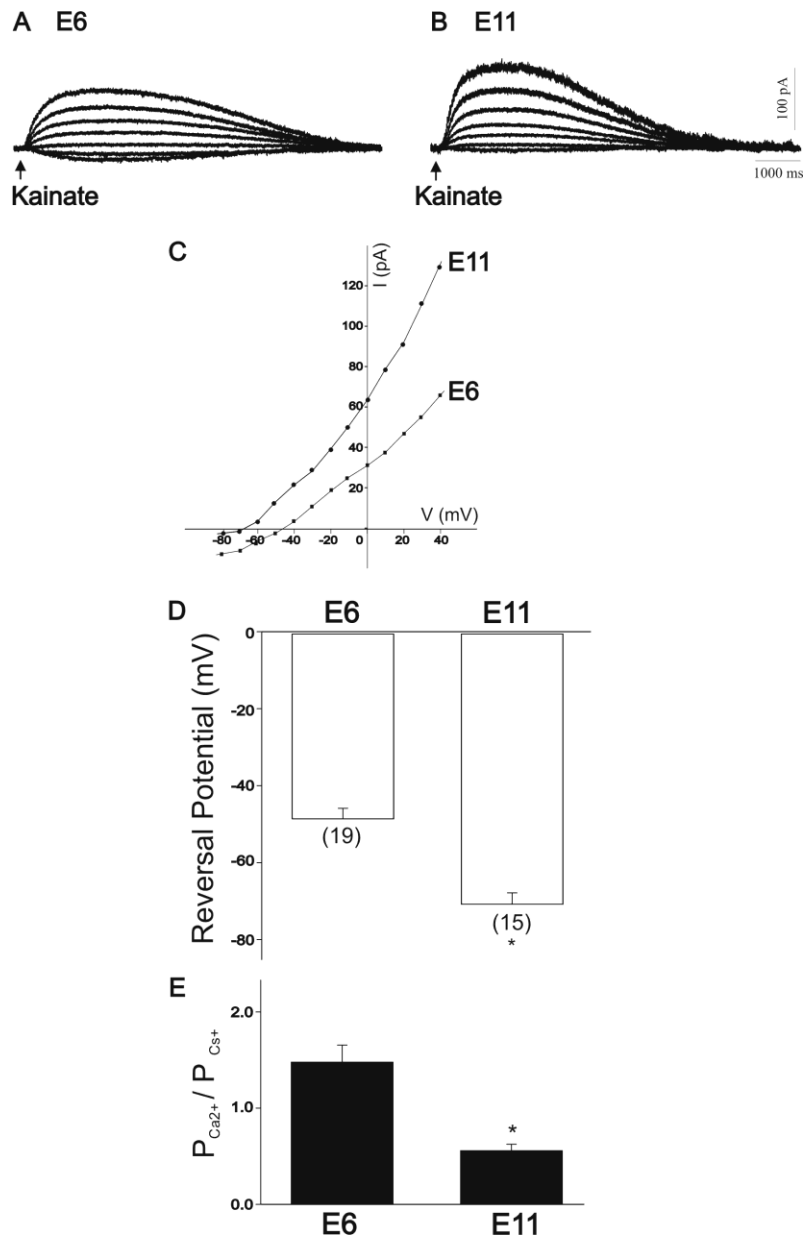


Figure 2.2. Developmental change in the Ca²⁺-permeability of AMPA receptors in chick LMNs. **A) & B)** Typical whole-cell currents in E6 and E11 LMNs evoked by kainate application in 10 mM Ca²⁺/Na⁺-free external solution, at holding potentials ranging from -80 to +40 mV. **C)** Current-voltage relationship of kainate-evoked

responses for the cells shown in A & 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^+ ($P_{\text{Ca}^{2+}}/P_{\text{Cs}^+}$) at E6 and E11. $P_{\text{Ca}^{2+}}/P_{\text{Cs}^+}$ 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.

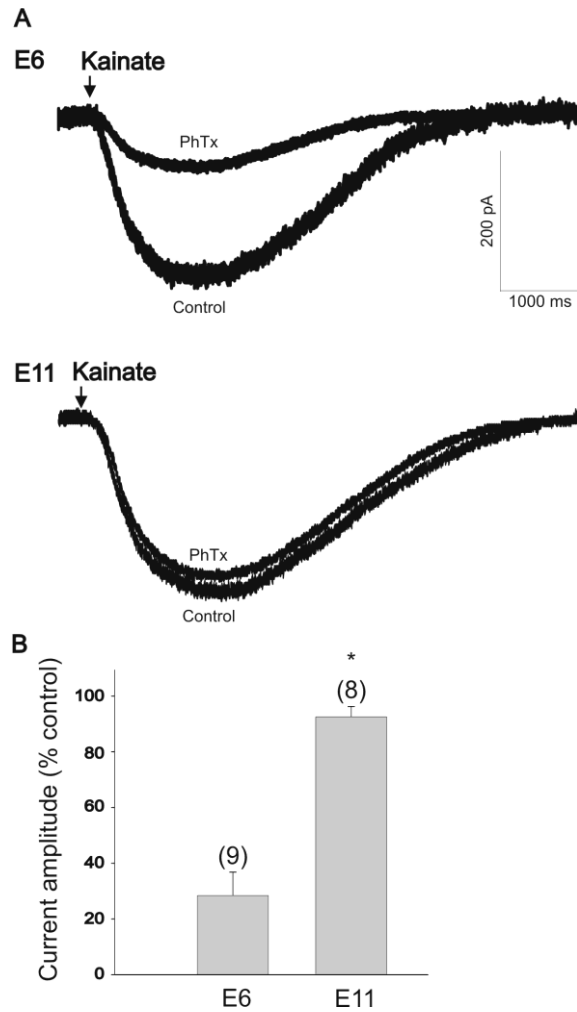


Figure 2.3. Effect of philanthotoxin (PhTx) on kainate-evoked currents in E6 and E11 motoneurons. **A)** Example of kainate-evoked currents in E6 and E11 motoneurons before (control) and after a 5 min bath application of philanthotoxin (1 μ M). Kianate-evoked currents were recorded at a holding potential 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).

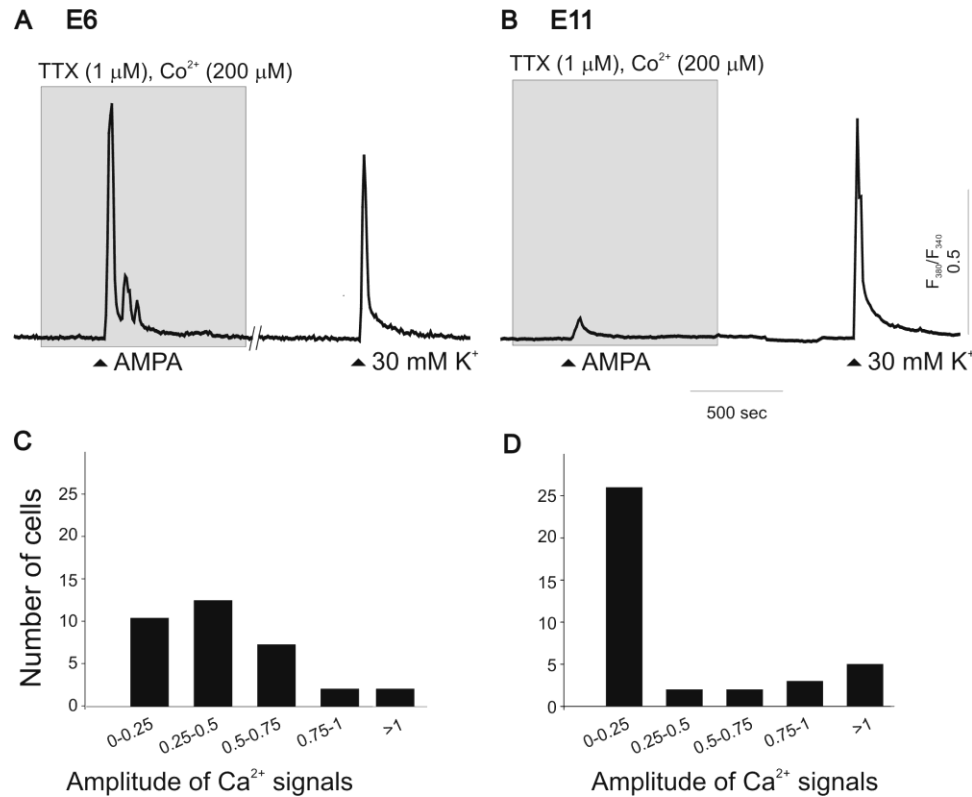


Figure 2.4. Developmental changes in the Ca²⁺ response of acutely isolated LMNs following AMPA receptor stimulation between E6 and E11. **A, B)** Typical Ca²⁺ responses recorded at E6 and E11. **C, D)** Graphic representation showing the range of Ca²⁺ signals in DiI-labeled motoneurons. After stimulation 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 sec. 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.

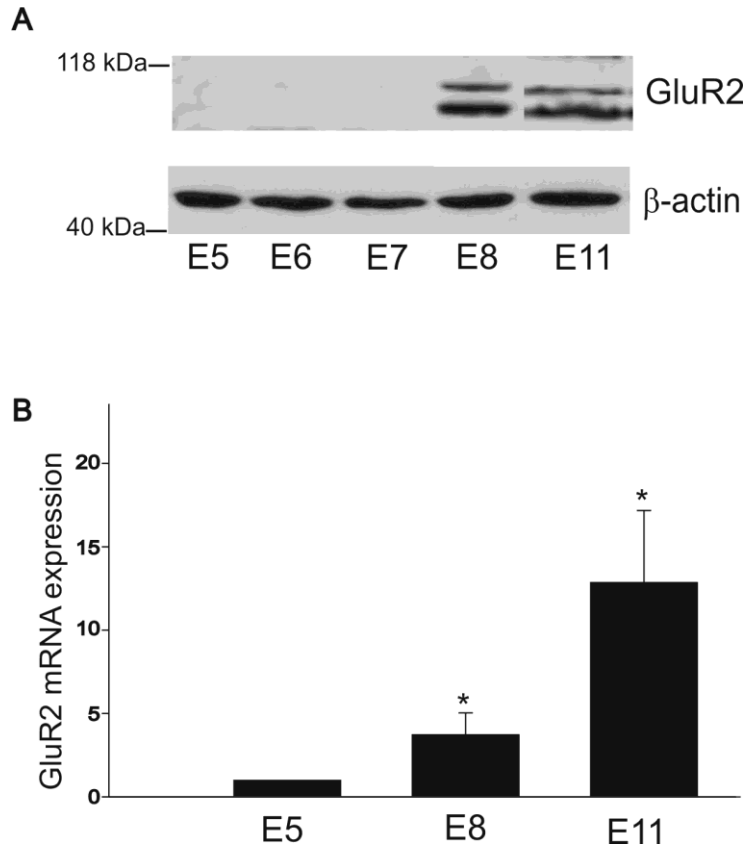


Figure 2.5. Developmental changes in GluR2 immunoreactivity and mRNA expression in chick ventral spinal cord. **A)** Representative example of western blot data collected from chick ventral spinal cords at various developmental stages (E5, E6, E7, E8, and E11). Each well was loaded with equal amounts of protein (30 μ g) as determined by the Bradford assay. To confirm equal loading, membranes were stripped following GluR2 immunoblot and re probed 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).

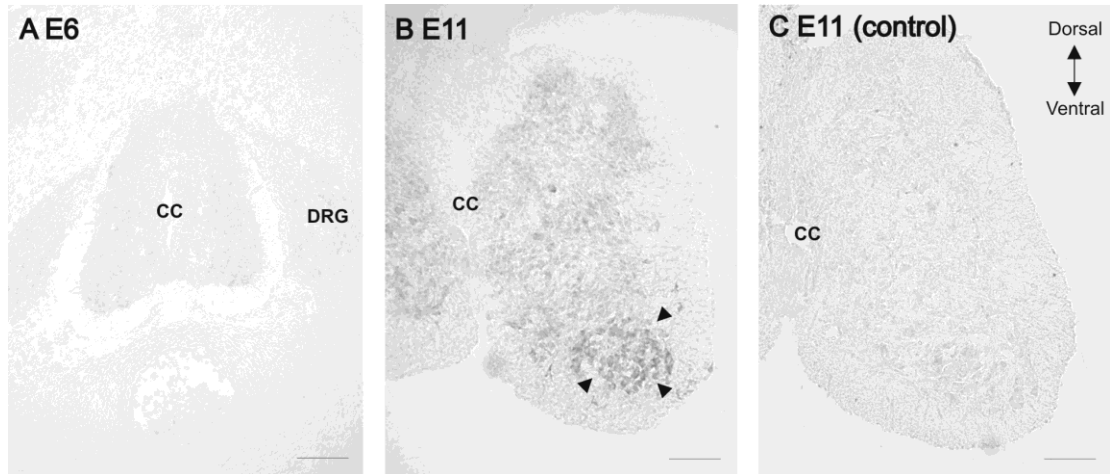


Figure 2.6. Developmental expression of GluR2 in chick spinal cord.

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, DRG=dorsal root ganglia. Scale bar=100 μ m.

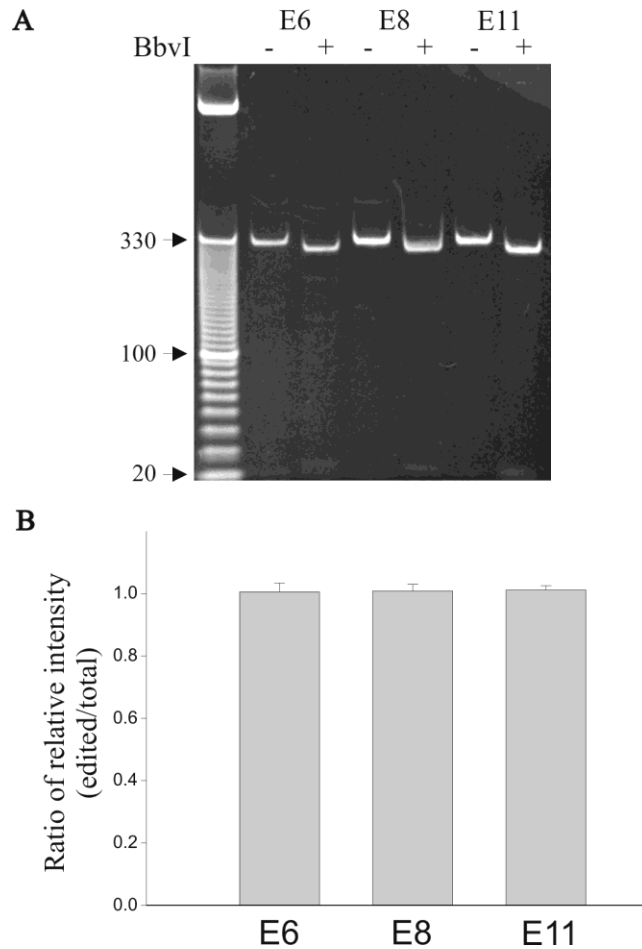


Figure 2.7. Extent of Q/R editing in chick ventral spinal cord between E6 and E11. GluR2 mRNA was amplified by PCR and the product containing the Q/R editing site was digested with the restriction enzyme BbvI. Digested products were separated on a 5% polyacrylamide gel (**A**). The amplified PCR products (331 bp) were cleaved into two fragments (311 bp 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.

CHAPTER 3

Subunit composition of AMPA receptors in the chicken spinal cord
during early embryonic development

ABSTRACT

AMPA receptors are tetramers, composed of various combinations of GluR1-4 subunits. We have demonstrated that chicken lumbar motoneurons express functional AMPA receptors by embryonic day (E) 5. AMPA receptors expressed at E5-E6 have a high Ca^{2+} permeability. By E11, however, AMPA receptors become Ca^{2+} -impermeable. These findings indicate that during a critical period of motoneuron development there is a significant switch in the Ca^{2+} permeability of AMPA receptors. The switch in the Ca^{2+} permeability of AMPA receptors is triggered by a significant increase in GluR2 expression but not by changes in the editing pattern of GluR2 subunits. Before the increased GluR2 expression occurs, it is unclear what receptor subunits are responsible for the formation of functional AMPA receptors at early stages of development. In this study, we investigated the expression pattern of AMPA receptor subunits in chicken lumbar motoneurons between E6 and E11. mRNA analysis indicates that GluR4 is the most abundant subunit in the chicken ventral spinal cord at both stages of development. Unlike GluR2 protein expression, which only becomes evident at E8, GluR4 protein expression can be found in ventral spinal cord samples isolated from E6 embryos. Immunohistochemistry analysis of spinal cord sections demonstrated that GluR4 protein expression is also detected in the ventral spinal cord where motoneurons are located. In addition to GluR4, GluR3 mRNA and protein expression is also found at E6 and E11. The levels of GluR3 and GluR4 mRNA expression did not change between E6 and E11, as revealed by real time PCR analysis. We conclude that at early stages of development, AMPA receptors are composed of GluR4 and GluR3 subunits.

INTRODUCTION

AMPA receptors are tetramers, composed of various combinations of four subunits (GluR1, 2, 3, and 4). Each AMPA receptor subunit can be found in multiple isoforms, resulting from RNA editing and alternative splicing (Sommer et al. 1990). The subunit composition of AMPA receptors can have important implications for receptor function. AMPA receptor subunits can form various combinations to generate heteromeric or homo-tetrameric receptors *in vitro* (Boulter et al. 1990). Assembly of GluR subunits into functional AMPA receptors occurs in a two-step process. First, two homomeric subunits undergo dimerization through interactions of their N terminal domains. Second, two GluR dimers will combine together to form homomeric or heteromeric tetramers (Greger et al., 2003). In most AMPA receptors, GluR2 exists in combination with either GluR1 or GluR3. Rarely, there will be AMPA receptors containing both GluR1 and GluR3 (Ritter et al., 2002). There are different combination patterns of AMPA receptor subunits in the CNS. GluR1 and 2 subunits are present in most AMPA receptors because they are ubiquitously expressed in the adult CNS. GluR2-containing AMPA receptors are expressed in most excitatory neurons in the adult brain. GluR3 mRNA is expressed in the Purkinje cell layer of the cerebellum and in the dentate gyrus and the pyramidal cell layer of the hippocampus. GluR3 is present at low levels in layer III and IV of the cerebral cortex. GluR4 mRNA is found in the granule cells of the olfactory bulb and in the molecular layer of the cerebellar cortex (Keinanen et al. 1990).

In addition to spatial differences, expression of AMPA receptor subunits is regulated in an age-dependent manner. GluR1, GluR3 and GluR4 are found before postnatal day (PND) 4 in rat primary auditory neurons. In contrast, GluR2 is not expressed until PND10 (Eybalin et al. 2004). Since different functional properties in neurons are based on synaptic receptor heterogeneity, it is important to know the compositions of AMPA receptor subunits in a certain neuronal type at various developmental stages (Grunder et al., 2000).

Changes in the subunit composition of AMPA receptors are very important in determining channel conductance properties, gating kinetics, and vesicular traffic to and from synaptic sites (Greger et al., 2007). The GluR1 subunit of AMPA receptors is involved in dendrite morphogenesis. It can control dendrite growth when it binds to a scaffolding protein called SAP97 (Zhou et al., 2008). In addition to GluR1, GluR2 also mediates dendrite outgrowth in spinal motor neurons. GluR2 can increase the length of dendritic arbor without increasing complexity (Prithviraj et al., 2008). The up-regulation of GluR3 mRNA in a model of sporadic amyotrophic lateral sclerosis (ALS) is believed to be involved in the selective vulnerability of motor neurons to kainate. This is due to the increased proportion of GluR2-lacking (Ca^{2+} -permeable) AMPA receptors (Sun et al., 2006). The percentage of spinal cord neurons in which each subunit is expressed varies. GluR4 can be detected in 81% of spinal cord neurons, whereas the other subunits are expressed in lower percentages of neurons (GluR1, 38%, GluR2, 13%, and GluR3, 13%). There is a correlation between subunit expression and neuronal properties. Due to the abundance of GluR4, AMPA receptors in spinal cord neurons deactivate and desensitize quickly (Dai et al., 2001).

In our previous study we found that chicken lumbar motoneurons express functional AMPA receptors as early as E5. Thus, application of kainate to isolated motoneurons generates a significant inward current that was sensitive to blockade by the specific AMPA receptor blocker GIKY 52466. Our earlier results also show that early expressed AMPA receptors are highly permeable to Ca^{2+} ions. The Ca^{2+} permeability of AMPA receptors is correlated with a lack of GluR2 mRNA and protein expression in the motoneuron pool. The question arises as to the exact subunit composition of these early expressed AMPA receptors in chicken lumbar motoneurons. This question is of particular importance considering that AMPA (or glutamatergic) synaptic transmission is not required for the generation of spontaneous electrical activity at early stages of development (E4-E8). This suggests that expression of AMPA receptors at early stages of development may be involved in the regulation of other developmental processes. Since GluR2 expression is minimal at E6, other subunits may be involved in the generation of functional AMPA receptors at early stages of development. The aim of this work is to determine the subunit composition of AMPA receptors at early stages of spinal cord development and possible changes in the subunit composition of AMPA receptors between E6 and E11 motoneurons.

METHODS

Motoneuron labeling: Chick lumbar motoneurons were retrogradely labeled *in ovo* with DiI which is a fluorescent carbocyanine dye (1 mg/ml in 20% ethanol and 80% saline). Dye injection into muscles of the thigh and foreleg was performed 24 hr (E6) or 4 days (E11) before spinal cord isolation.

Laser capture microdissection: DiI-labeled spinal cords were isolated at E6 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: RNAs from laser-captured samples of embryonic day 6 and 11 were isolated with the ArrayPure Nano-scale RNA purification kit according to the manufacturer's instructions. Extracted RNA was used for cDNA synthesis by reverse transcription with a Qiagen RNase reverse transcriptase system. cDNAs of GluR3, GluR4 and β -actin (used as a normalizer) were amplified in separate samples using their corresponding primers (supplied by Sigma Genosys).

Quantification of cDNAs of GluR3 and GluR4 was performed by quantitative real-time PCR using a SYBR probe (Power SYBR Green PCR master mix, Applied Biosystems) on an Applied Biosystems PRISM 7500 sequence detection system supplied in the COBRE Molecular/Cellular core facility. Quantification of β -actin cDNA was performed by quantitative real-time PCR using 5'-Fam3 and 3'-Black Hole Quencher (BHQ) probes on the same detection system. Primer design was based on the *G.gallus* mRNA published sequences and consisted of the following sequences: GluR3, forward primer (5'ATGGAGCCAAGGAATTGATA-3'), reverse primer (5'ATTTGTTCTCCGCCATA-3'); GluR4, forward primer (5'AAGACCTGGACCGAAGACA-3'), reverse primer (5'TGAAATCCCGTCACATTAGC-3'). The sequences for the forward and reverse primers for β -actin were 5'CACCTGAGCGCAAGTACTCTG-3' and 5'TCTGCTGGAAGGTGGACAG-3' respectively. The Taqman probe for β -actin was 5'TGGAGGCTCTATCCTGGCCTCCC-3'. Primers for GluR3 and GluR4 were designed using OLIGO primer analysis software (Molecular Biology Insights, Inc). Quantification of GluR3 and GluR4 cDNA was performed by quantitative real-time PCR using a SYBR green dye (Power SYBR Green PCR master mix, Applied Biosystems) on an Applied Biosystems PRISM 7500 sequence detection system. PCR reactions consisted of stage one with one cycle of 95° C for 10 min, stage two with forty-five cycles of 95° C for 15 sec, followed by 60° C for 1 min, and stage three with one cycle of 95° C for 15 sec, followed by one cycle of 60° C for 1 min, one cycle of 95° C for 15 sec and one cycle of 60° C for 15 sec. PCR reactions for β -actin consisted of one cycle of 95° C for 10 min, 45 cycles of 95° C for 15 sec followed by

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 GluR3, GluR4 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. GluR3 and GluR4 mRNA expression was normalized to β -actin to correct for differences in RNA concentration according to the delta-delta C_T method (Livak & Schmittgen, 2001). PCR products were separated on 1.5% low melting point agarose gels. Bands were excised and submitted for sequencing at the Vermont Cancer Center DNA facility on an Applied Biosystems DNA sequencer.

Western blot: Spinal cords from different ages of embryos were washed in ice-cold Ca^{2+}/Mg^{2+} free saline and lysed in RIPA buffer supplemented with a protease inhibitor cocktail. After determining their protein concentrations, lysates were combined with 2X Laemmli sample buffer. Proteins from all ages of preparations were resolved by SDS-PAGE (8% polyacrylamide) and transferred to a nitrocellulose membrane. Blots were probed with the GluR1, GluR2 and GluR2/3 antibodies (Chemicon). Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase and a chemiluminescent substrate (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL) were used to analyze the blots. 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 reprobed with a β -actin specific antibody (at 1:20000 dilution, Sigma) followed by incubation with the corresponding secondary antibody and immunodetection. The ratio of specific subunits to total protein content was determined by densitometry using Scion Image software. To detect the GluR4 subunit, the fluorescent secondary anti-rabbit antibody (Rockland) was used. After four washes, images of the membrane were taken using the 700 and 800nm channels of the Odyssey infrared imaging system (LICOR Biosciences GmbH) and detection was based on fluorescent secondary antibody binding. The membrane was reprobed with a fluorescent secondary anti-mouse to measure amounts of β -actin and confirm that an equal amount of protein had been loaded in each well. All experiments were done in triplicate.

Immunohistochemistry: Spinal cords were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. The next day, the spinal cords were washed in PBS and allowed to sink in 15% sucrose. Spinal cords were incubated overnight in 30% sucrose and embedded in tissue freezing medium before cryostat sectioning. Before incubation with the primary antibody, slides containing spinal cord sections were blocked in PBS with 0.5% Triton X-100 and 5% horse serum. After incubation with GluR4-specific primary antibodies (at 1:600 dilution, Chemicon), the sections were washed three times and incubated in 0.5% H₂O₂ for 30 minutes to block their endogenous peroxidase activity. After another three washes with PBS, the slides were exposed to a biotinylated anti-rabbit secondary antibody conjugated to HRP for

1 h at room temperature. A peroxidase reaction with diaminobenzidine was performed using the ABC kit from Vector Laboratories (Burlingame, CA). Control immunostaining was performed by omitting the primary antibody.

Analysis of relative abundance of AMPA receptor subunits: PCR of GluR1-4 from the ventral spinal cord region was performed using PCR master mix (Applied Biosystems). The pan-AMPA degenerate primers consisted of (forward) 5'GGATGYGATATTTCCSCCAAG-3' and (reverse) 5'CYTTRTCGTACCACCWTTTG-3'. A 550 base pair (bp) fragment of GluR1 (bp 1853-2403), a 549 bp fragment of GluR2 (bp 2040-2589), a 553 bp fragment of GluR3 (bp 1950-2503) and a 539 bp fragment of GluR4 (bp 1841-2380) were amplified. PCR reactions started with one cycle of 94°C for 2 min; the cycling parameters included 45 cycles of 91°C for 30 s, 53°C for 30 s, and 72°C for 1 min; the last step was a final extension of 5 min at 72°C. Equal amounts of the pan-AMPA PCR product were digested by using subunit-specific restriction endonucleases (Pvu II cuts GluR1 at bp 2120, BamH I cuts GluR2 at bp 2493, EcoR I cuts GluR3 at bp 2161 and PstI cuts GluR4 at bp 2139). The fragments were resolved on agarose gels after digestion and stained with ethidium bromide. Pictures of the gels were taken with a digital imaging system (ChemiDoc RXS System, Bio-Rad). The intensity of the undigested product band of each subunit-specific digestion, relative to the intensity of the total undigested control product, was used to determine the relative abundance of each individual subunit.

Chemicals and drugs: Pvu II, BamH I, EcoR I , and PstI were obtained from New England Biolabs (Beverly, MA). Anti-GluR2 and anti-GluR2/3 antibodies were purchased from Chemicon (Billerica, MA).

RESULTS

Relative abundance of AMPA receptor subunits between E6 and E11

To investigate the subunit composition of AMPA receptors in the chicken spinal cord, we examined the relative abundance of each AMPA subunit's mRNA in samples obtained from the ventral spinal cord. The mRNA from ventral spinal cords was extracted from chicken embryos at E6 and E11, reverse transcribed, and a pan-AMPA degenerate set of primers was used to amplify the cDNAs. Amplified PCR products were divided into five independent aliquots containing equal amounts of the cDNA. Four of the aliquots were digested with a single subunit-specific restriction endonuclease (Pvu II cuts GluR1 at bp 2120, BamH I cuts GluR2 at bp 2493, EcoR I cuts GluR3 at bp 2161 and PstI cuts GluR4 at bp 2139) and the final aliquot was left undigested (labeled "U"). The five samples were run on a gel and stained with ethidium bromide (Fig.3.1. A). Digestion of GluR1 resulted in one or more faint bands at E6 and E11, indicating relatively low levels of GluR1. GluR4 digestion resulted in two bright bands at both ages, indicating high levels of the subunit. Two bright bands were also present in the GluR2 and GluR3 digestions at both ages. Each digestion's undigested band intensity was measured. This value, relative to the undigested control's intensity value, was used to determine the relative abundance of each subunit (Fig.3.1. B). At each age, GluR4 was much more highly expressed than GluR1. At E6, GluR4 was more abundant than GluR2 and GluR3. In contrast to GluR1, GluR2 and GluR3, GluR4 was expressed in greater levels at E11. At E11, GluR2 was also found to be more abundant than GluR1. These results suggest that

most AMPA receptors consist of GluR4 subunits at early stages of development with a small contribution from GluR3 subunits.

Developmental changes in the composition of AMPA receptor subunits in the chicken ventral spinal cord

The previous results indicate that GluR4 is the most abundant subunit in the chick spinal cord at the mRNA level. To investigate whether the GluR4 protein is as abundant in the motoneuron pool, we performed immunoblot and immunohistochemistry analyses of chicken spinal cords isolated at various stages of development. Immunoblot analysis revealed GluR4 protein to be present in the ventral spinal cord tissue of chicken embryos at all stages of development (E6 to E11, Fig. 3.2.A). The gel was reprobed with β -actin to confirm that the same amount of protein was loaded into each well. To confirm the existence of GluR4 in the motoneuron pool in the ventral spinal cord, we performed immunohistochemistry analysis of spinal cord sections isolated from E6 and E11 chicken embryos. As represented in Fig. 3.2.B and C, a significant level of staining with a specific GluR4 antibody was detected in the motoneuron pool of E6 and E11 spinal cords. Most of the staining is limited to the motoneuron pool and can not be seen in the white matter or more dorsal portions of the spinal cord. There is no significant staining in the motoneuron pool of the spinal cord section not exposed to the GluR4 antibody (Fig. 3.2.D). These results suggest that GluR4 subunits are an important component of AMPA receptors in chicken lumbar motoneurons.

In addition to GluR4 mRNA, GluR3 mRNA was also detected in significant

levels in the ventral spinal cord by our experiments. To determine whether the GluR3 receptor subunit is also expressed at the protein level in the chicken spinal cord, we used an antibody that recognizes both GluR2 and GluR3 subunits (GluR2/3 antibody). No antibody targeting the GluR3 subunit specifically is currently available. However, we also probed our samples with an antibody specific to GluR2, allowing us to detect not only the presence of GluR2 but also GluR3 by comparing the results to the GluR2/3 staining. In agreement with our previous results (Ni et al., 2007), immunoblot analysis showed that GluR2 subunit expression was absent at early stages of spinal cord development (E6-E7) but present beginning at E8 (Fig. 3.3.A). Bands with a relative molecular weight of ~100 kD were detected at E8 and E11 with the anti-GluR2 antibody (Fig. 3.3.A). The two distinct bands detected with the GluR2/3 antibody are likely due to differences in the glycosylation levels of GluR2/3 subunits (Hall et al., 1997). With the GluR2/3 antibody, bands were detected at all stages of development (E6-E11, Fig. 3.3.A). This indicates that GluR3 may be present at early stages of development. Expression of GluR3 in the motoneuron pool was also detected by immunostaining of spinal cord sections at E6 and E11 (Fig. 3.3.B-C).

GluR1 protein was not detected in the chicken spinal cord at any stage of development (data not shown). This could be due to the low levels of GluR1 expression in the chicken spinal cord. Another possible reason is that the GluR1 antibody used in this study does not recognize the GluR1 subunit in the chicken spinal cord.

Developmental changes of AMPA receptor subunit mRNA in chick ventral spinal cord

The above experiments indicate that both the protein and the mRNA of GluR3 and GluR4 are present from E6 to E11 in the ventral spinal cord. Is the expression pattern in ventral spinal cord parallel with that in the motoneuron pool and is there any change of the expression pattern? To answer this question, we performed real time PCR analysis of isolated lumbar motoneurons. Lumbar motoneurons were retrogradely labeled with DiI and captured using laser microdissection (see Methods). Quantitative PCR analysis indicates no change in GluR3 or GluR4 mRNA expression between E6 and E11 lumbar motoneurons (Fig.3.4. A, B). The results demonstrate that both GluR3 and GluR4 mRNA are present at early stages of development in the motoneuron pool and their levels of expression do not change significantly by E11.

DISCUSSION

This study was conducted to shed light on the expression patterns of AMPA receptor subunits in the chicken spinal cord. AMPA receptor subunit expression was examined at both the mRNA and protein levels in chicken lumbar motoneurons. Our results demonstrate that GluR4 was the most highly expressed AMPA receptor subunit among the four subunits. GluR3 and GluR4 were found throughout all stages of development included in this study (E6 to E11). The levels of protein expression were consistent with the levels of mRNA expression detected for each subunit. GluR3 and GluR4 mRNA expression is not regulated in an age-dependent manner.

Relative abundance of AMPA receptor subunits

Because the subunit composition of an AMPA receptor determines the properties of the receptor, it is of interest to determine the relative amounts of each subunit that compose the AMPA receptors at different developmental stages. Ventral spinal cords were dissected and used to investigate the abundance of each AMPA receptor subunit. Our results indicated that GluR4 was most highly expressed from E6 to E11. Furthermore, GluR4 mRNA was present at higher levels at E11 than at E6. This result was not seen in motoneuron pools extracted through laser capture microdissection. There are other neurons expressing AMPA receptors in whole ventral spinal cord, which may account for the difference between the two samples. There was no significant difference in the relative abundance of GluR1 and GluR3 between E6 and E11. GluR2 underwent a significant increase by E11. This result is

consistent with our previous studies regarding the developmental increase in GluR2 by E8 (Ni et al., 2007). The subunit composition of an AMPA receptor determines the channel's properties, including gating kinetics and channel conductance. The presence of GluR1, GluR3, GluR4 in a channel, alone or in combination, results in strong inwardly rectifying current-voltage relationship. The presence of GluR2 can switch the current-voltage relationship from inwardly rectifying to linear or outward rectifying (Mcbain and Dingledine, 1993). Relatively high expression of GluR2 subunit results in development of slowly gated AMPARs. By contrast, high expression of GluR4 promotes the formation of rapidly gated AMPARs (Geiger et al., 1995). An abundance of GluR3 and GluR4 subunits is responsible for rapid deactivation and desensitization rates in ventral cochlear nuclei (Martinez-Galan et al., 2007).

Protein level of AMPA receptor subunits

GluR2 expression is low at early developmental stages (E5-E7) in the chicken spinal cord based on our previous studies (Ni et al., 2007). It was, however, highly expressed at later ages and made AMPA receptors impermeable to Ca^{2+} . This indicates that GluR2 does not play a role in the functional activity of AMPA receptors at early stages while GluR1, GluR3 or GluR4 may contribute to the formation of functional channels at early stages of development. Our present findings indicate that GluR1 mRNA is expressed at very low levels during early stages of development and cannot be detected using a specific GluR1 antibody. Therefore, the present results

suggest that early expressed AMPA receptors most likely consist of GluR3 or GluR4 subunits, or a combination of two.

The goal of this study was to determine the developmental pattern of AMPA subunit expression in the chicken spinal cord. Our results indicate that prior to E8, AMPA receptors were composed of GluR3 and GluR4. From E8 to E11, all subunits were expressed in AMPA receptors except GluR1. GluR1, GluR3, and GluR4 have also been shown to be developmentally regulated in the rat ventral spinal cord. Protein levels were more abundant in the neonatal spinal cord than in the adult spinal cord (Jakowec et al., 1995). There is evidence showing that GluR3 and GluR4 are the main AMPA receptor subunits responsible for excitatory synaptic transmission in neurons of the chick tangential nucleus during the perinatal period. In contrast, GluR1 and GluR2 are expressed at very low levels before hatching (Popratiloff et al., 2004). The various compositions of GluR subunits confer the synaptic transmission and neuronal activity of AMPA receptors. The lack of GluR2 expression at early developmental stages of chick LMNs makes the AMPA receptor permeable to Ca^{2+} . Although GluR1, GluR3, and GluR4 do not play roles in determining ion permeability, they may play other roles. The GluR1 subunit plays a role in NMDA-dependent synaptic delivery of AMPARs, which results in increased synaptic transmission during long-term potentiation (LTP) (Shi et al., 1999; Hayashi et al., 2000). GluR3 subunits are involved in activity-independent movements of AMPAR, which is important for stable basal synaptic responses (Meng et al., 2003). GluR4-containing receptors are inserted to synapse by spontaneous activity during early development stages (Zhu et al., 2000).

mRNA level of AMPA receptor subunits

In order to know whether mRNA expression is a reflection of protein expression, we extracted mRNA from labeled motoneuron pools in chick lumbar spinal cord and performed the RT-PCR amplification. It suggested that GluR3 and GluR4 mRNAs were expressed at both E6 and E11. There was no significant difference between the two ages. Because we were unable to detect GluR1 protein, we made no attempt to identify the presence or absence of GluR1 mRNA. This experiment further supported our previous results that GluR3 and GluR4 were the main subunits composing AMPA receptors at early developmental stages of chick LMNs. A similar AMPA receptor subunit pattern has been observed in normal adult rat spinal cord. It was found that GluR1 mRNA was not detectable in the ventral horn of the spinal cord, while GluR3 and GluR4 were strongly expressed in ventral motor neurons. This pattern was changed under the pathological state of contusive spinal cord injury (SCI). GluR2 and GluR4 decreased significantly after SCI (Grossman et al., 1999). This indicates that the normal expression pattern in chick LMNs may also undergo considerable changes under certain circumstances, including pathological or experimental spinal cord injuries.

REFERENCES

- Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* 249:1033-1037.
- Chub N, O'Donovan (1998) Blockade and recovery of spontaneous rhythmic activity after application of neurotransmitter antagonists to spinal networks of the chick embryo. *The Journal of Neuroscience* 18(1):294-306.
- Dai WM, Egebjerg J, Lambert JD (2001) Characteristics of AMPA receptor-mediated responses of cultured cortical and spinal cord neurones and their correlation to the expression of glutamate receptor subunits, GluR1-4. *Br J Pharmacol* 132(8):1859-1875.
- Eybalin M, Caicedo A, et al (2004) Transient Ca²⁺-permeable AMPA receptors in postnatal rat primary auditory neurons. *European Journal of Neuroscience* 20(11):2981-2989.
- Geiger JRP, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca²⁺ permeability of AMPA receptors in principal neurons and interneurons in rat CAN. *Neuron* 15:193-204.
- Greger IH, Khatri L, Kong X, Ziff EB (2003) AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* 40:763-774.
- Greger IH, Ziff EB, Penn AC (2007) Molecular determinants of AMPA receptor subunit assembly. *TRENDS in Neurosciences* 30(8):407-416.
- Grossman SD, Wolfe BB, Yasuda RP, Wrathall JR (1999) Alterations in AMPA receptor subunit expression after experimental spinal cord contusion injury. *The Journal of Neuroscience*. 19(14):5711-5720.
- Grunder T, Kohler K, Guenther E (2000) Distribution and developmental regulation of AMPA receptor subunit proteins in rat retina. *Invest ophthalmol vis sci*. 41: 3600-3606.
- Hayashi Y, Shi SH, Esteban JA, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* 287:2262-2267.
- Hall RA, Hansen A, Andersen PH, Soderling TR (1997) Surface expression of the

- AMPA receptor subunits GluR1, GluR2, and GluR4 in stably transfected baby hamster kidney cells. *J Neurochem.* 68(2):625-630.
- Jakowec MW, Fox AJ, Martin LJ, Kalb RG (1995) Quantitative and qualitative changes in AMPA receptor expression during spinal cord development. *Neuroscience* 67(4):893-907.
- Livak KJ, Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* 25: 402-408.
- Keinanen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. *Science* 249:556-560.
- Martinez-galan JR, Caminos E, Vale C, Juiz JM (2007) Auditory nerve inputs is not an absolute requirement for the expression, distribution and calcium permeability of AMPA receptors in the adult rat ventral cochlear nucleus. *Brain research* 1138:21-29.
- Mcbain CJ, Dingledine R (1993) Heterogeneity of synaptic glutamate receptors on CA3 stratum radiatum interneurons of rat hippocampus. *Journal of Physiology* 462:373-392.
- Meng Y, Zhang Y, Jia Z (2003) Synaptic transmission and plasticity in the absence of AMPA glutamate receptor GluR2 and GluR3. *Neuron* 39:163-176.
- Ni X, Sullivan GJ, Martin-Caraballo M (2007) Developmental characteristics of AMPA receptors in chick lumbar motoneurons. *Developmental Neurobiology* 67: 1419-1432.
- Popratiloff A, Wang Y, Narvid J, Petralia RS, Giaume C, Peusner KD (2004) AMPA receptor subunit expression in chick vestibular nucleus neurons. *Journal of Neuroscience Research* 76:662-677.
- Prithviraj R, Kelly KM, Espinoza-Lewis R, Hexom T, Clark AB, Inglis FM (2008) Differential regulation of dendrite complexity by AMPA receptor subunits GluR1 and GluR2 in motor neurons. *Developmental Neurobiology.* 68(2):247-264.
- Ritter LM, Vazquez DM, Meador-Woodruff JH (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. *Developmental Brain Research* 139:227-236.
- Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R

- (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* 284:1811-1816.
- Sprengel R (2006) Role of AMPA receptors in synaptic plasticity. *Cell Tissue Res.* 326:447-455.
- Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Kohler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* 249:1580-1585.
- Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S, Kwak S (2005) Expression profile of AMPA receptor subunit mRNA in single adult rat brain and spinal cord neurons in situ. *Neuroscience Research* 52:228-234.
- Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S (2006) Slow and selective death of spinal motor neurons in vivo by intrathecal infusion of kainic acid: implications for AMPA receptor-mediated excitotoxicity in ALS. *J Neurochem* 98(3):782-791.
- Turman JR JE, Macdonald AS, Pawl KEW, Bringas P, Chandler SH (2000) AMPA receptor subunit expression in trigeminal neurons during postnatal development. *The journal of comparative neurology.* 427:109-123.
- Zhou W, Zhang L, Guoxiang X, Mojsilovic-Petrovic J, Takamaya K, Sattler R, Huganir R, Kalb R (2008) GluR1 controls dendrite growth through its binding partner, SAP97. *Journal of Neuroscience.* 28(41): 10220-10233.
- Zhu JJ, Esteban JA, Hayashi Y, Malinow R (2000) Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat. Neuroscience* 3:1098-1106.

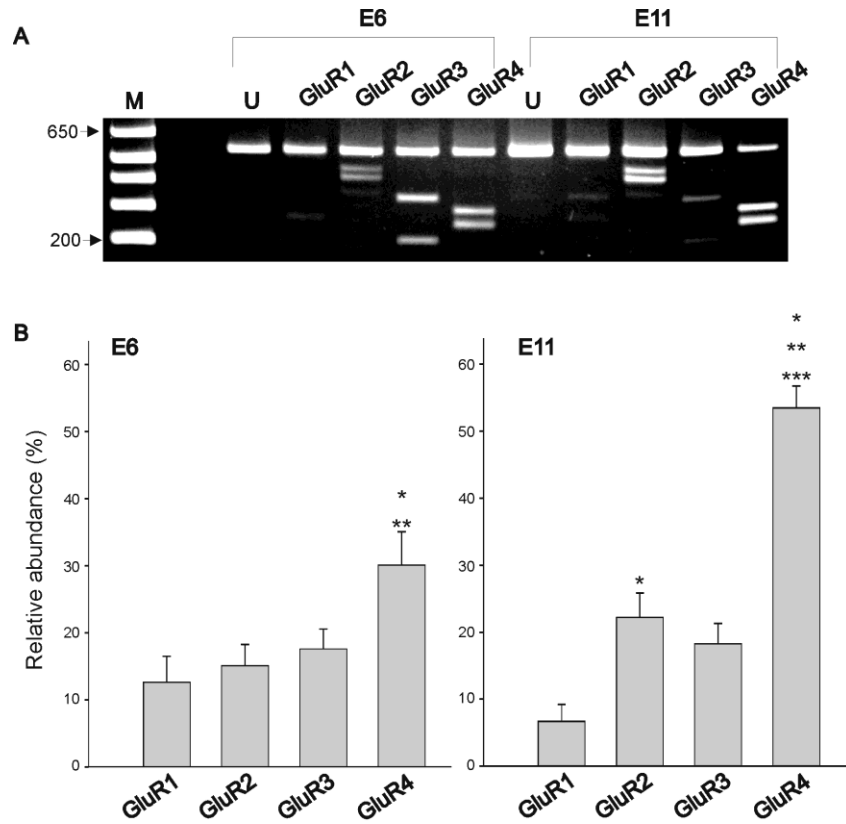


Figure 3.1. Relative abundance of AMPA receptor subunits in E6 and E11 lumbar spinal cords. A pan-AMPA degenerate set of primers were used to amplify products of the GluR1-4 subunits of ventral spinal cord region. The PCR products were digested by subunit-specific restriction endonucleases (Pvu II cuts GluR1 at bp 2120, BamH I cuts GluR2 at bp 2493, EcoR I cuts GluR3 at bp 2161 and PstI cuts GluR4 at bp 2139). Digested products were separated on a 5% polyacrylamide gel. **A)** Gel indicates the relative abundance of mRNA for GluR1-4 in chick lumbar motoneurons. The lane labeled with U shows the undigested PCR product generated by amplification with the pan-AMPA set of primers. Other lanes were digested with subunit-

specific restriction endonucleases. **B)** Relative abundance of AMPA receptor subunits in chick lumbar motoneurons. The results are summarized as mean percentages of the total AMPA receptor transcript expression accounted for by each subunit (* $p \leq 0.05$ vs. GluR1; ** $p \leq 0.05$ vs. GluR2; *** $p \leq 0.05$ vs. GluR3; n=3).

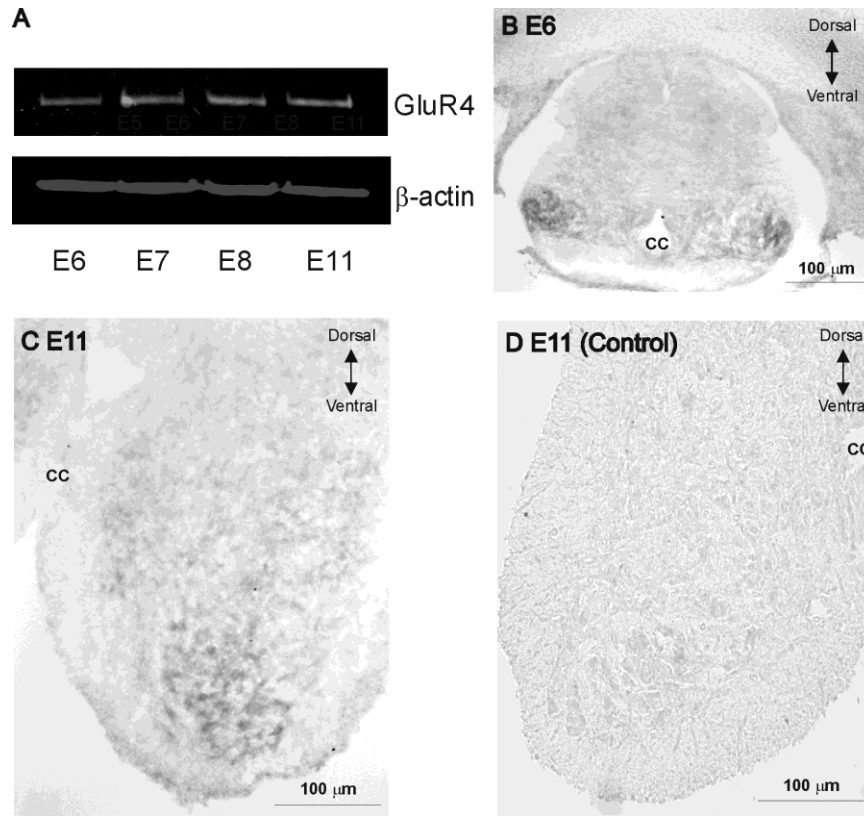


Figure 3.2. Developmental changes in GluR4 immunoreactivity in the chicken ventral spinal cord. **A)** Representative example of western blot data collected from chicken ventral spinal cords at various developmental stages (E6, E7, E8, and E11). β -actin was used to confirm equal loading in each well. Two-color western blot was used to detect GluR4 expression using the Odyssey infrared imaging system. **B)&C)** Developmental expression of GluR4 in chick spinal cord. Immunostaining of spinal cord sections for GluR4 expression at E6 and E11 demonstrate significant staining in the motoneuron pool. CC= central canal. Scale bar=100 μ m. **D)** Control immunostaining of spinal cord section excluding primary antibody shows no significant staining in the motoneuronal pool.

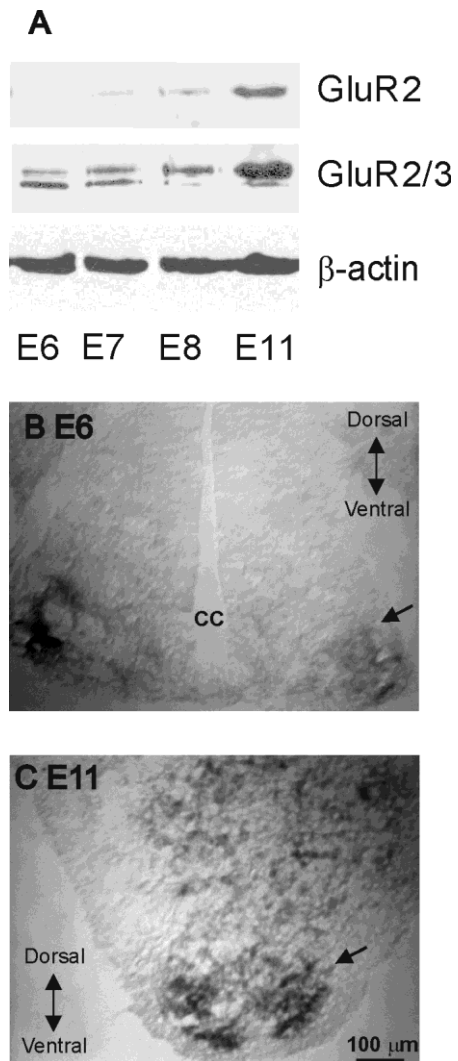


Figure 3.3. Developmental changes in GluR2 and GluR2/3 immunoreactivity in the chicken ventral spinal cord. **A)** Representative example of western blot data collected from chicken ventral spinal cords at various developmental stages (E6, E7, E8, and E11). Each well was loaded with equal amounts of protein (30 μ g) as determined by the Bradford assay. To confirm equal loading, membranes were stripped following GluR2 or GluR2/3 immunoblot and reprobbed for β -actin. **B)&C)** Developmental expression of GluR2/3 in chick spinal cord. Immunostaining of spinal cord sections

for GluR2/3 expression at E6 and E11 demonstrate significant staining in the motoneuron pool (arrow). At E11, significant staining can also be observed in the ventro-medial region of the spinal cord. CC= central canal. Scale bar=100 μ m.

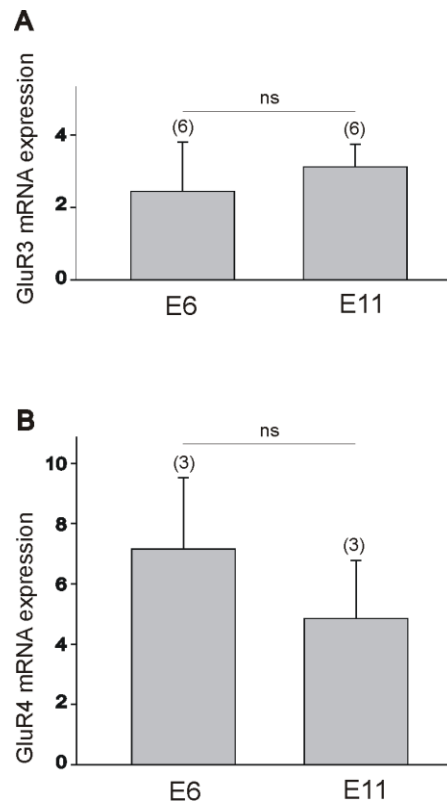


Figure 3.4. Developmental changes in GluR3 and GluR4 mRNA expression in the chicken motoneuron pool. **A)** Expression of GluR3 mRNA in laser microdissected motoneurons as detected by real time PCR (n=6). **B)** Expression of GluR4 mRNA in laser microdissected motoneurons as detected by real time PCR (n=3). NS indicates no significant differences between the E6 and E11 samples ($p>0.05$).

Chapter 4

Differential effect of glutamate receptor activation on the maturation of dendritic morphology in lumbar motoneurons

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Abbreviations

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione

E, embryonic day

MK-801, dizocilpine

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ABSTRACT

Glutamate receptor-mediated changes in intracellular Ca^{2+} may have important implications for activity-dependent regulation of early embryonic development. NMDA receptors were originally considered to be the sole source of glutamate-mediated Ca^{2+} influx. However, AMPA receptors lacking the GluR2 subunit also allow a significant influx of Ca^{2+} ions. Although Ca^{2+} -permeable AMPA receptors are a familiar feature in developing neurons, the developmental function of these receptors during the formation of the nervous system remains to be established. Previously, we have demonstrated that chicken lumbar motoneurons express Ca^{2+} -permeable AMPA receptors at embryonic day (E) 6. The Ca^{2+} permeability of AMPA receptors decreases three-fold by E11. In this study we explored the role of transiently expressed Ca^{2+} -permeable AMPA receptors in regulating the dendritic morphology of developing motoneurons *in ovo*. The AMPA receptor blocker CNQX (1 mg/day), when applied between E5 and E8, causes a significant increase in dendritic outgrowth and branching as compared with vehicle-treated embryos. Inhibition of NMDA receptor activity with MK-801 (100 $\mu\text{g}/\text{day}$) during this period has no effect on dendritic morphology. Treatment of chicken embryos with CNQX between E8 and E11 (when most receptors become Ca^{2+} -impermeable) has no significant effect on dendritic morphology. However, MK-801 application between E8 and E11 causes a significant reduction in dendritic length and branching. These findings indicate that AMPA receptor activation between E5 and E8 limits dendritic outgrowth in developing motoneurons, whereas NMDA receptor activation is involved in dendritic remodeling after the establishment of synaptic contacts with sensory afferents.

INTRODUCTION

Ca^{2+} influx through ionotropic glutamate receptors constitutes one important source of Ca^{2+} entry required for activity-dependent regulation of early embryonic development. NMDA receptors were originally thought to be the sole source of Ca^{2+} influx through glutamate receptors. However, AMPA receptors lacking the GluR2 subunit also allow a significant Ca^{2+} influx (Jonas et al., 1994; Greger et al., 2003). Ca^{2+} -permeable AMPA receptors are typically found at early stages of neuronal development (Gleason & Spitzer, 1998; Lee et al., 2001; Kumar et al., 2002). Although transiently expressed Ca^{2+} -permeable AMPA receptors are a familiar feature in developing neurons, very little is known about the developmental function of these receptors during the formation of the nervous system. Using the chicken spinal cord motoneurons as a model, this work was designed to investigate whether Ca^{2+} -permeable AMPA receptors regulate the dendritic morphology of chicken lumbar motoneurons.

We have demonstrated that chicken lumbar motoneurons express functional AMPA receptors by E5 (Ni et al., 2007). Our electrophysiological and pharmacological studies have demonstrated a high Ca^{2+} permeability of AMPA receptors at E5-E6. However, by E11, AMPA receptors become Ca^{2+} -impermeable. These findings indicate that during a critical period of motoneuron development there is a significant switch in the Ca^{2+} permeability of AMPA receptors (Ni et al., 2007). The switch in the Ca^{2+} permeability of AMPA receptors is triggered by a significant

increase in GluR2 expression but not by changes in the editing pattern of GluR2 subunits.

The presence of Ca^{2+} -permeable AMPA receptors at early stages of motoneuron development raises the question as to what functional role these receptors play in the overall maturation of the nervous system. Changes in AMPA receptor function during a critical period of neuronal development could potentially regulate several developmental processes *in ovo* including dendritic morphology and neuronal survival. Dendritic outgrowth is a highly dynamic process that occurs soon after neurogenesis. Initially, neurons extend dendrites in order to make appropriate synaptic connections with their intended presynaptic inputs. Synaptic activity, often generated by activation of glutamate receptors, further contributes to dendritic remodeling by regulating intracellular Ca^{2+} in already formed dendrites (Ramoia et al., 1988; Okada et al., 1999). For example, AMPA receptor activation is involved in dendritic remodeling of neonatal spinal motoneurons (Inglis et al., 2002; Prithviraj et al., 2007). While activity-dependent changes in dendritic morphology have been mostly studied in the context of dendritic remodeling (and plasticity), less is known about changes in dendritic outgrowth that occur as dendrites navigate to their appropriate targets. In this study we have taken advantage of the easy accessibility of chicken embryos to *in ovo* pharmacological manipulations in order to probe the role of Ca^{2+} -permeable AMPA receptors at early stages of motoneuron development.

Our present results demonstrate that blockade of AMPA receptor activation with CNQX between E5 and E8 causes a significant increase in dendritic outgrowth in lumbar motoneurons. Treatment of chicken embryos with CNQX between E8 and

E11, when AMPA receptors become Ca^{2+} -impermeable, has no effect on dendritic morphology, whereas MK-801 treatment causes a significant reduction in dendritic outgrowth. These results demonstrate that inhibition of glutamate receptor activity has a differential effect on maturation of the dendritic morphology at different stages of motoneuron development.

METHODS

In ovo manipulations of embryonic development: Embryos were windowed at E5 or E8 and sealed with Blendoderm surgical tape (3M Corp). CNQX (1 mg/mL) and MK-801 (100 µg/mL) were dissolved in sterile Tyrode's buffer containing (in mM): NaCl (139), KCl (3), MgCl₂ (1), CaCl₂ (3), NaHCO₃ (17). Controls consisted of embryos treated with vehicle (Tyrode's buffer). A 50-µL volume of each drug or vehicle was applied daily onto the vascularized chorioallantoic membrane as previously described by Martin-Caraballo & Dryer (2002). Considering a passive distribution of CNQX throughout the egg and an egg's volume equal to 60 mL, the concentration of CNQX used is equivalent to ~50 µM. The dose of MK-801 applied corresponds to a final concentration of 5 µM in the egg, which is sufficient to block native NMDA receptors *in vitro*. Chicken embryos have been shown to tolerate these doses of glutamate receptor antagonists (Caldero et al., 1997; Solum et al., 1997; Llado et al., 1999).

Assessment of dendritic morphology: Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight. After fixation, a small amount of DiI (2-4 µL) was applied using a picospritzer (Parker, Fairfield, NJ) onto the nerves in the ischiadic plexus and embryos were returned to the incubator for up to 8 weeks to allow time for complete neuronal labeling (Allan & Greer, 1997). Motoneurons of the ischiadic plexus can be found throughout lumbar segments L4 to L8 (Landmesser & Morris, 1975; Matise & Lance-Jones, 1996). The spinal cord tissue was sectioned into 200 µm slides and imaged with a Nikon Eclipse E600 microscope. The use of thicker

sections (200 μm) allowed us to obtain labeled motoneurons with their transverse dendritic tree intact (at least in the cross sectional plane). Therefore, our present analysis of dendritic morphology refers only to a portion of the dendritic tree, which should be taken into consideration when interpreting the data. We should point out, however, that many investigators have used this approach in order to study the maturation of the dendritic morphology in developing neurons (Lee et al., 1988; Kalb, 1994; Allan & Greer, 1997). Individually labeled motoneurons were traced using a computer-assisted camera morphometric program (NeuroLucida, MicroBrightfield, Colchester VT).

Three criteria were used in selecting appropriate motoneurons for tracing. First, only motoneurons with intact dendrites on the cross-sectional plane of the 200 μm section were included in our analysis (we did not analyze dendrites extending in the rostro-caudal direction, see below). Second, only motoneurons sufficiently separated from their neighbors were used for tracing. We have determined that the key to successful tracing is to apply a small amount of DiI in each nerve, which will only result in the labeling of at most 3 motoneurons/section. Extensive application of DiI resulted in labeling of a large number of neurons, which hindered the visualization and tracing processes of single motoneurons (as represented in Fig. 1A-B). Third, only motoneurons with primary dendrites emanating >180 degrees from the cell body were considered. This was an indication that DiI has spread evenly in all directions within the cell. In each DiI-labeled motoneuron, the following parameters were measured: dendritic arbor/cell, the longest dendritic tree derived from a primary dendrite (represented as the longest dendrite), number of primary dendrites, number

of nodes (branch points), and number of ends. We should mention that our technique for assessing dendritic morphology only included dendrites located in the transverse plane of the spinal cord but obviously did not include dendrites extending in the rostral-caudal direction. Therefore, our definition of dendritic arbor/cell refers to the length of all dendritic segments lying exclusively in the transverse plane of the spinal cord section. To investigate whether changes in the dendritic tree are localized to a particular area, we assessed dendritic length and/or number according to branch order. In this analysis, we compared the total length of primary dendrites, followed by second order dendrites (or dendrites bifurcating from primary dendrites) and so on. This allowed us to determine whether any changes in dendritic length may occur in proximal dendrites or more distal dendrites. Changes in soma morphology were assessed by measuring cell body perimeter and somatic surface area. The cell body perimeter was measured by focusing on the plane of the cell body, where cell dimensions were the greatest, and outlining the cell body contour.

Motoneuron labeling, dissociation and cell culture: Labeling, dissociation and culture of chick lumbar motoneurons were performed as previously described by Martin-Caraballo and Dryer (2002). 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 (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 (EMEM, BioWhittaker, Walkersville, MA) supplemented with 10% heat-inactivated horse serum, 2 mM

glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 ng/mL glial derived neurotrophic factor (GDNF). Recording of Ca²⁺ signals was performed 3-4 hr after dissociation of spinal cord tissue.

Intracellular free Ca²⁺ measurements: Changes in intracellular [Ca²⁺] resulting from activation of AMPA receptors was detected with the ratiometric dye Fura-2 as previously described by Ni et al., 2007. Briefly, cell cultures were bathed for 30 min in standard chick saline solution containing 5 µM Fura-2 AM (Molecular Probes, OR) and 0.2% pluronic acid under dark conditions. The standard chick saline contained (in mM): NaCl (145), KCl (5.4), MgCl₂ (0.8), CaCl₂ (5.4), glucose (5), and HEPES (13)(pH 7.4 with NaOH). 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 40x 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 Inc). Drugs were applied for 30-60 sec with a ValveLink 8 perfusion system (AutoMate Scientific Inc, San Francisco, CA). Recorded Ca²⁺ signals were corrected for background fluorescence and presented as the ratio of the fluorescent peak signals generated at 340 and 380 (F₃₄₀/F₃₈₀). This ratio represents relative changes in intracellular [Ca²⁺] without conversion to absolute values of intracellular-free Ca²⁺.

Data Analysis: Values are presented as mean \pm SEM where indicated. Statistical analyses consisted of one-way ANOVA followed by *post hoc* analysis using Tukey's honest significant difference test for unequal *n* for comparisons between multiple groups (SigmaStat software). Throughout, $p \leq 0.05$ was regarded as significant.

RESULTS

Dendritic morphology was investigated following injection of a small amount of DiI into the ischiadic plexus, which innervates the muscles of the hindlimb, lower leg and foot in the chicken embryo (Landmesser & Morris, 1975). DiI labeling of lumbar motoneurons between E6 and E11 reveals significant differences in dendritic outgrowth during this developmental period. Between E6 and E8, most dendrites are still located in the ventral portion of spinal cord and have hardly extended into the middle portions of the spinal cord (Fig.4.1. A-B). By E11, there is a significant increase in the dendritic complexity of the motoneurons (Fig.4.1. C). At this stage there is also a significant overlap between the dendrites of motoneurons and the projections of sensory neurons, suggesting that synapse formation is well underway at this stage (arrow in Fig.4.1. C, Lee et al., 1988). To characterize age-dependent changes in the morphology of lumbar motoneurons, we traced individual DiI-labeled motoneurons (Fig.4.1. D) using Neurolucida according to our exclusion criteria (see Methods). Examples of DiI-traced motoneurons from E6, E8 and E11 chicken embryos are represented in Fig.4.2. A. Quantification of the dendritic morphology of motoneurons between E6 and E11 is represented in Fig.4.2. B-E. Between E6 and E8 there is a significant increase in the length of the dendritic arbor/cell (E6, 1023 ± 54 μm , $n=29$ vs. E8, 1631 ± 57 μm , $n=33$; Fig.4.2. B) but not in the length of the longest dendrite (E6, 460 ± 22 μm vs. E8, 603 ± 35 μm , Fig.4.2. C). Changes in the length of the dendritic arbor/cell between E6 and E8 are likely mediated by a significant

increase in the number of branch points or nodes (E6, 11.8 ± 0.8 vs. E8, 16.4 ± 0.8 ; Fig.4.2. D) and the number of terminal dendrites (E6, 16 ± 0.9 vs. E8, 21.5 ± 0.9 ; Fig.4.2. E). Changes in the length of the dendritic arbor/cell between E6 and E8 may also involve a significant increase in the number of primary dendrites (E6, 4.3 ± 0.2 vs. E8, 5.2 ± 0.2 ; Fig.4.3. A). The cell body perimeter and somatic surface area also underwent a significant increase between E6 and E8 (Fig.4.3. B & C). Cell body perimeter increased 47% (E6, $53.5 \pm 1.7 \mu\text{m}$ vs. E8, $78.9 \pm 2.0 \mu\text{m}$; Fig.4.3. B), whereas somatic surface area increased by 106% (E6, $192 \pm 11 \mu\text{m}^2$ vs. E8, $396 \pm 17 \mu\text{m}^2$; Fig.4.3. C).

Comparison of the dendritic morphology of motoneurons between E8 and E11 also reveals significant differences in the extent of dendritic outgrowth. Between E8 and E11 there is a 68% increase in the length of the dendritic arbor/cell (E8, $1631 \pm 57 \mu\text{m}$, $n=33$ vs. E11, $2737 \pm 169 \mu\text{m}$, $n=45$; Fig.4.2. B) and a 63% increase in the longest dendrite (E8, $603 \pm 35 \mu\text{m}$ vs E11, $983 \pm 78 \mu\text{m}$; Fig.4.2. C). These changes in dendritic length are caused by a significant increase in the number of branch points or nodes (E8, 16.4 ± 0.8 vs. E11, 23.9 ± 1.4 ; Fig.4.2. D) and the number of terminal dendrites (E8, 21.5 ± 0.9 vs. E11, 29.6 ± 1.5 ; Fig.4.2. E). Interestingly, no changes in the number of primary dendrites were observed between E8 and E11 in lumbar motoneurons (E8, 5.1 ± 0.2 vs. E11, 5.8 ± 0.2 ; Fig.4.3. A). This data suggests that the number of primary dendrites is determined between E6 and E8 (Fig.4.3. A). The cell body perimeter and somatic surface area also underwent a significant increase between E8 and E11 (Fig.4.3. B & C). Cell body perimeter increased 15% (E8,

78.9±2.0 μm vs. E11, 90.7±2.1 μm; Fig.4.3. B), whereas somatic surface area increased by 27% (E8, 396±17 μm² vs. E11, 505±22 μm²; Fig.4.3. C).

To investigate whether the increase in the length of the dendritic arbor/cell occurs in specific segments of the dendritic tree, we analyzed changes in both dendritic length and number of dendrites as a function of dendritic order. The length of each dendritic segment (or total number of dendrites within a segment) was plotted as a function of their branch order (Fig.4.4. A & B). As represented in Fig.4.4. A-B, the dendritic arbor of E6 motoneurons extends until the 7th dendritic order, whereas at E8 dendrites branched out until the 8th dendritic order. The main differences in the length of the dendritic length as a function of branch order between E6 and E8 motoneurons was observed in proximal dendrites (between the 2nd and 4th order dendritic segments). However, major differences in the extent of the dendritic length and number of dendrites occurred between E8 and E11. As represented in Fig.4.4. A-B, there is a significant increase in the length of each dendritic segment between E8 and E11. We also saw that E11 motoneurons had higher order dendrites that were not found in E8 motoneurons ($\geq 9^{\text{th}}$, Fig.4.4. A). For example, we never found E8 motoneurons with branches at the 9th, 10th and 11th dendritic orders (Fig.4.4. A). A similar pattern of dendritic outgrowth was also observed when we plotted the number of dendritic branches as a function of dendritic order (Fig.4.4. B). Significantly more dendritic segments were found in the 3rd or higher branches in E11 motoneurons than in E8. More dendritic segments were also found in E11 motoneurons at higher branch orders ($\geq 9^{\text{th}}$, Fig.4.4. B). This data suggests that the increase in the dendritic arbor of

E11 motoneurons is the result of significant rearrangements of dendritic length and branching within each dendritic order.

In order to determine whether inhibition of Ca^{2+} -permeable AMPA receptor activity alters the dendritic morphology of motoneurons, we applied the AMPA receptor blocker CNQX (1 mg/day) daily to the chorioallantoic membrane of chicken embryos developing *in ovo*. Another set of embryos was treated with the NMDA receptor blocker MK-801 (100 $\mu\text{g}/\text{day}$). Controls consisted of chicken embryos treated with vehicle (Tyrode's buffer used to dissolve the drugs). Drugs were applied between E5 and E8 and embryos were isolated 6 hr after the last drug application on E8. There were no significant changes in dendritic length and dendritic complexity between non-treated E8 and vehicle-treated embryos. For example, the length of the dendritic arbor in E8 non-treated and vehicle-treated embryos was $1631 \pm 57 \mu\text{m}$ and $1713 \pm 82 \mu\text{m}$, respectively, whereas the number of branch points was 16.4 ± 0.8 and 14.5 ± 0.8 . Thus, treatment of chicken embryos with the vehicle buffer solution has no effect on the overall dendritic architecture when compared with non-treated embryos. Treatment of chicken embryos with CNQX between E5 and E8 caused a significant increase in the dendritic complexity of motoneurons (Fig.4.5. A-F). Application of CNQX between E5 and E8 induced a ~30% increase in the dendritic arbor/cell (Vehicle, $1713 \pm 82 \mu\text{m}$, $n=35$ vs. CNQX, $2226 \pm 108 \mu\text{m}$, $n=33$; Fig.4.5. B) and the length of the longest dendrite (Vehicle, $667 \pm 36 \mu\text{m}$ vs. CNQX, $856 \pm 60 \mu\text{m}$; Fig.4.5. C) when compared with vehicle-treated embryos. Changes in the dendritic arbor/cell were the result of increased complexity of dendritic morphology as indicated by the increased number of branch points (Vehicle, 14.5 ± 0.8 vs. CNQX, 22.6 ± 1.4 ; Fig.4.5. D) and the increased

number of ends (Vehicle, 18.7 ± 0.9 vs. CNQX, 27.5 ± 1.4 ; Fig.4.5. E). To determine whether the effect of CNQX was concentration-dependent we treated the chicken embryos with a 100-fold lower concentration of CNQX ($10 \mu\text{g}/\text{day}$, Fig.4.5. B-E) between E5 and E8. As represented in Fig.4.5. B-E, treatment of chicken embryos with $10 \mu\text{g}/\text{day}$ of CNQX between E5 and E8 did not have any effect on the overall dendritic morphology of E8 motoneurons suggesting that the stimulatory effect of CNQX ($1 \text{ mg}/\text{day}$) on dendritic outgrowth is specific.

The results with CNQX indicate that blockade of AMPA receptor activity between E5 and E8 causes a significant increase in dendritic complexity. Thus, the question arises as to which dendrites are most likely affected by inhibition of AMPA receptor activity. In order to investigate which dendritic branches are most likely affected by CNQX application to chicken embryos between E5 and E8, we plotted the dendritic length as a function of branch order (Fig.4.5. F). Our results show that treatment of chicken embryos with CNQX between E5 and E8 causes a significant increase in high-level dendritic branches ($\geq 4^{\text{th}}$ dendritic order) when compared with vehicle-treated embryos. Blockade of glutamate receptor activity with CNQX or MK-801 did not have any effect on the number of primary dendrites (Fig.4.6. A) or the overall properties of the cell bodies (including cell body perimeter and somatic surface area, Fig.4.6. B & C). This data suggests that blockade of Ca^{2+} -permeable AMPA receptor activity during a specific period of motoneuron development has important implications for dendritic morphology.

Previously, it has been reported that CNQX becomes a partial agonist of AMPA receptors when these receptors become associated with regulatory proteins

like TARP (also known as transmembrane AMPA receptor regulatory protein) (Menuz et al., 2007). CNQX stimulation of AMPA receptors co-associated with TARP evokes a significant inward current in cerebellar granular cells (Menuz et al., 2007). Thus, the question arise as to whether CNQX behaves as a partial agonist of AMPA receptors in lumbar motoneurons. To investigate this possibility, we measured the changes in intracellular Ca^{2+} generated by stimulation of acutely isolated E8 and E11 motoneurons with AMPA (50 μM) or CNQX (50 μM). Recordings of intracellular Ca^{2+} signals were conducted in normal saline solution. Since no voltage-gated channels were blocked under these experimental conditions, Ca^{2+} signals were likely generated by the influx of Ca^{2+} ions through voltage-gated calcium channels and/or Ca^{2+} -permeable AMPA receptors. If CNQX behaves as a partial agonist of AMPA receptors, then exposure of lumbar motoneurons to CNQX should generate a significant intracellular Ca^{2+} response. As represented in Fig.4.7. (A, B), stimulation of E8 lumbar motoneurons with 50 μM AMPA evoked a significant increase in intracellular Ca^{2+} . However, CNQX stimulation alone did not have any noticeable effect on the intracellular Ca^{2+} signal. The AMPA-evoked increase in intracellular Ca^{2+} was eliminated in the presence of CNQX, suggesting that in our model CNQX behaves as an inhibitor of AMPA-mediated responses (Fig.4.7. A, B). Recordings from multiple motoneurons isolated from chicken embryos at E6, E8 or E11 indicate that CNQX, unlike AMPA, has little effect on the intracellular Ca^{2+} signal (Fig.4.7. B). Furthermore, at all ages tested, pretreatment of lumbar motoneurons with CNQX eliminates the AMPA-evoked increase in intracellular Ca^{2+} , confirming the antagonistic role of CNQX on AMPA receptor activation (Fig.4.7. B).

Treatment of chicken embryos with the NMDA receptor blocker MK-801 between E5 and E8 results in no significant change in the dendritic morphology of E8 motoneurons (Fig.4.5. B-E). Thus, no changes in dendritic length or branching patterns were detected in the motoneurons of chicken embryos treated with 1 µg/day or 100 µg/day MK-801 (Fig.4.5. B-E). MK-801 (100 µg/day) treated embryos also showed no significant change in the number of primary dendrites (Fig.4.6. A) or soma morphology (Fig.4.6. B-C). No change in the distribution of dendritic length as a function of dendritic order was found in E8 motoneurons following treatment of chicken embryos with MK-801(100 µg/day) when compared with vehicle-treated embryos (results not shown). These findings suggest that NMDA receptor activation plays no significant role in the maturation of the dendritic morphology of motoneurons between E5 and E8.

Does inhibition of glutamate receptor activity between E8 and E11 have any effect on motoneuron morphology? To investigate this possibility, chicken embryos were treated *in ovo* with daily doses of CNQX or MK-801 between E8 and E11. Controls consisted of chicken embryos treated with Tyrode's buffer (vehicle). Drugs were applied between E8 and E11 and embryos were isolated 6 hr after the last drug application on E11. There were no significant changes in dendritic length and dendritic complexity between non-treated E11 and vehicle-treated embryos. As represented in Fig.4.8. (B-E), treatment of chicken embryos with CNQX (1 mg/day) has no significant effect on the dendritic morphology of E11 lumbar motoneurons, suggesting activation of Ca²⁺-impermeable AMPA receptors is not involved in the maturation of the dendritic morphology of motoneurons. Thus, no changes in the

dendritic arbor/cell (Fig.4.8. B), longest dendrite (Fig.4.8. C), number of branch points (Fig.4.8. D) or number of ends (Fig.4.8. E) were detected when 1 mg/day CNQX was applied between E8 and E11. CNQX (1 mg/day) application to chicken embryos between E8 and E11 did not alter the number of primary dendrites or cell body morphology (Fig.4.9. A-C). No change in the distribution of dendritic length as a function of dendritic order was found in E11 motoneurons following treatment of chicken embryos with CNQX (1 mg/day) when compared with the length of motoneurons of vehicle-treated embryos (results not shown).

Surprisingly, treatment of chicken embryos with the NDMA receptor inhibitor MK-801 (100 $\mu\text{g}/\text{day}$) between E8 and E11 caused a significant reduction in dendritic outgrowth (Fig.4.8. A-F). Thus, application of MK-801 to chicken embryos between E8 and E11 led to a significant decrease in the dendritic arbor/cell (Vehicle, $3168 \pm 156 \mu\text{m}$, $n=48$ vs. MK-801, $2419 \pm 109 \mu\text{m}$, $n=55$; Fig.4.8. B) and the length of the longest dendrite (Vehicle, $1084 \pm 65 \mu\text{m}$ vs. MK-801, $853 \pm 39 \mu\text{m}$; Fig.4.8. C). These changes in dendritic length were likely caused by a significant reduction in the number of branching points (Vehicle, 21.7 ± 1.2 vs. MK-801, 16.3 ± 0.8 ; Fig.4.8. D) and an overall reduction in the number of dendritic ends (Vehicle, 26.6 ± 1.3 vs. MK-801, 21.1 ± 0.9 ; Fig.4.8. E). In order to investigate which dendritic branches are most likely affected by MK-801 application to chicken embryos between E8 and E11, we plotted dendritic length as a function of branch order (Fig.4.8. F). Our results show that application of MK-801 between E8 and E11 causes a significant reduction in certain branches (5th and 6th order branches) and a complete elimination of dendritic branches above the 7th dendritic order (Fig.4.8. F). The inhibitory effect of MK-801

(100 $\mu\text{g}/\text{day}$) was specific since application of a 100-fold lower concentration of the drug did not have any significant effect on the dendritic complexity of E11 motoneurons (Fig.4.8. B-E). Thus, treatment of chicken embryos with 1 $\mu\text{g}/\text{day}$ of MK-801 did not have any noticeable effect on the dendritic length or branching pattern in E11 motoneurons when compared with vehicle (Fig.4.8. B-E). Curiously, treatment of chicken embryos with MK-801 also caused a significant reduction in the number of primary dendrites (Vehicle, 5.8 ± 0.2 vs. MK-801, 4.8 ± 0.1 ; Fig.4.9. A) without any alterations in cell body morphology (Fig.4.9. B & C).

DISCUSSION

The period spanning from E4 to E11 is critical to the development of chicken lumbar motoneurons. During this period, motoneurons begin the process of target innervation of hindlimb muscles, which ultimately leads to the formation of functional synapses and the generation of spontaneous motor activity (Dahm & Landmesser, 1991; Chub & O'Donovan, 1998; Milner & Landmesser, 1999). Motoneurons also undergo considerable changes in their electrophysiological properties between E4 and E11 (McCobb et al., 1989, 1990; Martin-Caraballo & Dryer, 2002). During this period of differentiation, there are also considerable changes in the development of sensorimotor synaptic connections between motoneurons and their sensory afferents (Lee et al., 1988). Our present results demonstrate that functional maturation of lumbar motoneurons also involves a significant increase in dendritic complexity. Changes in the dendritic morphology of motoneurons included an increased number of branch points and dendritic ends between E6 and E11, which result in an overall increase in dendritic length. Increased dendritic length is likely caused by a significant increase in the length of proximal and distal dendritic orders, as evidenced by plots of dendritic length as a function of dendritic order in E8 and E11 motoneurons. Another contributing factor to the increase in total length is the increased branching pattern found in more distal dendrites in E11 motoneurons.

As our results demonstrated, CNQX-mediated inhibition of AMPA/kainate receptor activity between E5 and E8 causes a significant increase in dendritic length and complexity, suggesting that glutamatergic neurotransmission in the chicken spinal

cord plays a significant role in regulating early dendritic development. The CNQX-evoked effect on dendritic morphology alters dendritic length and branching without having any significant effect on cell body morphology or the number of primary dendrites. Two different mechanisms could explain the stimulatory role of CNQX on dendritic outgrowth observed between E6 and E8. First, CNQX can potentially inhibit dendritic regression between E6 and E8, resulting in stimulation of dendritic outgrowth. This possibility can be refuted by our data showing a significant increase in the dendritic outgrowth of normally-developing motoneurons between E6 and E8. The second possibility is that during normal development between E6 and E8, AMPA receptor activation limits dendritic outgrowth. Therefore, inhibition of AMPA receptor activity during this developmental period will promote dendritic outgrowth. Indeed, our analysis of dendritic length as a function of dendritic order reveals that the CNQX-generated increase in dendritic length mainly affects distal dendrites, suggesting that the AMPA receptor activation acts as a stop signal for dendritic outgrowth. The ability of AMPA receptor activation to limit dendritic growth between E5 and E8 coincides with the period when these receptors are Ca^{2+} -permeable due to the lack of GluR2 expression in the motoneurons (Ni et al., 2007). Previous findings have demonstrated that increased intracellular $[\text{Ca}^{2+}]$ acts as a stop signal for growing dendrites, which prevents synapse formation (Lohmann et al., 2005). Thus, the ability of AMPA receptor activation to limit dendritic growth could be mediated by the high Ca^{2+} permeability of these receptors prior to E8. If activation of Ca^{2+} -permeable AMPA receptors were acting as a stop signal for growing dendrites, then this effect should be absent in motoneurons expressing Ca^{2+} -impermeable receptors. Indeed, our present

findings reveal that treatment of chicken embryos with CNQX between E8 and E11 when AMPA receptors become Ca²⁺-impermeable has no effect on dendritic morphology. Thus, our present results demonstrate that blockade of Ca²⁺-permeable AMPA receptors regulates the maturation of dendritic morphology during a critical period of development, whereas inhibition of Ca²⁺-impermeable AMPA receptors has no effect on dendritic morphology. Our results are consistent with previous findings indicating that activation of Ca²⁺-permeable AMPA receptors downregulates the dendritic outgrowth of immature neurons in culture (Metzger et al., 1998; Mattson et al., 1990), whereas AMPA receptor activation enhances dendritic outgrowth in cultured cortical neurons (Monnerie & Le Roux, 2006).

Although the CNQX-evoked effect on the dendritic morphology of motoneurons is limited to a specific developmental period (E5-E8), when most AMPA receptors are Ca²⁺-permeable, the question arises as to whether other factors could potentially account for the drug-induced changes in dendritic morphology. CNQX is a broad inhibitor of both AMPA and kainate receptors. Therefore, the stimulatory effect of CNQX on dendritic outgrowth at E8 could be mediated by the blockade of kainate receptors *in ovo*. However, this seems unlikely since we have previously demonstrated that nearly 100% of kainate-evoked currents in chicken lumbar motoneurons are generated by activation of AMPA receptors (Ni et al., 2007). It is also unlikely that the stimulatory effect of CNQX on dendritic outgrowth was due to a non-specific effect of the drug on early motoneurons since the present findings indicate that treatment of motoneurons between E8 and E11 with the same dose of CNQX has no effect on dendritic morphology. Our present results also demonstrate that CNQX does not

behave like a partial agonist of AMPA receptors in motoneurons. Thus, stimulation of E8 or E11 motoneurons with 50 μ M CNQX did not cause any significant elevation in intracellular Ca^{2+} . Therefore, CNQX does not appear to behave like a partial agonist of AMPA receptors as previously reported for granular cells in the hippocampus and cerebellum (Brickley et al., 2001; Maccaferri & Dingledine, 2002; Menuz et al., 2007). It is also unlikely that the stimulatory effect of CNQX on dendritic outgrowth at E8 but not at E11 could be due to a secondary effect on NMDA receptors (Yamada et al., 1989; Piochon et al., 2007) since our results demonstrate that CNQX and MK-801 have different patterns of regulation of dendritic outgrowth *in ovo*.

Inhibition of NMDA receptors plays a significant role in regulating the dendritic morphology of motoneurons after E8, when motoneurons are establishing appropriate synaptic connections with sensory inputs. Blockade of NMDA receptors with MK-801 causes a significant decrease in dendritic morphology including an overall reduction in total length and branching, suggesting that NMDA receptor activation during normal development promotes dendritic outgrowth. The inhibitory effect of MK-801 in dendritic length is likely caused by a significant reduction in dendritic branching and pruning of primary and high order dendrites. The MK-801-mediated effect on dendritic outgrowth coincides with the period of synapse formation and rearrangements that occur in the chicken spinal cord between motoneurons and sensory afferents after E8 (Lee et al., 1988). Our findings are in agreement with previous reports demonstrating that inhibition of NMDA receptor activity *in vivo* also evokes a significant reduction in dendritic length and arborization in postnatal rat motoneurons (Kalb, 1994; Inglis et al., 1998) and developing tectal neurons (Rajan &

Cline, 1998). Interestingly, inhibition of NMDA receptor activation with MK-801 also generates a significant reduction in the number of primary dendrites without any further effect on cell body morphology. The MK-801-evoked effect in E11 motoneurons could be due to a trophic effect of NMDA receptor activation on lumbar motoneurons. If this is the case, then inhibition of NMDA receptor activation will disrupt maturation of the dendritic morphology in the motoneurons. Indeed, previous results indicate that daily applications of NMDA (0.5 mg) to chicken embryos between E5 and E9 result in a significant increase in motoneuron survival, whereas NMDA receptor blockade with MK-801 ($\geq 500 \mu\text{g}$) during this period causes a significant increase in the number of apoptotic motoneurons (Llado et al., 1999). However, we should not discard the possibility that the MK-801-evoked effect in E11 motoneurons could be due to a toxic effect of the drug on motoneuron survival and dendritic morphology. The MK-801-evoked effect in E11 motoneurons is more puzzling considering that we did not detect any significant changes in the number of primary dendrites between E8 and E11 motoneurons during normal development. These results suggest that at later stages of development, glutamatergic signaling through NMDA receptors may be required for maintenance of the initial number of primary dendrites.

In central synapses, membrane depolarization generated by AMPA receptor activation is required to relieve the Mg^{2+} blockade of NMDA receptors (Koh et al., 1995). If this is the case, then it is puzzling that only blockade of NMDA receptors with MK-801 between E8 and E11 causes a significant reduction in dendritic outgrowth. CNQX inhibition of AMPA receptor activity between E8 and E11 has no effect on dendritic outgrowth. One possibility is that multiple factors generate

membrane depolarizations required for activation of NMDA receptors *in ovo*. For example, GABA receptor activation generates membrane depolarizations in the embryonic chicken spinal cord (Sernagor et al., 1995; Yoon et al., 2008). Furthermore, a GABA-generated tonic current is required for the generation of spontaneous bursts of activity in the chicken spinal cord (Chub & O'Donovan, 1998; 2001).

While several mechanisms can potentially regulate dendritic outgrowth during neuronal differentiation, changes in glutamate receptor function and patterns of activity in the spinal cord likely underlie the changes in dendritic outgrowth caused by glutamate antagonists. Spontaneous synaptic activity is an early feature of developing spinal cord networks (Milner & Landmesser, 1999), which underlies the generation of rhythmic limb movements (O'Donovan & Landmesser, 1987). In chicken embryos, bursts of spontaneous activity in the motoneurons can be recorded from ventral roots as early as E4, 48 hrs prior to the onset of target innervation (Milner & Landmesser, 1999). Although glutamatergic neurotransmission is not required for the early generation of spontaneous activity (prior to E8), both AMPA and NMDA receptors appear to be functional as early as E4.5. For example, activation of NMDA or kainate receptors at E4.5 elicits an increase in spontaneous bursting activity in isolated spinal cord preparations (Milner & Landmesser, 1999). We have also shown that chicken spinal motoneurons expressed functional AMPA receptors at E5 (Ni et al., 2007). Although glutamate receptors are not involved in the generation of early spontaneous activity in the spinal cord, they nonetheless play a critical role in regulating the morphological differentiation of motoneurons. Thus, AMPA receptor activation limits

dendritic outgrowth between E5 and E8, indicating that AMPA receptor activation is a critical factor regulating dendritic morphology at early stages of development.

REFERENCES

- Allan DW, Greer JJ. 1997. Development of phrenic motoneuron morphology in the fetal rat. *J. Comp Neurol.* 382(4):469-79.
- Brickley SG, Farrant M, Swanson GT, Cull-Candy SG. 2001. CNQX increases GABA-mediated synaptic transmission in the cerebellum by an AMPA/kainate receptor-independent mechanism. *Neuropharmacol.* 41(6):730-6.
- Calderó J, Ciutat D, Lladó J, Castán E, Oppenheim RW, Esquerda JE. 1997. Effects of excitatory amino acids on neuromuscular development in the chick embryo. *J Comp Neurol.* 387(1):73-95.
- Chub N, O'Donovan MJ. 2001. Post-episode depression of GABAergic transmission in spinal neurons of the chick embryo. *J Neurophysiol.* 85(5), 2166-76.
- Chub N, O'Donovan MJ. 1998. Blockade and recovery of spontaneous rhythmic activity after application of neurotransmitter antagonists to spinal networks of the chicken embryo. *J Neurosci.* 18(1), 294-306.
- Chu-Wang I-W, Oppenheim RW. 1978. Cell death of motoneurons in the chicken embryo spinal cord. I. A light and electron microscopy study of naturally occurring and induced cell loss during development. *J Comp Neurol* 177, 33-58.
- Dahm LM, Landmesser LT. 1991. The regulation of synaptogenesis during normal development and following activity blockade. *J Neurosci.* 11(1):238-55.
- Gleason EL, Spitzer NC. 1998. AMPA and NMDA receptors expressed by differentiating *Xenopus* spinal neurons. *J Neurophysiol.* 79(6), 2986-2998.
- Greger IH, Khatri L, Kong X, Ziff EB. 2003. AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* 40(4), 763-774.
- Hollyday M, Hamburger V. 1977. An autoradiographic study of the formation of the lateral motor column in the chicken embryo. *Brain Res.* 132(2), 197-208.
- Inglis FM, Crockett R, Korada S, Abraham WC, Hollmann M, Kalb RG. 2002. The AMPA receptor subunit GluR1 regulates dendritic architecture of motor neurons. *J Neurosci.* 22(18), 8042-51.

- Inglis FM, Furia F, Zuckerman KE, Strittmatter SM, Kalb RG. 1998. The role of nitric oxide and NMDA receptors in the development of motor neuron dendrites. *J Neurosci.* 18(24), 10493-501.
- Jeong GB, Werner M, Gazula VR, Itoh T, Roberts M, David S, Pfister B, Cohen A, Neve RL, Hollmann M, Kalb R. 2006. Bi-directional control of motor neuron dendrite remodeling by the calcium permeability of AMPA receptors. *Mol Cell Neurosci.* 32(3):299-314.
- Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H. 1994. Differences in Ca²⁺-permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* 12(6), 1281-1289.
- Kalb RG. 1994. Regulation of motor neuron dendrite growth by NMDA receptor activation. *Development.* 120(11), 3063-71.
- Koh DS, Geiger JR, Jonas P, Sakmann B. 1995. Ca²⁺-permeable AMPA and NMDA receptor channels in basket cells of rat hippocampal dentate gyrus. *J Physiol.* 485, 383-402.
- Kumar SS, Bacci A, Kharazia V, Huguenard JR. 2002. A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci.* 22(8), 3005-3015.
- Landmesser L, Morris DG. 1975. The development of functional innervation in the hind limb of the chick embryo. *J Physiol.* 249(2), 301-26.
- Lee CJ, Kong H, Manzini MC, Albuquerque C, Chao MV, MacDermott AB. 2001. Kainate receptors expressed by a subpopulation of developing nociceptors rapidly switch from high to low Ca²⁺ permeability. *J Neurosci.* 21(13), 4572-81.
- Lee MT, Koebbe MJ, O'Donovan MJ. 1988. The development of sensorimotor synaptic connections in the lumbosacral cord of the chicken embryo. *J Neurosci.* 8(7), 2530-2543.
- Llado J, Caldero J, Ribera J, Tarabal O, Oppenheim RW, Esquerda JE. 1999. Opposing effects of excitatory amino acids on chicken embryo spinal cord motoneurons: excitotoxic degeneration or prevention of programmed cell death. *J Neurosci.* 19(24), 10803-12.
- Lohmann C, Finski A, Bonhoeffer T. 2005. Local calcium transients regulate the spontaneous motility of dendritic filopodia. *Nat Neurosci.* 8(3), 305-312.

- Maccaferri G, Dingledine R. 2002. Complex effects of CNQX on CA1 interneurons of the developing rat hippocampus. *Neuropharmacology*. 43(4):523-9.
- Martin-Caraballo M, Dryer, SE. 2002. Activity- and target-dependent regulation of large-conductance Ca^{2+} -activated K^{+} channels in developing chicken lumbar motoneurons. *J Neurosci*. 22(1), 73-81.
- Matise MP, Lance-Jones C. 1996. A critical period for the specification of motor pools in the chicken lumbosacral spinal cord. *Development*. 122(2), 659-69.
- Mattson MP. 1990. Excitatory amino acids, growth factors, and calcium: a teeter-totter model for neural plasticity and degeneration. *Adv Exp Med Biol*. 268, 211-20.
- McCobb DP, Best PM, Beam KG. 1990. The differentiation of excitability in embryonic chicken limb motoneurons. *J Neurosci* 10, 2974-2984.
- McCobb DP, Best PM, Beam KG. 1989. Development alters the expression of calcium currents in chicken limb motoneurons. *Neuron* 2, 1633-1643.
- Menuz K, Stroud RM, Nicoll RA, Hays FA. 2007. TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists. *Science* 318(5851):815-7.
- Metzger F, Wiese S, Sendtner M. 1998. Effect of glutamate on dendritic growth in embryonic rat motoneurons. *J Neurosci*. 18(5), 1735-1742.
- Milner LD, Landmesser LT. 1999. Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J Neurosci*. 19(8), 3007-3022.
- Monnerie H, Le Roux PD. 2006. Glutamate receptor agonist kainate enhances primary dendrite number and length from immature mouse cortical neurons in vitro. *J Neurosci Res*. 83(6), 944-56.
- Ni X, Sullivan GJ, Martin-Caraballo M. 2007. Developmental characteristics of AMPA receptors in chick lumbar motoneurons. *Dev Neurobiol*. 67(11), 1419-32.
- O'Donovan MJ. 1999. The origin of spontaneous activity in developing networks of the vertebrate nervous system. *Curr Opin Neurobiol*. 9(1), 94-104.
- O'Donovan MJ, Chub N, Wenner P. 1998. Mechanisms of spontaneous activity in developing spinal networks. *J Neurobiol*. 37(1), 131-45.
- O'Donovan MJ, Landmesser L. 1987. The development of hindlimb motor activity

- studied in the isolated spinal cord of the chicken embryo. *J Neurosci.* 7(10), 3256-3264.
- Okada T, Schultz K, Geurtz W, Hatt H, Weiler R. 1999. AMPA-preferring receptors with high Ca²⁺ permeability mediate dendritic plasticity of retinal horizontal cells. *Eur J Neurosci.* 11(3), 1085-95.
- Piochon C, Irinopoulou T, Bruscianno D, Bailly Y, Mariani J, Levenes C. 2007. NMDA receptor contribution to the climbing fiber response in the adult mouse Purkinje cell. *J Neurosci.* 27(40):10797-809.
- Prithviraj R, Kelly KM, Espinoza-Lewis R, Hexom T, Clark AB, Inglis FM. 2007. Differential regulation of dendrite complexity by AMPA receptor subunits GluR1 and GluR2 in motor neurons. *Dev Neurobiol.* 68(2), 247-64.
- Rajan I, Cline HT. 1998. Glutamate receptor activity is required for normal development of tectal cell dendrites in vivo. *J Neurosci.* 18(19), 7836-46.
- Ramoas AS, Campbell G, Shatz CJ. 1988. Dendritic growth and remodeling of cat retinal ganglion cells during fetal and postnatal development. *J Neurosci.* 8(11), 4239-61.
- Sernagor E, Chub N, Ritter A, O'Donovan MJ. 1995. Pharmacological characterization of the rhythmic synaptic drive onto lumbosacral motoneurons in the chick embryo spinal cord. *J Neurosci.* 15(11), 7452-64.
- Solum D, Hughes D, Major MS, Parks TN. 1997. Prevention of normally occurring and deafferentation-induced neuronal death in chicken brainstem auditory neurons by periodic blockade of AMPA/kainate receptors. *J Neurosci.* 17(12), 4744-4751.
- Yamada KA, Dubinsky JM, Rothman SM. 1989. Quantitative physiological characterization of a quinoxalinedione non-NMDA receptor antagonist. *J Neurosci.* 9(9):3230-6.
- Yoon YJ, Kominami H, Trimarchi T, Martin-Caraballo M. 2008. Inhibition of electrical activity by retroviral infection of chicken spinal cord with Kir2.1 transgenes disrupts electrical differentiation of motoneurons. *PloS ONE* 3(8), e2971.

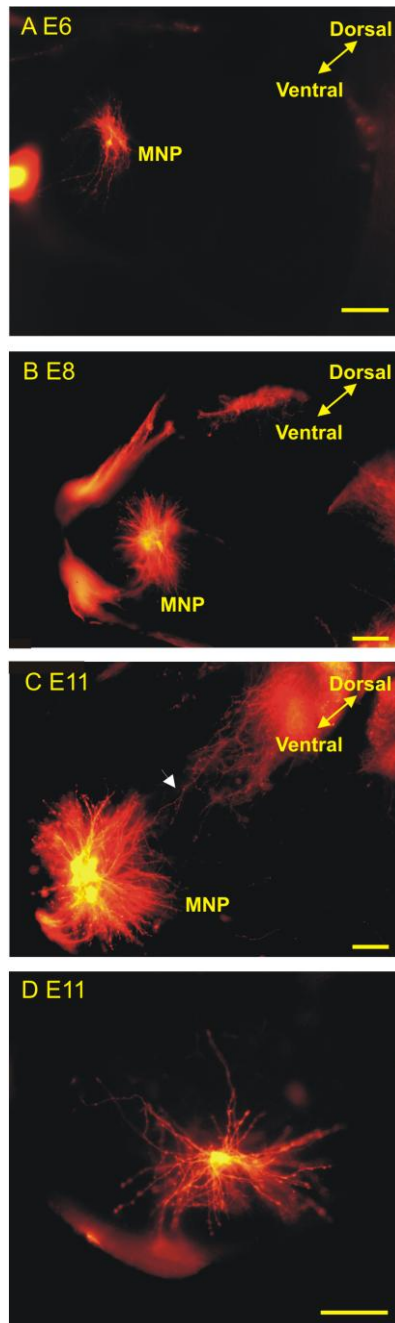


Figure 4.1. DiI labeling of chicken lumbar motoneurons at E6 (A), E8 (B) and E11 (C). Embryos were isolated between E6 and E11 and fixed overnight in 4% paraformaldehyde. Labeling was performed by application of a small amount of DiI into the ischiadic plexus. In A-C the whole motoneuron pool was labeled with DiI

(multiple motoneurons) to indicate the scope of dendritic outgrowth (MNP indicates the motoneuron pool). **D)** Representative E11 spinal cord section with one DiI-labeled motoneuron captured with a 10X objective. Only spinal cord sections containing no more than three DiI-labeled motoneurons/section were used for tracing of dendritic arbor using a 40X magnification objective. Scale bar=100 μm .

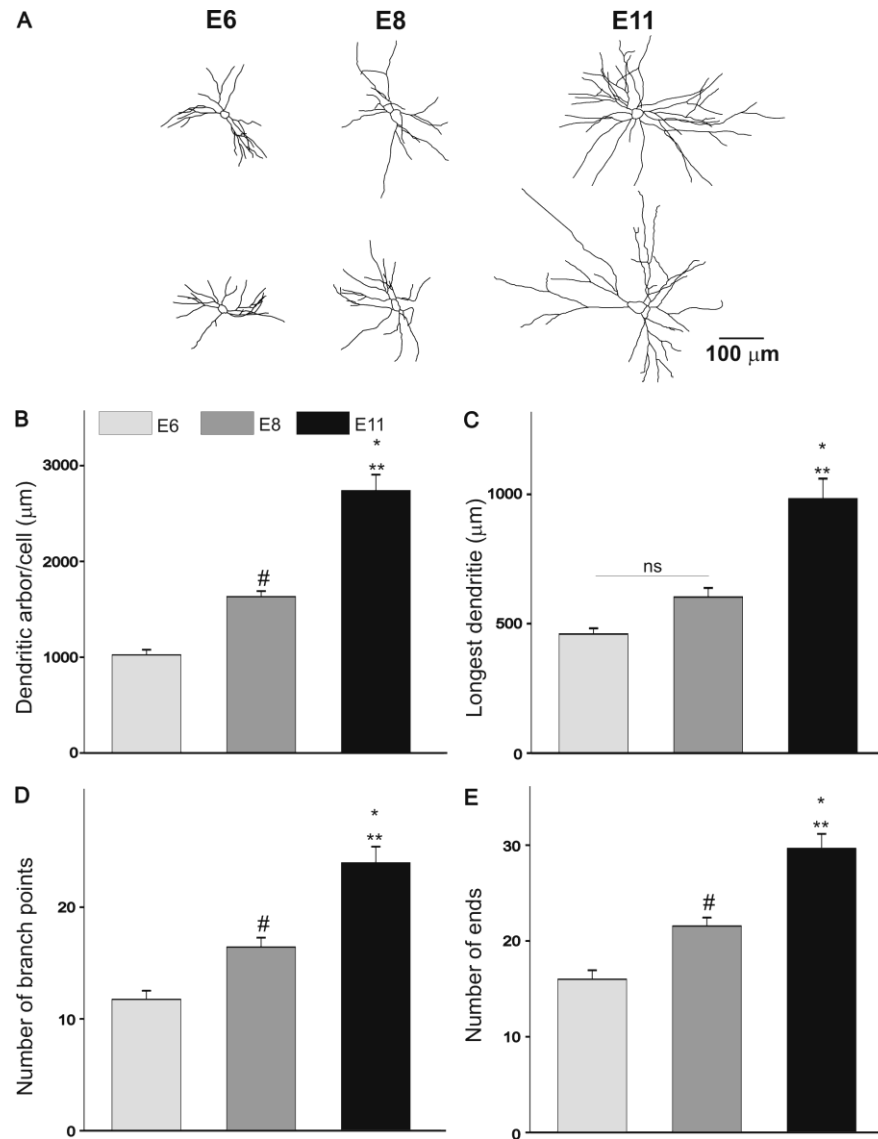


Figure 4.2. Developmental changes in dendritic morphology of lumbar motoneurons between E6 and E11. **A)** Neurolucida drawing of DiI-labeled lumbar motoneurons showing the typical dendritic structure of lumbar motoneurons between E6 and E11. Notice significant increase in dendritic architecture between E6 and E11. **B-E)** Overall changes in dendritic arbor/cell (**B**), length of the longest dendrite (**C**), number of branch points (**D**) and number of dendritic ends (**E**) of motoneurons between E6

and E11. Notice significant increase in dendritic arbor/cell, length of the longest dendrite, number of branch points and number of ends between E6 and E11 motoneurons (E6, n=29, E8, n=33, E11 n=45; # $p < 0.05$ vs. E6; * $p < 0.01$ vs. E6; ** $p < 0.01$ vs. E8; ns= no significant difference).

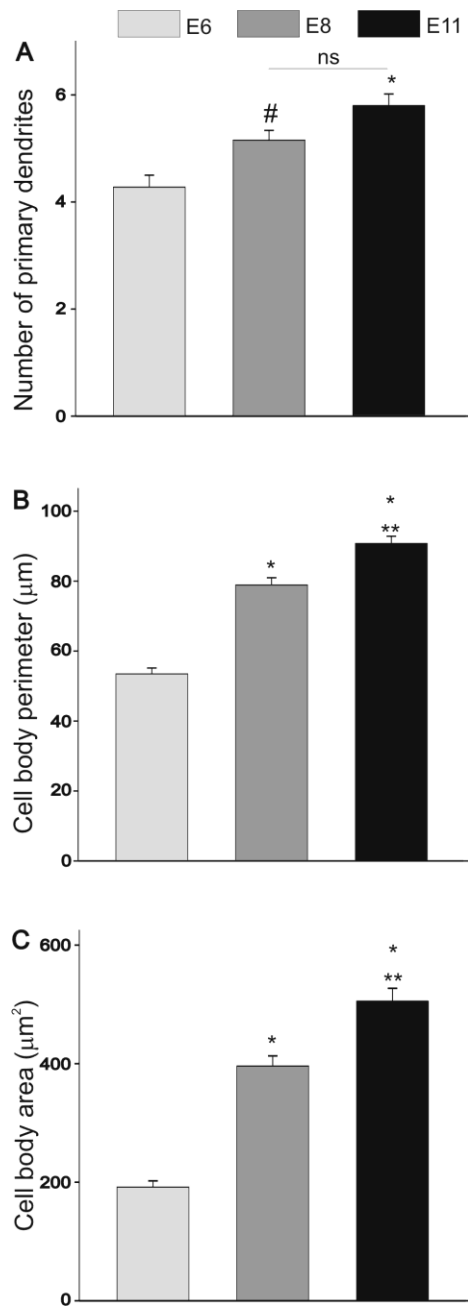


Figure 4.3. Age-dependent changes in cell body morphology between E6 and E11 in DiI-labeled lumbar motoneurons. Notice a significant increase in the number of primary dendrites between E6 and E8, whereas the number of primary dendrites was

unchanged between E8 and E11 (**A**). Maturation of lumbar motoneurons is accompanied by a significant increase in cell body perimeter (**B**) and somatic surface area (**C**) (E6, n=29, E8, n=33, E11 n=45; # $p < 0.05$ vs. E6; * $p < 0.01$ vs. E6; ** $p < 0.01$ vs. E8; ns=no significant difference).

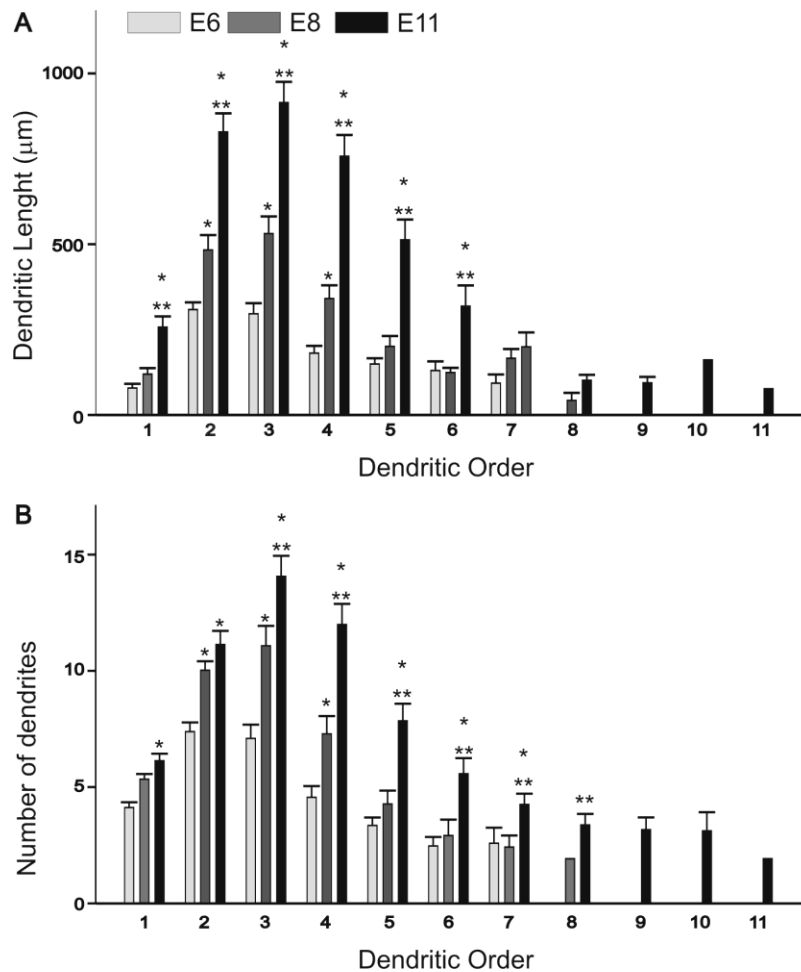


Figure 4.4. Analysis of dendritic length (**A**) and number of dendritic segments (**B**) according to dendritic order in E6, E8 and E11 motoneurons. At E6, dendrites extend until the 7th dendritic order, whereas at E8 they extend until the 8th dendritic order with significant differences only in dendritic length and number of dendrites between the 2nd and 4th dendritic orders. Between E8 and E11 there is a significant increase in the length of each dendritic segment within each dendritic order, whereas there is only a significant increase in the number of dendrites in 3rd or higher order dendrites (E6, n= 29; E8, n=30, E11 n=43; *p<0.05 vs. E6, **p<0.05 vs. E8).

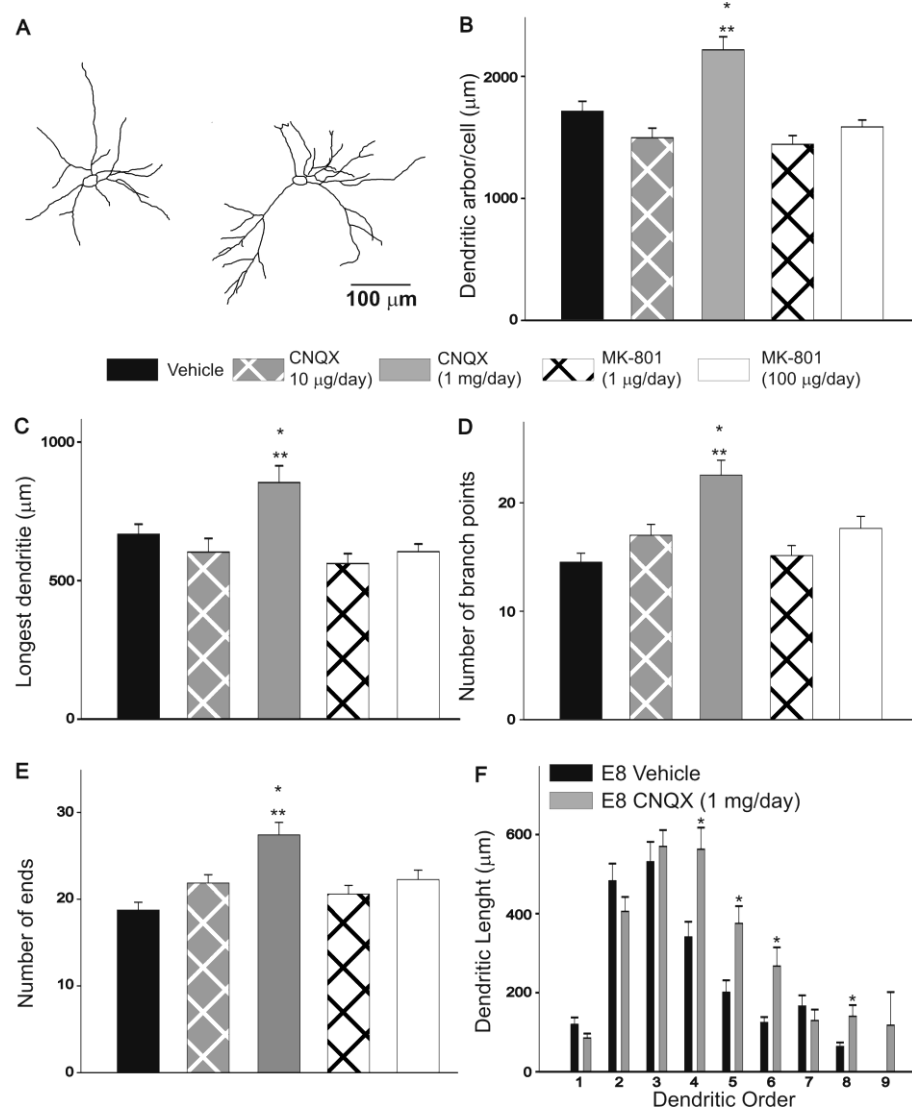


Figure 4.5. Effect of CNQX or MK-801 application to chicken embryos between E5 and E8. **A)** Neurolucida drawing of DiI-labeled E8 lumbar motoneurons showing the typical dendritic structure of a vehicle-treated (left) and a CNQX-treated motoneuron (right). **B-E)** CNQX but not MK-801 treatment of chicken embryos between E5 and E8 causes a significant increase in dendritic complexity as indicated by changes in the

extent of the dendritic arbor/cell (**B**), the length of the longest dendrite (**C**), number of branches (**D**) and number of dendritic ends (**E**). **F**) Comparison of dendritic morphology as a function of dendritic order in E8 embryos treated with CNQX between E5 and E8. Application of CNQX between E5 and E8 causes a significant increase in higher order (≥ 4) dendritic length. * $p < 0.01$ vs. vehicle; ** $p < 0.01$ vs. CNQX 10 $\mu\text{g}/\text{day}$; ns=not significant. Vehicle, $n=35$; CNQX 10 $\mu\text{g}/\text{day}$, $n=24$; CNQX 1 mg/day , $n=33$; MK-801 1 $\mu\text{g}/\text{day}$, $n=19$; MK-801 100 $\mu\text{g}/\text{day}$, $n=46$.

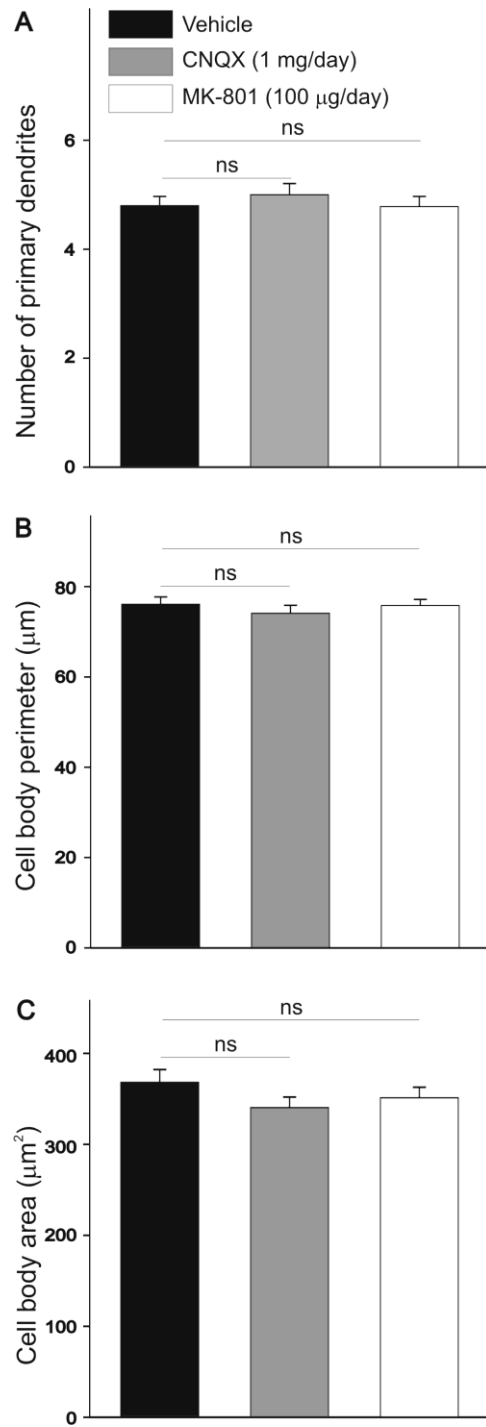


Figure 4.6. Effect of CNQX or MK-801 on cell body morphology in motoneurons treated with CNQX or MK-801 between E5 and E8 (vehicle,

n=35; CNQX, n=33; MK-801, n=46; ns=not significant.). Treatment of lumbar motoneurons with CNQX or MK-801 between E5 and E8 had no noticeable effect on the number of primary dendrites (**A**), cell body perimeter (**B**) and somatic surface area (**C**).

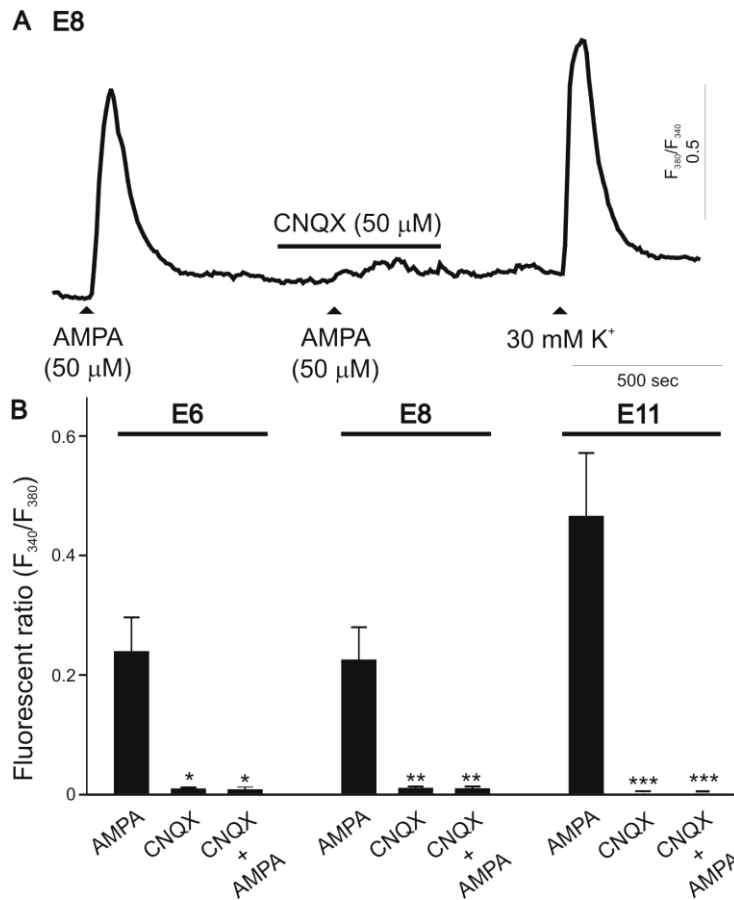


Figure 4.7. Changes in the Ca²⁺ response of acutely isolated motoneurons following stimulation with AMPA or CNQX. **A)** Typical Ca²⁺ responses recorded in an E8 motoneuron following stimulation with AMPA (50 μM) or CNQX (50 μM). Control Ca²⁺ signals generated by activation of voltage-gated Ca²⁺ channels were induced with 30 mM extracellular K⁺. Recordings were performed in normal saline solution without any blockers of voltage-gated channels. **B)** Averaged Ca²⁺ signals generated in motoneurons isolated from chicken embryos at E6, E8 and E11 following stimulation with AMPA (50 μM) or CNQX (50 μM). Notice that CNQX application

alone causes no significant elevation of intracellular Ca^{2+} from baseline. At all ages, the AMPA-evoked increase in intracellular Ca^{2+} was inhibited by pre-treatment of motoneurons with CNQX. * $p < 0.05$ vs. AMPA at E6 (n=11); ** $p < 0.05$ vs. AMPA at E8 (n=16); *** $p < 0.05$ vs. AMPA at E11 (n=9).

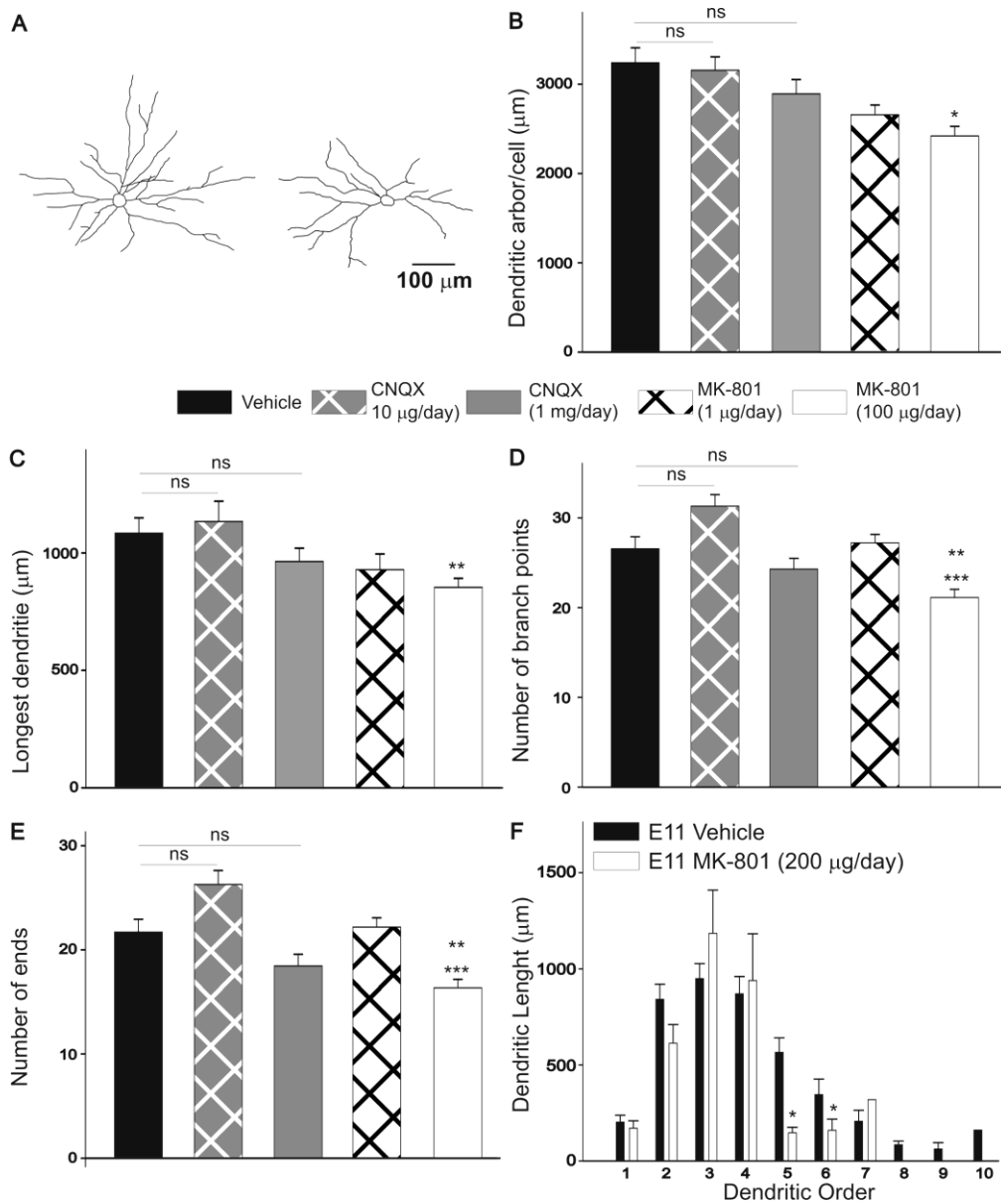


Figure 4.8. Effect of CNQX or MK-801 application to chicken embryos between E8 and E11. **A)** Neurolucida drawing of DiI-labeled E11 lumbar motoneurons showing the typical dendritic structure of a vehicle-treated (left) and a MK-801 treated motoneuron (right). **B-E)** MK-01 but not CNQX treatment of chicken embryos between E8 and E11 causes a significant decrease in dendritic complexity. **F)**

Comparison of dendritic morphology as a function of dendritic order in E11 embryos treated with MK0801 between E8 and E11. Application of MK-801 to chicken embryos between E8 and E11 causes a significant reduction of higher order dendrites. * $p < 0.01$ vs. Vehicle; ** $p < 0.05$ vs. Vehicle; ** $p < 0.05$ vs MK-801 1 $\mu\text{g}/\text{day}$; ns=not significant. Vehicle, $n=48$; CNQX 10 $\mu\text{g}/\text{day}$, $n=27$; CNQX 1 mg/day , $n=53$; MK-801 1 $\mu\text{g}/\text{day}$, $n=34$; MK-801 100 $\mu\text{g}/\text{day}$, $n=55$.

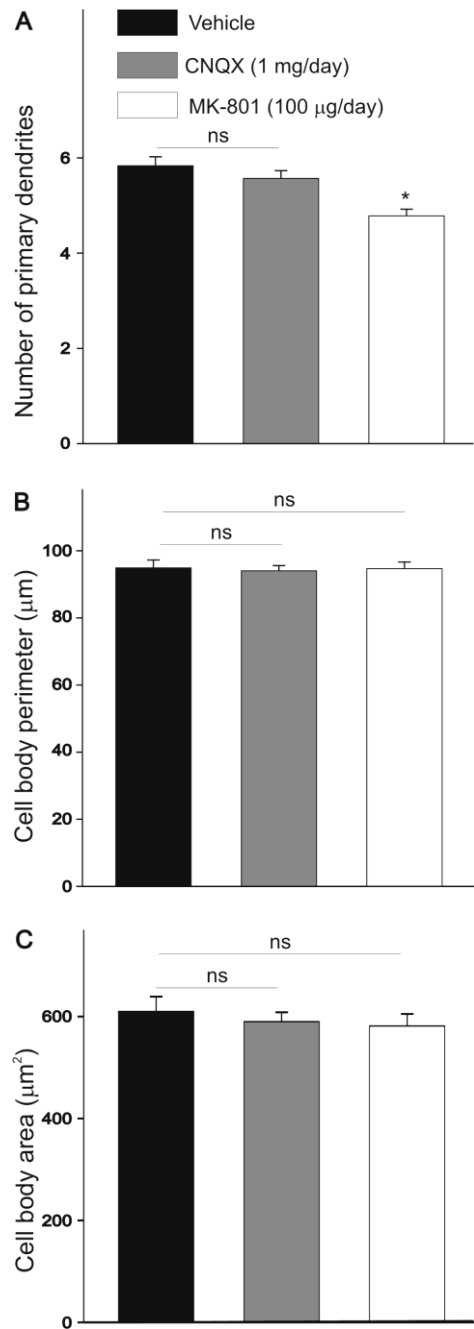


Figure 4.9. Effect of CNQX or MK-801 on cell body morphology in motoneurons treated with CNQX or MK-801 between E8 and E11 (vehicle, n=48; CNQX, n=53; MK-801, n=55). Exposure of chicken embryos to CNQX (1 mg/day) between E8 and E11 had no effect on the number of primary

dendrites or cell body morphology when compared with vehicle treatments (A-C). MK-801 (100 µg/day) treatment caused a significant decrease in the number of primary dendrites (A) without altering cell body morphology (B & C). * $p < 0.01$ vs. Vehicle; ns=not significant.

Chapter 5: General discussion and future aims

1. General discussion

1.1. Expression of AMPA receptors in chick lumbar motoneurons

Our present results demonstrate that AMPA receptors are expressed from E5 to E11 in chick lumbar motoneurons. This suggests that AMPA receptors are expressed at early stages of development in the chicken spinal cord. This is consistent with previous findings showing that motoneurons isolated between E5 and E6 in the chick spinal cord respond to AMPA and kainate receptor activation (O'Brien and Fischbach, 1986). In addition, our data reveal that AMPA receptor activation at E6 allows a significant influx of Ca^{2+} into the cell after the application of kainate. The kainate-evoked inward currents are greatly decreased by philanthotoxin, which blocks Ca^{2+} -permeable AMPA receptors specifically. By contrast, there are no inward currents at E11 in a Na^+ -free, high Ca^{2+} extracellular solution and kainate-generated currents are insensitive to philanthotoxin. These results indicate that AMPA receptors are an important source of Ca^{2+} influx at early developmental stages. This result challenged the earlier notion that NMDA receptors were the only source of Ca^{2+} influx through glutamate receptors. The differences in Ca^{2+} permeability at various stages indicate that there is developmental regulation of Ca^{2+} -permeable AMPA receptors in chick lumbar motoneurons.

The switch in the Ca^{2+} permeability of AMPA receptors is mediated by changes in the expression of the AMPA receptor subunit GluR2 but not by changes in the subunit's editing pattern. It is widely accepted that the insertion of one or more edited GluR2 subunits can determine the Ca^{2+} permeability of AMPA receptors. The presence of at least one edited GluR2 makes AMPA receptors Ca^{2+} -impermeable (Burnashev et al., 1992). A posttranscriptional modification in the pore-forming domain is responsible for the generation of edited GluR2 subunits (Lomeli et al., 1994). Our data support the idea that the presence of edited GluR2 at older developmental stages makes the AMPA receptor Ca^{2+} -impermeable in E11 motoneurons.

1.2. Role of GluR2 and regulation of AMPA receptors properties in chick lumbar motoneurons

Glutamate receptors are expressed at early stages of development. Early expression of the receptors may regulate multiple developmental processes. Our data shows that there is a transitional point for expression of the AMPA GluR2 subunit in chick lumbar motoneurons. At early ages such as E5, E6 and E7, there is no detectable GluR2 in the chicken spinal cord. It isn't until E8 that GluR2 can be detected at significant levels by immunoblot analysis. The expression of GluR2 at this stage coincides with other events that indicate E8 is a transition point for the neurochemistry of spinal networks. Before E8, the generation of spontaneous activity at early stages of development (between E4-E6) relies solely on cholinergic and GABAergic neurotransmission (Milner & Landmesser, 1999). After E8, however,

network activity is driven by glutamate and GABA synaptic transmission (Chub & O'Donovan, 1998). In addition, programmed cell death reaches its peak at E8 in chick lumbar motoneurons. The involvement of edited GluR2 subunits confers Ca^{2+} impermeability to the AMPA receptor. It is likely that the GluR2 expression and subsequent reduction in the receptors' Ca^{2+} permeability limit Ca^{2+} entry into the neurons and prevent cell death by Ca^{2+} overload. Our results are in agreement with other work which shows that the Ca^{2+} permeability of AMPA receptors is dramatically reduced following insertion of edited GluR2 subunits (Geiger et al., 1995; Burnashev et al., 1992; Hollmann et al., 1991).

Another important property of Ca^{2+} -permeable AMPA receptors is their inward rectification, caused by endogenous polyamines blocking the outward flow of ions (Bowie and Myer, 1995; Donevan and Rogawski, 1995). Our results revealed that there is no inward rectification in Ca^{2+} -permeable AMPA receptors at E6 even with addition of high concentrations of spermine to the pipette solution. This result contradicts other findings, suggesting that the Ca^{2+} permeability and inward rectification of AMPA receptors are not intrinsically correlated in chick spinal motoneurons.

1.3. Posttranslational modification of GluR2-containing AMPA receptor in chick spinal motoneurons

GluR2 subunits may exist in two forms, edited and unedited. The expression of the edited form of GluR2 is determined by a posttranscriptional modification in the pore-forming domain. The replacement of glutamine (Q) with arginine (R) shapes the

Ca²⁺ permeability of the AMPA receptor. Arginine is positively charged and it repels Ca²⁺ from the channel's pore, which results in Ca²⁺-impermeable AMPA receptors (Carlson et al., 2000). We examined the extent of Q/R editing in chick ventral spinal cord between E6 and E11. Our data shows that almost 100% of GluR2 mRNA is edited in chick spinal cords from E6 to E11. This result is similar to the extent of GluR2 editing that has been found in rat spinal motoneurons (Greig et al., 2000). In general, the Ca²⁺ permeability of AMPA receptors can be regulated by changes in the expression level of GluR2, the levels at which edited GluR2 subunits are inserted in the receptor, or by changes in the level of editing of these subunits. Our present results demonstrate no changes in the level of editing in GluR2 subunits. Thus, our data indicates that the Ca²⁺ permeability of AMPA receptors in chick motoneurons is only related to the amount of edited GluR2 subunits. The levels at which edited GluR2 are expressed is altered during the developmental stages of chick spinal neurons according to our findings.

Changes in the GluR2 editing pattern or the levels of GluR2 expression are two ways of regulating the Ca²⁺ permeability of AMPA receptors in some disease states. A significant reduction of GluR2 subunits is observed in rat spinal motoneurons following ventral root avulsion (Nagano et al., 2003). The low expression of GluR2 AMPA receptors makes motoneurons vulnerable to calcium toxicity, which results in motoneuron injury in amyotrophic lateral sclerosis (ALS) (Bosch et al., 2000; Shaw and Eggett, 2000; Young et al., 2007). These results indicate that the GluR2 subunit contributes to the neurodegenerative process in some diseases involved in motoneuron loss. Although our data shows that the

posttranslational modification of GluR2 subunits at the Q/R site is not developmentally regulated in chicken motoneurons, this modification of the AMPA receptor subunit may be altered by various pathological states. Changes in the level of editing of GluR2 subunits may regulate the Ca^{2+} permeability of AMPA receptors in certain diseases. For example, the editing efficiency is only 56% in amyotrophic lateral sclerosis (ALS), which results in increased Ca^{2+} permeability of AMPA receptors and motoneuron loss (Kwak and Kawahara, 2005). Overall, editing of AMPA receptor subunits plays a crucial role in regulating the Ca^{2+} permeability of AMPA receptors and promotes motoneuronal survival.

1.4. Composition of AMPA receptors during development of spinal motoneurons

Previous results demonstrate that AMPA receptors consist of combinations of different subunits. For example, GluR2 coexists with GluR1 or GluR3 in most AMPA receptors in the rat brain. However, GluR1 and GluR3 are less likely to form heteromeric AMPA receptors (Ritter et al., 2002). The composition of the AMPA receptors can change during early embryonic development. Qualitative and quantitative changes in AMPA receptor subunit expression have a great influence on the activity-dependent development of motoneurons in spinal cords (Jakowec et al., 1995). Our results demonstrate that AMPA receptors mainly consist of GluR3 and GluR4 subunits at E6. GluR4 is the most abundant subunit throughout all developmental stages. A similar result is also observed in the adult rat spinal cord where GluR3 and GluR4 proteins are abundantly expressed (Polgar et al., 2008). This is also consistent with findings in the adult rat ganglia where GluR3 and GluR4 are

the dominant subunits (Jakowec et al., 1998). Our data demonstrate that GluR2 subunits are present at E8 and E11 in the chicken spinal cord. This suggests that GluR2 is regulated in an age-dependent manner. GluR1 protein is absent at all the ages investigated in the chick ventral spinal cord and GluR1 mRNA is expressed at very low levels in chick ventral spinal cords at both E6 and E11. In contrast to our findings, it has been shown that GluR1 is expressed in cultured rat motoneurons (O'Brien et al., 1997). It is likely that species differences and experimental conditions can account for the diverse expression of AMPA receptor subunits during the development of spinal motoneurons.

1.5. Role of AMPA receptor in dendritic outgrowth in chick spinal neurons

Between E4 and E11, lumbar motoneurons undergo significant changes in their electrophysiological properties (McCobb et al., 1989, 1990; Martin-Caraballo & Dryer, 2002). There are also changes in the development of sensorimotor synaptic connections between motoneuron and the sensory afferents during this developmental period (Lee et al., 1988). Our studies have demonstrated that there is a substantial change in the dendritic morphology of motoneurons as the chicken spinal cord develops during this critical period of time. We have also observed an increased dendritic complexity at E11 in chick spinal motoneurons compared to E6. There are greater numbers of branch nodes and dendritic ends at E11. In addition, the total dendritic length is increased significantly in E11 motoneurons.

Although AMPA and NMDA receptors are functionally expressed as early as

E4.5, glutamatergic neurotransmission is not required for the early generation of spontaneous activity (prior to E8) in the chicken spinal cord (Milner & Landmesser, 1999; Ni et al., 2007). However, it appears that glutamatergic neurotransmission in the chicken spinal cord plays a significant role in regulating early dendritic outgrowth. Activation of Ca^{2+} -permeable AMPA receptors results in the downregulation of dendritic outgrowth (but not axonal growth) of rat spinal motoneurons in culture (Metzger et al., 1998). This is caused by increased levels of intracellular Ca^{2+} ions which act as stop signals for growing dendrites. Excessive intracellular $[\text{Ca}^{2+}]$ prevents synapse formation in rat hippocampal neurons (Lohmann et al., 2005). Our data indicate that CNQX-mediated inhibition of AMPA/kainate receptor function between E5 and E8 causes a significant increase in dendritic length and complexity. This suggests that AMPA receptor activation limits dendritic outgrowth at early developmental stages in embryonic lumbar motoneurons. However, NMDA receptor function does not have any effect on dendritic morphology during this period, which implies that AMPA receptor function is a critical factor controlling dendritic outgrowth during a critical period of development. The ability of AMPA receptor activation to limit dendritic growth could be mediated by the high Ca^{2+} permeability of these receptors prior to E8. On the other hand, our data demonstrate that when AMPA receptors become Ca^{2+} -impermeable, inhibition of AMPA receptor from E8 to E11 does not alter the dendritic outgrowth in spinal motoneurons. Surprisingly, inhibition of NMDA receptors with MK-801 causes a significant decrease in dendritic outgrowth after E8, when AMPA receptors are mostly Ca^{2+} -impermeable. The inhibitory effect of NMDA receptors is reflected in

significant reductions in dendritic branching and total dendritic length. These data indicate that NMDA receptor activation promotes dendritic outgrowth when motoneurons are establishing appropriate synaptic connections with sensory neurons. Similar results were found in postnatal rat motoneurons where blockade of NMDA receptor function *in vivo* evokes a significant reduction in dendritic length and arborization (Kalb, 1994; Inglis et al., 1998). These findings indicate that AMPA and NMDA regulate dendritic outgrowth and dendritic morphology at different developmental stages.

2. Conclusions

Our data demonstrate that the period from E5-E11 is very important for the development of chicken lumbar motoneurons. AMPA receptors are functionally expressed as early as E5 and they undergo considerable changes during this critical period. There is a significant decrease in the Ca^{2+} permeability of AMPA receptors in chicken lumbar motoneurons between E6 and E11. Our results indicate that E8 is a transitional stage during the chicken spinal cord development where the GluR2 subunit appears and AMPA receptors become Ca^{2+} -impermeable. Changes in the Ca^{2+} permeability of AMPA receptors are not mediated by age-dependent changes in the editing pattern of the GluR2 subunit. Instead, changes in the Ca^{2+} permeability of AMPA receptors are regulated by increased expression of GluR2 mRNA and protein in the motoneuron pool. Unlike GluR2 subunit expression, GluR3 and GluR4 expression is not regulated in an age-dependent manner. GluR3 and GluR4 are highly expressed in chicken lumbar motoneurons throughout the ages investigated. Our other major finding is that activation of Ca^{2+} -permeable AMPA receptors downregulates dendritic outgrowth in spinal motoneurons while NMDA receptor activation promotes dendritic outgrowth when motoneurons are establishing appropriate synaptic connections with sensory neurons.

3. Future Aims

3.1. Effect of disrupting GluR2 expression on the maturation of dendritic morphology

Our data have demonstrated that Ca²⁺-permeable AMPA receptors are expressed at early stages of development. They are not responsible for the generation of early network activity. They may be involved in other developmental processes such as programmed cell death and dendritic outgrowth. Our pharmacological inhibition of Ca²⁺-permeable AMPA receptors increases neurite outgrowth, whereas blockade of Ca²⁺-impermeable AMPA receptors does not affect dendritic outgrowth. However, CNQX not only blocks the Ca²⁺ permeability of AMPA receptors but also inhibits glutamate synaptic transmission. An alternative approach to investigate whether changes in the Ca²⁺ permeability of AMPA receptors regulate dendritic outgrowth might be necessary. One option is to interfere with GluR2 expression. As a result, glutamate synaptic transmission and activity will not be affected. Chick embryos will be infected with a RCASBP virus expressing an RNAi against GluR2 mRNA. The vectors will also express an RFP marker, which allows infected neurons to be tracked in vivo (Das et al., 2006). The extent of dendritic outgrowth should be determined at E8 and E11 in control embryos and embryos treated with the RNAi against GluR2.

3.2. Molecular mechanisms regulating neurite growth by AMPA and NMDA receptors

Glutamate receptors are involved in the regulation of dendritic morphology in neurons. Our data show that inhibition of Ca²⁺-permeable AMPA receptors promotes dendritic outgrowth in chicken spinal motoneurons. By contrast, inhibition of NMDA receptors attenuates dendritic growth. The Ca²⁺ ions flowing through AMPA and NMDA receptors mediate the dendritic growth and branching. This is consistent with previous findings, which indicate that activity-dependent calcium signaling plays a role in regulating dendritic growth (Konur and Ghosh, 2005). Ca²⁺/calmodulin-dependent protein kinases (CaMKs) and mitogen-activated protein kinases (MAPKs) have been proposed as the key mediators of calcium-dependent neurite outgrowth (Redmond et al., 2002). Increased CaMKs activity results in a decrease in new branch additions (Cline, 2001). The nuclear target of CaMKs is cyclic-AMP-responsive-element-binding protein (CREB), which is a transcription factor. CREB targets the neurotrophin BDNF (Wong and Ghosh, 2002). BDNF is a key mediator involved in neurite growth initiated by AMPA receptors (Voss et al., 2007). It has been demonstrated that BDNF is involved in regulating dendritic growth in cortical and cerebellar neurons (McAllister et al., 1995; Schwartz et al., 1997). Since our data demonstrates that both AMPA and NMDA receptors play roles in the dendritic outgrowth of chick LMNs at distinct stages, it is of interest to know the signaling pathways involved in the regulation of dendritic outgrowth in chicken spinal neurons.

3.3. Structural exploration of AMPA receptor

In addition to the four subunits, AMPA receptors interact with other proteins which confer onto them diverse properties. It is important to explore the expression

pattern of those proteins so that we have better understanding of AMPA receptor function during development. Several auxiliary subunits regulate trafficking and expression of AMPA receptors. Stargazin is a member of the transmembrane AMPA receptor regulatory proteins (TARPs) which are AMPA receptor auxiliary subunits (Milstein and Nicoll, 2008; Menuz et al., 2007). TARPs appear with AMPA receptors early in the synthetic pathway and regulate their maturation and trafficking. TARPs play a critical role in interacting with PSD-95 to promote the synaptic targeting of AMPA receptors. Stargazin increases the expression of AMPA receptors at the cell surface (Bats et al., 2007). The strength and timing of synaptic transmission are determined by the number of AMPA receptors clustered at the synapse. Thus, it is crucial to find out the factors that regulate the gating and trafficking of AMPA receptors. This will facilitate the design of corresponding agonists or antagonists, which bind the exact sites of AMPA receptors. Eventually, this will be beneficial in treating AMPA-receptor-mediated neurodegenerative disorders.

3.4. Mechanisms involved in AMPA receptor mediated neurodegenerative disorders

Overactivation of AMPA receptors is involved in some neurodegenerative diseases, such as Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis (ALS) (Jayakar and Dikshit, 2004). Therefore, it is important to explore the mechanisms regulating AMPA receptors in order to reduce excitotoxicity in neurons. There are conflicting arguments regarding whether or not the GluR2 subunits are responsible for motoneuron degeneration in ALS. Calcium homeostasis is regulated

by multiple cellular factors. The factors causing ALS are yet to be determined (Laslo et al., 2001). There is growing evidence showing that Ca^{2+} -permeable AMPA receptors may be implicated in motoneuron degeneration (Shaw and Eggett, 2000). $[\text{Ca}^{2+}]$ is considered to be an important factor mediating the disorder, but not the only one. Other factors, including neurofilaments and cytoplasmic dynein, contribute to motoneuron diseases (Bruijn et al., 2004). Therefore, it is of interest to investigate these other factors implicated in motor neuron degenerative disorders.

REFERENCES

- Bats C, Groc L, Choquet D (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53:719-734.
- Bosch LVD, Vandenberghe W, Klaassen H, Houtte EV, Robberecht W (2000) Ca²⁺-permeable AMPA receptors and selective vulnerability of motor neurons. *Journal of the Neurological Sciences* 180:29-34.
- Bowie D, Mayer ML (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* 15:453-462.
- Brujin LI, Miller TM, Cleveland DW (2004) Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annual Review of Neuroscience* 27:723-749.
- Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 8:189-198.
- Burnashev N, Schoepfer R, Monyer H, Ruppersberg JP, Gunther W, Seeburg PH, Sakmann B (1992) Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. *Science* 257:1415-1419.
- Carlson NG, Howard J, Gahring LC, Rogers SW (2000) RNA editing (Q/R site) and flop/flip splicing of AMPA receptor transcript in young and old brains. *Neurobiol aging* 21:599-606.
- Chub N, O'Donovan MJ (1998) Blockade and recovery of spontaneous rhythmic activity after application of neurotransmitter antagonists to spinal networks of the chicken embryo. *J Neurosci.* 18(1): 294-306.
- Cline HT (2001) Dendritic arbor development and synaptogenesis. *Current Opinion in Neurobiology* 11:118-126.
- Dai WM, Egebjerg J, Lambert JD (2001) Characteristics of AMPA receptor-mediated responses of cultured cortical and spinal cord neurones and their correlation to the expression of glutamate receptor subunits, GluR1-4. *Br J Pharmacol* 132(8):1859-1875.
- Das RM, Hateren NJV, Howell GR, Farrell ER, Bangs FK, Porteous VC, Manning EM, McGrew MJ, Ohyama K, Sacco MA, Halley PA, Sang HM, Storey KG, Placzek M, Cheryll T, Nair VK, Wilson SA (2006) A robust system for RNA

- interference in the chicken using a modified microRNA operon. *Developmental Biology*. 294: 554-563.
- Donevan SD, Rogawski MA (1995) Intracellular polyamines mediate inward rectification of Ca²⁺-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Proc. Natl. Acad. Sci. USA* 92:9298-9302.
- Furuyama T, Kiyama H, Sato K, Park HT, Maeno H, Takagi H, Tohyama M(1993) Region-specific expression of subunits of subunits of ionotropic glutamate receptors (AMPA-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociception. *Brain Res Mol Brain Res* 18:141-151.
- Geiger JR, Melcher T, Koh DS, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca²⁺ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron* 15:193-204.
- Greig A, Donevan SD, Mujtaba TJ, Parks TN, Tao MS (2000) Characterization of the AMPA-activated receptors present on motoneurons. *Journal of Neurochem* 74:179-191.
- Hollmann M, Hartley M, Heinemann S (1991) Ca²⁺ permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 252:851-853.
- Inglis FM, Furia F, Zuckerman KE, Strittmatter SM, Kalb RG.(1998) The role of nitric oxide and NMDA receptors in the development of motor neuron dendrites. *J Neurosci*. 18(24):10493-501.
- Jakowec MW, Fox AJ, Martin LJ, Kalb RG (1995) Quantitative and qualitative changes in AMPA receptor expression during spinal cord development. *Neuroscience* 67(4):893-907.
- Jakowec MW, Jackson-Lewis V, Chen X, Langston JW, Przedborski S (1998) The postnatal developmental of AMPA receptor subunits in the basal ganglia of the rat. *Developmental Neuroscience* 20(1):19-33.
- Jayakar SS, Dikshit M (2004) AMPA receptor regulation mechanisms: future target for safer neuroprotective drugs. *Int J Neurosci* 114:695-734.
- Kalb RG (1994) Regulation of motor neuron dendrite growth by NMDA receptor activation. *Development*. 120(11):3063-71.
- Konur S, Ghosh A (2005) Calcium signaling and the control of dendritic development. *Neuron* 46:401-405.

- Kwak S, Kawahara Y (2005) Deficient RNA editing of GluR2 and neuronal death in amyotrophic lateral sclerosis. *J Mol Med* 83:110-120.
- Laslo P, Lipski J, Nicholson LFB, Miles GB, Funk GD (2001) GluR2 AMPA receptor subunit expression in motoneurons at low and high risk for degeneration in amyotrophic lateral sclerosis. *Experimental Neurology* 169:461-471.
- Lee MT, Koebbe MJ, O'Donovan MJ (1988) The development of sensorimotor synaptic connections in the lumbosacral cord of the chicken embryo. *J Neurosci.* 8(7): 2530-2543.
- Lohmann C, Finski A, Bonhoeffer T (2005) Local calcium transients regulate the spontaneous motility of dendritic filopodia. *Nat Neurosci.* 8(3): 305-312.
- Lomeli H, Mosbacher J, Melcher T, Hoyer T, Geiger JR, Kuner T, Monyer H, et al. (1994) Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266:1709-1713.
- Martin-Caraballo M, Dryer, SE (2002) Activity- and target-dependent regulation of large-conductance Ca^{2+} -activated K^{+} channels in developing chicken lumbar motoneurons. *J Neurosci.* 22(1): 73-81.
- McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15:791-803.
- McCobb DP, Best PM, Beam KG (1990) The differentiation of excitability in embryonic chicken limb motoneurons. *J Neurosci* 10:2974-2984.
- McCobb DP, Best PM, Beam KG (1989) Development alters the expression of calcium currents in chicken limb motoneurons. *Neuron* 2:1633-1643.
- Menuz K, Stroud RM, Nicoll RA, Hays FA (2007) TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists. *Science* 318:815-817.
- Metzger F, Wiese S, Sendtner M (1998) Effect of glutamate on dendritic growth in embryonic rat motoneurons. *J Neurosci.* 18(5): 1735-1742.
- Milner LD, Landmesser LT (1999) Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J Neurosci.* 19(8): 3007-3022.
- Milstein AD, Nicoll R (2008) Regulation of AMPA receptor gating and pharmacology by TARP auxiliary subunits. *Cell* 29(7):333-339.

- Nagano I, Murakami T, Shiote M, Abe K, Itoyama Y (2003) Ventral root avulsion leads to downregulation of GluR2 subunit in spinal motoneurons in adult rats. *Neuroscience* 117:139-146.
- Ni X, Sullivan GJ, Martin-Caraballo M (2007) Developmental characteristics of AMPA receptors in chick lumbar motoneurons. *Dev Neurobiol.* 67(11):1419-32.
- O'Brien R, Fischbach GD (1986) Characterization of excitatory amino acid receptors expressed by embryonic chick motoneurons in vitro. *The Journal of Neuroscience* 6(11):3275-3282.
- O'Brien RJ, Mammen AL et al. (1997) The development of excitatory synapses in cultures spinal neurons. *The Journal of Neuroscience* 17(19):7339-7350.
- Polgar E, Al-Khater KM, Shehab S, Watanabe M, Todd AJ (2008) Large projection neurons in laminal I of the rat spinal cord that lack the neurokinin 1 receptor are densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors. *The Journal of Neuroscience* 28(49):13150-13160.
- Redmond L, Kashani AH, Ghosh A (2002) Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron* 34:999-1010.
- Ritter LM, Vazquez DM, Meador-Woodruff JH(2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. *Developmental Brain Research* 139:227-236.
- Schwartz PM, Borghesani PR, Levy RL, Pomeroy SL, Segal RA (1997) Abnormal cerebellar development and foliation in BDNF^{-/-} mice reveals a role for neurotrophins in CNS patterning. *Neuron* 19:269-281.
- Shaw PJ, Eggett CJ (2000) Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. *J Neurol* 247(Suppl 1):I/17-I/27.
- Vandenberghe W, Robberecht W, Brorson JR (2000) AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability. *The Journal of Neuroscience* 20(1):123-132.
- Voss OP, Milne S, Sharkey J, O'Neill MJ, McCulloch J (2007) Molecular mechanisms of neurite growth with AMPA receptor potentiation. *Neuropharmacology* 52:590-597.

Williams TL, Day NC, Ince PG, Kamboj RK, Shaw PJ (1991) Calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors: a molecular determinant of selective vulnerability in amyotrophic lateral sclerosis. *Ann Neurol* 42(2):200-207.

Wong ROL, Ghosh A (2002) Activity-dependent regulation of dendritic growth and patterning. *Nature Review Neuroscience* 3(10):803-812.

Young KC, McGehee DS, Brorson JR (2007) Glutamate receptor expression and chronic glutamate toxicity in rat motor cortex. *Neurobiol Dis* 26(1):78-85.

COMPREHENSIVE BIBLIOGRAPHY

- Ammori JB, Zhang W, Newman EA, Mulholland MW (2007) Glutamate-induced calcium transients in rat neurons of the dorsal motor nucleus of the vagus. *Journal of Gastrointestinal Surgery* 11(8):1016-1024.
- Armstrong N, Gouaux E (2000) Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structure of the GluR2 ligand binding core. *Neuron* 28:165-181.
- Armstrong N, Sun Y, Chen G-Q, Gouaux E (1998) Structure of a glutamate-receptor ligand-binding core in complex with kainite. *Nature* 395:913-917.
- Arvanov VL, Seebach BS, Mendell LM (2000) NT-3 evokes an LTP-like facilitation of AMPA/kainite receptor-mediated synaptic transmission in the neonatal rat spinal cord. *Journal of Neurophysiology* 84(2):752-758.
- Ayalon G, Stern-Bach Y (2001) Functional assembly of AMPA and kainite receptors is mediated by several discrete protein-protein interactions. *Neuron* 31:103-113.
- Banke TG, Bowie D, Lee HK, Huganir RL, Schousboe A, Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. *Journal of Neuroscience* 20(1):89-102.
- Bats C, Groc L, Choquet D (2007) The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53:719-734.
- Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, Zastrow MV, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nature Neuroscience* 3(12):1291-1300.
- Bekoff A (1976) Ontogeny of leg motor output in the chick embryo: a neural analysis. *Brain Res* 106:271-291.
- Bettler B, Boulter J, Hermans-Borgmeyer I, O'Shea-Greenfield A, Deneris ES, Moll C, Borgmeyer U, Hollmann M, Heinemann S (1990) Cloning of a novel glutamate receptor subunit, GluR5-expression in the nervous-system during development. *Neuron* 5(5):583-595.
- Blaschke M, Keller BU, Rivosecchi R, Hollmann M, Heinemann S, Konnerth A (1993) Single amino acid determines the subunit-specific spider toxin block of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor channels. *Proc. Natl. Acad. Sci. USA* 90(14):6528-6532.

- Bochet P, Audinat E, Lambolez B, Crepel F, Rossier J, Iino M, Tsuzuki K, Ozawa S (1994) Subunit composition at the single-level explains functional properties of a glutamate-gated channel. *Neuron* 12:383-388.
- Bosch LVD, Vandenberghe W, Klaassen H, Houtte EV, Robberecht W (2000) Ca²⁺-permeable AMPA receptors and selective vulnerability of motor neurons. *Journal of the Neurological Sciences* 180:29-34.
- Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* 249:1033-1037.
- Bowie D, Mayer ML (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* 15:453-462.
- Brene S, Messer C, Okado H, Hartley M, Heinemann SF, Nestler EJ (2000) Regulation of GluR2 promoter activity by neurotrophic factors via a neuron-restriction silence element. *European Journal of Neuroscience* 12(5):1525-1533.
- Brickley SG, Farrant M, Swanson GT, Cull-Candy SG (2001) CNQX increases GABA-mediated synaptic transmission in the cerebellum by an AMPA/kainate receptor-independent mechanism. *Neuropharmacology* 41(6):730-6.
- Brill J, Huguenard JR (2008) Sequential changes in AMPA receptor targeting in the developing neocortical excitatory circuit. *The Journal of Neuroscience* 28(51):13918-13928.
- Brorson JR, Zhang Z, Vandenberghe W (1999) Ca²⁺ permeation of AMPA receptors in cerebellar neurons expressing glu receptor 2. *J Neurosci*. 19(21):9149-9159.
- Bruce M, Eric F (1991) Specific monosynaptic sensory-motor connections form in the absence of patterned neural activity and motoneuronal cell death. *Journal of Neuroscience* 11(5):1390-1403.
- Bruijn LI, Miller TM, Cleveland DW (2004) Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annual Review of Neuroscience* 27:723-749.
- Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single

subunit. *Neuron* 8:189-198.

- Burnashev N, Schoepfer R, Monyer H, Ruppersberg JP, Gunther W, Seeburg PH, Sakmann B (1992) Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. *Science* 257:1415-1419.
- Caicedo A, Kungel M, Pujol R, Friauf E (1998) Glutamate-induced Co^{2+} uptake in rat auditory brainstem neurons reveals developmental changes in Ca^{2+} permeability of glutamate receptors. *European Journal of Neuroscience* 10(3):941-954.
- Calderia MV, Melo CV, Pereira DB, Carvalho R, Correia SS, Backos DS, Carvalho AL, Esteban JA, Duarte CB (2007) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of α -Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. *Journal of Biological Chemistry* 282(17):12619-12628.
- Calderó J, Ciutat D, Lladó J, Castán E, Oppenheim RW, Esquerda JE (1997) Effects of excitatory amino acids on neuromuscular development in the chick embryo. *J Comp Neurol.* 387(1):73-95.
- Caldero J, Prevette D, Mei X, Oakley RA, Li L, Milligan C, Houenou L, Burek M, Oppenheim RW (1998) Peripheral target regulation of the development and survival of spinal sensory and motor neurons in the chick embryo. *J Neurosci.* 18(1):356-370.
- Caldero J, Ciutat D, Llado J, Castan E, Oppenheim RW, Esquerda JE (1997) Effects of excitatory amino acids on neuromuscular development in the chick embryo. *Journal of Comparative Neurology* 387(1):73-95.
- Carlson NG, Howard J, Gahring LC, Rogers SW (2000) RNA editing (Q/R site) and flop/flip splicing of AMPA receptor transcript in young and old brains. *Neurobiol aging* 21:599-606.
- Carvalho AL, Kameyama K, Huganir RL (1999) Characterization of phosphorylation sites on the glutamate receptor 4 subunit of the AMPA receptors. *Journal of Neuroscience* 19(12):4748-4754.
- Catsicas M, Allcorn S, Mobbs P (2001) Early activation of Ca^{2+} -permeable AMPA receptors reduce neurite outgrowth in embryonic chick retinal neurons. *Journal of Neurobiology* 49(3):200-211.
- Chan YS, Chen LW, Lai CH, Shum DKY, Yung KKL, Zhang FX (2003) Receptors of glutamate and neurotrophin in vestibular neuronal functions. *Journal of Biomedical Science* 10(6):577-587.

- Cheng B, Furukawa K, O'Keefe JA, Goodman Y, Kihiko M, Fabian T, Mattson MP (1995) Basic fibroblast growth-factor selectively increases AMPA-receptor subunit GluR1 protein level and differentially modulates Ca²⁺ response to AMPA and NMDA in hippocampal-neurons. *Journal of Neurochemistry* 65(6):2525-2536.
- Chub N, O'Donovan MJ (1998) Blockade and recovery of spontaneous rhythmic activity after application of neurotransmitter antagonists to spinal networks of the chick embryo. *J Neurosci.* 18(1):294-306.
- Chub N., O'Donovan M.J (2001) Post-episode depression of GABAergic transmission in spinal neurons of the chick embryo. *J Neurophysiol.* 85(5), 2166-76.
- Chu-Wang I-W., Oppenheim R.W (1978) Cell death of motoneurons in the chicken embryo spinal cord. I. A light and electron microscopy study of naturally occurring and induced cell loss during development. *J Comp Neurol* 177, 33-58.
- Chung HJ, Xia J, Scannevin RH, Zhang X, Huganir RL (2000) Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *Journal of Neuroscience* 20(19):7258-7267.
- Cline HT (2001) Dendritic arbor development and synaptogenesis. *Current Opinion in Neurobiology* 11:118-126.
- Cruce WLR (1974) The anatomical organization of hind limb motoneurons in the lumbar spinal cord of the frog *Rana-Catesbeiana*. *Journal of Comparative Neurology* 153(1):59-76.
- Dahm LM, Landmesser LT (1991) The regulation of synaptogenesis during normal development and following activity blockade. *J Neurosci.* 11(1):238-255.
- Das RM, Hateren NJV, Howell GR, Farrell ER, Bangs FK, Porteous VC, Manning EM, McGrew MJ, Ohyama K, Sacco MA, Halley PA, Sang HM, Storey KG, Placzek M, Cheryll T, Nair VK, Wilson SA (2006) A robust system for RNA interference in the chicken using a modified microRNA operon. *Developmental Biology.* 294: 554-563.
- Dingledine R, Hume RI, Heinemann SF (1992) Structural determinants of barium permeation and rectification in non-NMDA glutamate receptor channels. *Journal of Neuroscience* 12:4080-4087.
- Donevan SD, Rogawski MA (1995) Intracellular polyamines mediate inward rectification of Ca²⁺-permeable alpha-amino-3-hydroxy-5-methyl-4-

- isoxazolepropionic acid receptors. *Proc. Natl. Acad. Sci. USA* 92:9298-9302.
- Durand GM, Kovalchuk Y, Konnerth A (1996) Long-term potentiation and function synapse induction in developing hippocampus. *Nature* 381(6577):71-75.
- Eric F, Bruce M (1990) Specification of synaptic connections between sensory and motor neurons in the developing spinal cord. *Journal of Neurobiology* 21(1):33-50.
- Eybalin M, Caicedo A, Renard N, Ruel J, Puel JL (2004) Transient Ca²⁺-permeable AMPA receptors in postnatal rat primary auditory neurons. *European Journal of Neuroscience* 20(11):2981-2989.
- Fakler B, Brandle U, Glowatzki E, Weidemann S, Zenner HP, Ruppersberg JP (1995) Strong voltage-dependent inward rectification of inward rectifier K⁺ channels is caused by intracellular spermine. *Cell* 80(1):149-154.
- Feldmeyer D, Kask K, Brusa R, Kornau HC, Kolhekar R, Rozov A, Burnashev N, Jensen V, Hvalby O, Sprengel R, Seeburg PH (1999) Neurological dysfunctions in mice expression different levels of the Q/R site-unedited AMPAR subunit GluR-B. *Nature Neuroscience* 2(1):57-64.
- Ferguson BA (1983) Development of motor innervation of the chick following dorsal-ventral limb bud rotations. *Journal of Neuroscience* 3(9):1760-1772.
- Fouvet PB (1973) Innervation et morphogenese de la patte chez l'embryon de poulet. I. Mise en place de l'innervation normale. *Arch. Anat Microsc. Morphol. Exp* 62:269-280.
- Friedman LK, Veliskova J (1998) GluR2 hippocampal knockdown reveals developmental regulation of epileptogenicity and neurodegeneration. *Molecular brain research* 61(1-2):224-231.
- Fryer HJ, Knox RJ, Strittmatter SM, Kalb RG (1999) Excitotoxic death of a subset of embryonic rat motor neurons in vitro. *J Neurochem.* 72(2): 500-513.
- Fujiyanma F, Kuramoto E, Okamoto K, Hioki H, Furuta T, Zhou L, Nomura S, Kaneko T (2004) Presynaptic localization of an AMPA-type glutamate receptor in corticostriatal and thalamostriatal axon terminals. *European Journal of Neuroscience* 20(12):3322-3330.
- Furuyama T, Kiyama H, Sato K, Park HT, Maeno H, Takagi H, Tohyama M(1993) Region-specific expression of subunits of subunits of ionotropic glutamate receptors (AMPA-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociception. *Brain Res Mol Brain Res* 18:141-151.

- Gardner SM, Trussell LO, Oertel D (1999) Time course and permeation of synaptic AMPA receptor in cochlear nuclear neurons correlate with input. *Journal of Neuroscience* 19(20):8721-8729.
- Gardner SM, Takamiya K, Xia Jun, Suh J, Johnson R, Yu S, Huganir RL (2005) Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF. *Neuron* 45:903-915.
- Geiger JPR, Melcher T, Koh D-S, Sakmann B, Seeburg PH, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron* 15:193-204.
- Gleason EL, Spitzer NC (1998) AMPA and NMDA receptor expressed by differentiating *Xenopus* spinal neurons. *Journal of Neurophysiology* 79(6):2986-2998.
- Gouaux E (2004) Structure and function of AMPA receptors. *Journal of Physiology* 554:249-253.
- Greger IH, Khatri L, Kong X, Ziff EB (2003) AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* 40(4):763-774.
- Greger IH, Ziff EB, Penn AC (2007) Molecular determinants of AMPA receptor subunit assembly. *TRENDS in Neurosciences* 30(8):407-416.
- Greig A, Donevan SD, Mujtaba TJ, Parks TN, Rao MS (2000) Characterization of the AMPA-activated receptors present on motoneurons. *Journal of Neurochemistry* 74(1):179-191.
- Grossman SD, Wolfe BB, Yasuda RP, Wrathall JR (1999) Alterations in AMPA receptor subunit expression after experimental spinal cord contusion injury. *The Journal of Neuroscience*. 19(14):5711-5720.
- Grunder T, Kohler K, Guenther E (2000) Distribution and developmental regulation of AMPA receptor subunit proteins in rat retina. *Investigative Ophthalmology & Visual Science* 41(11):3600-3606.
- Guthrie S (2002) Neuronal development: sorting out motor neurons. *Current Biology* 12:R488-R490.
- Hall RA, Hansen A, Andersen PH, Soderling TR (1997) Surface expression of the AMPA receptor subunits GluR1, GluR2, and GluR4 in stably transfected baby hamster kidney cells. *J Neurochem*. 68(2):625-630.

- Hamburger V, Balaban M (1963) Observations and experiments on spontaneous rhythmic behavior in the chick embryo. *Dev Biol* 7:533-545.
- Hamburger V (1975) Cell death in the development of the lateral motor column of the chick embryo. *J. Comp. Neurol* 160:535-546.
- Hanson MG, Landmesser LT (2003) Characterization of the circuits that generate spontaneous episodes of activity in the early embryonic mouse spinal cord. *J Neurosci.* 23(2): 587-600.
- Hanson MG, Landmesser LT (2006) Increasing the frequency of spontaneous rhythmic activity disrupts pool-specific axon fasciculation and pathfinding of embryonic spinal motoneurons. *Journal of Neuroscience* 26(49):12769-12780.
- Hanson MG, Milner LD, Landmesser LT (2008) Spontaneous rhythmic activity in early chick spinal cord influences distinct motor axon pathfinding decision. *Brain Research reviews* 57:77-85.
- Hartmann B, Ahmadi S, Heppenstall PA, Lewin GR, Schott C, Borchardt T, Seeburg PH, Zeihofer HU, Sprengel R, Kuner R (2004) The AMPA receptor subunits GluR-A and GluR-B reciprocally modulate spinal synaptic plasticity and inflammatory pain. *Neuron* 44(4):637-650.
- Hayashi Y, Shi SH, Esteban JA, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* 287:2262-2267.
- Herin GA, Aizenman E (2004) Amino terminal domain regulation of NMDA receptor function. *Eur J Pharmacol* 500:101-111.
- Herlitze S, Raditsch M, Ruppertsberg JP, Jahn W, Monyer H, Schoepfer R, Witzemann V. 1993. Argitoxin detects molecular differences in AMPA receptor channels. *Neuron* 10(6):1131-1140.
- Hollmann M, Hartley M, Heinemann S (1991) Ca²⁺ permeability of Ka-AMPA gated glutamate receptor channels depends on subunit composition. *Science* 252(5007):851-853.
- Hollmann M, Heinemann S (1994) Cloned glutamate receptors. *Annual Review of Neuroscience* 17: 31-108.
- Hollyday M, Hamburger V (1976) Reduction of naturally-occurring motor neuron loss by enlargement of the periphery. *J. Comp. Neuro* 170:311-320.

- Hollyday M., Hamburger V (1977) An autoradiographic study of the formation of the lateral motor column in the chicken embryo. *Brain Res.* 132(2), 197-208.
- Hollyday M, Hamburger V (1997) An autoradiographic study of the formation of the lateral motor column in the chick embryo. *Brain Research* 132:197-208.
- Hunter C, Petralia RS, Vu T, Wenthold RJ (1993) Expression of AMPA-selective glutamate receptor subunits in Morphologically defined neurons of the mammalian cochlear nucleus. *Journal of Neuroscience* 13(5):1932-1946.
- Inglis FM, Crockett R, Korada S, Abraham WC, Hollmann M, Kalb RG (2002) The AMPA receptor subunit GluR1 regulates dendritic architecture of motor neurons. *J Neurosci.* 22(18):8042-51.
- Inglis FM, Furia F, Zuckerman KE, Strittmatter SM, Kalb RG (1998) The role of nitric oxide and NMDA receptors in the development of motor neuron dendrites. *J Neurosci.* 18(24):10493-501.
- Jakowec MW, Fox AJ, Martin LJ, Kalb RG (1995) Quantitative and qualitative changes in AMPA receptor expression during spinal cord development. *Neuroscience* 67(4):893-907.
- Jakowec MW, Jackson-Lewis V, Chen X, Langston JW, Przedborski S (1998) The postnatal developmental of AMPA receptor subunits in the basal ganglia of the rat. *Developmental Neuroscience* 20(1):19-33.
- Jakowec MW, Yen L, Kalb RG (1995) In-Situ hybridization analysis of AMPA receptor subunits gene-expression in the developing rat spinal-cord. *Neuroscience* 67(4):909-920.
- Janssens N, Lesage ASJ (2001) Glutamate receptor subunit expression in primary neuronal and secondary glial cultures. *Journal of Neurochemistry* 77(6):1457-1474.
- Jayakar SS, Dikshit M (2004) AMPA receptor regulation mechanisms: future target for safer neuroprotective drugs. *Int J Neurosci* 114:695-734.
- Jeong GB, Werner M, Gazula VR, Itoh T, Roberts M, David S, Pfister B, Cohen A, Neve RL, Hollmann M, Kalb R (2006) Bi-directional control of motor neuron dendrite remodeling by the calcium permeability of AMPA receptors. *Mol Cell Neurosci.* 32(3):299-314.
- Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H (1994) Differences in Ca²⁺

- permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* 12(6): 1281-1289.
- Jonas P, Racca C, Sakmann B, Seeburg PH, Monyer H (1994) Differences in Ca²⁺ permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* 12(6), 1281-1289.
- Kumar SS, Bacci A, Kharazia V, Huguenard JR (2002) A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci.* 22(8), 3005-3015.
- Kalb RG (1994) Regulation of motor neuron dendrite growth by NMDA receptor activation. *Development.* 120(11):3063-71.
- Kalb RG, Fox AJ (1997) Synchronized overproduction of AMPA, kainite, and NMDA glutamate receptors during human spinal cord development. *Journal of Comparative Neurology* 384(2):200-210.
- Kamboj SK, Swanson GT, Cull-Candy SG (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainite receptors. *Journal of Physiology-London* 486(2):297-303.
- Keinanen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. *Science* 249:556-560.
- Keller BU, Hollmann M, Heinemann S, Konnerth A (1992) Calcium influx through subunits GluR1 GluR3 of Kainate AMPA receptor channels is regulated by cAMP dependent protein-kinase. *Embo Journal* 11(3):891-896.
- Kim EJ, Sheng M (2004) PDZ domain proteins of synapse. *Nature Reviews Neuroscience* 5(10):771-781.
- Koh D.S., Geiger J.R., Jonas P., Sakmann B. (1995) Ca²⁺-permeable AMPA and NMDA receptor channels in basket cells of rat hippocampal dentate gyrus. *J Physiol.* 485: 383-402.
- Koh DS, Burnashev N, Jonas P (1995) Block of native Ca²⁺-permeable AMPA receptors in rat-brain by intracellular polyamines generates double rectification. *Journal of Physiology* 486(2):305-312.
- Kohler M., Kornau H.-C. and Seeburg P. H. (1994) The organization of the gene for the functionally dominant α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic

- acid receptor subunit GluR-B. *J. Biol. Chem.* 269: 17367–17370
- Konur S, Ghosh A (2005) Calcium signaling and the control of dendritic development. *Neuron* 46:401-405.
- Kossel AH, Williams CV, Schweizer M, Kater SB (1997) Afferent innervation influences the development of dendritic branches and spines via both activity-dependent and non-activity-dependent mechanisms. *The Journal of Neuroscience* 17(16):6314-6324.
- Kumar SS, Bacci A, Kharazia V, Huguenard JR (2002) A development switch of AMPA receptor subunits in neocortical pyramidal neurons. *Journal of Neuroscience* 22(8):3005-3015.
- Kwak S, Kawahara Y (2005) Deficient RNA editing of GluR2 and neuronal death in amyotrophic lateral sclerosis. *J Mol Med* 83:110-120.
- Lambolez B, Audinat E, Bochet P, Crepel F, Rossier J (1992) AMPA receptor subunits expressed by single Purkinje-cells. *Neuron* 9(2):247-258.
- Lance-Jones C, Landmesser L (1981) Pathway selection by chick lumbo sacral moto neurons during normal development. *Proceedings of the Royal Society of London Series B Biological Biological Sciences* 214(1194):1-18.
- Landmesser L (1978) The development of motor projection patterns in the chick hind limb. *Journal of Physiology (Cambridge)* 284:391-414.
- Landmesser L (1978) The distribution of moto neurons supplying chick hind limb muscles. *Journal of Physiology (Cambridge)* 284:371-390.
- Landmesser L, Morris DG (1975) The development of function innervation in the hind limb of the chick embryo. *Journal of Physiology (Cambridge)* 249(2):301-326.
- Landmesser LT (1980) The generation of neuromuscular specificity. *Ann. Rev. Neurosci.* 3:279-302.
- Landmesser LT (2001) The acquisition of motoneuron subtype identity and motor circuit formation. *International Journal of Development Neuroscience* 19(2):175-182.
- Laslo P, Lipski J, Nicholson LFB, Miles GB, Funk GD (2001) GluR2 AMPA receptor subunit expression in motoneurons at low and high risk for degeneration in amyotrophic lateral sclerosis. *Experimental Neurology* 169:461-471.

- Lawrence JJ, Trussell LO (2000) Long-term specification of AMPA receptor properties after synapse formation. *Journal of Neuroscience* 20(13):4864-4870.
- Lee C.J., Kong H., Manzini M.C., Albuquerque C., Chao M.V., MacDermott A.B (2001) Kainate receptors expressed by a subpopulation of developing nociceptors rapidly switch from high to low Ca^{2+} permeability. *J Neurosci.* 21(13), 4572-81.
- Lee JC, Greig A, Ravindranathan A, Parks TN, Rao MS (1998) Molecular analysis of AMPA-specific receptors: subunit composition, editing, and calcium influx determination in small amounts of tissue. *Brain Res Brain Res Protoc.* 3(2):142-154.
- Lee MT, Koebbe MJ, O'Donovan MJ (1988) The development of sensorimotor synaptic connections in the lumbosacral cord of the chicken embryo. *J Neurosci.* 8(7): 2530-2543.
- Lee MT, O'Donovan MJ (1991) Organization of hindlimb muscle afferent projections to lumbosacral motoneurons in the chick embryo. *Journal of Neuroscience* 11(8):2564-2573.
- Lee YH, Lin CH, Hsu LW, Hu SY, Hsiao WT, Ho YS (2003) Roles of ionotropic glutamate receptors in early developing neurons derived from the P19 mouse cell line. *Journal of Biomedical Science* 10:199-207.
- Liu SQJ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. *Nature* 405(6785):454-458.
- Liu SQJ, Zukin RS (2007) Ca^{2+} -permeable AMPA receptors in synaptic plasticity and neuronal death. *TRENDS in Neurosciences* 30(3):126-134.
- Livak KJ, Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* 25: 402-408.
- Llado J, Caldero J, Ribera J, Tarabal O, Oppenheim RW, Esquerda JE (1999) Opposing effects of excitatory amino acids on chick embryo spinal cord motoneurons: Excitotoxic degeneration or prevention of programmed cell death. *Journal of Neuroscience* 19(24):10803-10812.
- Lohmann C, Finski A, Bonhoeffer T (2005) Local calcium transients regulate the spontaneous motility of dendritic filopodia. *Nat Neurosci.* 8(3): 305-312.
- Lomeli H, Mosbacher J, Melcher T, Hoyer T, Geiger JRP, Kuner T, Monyer H,

- Higuchi M, Bach A, Seeburg PH (1994) Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 266(5191):1709-1713.
- Longone P, Impagnatiello F, Mienville JM, Costa E, Guidotti A (1998) Changes in AMPA receptor-spliced variant expression and shift in AMPA receptor spontaneous desensitization pharmacology during cerebellar granule cell maturation in vitro. *J Mol Neurosci.* 11(1): 23-41.
- Lowe DL, Jahn K, Smith DO (1997) Glutamate receptor editing in the mammalian hippocampus and avian neurons. *Molecular Brain Research* 48:37-44.
- Lu CR, Hwang J, Phend KD, Rustioni A, Valtschanoff JG (2002) Primary afferent terminals in spinal cord express presynaptic AMPA receptors. *Journal of Neuroscience* 22(21):9522-9529.
- Lu YM, Yin HZ, Chiang J, Weiss JH (1996) Ca²⁺-permeable AMPA/kainite and NMDA channels: High rate of Ca²⁺ influx underlies potent induction of injury. *Journal of Neuroscience* 16(17):5457-5465.
- Luscher C, Frerking M (2001) Restless AMPA receptors: implications for synaptic transmission and plasticity. *Trends in Neurosciences* 24(11):665-670.
- Maccaferri G, Dingledine R (2002) Complex effects of CNQX on CA1 interneurons of the developing rat hippocampus. *Neuropharmacology* 43(4):523-9.
- Martin LJ, Furuta A, Blackstone CD (1998) AMPA receptor protein in developing rat brain: Glutamate receptor-1 expression and localization change at regional, cellular, and subcellular levels with maturation. *Neuroscience* 83(3):917-928.
- Martin-Caraballo M, Dryer, SE (2002a) Activity- and target-dependent regulation of large-conductance Ca²⁺-activated K⁺ channels in developing chick lumbar motoneurons. *J Neurosci.* 22(1):73-81.
- Martin-Caraballo M, Dryer, SE (2002b) Glial-cell line-derived neurotrophic factor and target-dependent regulation of large-conductance K_{Ca} channels in developing chick lumbar motoneurons. *J Neurosci.* 22(23):10201-10208.
- Mattson MP, Lance-Jones C (1996) A critical period for the specification of motor pools in the chicken lumbosacral spinal cord. *Development* 122(2):659-69.
- Mattson MP (1990) Excitatory amino acids, growth factors, and calcium: a teeter-totter model for neural plasticity and degeneration. *Adv Exp Med Biol.* 268:211-20.

- Mattson MP, Dou P, Kater SB (1998) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *Journal of Neuroscience* 8:2087-2100.
- Mayer ML, Westbrook GL (1987) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. *J Physiol.* 394:501-527.
- Meldrum BS (1994) The role of glutamate in epilepsy and other CNS disorders. *Neurology* 44:S14-S23.
- Martinez-galan JR, Caminos E, Vale C, Juiz JM (2007) Auditory nerve inputs is not an absolute requirement for the expression, distribution and calcium permeability of AMPA receptors in the adult rat ventral cochlear nucleus. *Brain research* 1138:21-29.
- Mayer ML, Armstrong N (2004) Structure and function of glutamate receptor ion channels. *Annu. Rev. Physiol* 66:161-181.
- McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15:791-803.
- Mcbain CJ, Dingledine R (1993) Heterogeneity of synaptic glutamate receptors on CA3 stratum radiatum interneurons of rat hippocampus. *Journal of Physiology* 462:373-392.
- McCobb DP, Best PM, Beam KG (1990) The differentiation of excitability in embryonic chicken limb motoneurons. *J Neurosci* 10:2974-2984.
- McCobb DP, Best PM, Beam KG (1989) Development alters the expression of calcium currents in chicken limb motoneurons. *Neuron* 2:1633-1643.
- McDonald JW, Johnston MV (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Research Review* 15:41-70.
- Mendelson B, Frank E (1991) Specific monosynaptic sensory-motor connections form in the absence of patterned neural activity and motoneuronal cell death. *J Neurosci.* 11(5): 1390-1403.
- Meng Y, Zhang Y, Jia Z (2003) Synaptic transmission and plasticity in the absence of AMPA glutamate receptor GluR2 and GluR3. *Neuron* 39:163-176.
- Menuz K, Stroud RM, Nicoll RA, Hays FA (2007) TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists. *Science* 318:815-817.

- Mettling C, Gouin A, Robinson M, el M'Hamdi H, Camu W, Bloch-Gallego E, Buisson B, Tanaka H, Davies AM, Henderson CE (1995) Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support. *J Neurosci.* 15(4):3128-3137.
- Metzger F, Wiese S, Sendtner M (1998) Effect of glutamate on dendritic growth in embryonic rat motoneurons. *Journal of Neuroscience* 18(5):1735-1742.
- Meucci O, Fatatis A, Holzwarth JA, Miller RJ (1996) Development regulation of the toxin sensitivity of Ca²⁺-permeable AMPA receptors in cortical glia. *Journal of Neuroscience* 16(2):519-530.
- Meucci O, Miller RJ (1998) Dissociation between the Joro spider toxin sensitivity of recombinant alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and their ability to increase intracellular calcium. *Neuropharmacol.* 37(10-11):1431-1443.
- Milner LD, Landmesser LT (1999) Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J Neurosci.* 19(8): 3007-3022.
- Milstein AD, Nicoll R (2008) Regulation of AMPA receptor gating and pharmacology by TARP auxiliary subunits. *Cell* 29(7):333-339.
- Monnerie H, Le Roux PD (2006) Glutamate receptor agonist kainite enhances primary dendrite number and length from immature cortical neurons in vitro. *Journal of Neuroscience Research* 83(6):944-956.
- Monyer H, Seeburg PH, Wisden W (1991) Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. *Neuron* 6:799-810.
- Morkve SH, Veruki ML, Hartveit E (2002) Functional characteristics of non-NMDA-type ionotropic glutamate receptor channels in AII amacrine cells in rat retina. *J Physiol.* 542(Pt 1):147-165.
- Mosbacher J, Schoepfer R, Monyer H, Burnashev N, Seeburg PH, Ruppersberg JP (1994) A molecular determinant for submillisecond desensitization in glutamate receptors. *Science* 266(5187):1059-1062.
- Nagano I, Murakami T, Shiote M, Abe K, Itoyama Y (2003) Ventral root avulsion leads to downregulation of GluR2 subunit in spinal motoneurons in adult rats. *Neuroscience* 117:139-146.

- Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* 258(5082):597-603.
- Ni X, Sullivan GJ, Martin-Caraballo M (2007) Developmental characteristics of AMPA receptors in chick lumbar motoneurons. *Dev Neurobiol.* 67(11):1419-32.
- Niedzielski AS, Wenthold RJ (1995) Expression of AMPA, kainite, and NMDA receptor subunits in cochlear and vestibular ganglia. *Journal of Neuroscience* 15(3):2338-2353.
- Nutt SL, Kamboj RK (1994) Differential RNA editing efficiency of AMPA receptor subunit GluR-2 in human brain. *Neuroreport* 5:1679-1683.
- O'Brien RJ, Fischbach GD (1986) Modulation of embryonic chick motoneuron glutamate sensitivity by interneurons and agonists. *Journal of Neuroscience* 11:3290-3296.
- O'Brien RJ, Mammen AL, Blackshaw S, Ehlers MD, Rothstein JD, Huganir RL (1997) The development of excitatory synapses in cultured spinal neurons. *Journal of Neuroscience* 17(19):7339-7350.
- O'Brien RJ, Kamboj S, Ehlers MD, Rosen KG, Fischbach GD, Huganir RL (1998) Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21(5):1067-1078.
- O'Brien R, Fischbach GD (1986) Characterization of excitatory amino acid receptors expressed by embryonic chick motoneurons in vitro. *The Journal of Neuroscience* 6(11):3275-3282.
- O'Brien RJ, Lau LF, Huganir RL (1998) Molecular mechanisms of glutamate receptor clustering at excitatory synapses. *Current Opinion in Neurobiology* 8:364-369.
- O'Brien RJ, Xu DS, Petralia RS, Steward O, Huganir RL, Worley P (1999) Synaptic clustering of AMPA receptors by the extracellular immediate-early gene product *Narp*. *Neuron* 23(2):309-323.
- O'Connell MA (1997) RNA editing: rewriting receptors. *Curr. Biol* 7:R437-R439.
- O'Donovan M, Ho S, Yee W (1994) Calcium imaging of rhythmic network activity in the developing spinal cord of the chick embryo. *Journal of Neuroscience* 14(11):6354-6369.
- O'Donovan MJ, Landmesser L (1987) The development of hindlimb motor activity

- studied in the isolated spinal cord of the chick embryo. *J Neurosci.* 7(10):3256-3264.
- O'Donovan MJ (1999) The origin of spontaneous activity in developing networks of the vertebrate nervous system. *Current Opinion in Neurobiology* 9:94-104.
- O'Donovan M.J., Chub N., Wenner P (1998) Mechanisms of spontaneous activity in developing spinal networks. *J Neurobiol.* 37(1):131-45.
- Okado N, Oppenheim RW (1984) Cell-death of motoneurons in the chick-embryo-spinal-cord .9. the loss of motoneurons following removal of afferent inputs. *Journal of Neuroscience* 4(6):1639-1652.
- Okada T., Schultz K., Geurtz W., Hatt H., Weiler R (1999) AMPA-preferring receptors with high Ca^{2+} permeability mediate dendritic plasticity of retinal horizontal cells. *Eur J Neurosci.* 11(3):1085-95.
- Oppenheim RW(1981) Cell death of motoneurons in the chick embryo spinal cord V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connections. *Journal of Neuroscience* 1(2):141-151.
- Oppenheim RW, Bursztajn S et al. (1989) Cell-death of motoneurons in the chick-embryo-spinal-cord.11. Acetylcholine-receptors and synaptogenesis in skeletal-muscle following the reduction of motoneuron death by neuromuscular blockade. *Development* 107(2):331-341.
- Oppenheim RW, Prevet D, Houenou LJ, Pincon-Raymond M, Dimitriadou V, Donevan A, O'Donovan M, Wenner O, Mckemy DD, Allen PD (1997) Neuromuscular development in the avian paralytic mutant crooked neck dwarf (cn/cn): Further evidence for the role of neuromuscular activity in motoneuron survival. *Journal of Comparative Neurology* 381(3):353-372.
- Otis TS, Raman IM, Trussell LO (1995) AMPA receptors with high Ca^{2+} permeability mediate synaptic transmission in the avian auditory pathway. *Journal of Physiology-London* 482(2):309-315.
- Palla SL, Wenner P, Gonzalez-Islas C, Fagiolini M, Razak KA, Kim G, Sanes D, Roerig B (2006) Developmental plasticity of inhibitory circuitry. *Journal of Neuroscience* 26(41):10358-10361.
- Paternain AV, Morales M, Lerma J (1995) Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron* 14(1):185-189.

- Pemberton KE, Belcher SM, Ripellino JA, Howe JR (1998) High-affinity kainate-type ion channels in rat cerebellar granule cells. *J Physiol.* 510 (Pt 2):401-420.
- Pickard L, Noel J, Henley JM, Collingridge GL, Molnar E (2000) Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA receptor subunit composition in living hippocampal neurons. *Journal of Neuroscience* 20(21):7922-7931.
- Piochon C, Irinopoulou T, Bruscianno D, Bailly Y, Mariani J, Levenes C (2007) NMDA receptor contribution to the climbing fiber response in the adult mouse Purkinje cell. *J Neurosci.* 27(40):10797-809.
- Pittman R, Oppenheim RW (1978) Neuromuscular blockade increases motoneuron survival during normal cell death in the chick embryo. *Nature* 271:364-366.
- Placzek M, Yamada T, Tessier-lavigne M, Jessell T, Dodd J (1991) Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development supplement* 2:105-122.
- Poh A, Karunaratne A, Kolle G, Huang N, Smith E, Starkey J, Wen D, Wilson I, Yamada T, Hargrave M (2002) Patterning of the vertebrate ventral spinal cord. *Int. J. Dev. Biol.* 46:597-608.
- Polgar E, Al-Khater KM, Shehab S, Watanabe M, Todd AJ (2008) Large projection neurons in laminal I of the rat spinal cord that lack the neurokinin 1 receptor are densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors. *The Journal of Neuroscience* 28(49):13150-13160.
- Poluch S, Drian MJ, Durand M, Astier C, Benyamin Y, Konig N (2001) AMPA receptor activation leads to neurite retraction in tangentially migrating neurons in the intermediate zone of the embryonic rat neocortex. *Journal of Neuroscience Research* 63(1):35-44.
- Popratiloff A, Wang Y, Narvid J, Petralia RS, Giaume C, Peusner KD (2004) AMPA receptor subunit expression in chick vestibular nucleus neurons. *Journal of Neuroscience Research* 76:662-677.
- Prithviraj R, Kelly KM, Espinoza-Lewis R, Hexom T, Clark AB, Inglis FM (2008) Differential regulation of dendrite complexity by AMPA receptor subunits GluR1 and GluR2 in motor neurons. *Developmental Neurobiology.* 68(2):247-264.
- Ragnarson B, Ornung G, Grant G, Ottersen OP, Ulfhake B (2003) Glutamate and AMPA receptor immunoreactivity in la synapse with motoneurons and

- neurons of the central cervical nucleus. *Experimental Brain Research* 149(4):447-457.
- Rajan I, Cline HT (1998) Glutamate receptor activity is required for normal development of tectal cell dendrites in vivo. *J Neurosci.* 18(19):7836-46.
- Ramoia AS, Campbell G, Shatz CJ (1988) Dendritic growth and remodeling of cat retinal ganglion cells during fetal and postnatal development. *J Neurosci.* 8(11):4239-61.
- Rao A, Kim E, Sheng M, Craig AM (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *Journal of Neuroscience* 18(4):1217-1229.
- Ravindranathan A, Donevan SD, Sugden SG, Greig A, Rao MS, Parks TN (2000) Contrasting molecular composition and channel properties of AMPA receptors on chick auditory and brainstem motor neurons. *J Physiol.* 523 Pt 3:667-684.
- Redmond L, Kashani AH, Ghosh A (2002) Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron* 34:999-1010.
- Ritter LM, Vazquez DM, Meador-Woodruff JH (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. *Development Brain Research* 139(2):227-236.
- Rueter SM, Burns CM, Coode SA, Mookherjee P, Emeson RB (1995) Glutamate receptor RNA editing in vitro by enzymatic conversion of adenosine to inosine. *Science* 267:1491-1494.
- Sands SB, Barish ME (1992) Neuronal Nicotinic Acetylcholine-receptor currents in pheochromocytoma (Pc12) cells-dual mechanisms of rectification. *Journal of Physiology-London* 447:467-487.
- Sanes DH, Song J, Tyson J (1992) Refinement of dendritic arbors along the tonotopic axis of the gerbil lateral superior olive. *Developmental Brain Research* 67(1):47-55.
- Santos AE, Duarte CB, Iizuka M, Barsoumian EL, Ham J, Lopes MC, Carvalho AP, Carvalho AL (2006) Excitotoxicity mediated by Ca²⁺-permeable GluR4-containing AMPA receptors involves the AP-1 transcription factor. *Cell Death and Differentiation* 13(4):652-660.
- Schwartz PM, Borghesani PR, Levy RL, Pomeroy SL, Segal RA (1997) Abnormal

- cerebellar development and foliation in BDNF^{-/-} mice reveals a role for neurotrophins in CNS patterning. *Neuron* 19:269-281.
- Seifert G, Zhou M, Dietrich D, Schumacher TB, Dybek A, Weiser T, Wienrich M, Wilhelm D, Steinhauser C (2000) Development regulation of AMPA-receptor properties in CA1 pyramidal neurons of rat hippocampus. *Neuropharmacology* 39(6):931-942.
- Sernagor E, Chub N, Ritter A, O'Donovan M.J (1995) Pharmacological characterization of the rhythmic synaptic drive onto lumbosacral motoneurons in the chick embryo spinal cord. *J Neurosci.* 15(11):7452-64.
- Shaw PJ, Eggett CJ (2000) Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. *J Neurol* 247(Suppl 1):I/17-I/27.
- Sheng M, Lee SH (2001) AMPA receptor trafficking and the control of synaptic transmission. *Cell* 105(7):825-828.
- Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* 284:1811-1816.
- Shin J, Shen F, Huguenard JR (2005) Polyamines modulate AMPA receptor-dependent synaptic responses in immature layer V pyramidal neurons. *J Neurophysiol.* 93(5):2634-2643.
- Sholomenko GN, O'Donovan MJ (1995) Development and characterization of pathways descending to the spinal cord in the embryonic chick. *Journal of Neurophysiology* 73(3):1223-1233.
- Simon CM, Eric F(1997) Formation of specific monosynaptic connections between muscle spindle afferents and motoneurons in the mouse. *Journal of Neuroscience* 17(9):3128-3135.
- Solum D, Hughes D, Major MS, Parks TN (1997) Prevention of normally occurring and deafferentation-induced neuronal death in chick brainstem auditory neurons by periodic blockade of AMPA/kainite receptors. *Journal of Neuroscience* 17(12):4744-4751.
- Sommer B, Keinänen K, Verdoorn TA, Wisden W, Burnashev N, Herb A, Kohler M, Takagi T, Sakmann B, Seeburg PH (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* 249(4976):1580-1585.

- Sommer B., Kohler M., Sprengel R. and Seeburg P. H. (1991) RNA editing in brain controls a determinant of ion flow in glutamate-gated channel. *Cell* 67(1):11–19.
- Song I, Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. *Trends in Neuroscience* 25(11):578-588.
- Sprengel R (2006) Role of AMPA receptors in synaptic plasticity. *Cell Tissue Res.* 326:447-455.
- Strack S, Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl-D-aspartate receptor. *Journal of Biological Chemistry* 273(33):20689-20692.
- Stromgaard K, Mellor I (2004) AMPA receptor ligands: synthetic and pharmacological studies of polyamines and polyamine toxins. *Medicinal research reviews* 24(5):589-620.
- Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S (2005) Expression profile of AMPA receptor subunit mRNA in single adult rat brain and spinal cord neurons in situ. *Neuroscience Research* 52(3):228-234.
- Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S (2006) Slow and selective death of spinal motor neurons in vivo by intrathecal infusion of kainic acid: implications for AMPA receptor-mediated excitotoxicity in ALS. *J Neurochem* 98(3):782-791.
- Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. *The Journal of Neuroscience* 17(1):58-69.
- Tanaka H, Grooms SY, Bennett MVL, Zukin RS (2000) The AMPAR subunit GluR2: still front and center-stage. *Brain Research* 886:190-207.
- Tang J, Landmesser LT (1993) Reduction of intramuscular nerve branching and synaptogenesis is correlated with decreased motoneuron survival. *J Neurosci.* 13:3095-3103.
- Temkin R, Lowe D, Jensen P, Hatt H, Smith DO (1997) Expression of glutamate receptor subunits in alpha-motoneurons. *Brain Res Mol Brain Res.* 52(1): 38-45.
- Thoenen H (1995) Neurotrophins and neuronal plasticity. *Science* 270(5236):593-598.

- Threadgill R, Bobb K, Ghosh A (1997) Regulation of dendritic growth and remodeling by Rho, Rac, Cdc42. *Neuron* 19:625-634.
- Toth K, McBain CJ (1998) Afferent-specific innervation of two distinct AMPA receptor subtypes on single hippocampal interneurons. *Nat Neurosci.* 1(7):572-578.
- Tsuchida T, Ensini M, Morton SB, Baldassare M, Edlund T, Jessell TM, Pfaff SL (1994) Topographic organization of embryonic motor-neurons defined by expression of lim homeobox genes. *Cell* 79(6):957-970.
- Tsui CC, Copeland NG, Gilbert DJ, Jenkins NA, Barnes C, Worley PF (1996) Narp, a novel member of the pentraxin family, promotes neurite outgrowth and is dynamically regulated by neuronal activity. *Journal of Neuroscience* 16(8):2463-2478.
- Turman JE, Macdonald AS, Pawl KE, Bringas P, Chandler SH (2000) AMPA receptor subunit expression in trigeminal neurons during postnatal development. *Journal of comparative neurology* 427:109-123.
- Van Damme P, Bracken D, Callewaert G, Robberecht W, Van Den Bosch L (2005) GluR2 deficiency accelerates motor neuron degeneration in a mouse model of amyotrophic lateral sclerosis. *Journal of Neuropathology and Experimental Neurology* 64(7):605-612.
- Van Damme P, Van den Bosch L, Van Houtte E, Callewaert G, Robberecht W (2002) GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. *Journal of Neurophysiology* 88(3):1279-1287.
- Vandenbergh W, Robberecht W, Brorson JR (2000) AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability. *The Journal of Neuroscience* 20(1):123-132.
- Vandenbergh W, Bindokas VP, Miller RJ, Robberecht W, Brorson JR (2001) Subcellular localization of calcium-permeable AMPA receptors in spinal motoneurons. *Eur J Neurosci.* 14(2):305-314.
- Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 252:1751-1718.
- Vitten H, Reusch M, Friauf E, Lohrke S (2004) Expression of functional kainate and

- AMPA receptors in developing lateral superior olive neurons of the rat. *J Neurobiol.* 59(3):272-288.
- Voss OP, Milne S, Sharkey J, O'Neill MJ, McCulloch J (2007) Molecular mechanisms of neurite growth with AMPA receptor potentiation. *Neuropharmacology* 52:590-597.
- Washburn MS, Numberger M, Zhang S, Dingledine R (1997) Differential dependence on GluR2 expression of three characteristic features of AMPA receptors. *J Neurosci.* 17(24):9393-9406.
- Wenger BS (1995) Determination of structural patterns in the spinal cord of the chick embryo studied by transplantations between brachial and adjacent levels. *J of Exp Zool* 116:123-163.
- Wenger EL (1950) An experimental analysis of relations between parts of the brachial spinal cord of the embryonic chick. *J of Exp Zool* 114:51-86.
- Wenner P, O'Donovan MJ (1999) Identification of an interneuronal population that mediates recurrent inhibition of motoneurons in the developing chick spinal cord. *Journal of Neuroscience* 19(17):7557-7567.
- Wenner P, O'Donovan MJ, Matisse MP (2000) Topographical and physiological characterization of interneurons that express engrailed-1 in the embryonic chick spinal cord. *Journal of Neurophysiology* 84(5):2651-2657.
- Williams TL, Day NC, Ince PG, Kamboj RK, Shaw PJ (1991) Calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors: a molecular determinant of selective vulnerability in amyotrophic lateral sclerosis. *Ann Neurol* 42(2):200-207.
- Wilson MT, Kisaalita WS, Keith CH (2000) Glutamate-induced changes in the pattern of hippocampal dendrite outgrowth: a role for calcium-dependent pathways and the microtubule cytoskeleton. *Journal of Neurobiology* 43(2):159-172.
- Wollmuth LP, Sobolevsky AI (2004) Structure and gating of the glutamate receptor ion channel. *Trends Neurosci* 27:321-328.
- Wong ROL, Ghosh A (2002) Activity-dependent regulation of dendritic growth and patterning. *Nature Review Neuroscience* 3(10):803-812.
- Wong W.T., Faulkner-Jones B.E., Sanes J.R., Wong R.O (2000) Rapid dendritic remodeling in the developing retina: dependence on neurotransmission and reciprocal regulation by Rac and Rho. *J Neurosci.* 20(13), 5024-36.

- Xu HY, Clement A, Wright TM, Wenner P (2007) Developmental reorganization of the output of a GABAergic interneuronal circuit. *Journal of Neurophysiology* 97(4):2769-2779.
- Yamada KA, Dubinsky JM, Rothman SM (1989) Quantitative physiological characterization of a quinoxalinedione non-NMDA receptor antagonist. *J Neurosci.* 9(9):3230-6.
- Yin QW, Johnson J, Prevette D, Oppenheim RW (1994) Cell death of spinal motoneurons in the chick embryo following deafferentation: rescue effects of tissue extracts, soluble proteins, and neurotrophic agents. *Journal of Neuroscience* 14(12):7629-7640.
- Yoon Y.J., Kominami H., Trimarchi T., Martin-Caraballo M., 2008. Inhibition of electrical activity by retroviral infection of chicken spinal cord with Kir2.1 transgenes disrupts electrical differentiation of motoneurons. *PloS ONE* 3(8), e2971.
- Young KC, McGehee DS, Brorson JR (2007) Glutamate receptor expression and chronic glutamate toxicity in rat motor cortex. *Neurobiol Dis* 26(1):78-85.
- Zhou W, Zhang L, Guoxiang X, Mojsilovic-Petrovic J, Takamaya K, Sattler R, Haganir R, Kalb R (2008) GluR1 controls dendrite growth through its binding partner, SAP97. *Journal of Neuroscience.* 28(41): 10220-10233.
- Zhu JJ, Esteban JA, Hayashi Y, Malinow R (2000) Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nature Neuroscience* 3(11):1098-1106.
- Zorumski CF, Yang J (1988) AMPA, kainate, and quisqualate activate a common receptor-channel complex on embryonic chick motoneurons. *J Neurosci.* 8(11):4277-4286.