Chapter 2
The Molecular Basis of Experience-Dependent Motor System Development

Robert G. Kalb, Weiguo Zhou and Lei Zhang

Overview

Nervous system operation depends on the ability of neurons to process a panoply of excitatory and inhibitory signals and generate meaningful output. The size and complexity of the dendritic tree is a critical determinant of the computational work of neurons (Stuart et al. 1999). In addition, the geometry of the dendritic tree can regulate who communicates with a neuron by controlling the quantitative and qualitative nature of the afferent input (Hume and Purves 1981). Theorists, taking a wiring optimization approach, view synaptic connectivity and neuronal morphology as inextricably linked because it is the most efficient fit of network wiring within a given volume of neuropil (Chklovskii 2004). The spectrum of animal behavior, from a 0.5 mm roundworm wiggling on a Petri dish to Glen Gould playing Bach, reflects the precision with which neurons elaborate their dendritic tree and are innervated.

R. G. Kalb
Department of Neurology, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, 3615 Civic Center Boulevard, Philadelphia, PA 19104, USA
e-mail: kalb@email.chop.edu

R. G. Kalb · W. Zhou · L. Zhang
Research Institute and Division of Neurology, Department of Pediatrics, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, 3615 Civic Center Boulevard, Philadelphia, PA 19104, USA

W. Zhou
e-mail: weiguozhou@gmail.com

L. Zhang
e-mail: ZhangL@email.chop.edu

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Cell and Molecular Biology of Activity-Dependent Development

The process of dendrite elaboration is often divided into an initial, synaptic activity-independent phase and a subsequent, synaptic activity-dependent phase (Goodman and Shatz 1993; Shatz 1990). The first phase sets up the basic architecture of the tree and is likely to be under strong genetic control (Gao and Bogert 2003; Jan and Jan 2003). The synaptic activity-dependent phase of dendrite elaboration is believed to fine tune structure into a precise configuration (Cline 2001; Constantine-Paton et al. 1990). There is much evidence to support the "synaptotrophic hypothesis" as a mechanism for activity-dependent regulation of dendrite arbor development (Vaughn 1989; Vaughn et al. 1988; reviewed by Cline and Haas 2008). Developing axons and dendrites undergo exploratory growth and make nascent synapses. Adhesion molecules such as neurexins (NRX) and neuroligins (NLG; Chen et al. 2010; Thyagarajan and Ting 2010) are likely to be involved. Repeated use of a synapse leads to stabilization of the synapse if pre- and postsynaptic elements are coincidently active (Bi and Poo 2001; Engert et al. 2002; Ruthazer et al. 2003). Axons and dendrites that bear stable synapses are retained (or perhaps grow) and conversely, portions of axons and dendrites that do not bear stable synapses are withdrawn (Katz and Constantine-Paton 1988). Spontaneous synaptic activity is the initial driver of these events and subsequently environmentally evoked synaptic activity does the heavy lifting. Experience-dependent refinement of neuronal architecture and synaptic connectivity sculpts each nervous system to perform best in the environment in which the animal was reared (Zhang et al. 2000).

One form of activity-dependent development involves activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor (Cline et al. 1987; Kalb 1994; Kleinschmidt et al. 1987). This leads to a substantial rise in dendritic calcium, which is thought to be the key trigger for subsequent events. The precise ordering of what occurs next is not entirely clear, but the literature supports the view that there are three linked main events. Event #1 is the secretion/elaboration of extracellular factors such as BDNF, Wnts, and nitric oxide (Cramer et al. 1996; Inglis et al. 1998; Wu et al. 1994). There is evidence that secretion/elaboration is activity-dependent and blocking their action can prevent synapse stablization and dendrite growth (McAllister et al. 1996, 1997; also see Lu 2003). Event #2 is the activation of intracellular signaling molecules such as Ca^{++}/calmodulin-dependent kinase (CamK) type I (Wayman et al. 2006), CamK II (Wu and Cline 1998; Zou and Cline 1999; Gaudilliere et al. 2004) and CamK IV (Redmond et al. 2002), mitogen-activated protein (MAP) kinase (Ha and Redmond 2008; Redmond et al. 2002; Wu et al. 2001), βcatenin (Yu and Malenka 2003; Peng et al. 2009), and RhoA GTPases (Li et al. 2000, 2002; Sin et al. 2002). The role of these molecules has been studied using pharmacological inhibitors and expression of dominant-negative and constitutively-active forms of these proteins. Event #3 is a new gene expression and the list of contributing transcription factors includes cAMP response element-binding protein (CREB; Li et al. 2009; Redmond et al. 2002; Wayman et al. 2006), CREST (Aizawa et al. 2004), NeuroD (Gaudilliere et al. 2004), and myocyte-specific enhancer factor

2A (MEF2A; Shalizi et al. 2006). As discussed above, the role of these molecules has been studied employing molecular genetic techniques. Modification of cytoskeletal elements, maturation of silent synapses (NMDA-R only → AMPA-R + NMDA-R), and the precise apposition of pre- and postsynaptic membranes incorporating adhesion molecules are all necessary steps in this process. With so many events apparently occurring simultaneously, it is difficult to discern the epistatic relationships. How activity-dependent processes dovetail with dendrite growth-promoting processes not-shown-to-be-activity-dependent such as activation of PI3K and mammalian target of rapamycin (mTOR; Jaworski et al. 2005; Kumar et al. 2005) and Notch signaling (Redmond et al. 2000) only complicates matters more.

Experience-Dependent Motor System Development

The normal development of the locomotor system (from behavior, to connectivity within the segmental spinal cord, to motor neuron dendrite architecture) emerges during prenatal and early postnatal life (Altman and Sudarshan 1975; Curfs et al. 1993, 1994; Donatelle 1977; Pellis et al. 1991; Seebach and Ziskind-Conhaim 1994; Snider et al. 1992). In the next section, we outline the evidence that locomotor development is experience-dependent and that the molecular machinery that drives this process can involve activation of NMDA-Rs. In addition, we will provide evidence for a second set of molecules that appear to act in parallel with NMDA-Rs to drive motor system development. We have found that GluA1 subunit of the 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA)-R, in concert with an intracellular-binding partner called SAP97, promote motor system development by an NMDA-R-independent mechanism.

Experience-Dependent Motor System Development and the NMDA-R

We begin with studies by Kerry Walton's group of neonatal rats reared in space. The force of gravity at the surface of the earth is called "1G" and anything less than that is referred to as "microgravity". Walton's group studied a cohort of animals that spent about 2 weeks of early postnatal life in the space shuttle. She showed that young rats that develop in microgravity have demonstrably different locomotor behavior than those that develop on earth (Walton et al. 2005). These observations echo her previous work using the tail suspension model (Walton et al. 1992). Work from my laboratory using these mice demonstrated that the motor neuron dendritic tree also undergoes experience-dependent development (Inglis et al. 2000). The parsimonious construct is that at least some on the alterations in motor neuron dendrite structure subserve the alterations in locomotor function that follow microgravity rearing.

These behavioral and anatomical studies prompted us to ask whether NMDA-Rs were involved in activity-dependent maturation of motor neuron dendritic architecture. We began by asking whether NMDA-R components were expressed by developing motor neurons. In situ hybridization studies show that newborn motor neurons express NR1, NR2A, and NR2C at particularly high levels and over the subsequent next few weeks of life, the abundance of these messenger RNAs (mRNAs) falls off considerably (Stegenga and Kalb 2001). The NR1 subunit undergoes alternative splicing, and an analysis of specific NR1 variants reveals that NR1A, NR1B, NR1-2, and NR1-4 are expressed at particularly high levels in newborn motor neurons. The abundance of these splice variants falls subsequently in early postnatal life (Stegenga and Kalb 2001). This work demonstrates that motor neurons express a unique repertoire of NMDA-R subunits in early postnatal life.

Coincident with the period when motor neurons express a distinct type of NMDA-R, the dendrites of motor neurons are undergoing substantial growth (Curfs et al. 1993; Lindsay et al. 1991; Núñez-Abades et al. 1994). Overall tree size and number of branches increase approximately twofold to threefold between postnatal day 7 (P7) and P21. Antagonism of NMDA-Rs with (2*R*)-amino-5-phosphonopentanoate (APV) or MK-801 inhibits the growth of motor neuron dendrites of NMDA-R (Kalb 1994). In contrast to their effects on developing dendrites, antagonism of NMDA-Rs in adult animals has no effect on motor neuron dendrite architecture. These results indicate that during a critical period in early postnatal life, activation of NMDA-Rs promotes the elaboration of motor neurons dendrites. In subsequent work, we showed that the dendrite growth-promoting actions of NMDA-Rs are mediated by the second messenger, nitric oxide (Inglis et al. 1998). Overall, this work highlights the distinct parallel between the experience-dependent development of sensory and motor systems.

A Novel Form of Activity-Dependent Development Utilizes GluA1-Containing AMPA-R

In addition to characterizing the expression pattern of NMDA-R subunits, we also examined the expression pattern of AMPA receptors (AMPA-R) subunits. Although all subunits undergo developmentally regulated expression, we were impressed that neonatal motor neurons express particularly high levels of the GluA1 (mRNA and protein; Jakowec et al. 1995a, b). (This is the unedited version of the protein that contains the "flip" alternatively spliced exon and unless otherwise stated, we will use "GluA1" to denote "GluA1(Q)flip.") Electrophysiologic studies show that neonatal motor neurons display Ca^{++}-permeable AMPA receptors (as would be expected from assembled tetramers enriched with GluA1 or even homomeric GluA1 tetramers; Carriedo et al. 1996; Vandenberghe et al. 2000). This raises the possibility that the special type of AMPA receptors expressed by neonatal motor neurons is part of the molecular mechanism of experience-dependent dendrite development. The

first good clue that this could be the case was a study in which we overexpressed (OE) GluA1 in mature motor neurons (after the period of developmental dendrite growth; Inglis et al. 2002). We found that this led to large-scale remodeling of the dendritic tree with a marked increase in dendrite branching. Expression of a version of GluR1 with an arginine in the critical "Q/R editing site" (GluA1(R)) had no effects on dendrite architecture. AMPA-R assembled from GluA1(R) are calcium impermeable and pass very little current upon activation with glutamate. These in vivo observations suggest that GluA1 can promote dendrite growth and this depends on the ability of GluA1-containing AMPA-R to depolarize cells. Subsequent in vivo and in vitro works provide strong support for the idea that calcium permeability of GluA1-containing AMPA-R is a major determinant of its effect on dendrite growth (Jeong et al. 2006).

One could imagine at least two ways in which increasing the expression of AMPA-R assembled with GluA1 might promote dendrite growth: (1) By enhancing the ability of cells to depolarize upon afferent stimulation, GluA1 might facilitate the activation of NMDA-Rs. In this scenario, GluA1 acts upstream of NMDA-R-mediated events; (2) GluA1 may act through an NMDA-R-independent pathway to regulated dendrite growth. In this scenario, GluA1 acts in parallel to NMDA-R-mediated events. There are several reasons for favoring the second scenario. First, administration of MK-801 to rats OE GluA1 did not block the prodendrite growth effect. We know MK-801 was at an effective concentration in the brain because we could not evoke LTP in the dentate gyrus from animals treated with MK-801 (see Fig. 6 of Inglis et al. 2002). So, OE GluA1 led to increase dendrite branching even though NMDA-Rs were effectively antagonized. Second, NMDA-R mRNAs are developmentally regulated and not expressed by mature motor neurons (Stegenga and Kalb 2001). So, GluA1-mediated dendrite remodeling can occur in neurons that do not express NMDA-R subunits. Finally, we more formally examined the role of NMDA-Rs in GluA1-dependent dendrite growth in vitro.

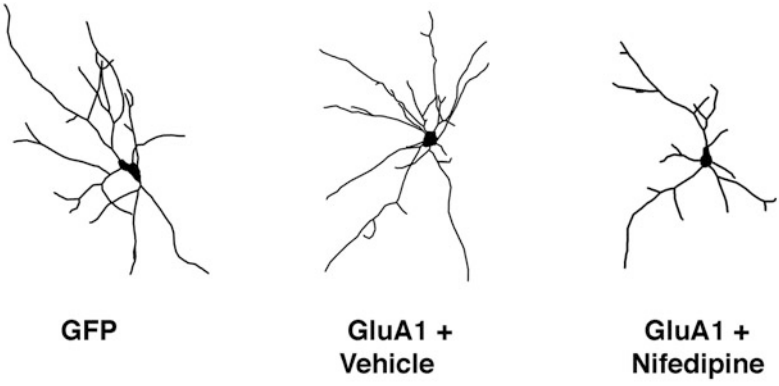
We grew dissociated spinal cord neurons in vitro and expressed GluA1 by transfection and treated some cells with the NMDA-R open-channel blocker MK-801 (10 μM). Three groups of neurons were studied morphologically: (1) green fluorescent protein (GFP), (2) GFP + GluA1 + vehicle, and (3) GFP + GluA1 + MK-801. MK-801 or vehicle was administered daily to the cultures and after 5 days in vitro (DIV), the cultures were fixed, immunostained for GFP to enhance the cell labeling, camera lucida drawings generated and quantitatively analyzed (Fig. 2.1). Compared with GFP alone, GluA1 led to an ~30% increase in dendrite branches ($F_{(2,60)} = 3.507$, $p = 0.03$), ~60% increase in overall arbor size ($F_{(2,60)} = 17.005$, $p < 0.0001$), ~60% increase in average dendrite length ($F_{(2,60)} = 9.370$, $p = 0.0003$), and a 40 % increase in the length of the longest dendrite ($F_{(2,60)} = 10.817$, $p < 0.0001$). Treatment with MK-801 did not influence the progrowth effects of GluA1; the dendritic arbor of neurons in the "GluA1 + MK-801" were not statistically different from the dendrite arbor of the neurons in the "GluA1" group. These observations establish that overexpression of GluA1 stimulates dendrite growth in a manner that is independent of NMDA-Rs.

Cell Parameter	GFP Control (n = 20)	GluA1 (n = 21)	MK-801 and GluA1 (n = 22)	$F_{(2,60)}$, p
Soma area (μm^2)	308.7 ± 89.9	349.8 ± 77.5	359.2 ± 80.1	2.187; 0.1211
1° dendrites	6.7 ± 1.3	6.8 ± 1.4	6.9 ± 1.9	0.098; 0.9071
Branches, #	13.3 ± 5.2	17.0 ± 4.8*	16.1 ± 4.3	3.507; 0.0363
Σlength (μm)	998.8 ± 236.0	1611.5 ± 431.6***	1581.0 ± 426.5***	17.005; <0.0001
Average dendrites	156.1 ± 46.6	246.3 ± 78.4***	246.6 ± 95.6***	9.370; 0.0003
Longest dendrite (μm)	178.9 ± 57.0	251.9 ± 50.9***	237.3 ± 51.4***	10.817; <0.0001

*Differs from GFP Control: *p<0.05; ** p<0.01; *** p<0.001;
†Differs from GluR1, †p<0.05; †† p<0.01; ††† p<0.001

Fig. 2.1 Overexpression of wild-type (WT) GluA1 stimulates dendrite growth in vitro in an NMDA-R-independent manner. *Top*, representative camera lucida images of neurons expression GFP alone, or GFP + GluA1 (treated or not with MK-801). The chart *below* provides a quantitative analysis of dendrites as well as a statistical analysis using ANOVA. The number of neurons drawn is noted in parentheses next to the column title. There is statistically significant increase in branching and overall tree size in neurons overexpressing GluA1 and these effects are not influenced by NMDA-R antagonism

In addition to NMDA-R, voltage-gated calcium channels are activated by membrane depolarization, and so these channels might participate in the GluA1 form of dendrite growth. To examine this issue we undertook a second set of experiments; we used the L-type calcium channel blocker nifedipine (20 μM). Three groups of neurons were studied morphologically: (1) GFP, (2) GFP + GluA1 + vehicle, and (3) GFP + GluA1 + nifedipine. Nifedipine or vehicle was administered daily and cells were prepared for analysis as discussed above (Fig. 2.2). Compared with GFP alone, GluA1 led to an ~40% increase in dendrite branches ($F_{(2,63)} = 8.396$, $p = 0.0006$), ~40% increase in overall arbor size ($F_{(2,63)} = 12.99$, $p < 0.0001$), and ~20 % increase in average dendrite length ($F_{(2,63)} = 9.085$, $p = 0.0004$). Treatment with nifedipine blocked all the prodendrite growth effects of GluA1 overexpression on branching and even suppressed overall tree growth and elaboration of the longest dendrite in comparison with neurons treated with vehicle. It is interesting that recent work has shown that L-type calcium channels play a critical role in homeostatic synaptic plasticity (Goold and Nicoll 2011). Thus, the activity-dependent dendrite growth elicited by GluA1 is NMDA-R independent and requires activation of voltage-gated calcium channels.



Cell Parameter	GFP Control (n=22)	GluA1 (n=22)	GluA1 and Nifedipine (n=21)	$F_{(2,63)}$, p
Soma area (μm^2)	207.7 ± 50.3	231.9 ± 44.4	213.1 ± 44.5	1.588; 0.2126
1° dendrites	6.0 ± 1.0	6.8 ± 1.2	6.3 ± 1.4	2.110; 0.1300
Branches, #	13.6 ± 4.8	18.7 ± 6.2	12.1 ± 5.4†††	8.396; $p = 0.0006$
∑length (μm)	1125.5 ± 326.1	1542.9 ± 625.2	875.3 ± 242.0†††	12.99; $p < 0.0001$
Average dendrites	192.3 ± 63.7	230.1 ± 89.3	141.6 ± 41.1*†††	9.085; $p = 0.0004$
Longest dendrite (μm)	239.6 ± 82.3	258.8 ± 79.5	177.6 ± 65.5*††	6.531; $p = 0.0027$

*Differs from GFP Control: *p<0.05; ** p<0.01; *** p<0.001;
†Differs from GluA1 Treatment, †p<0.05; †† p<0.01; ††† p<0.001

Fig. 2.2 Overexpression of WT GluA1 stimulates dendrite growth in vitro in a voltage-gated calcium channel-dependent manner. *Top*, representative camera lucida images of neurons expression GFP alone, or GFP + GluA1 (treated or not with nifedipine). The chart *below* provides a quantitative analysis of dendrites as well as a statistical analysis using ANOVA. The number of neurons drawn is noted in parentheses next to the column title. There is statistically significant increase in branching and overall tree size in neurons overexpressing GluA1 and these effects are blocked by voltage-gated calcium channel antagonism

The above-described work argues that expression of GluA1 is sufficient to promote dendrite growth. Simply overexpressing the protein can trigger the elaboration of dendrites (either in vitro or in vivo). To determine if GluA1 is necessary for dendrite growth under endogenous conditions requires that we reduced or eliminated its expression in neurons and determined the effects on morphogenesis. In vitro studies using RNA interference (RNAi) technology reveal that reducing GluA1 in neurons inhibits the normal elaboration of dendrites (Zhang et al. 2008). The effects are dose dependent and cell autonomous. These observations support the view that expression of GluA1 by neurons is necessary for the normal morphogenesis of the dendritic tree.

The In Vivo Role of GluA1 in Motor System Development

Activity-dependent growth of dendrites is a component of experience-dependent motor system development. In light of this, we wondered if the above in vitro observations were also seen in vivo. If so, we would be well positioned to determine

the broader impact of GluA1 on other features of experience-dependent motor development such as behavior and circuit connectivity.

GluA1 null mice (GluA1$^{-/-}$) are viable and have defects in cognitive/affective behavioral realms (Bannerman et al. 2004; Zamanillo et al. 1999). We began our studies of these animals by asking if GluA1$^{-/-}$ neonatal motor neurons elaborate a normal dendritic tree. Both at P10 and P23, the dendritic tree of GluA1$^{-/-}$ motor neurons is smaller than wild-type (WT) control mice (Zhang et al. 2008). It is worth noting that in addition to motor neurons, interneurons within the neonatal ventral horn also express GluA1 at very high levels (Jakowec et al. 1995a, b). And so, it is possible that loss of GluA1 from interneurons that innervate motor neurons influences the capacity of motor neurons to elaborate a normal dendritic tree. To examine this issue directly we generated conditional knock-out mice. Mating Hb9-Cre mice with mice bearing a LoxP-flanked allele of GluA1 generates mice with loss of one copy of GluA1. Using an appropriate breeding we generated mice that are homozygous for the LoxP-flanked allele and Cre recombinase and these mice have ablation of GluA1 expression restricted to motor neurons. Analysis of dendrite from these animals (*GluA1^{deltaHb9}*) revealed a reduction of dendrite size and branching, similar but not as severe as what we observed in the GluA1$^{-/-}$ mice (Zhang et al. 2008). There was no effect on dendrite structure in various control mice (i.e., Hb9-Cre alone or GluA1^{LoxP} alone). These in vivo observations support the view that the expression of endogenous GluA1 by motor neurons is required for the normal elaboration of the dendritic tree during development.

Does the loss of GluA1 influence other aspects of the motor system such as circuitry within the segmental spinal cord? To address this question, we used the pseudorabies virus (PRV)-tracing system. A recombinant PRV was generated that expressed GFP in cells. Upon injection into the hamstring leg muscle, PRV-GFP particles are retrogradely transported to motor neurons. PRV-GFP particles are then exported into dendrites where they cross synapses from motor neurons into innervating premotor interneurons. Thus, the distribution of GFP-labeled cells in the spinal cord reflect the pattern of premotor innervation of motor neurons. When we applied this approach to the GluA1$^{-/-}$ and the GluA1$^{+/+}$ mice, we found that much of the GFP labeling was identical between groups. However, fewer labeled interneurons were seen in the ipsilateral Rexed lamina VIII of lumbar segment 4 of the GluA1$^{-/-}$ than the GluA1$^{+/+}$ mice. In addition, there were fewer labeled interneurons in the multiple contralateral Rexed lamina of lumbar segment 2–5 of the GluA1$^{-/-}$ than the GluA1$^{+/+}$ mice (Zhang et al. 2008). These results suggest that segmental spinal cord connectivity is different between the GluA1$^{-/-}$ and the GluA1$^{+/+}$ mice.

Do these alterations in dendrite structure and segmental spinal cord interneuronal connectivity manifest in behavioral differences between the GluA1$^{-/-}$ and the GluA1$^{+/+}$ mice? To examine this, we subjected the two strains of mice to a battery of locomotor tasks including treadmill running, rotarod and fore- and hind-limb grip strength. At P23 and in adulthood, the GluA1$^{-/-}$ mice performed poorer in every single test in comparison with the GluA1$^{+/+}$ mice (Zhang et al. 2008). Similar trends were seen when we studied the *GluA1^{deltaHb9}* although the degree of locomotor impairment was less than seen in the GluA1$^{-/-}$ mice. These differences in motor

function could not be ascribed to a difference in motor neuron number. The weakness phenotype of the GluA1$^{-/-}$ mice was associated with an increase in type I muscle fibers in the gastrocnemius. Thus, elimination of GluA1 from motor neurons (as well as other neurons presumably in the ventral horn) leads to abnormal development of the neuromuscular unit. The dendritic tree of GluA1$^{-/-}$ motor neurons is stunted, the pattern of premotor interneuron connectivity is perturbed, muscle fiber-type specification is distorted and this leads to poorer locomotor performance in comparison with WT animals. These observations point to the critical role that GluA1 plays in the normal activity-dependent development of the motor system.

SAP97 Translates the GluA1-Generated Signal into Dendrite Growth

Our working hypothesis is that synaptic activation of AMPA-Rs assembled with the GluA1 subunit initiate an activity-dependent prodendrite growth signal. Some data suggest that the electrophysiological properties of GluA1-containing AMPA-R regulate how GluA1 influences dendrite morphology, and this is linked to AMPA-R calcium permeability (Jeong et al. 2006). We wondered if, in addition, intracellular proteins that bind GluA1 are also important determinants.

The extreme C-terminal four amino acids of GluA1 act as a ligand for the synapse-associated protein of 97 kDa molecular weight called SAP97 (Cai et al. 2002). SAP97 is a membrane-associated guanylate kinases (MAGUK)-class-scaffolding protein and is the only known binding partner of the extreme C-terminus of GluA1. MAGUK proteins are enriched in the postsynaptic density where they play a variety of roles in synaptic function including chaperoning glutamate receptor subunits into and out of the synapse, receptor clustering and modulation of receptor electrophysiological function (Palmer et al. 2005; Sheng and Sala 2001; Shepherd and Huganir 2007). SAP97 is a modular protein with multiple protein–protein interaction domains. As detailed below, we have explored the dendrite growth-promoting role of SAP97 and its binding to GluA1 in a series of in vitro and vivo experiments.

In co-immunoprecipitation (co-IP) experiments using spinal cord or cerebral cortex tissue, we find that GluA1 and SAP97 are part of a physical complex (Zhou et al. 2008). When the two full-length proteins are expressed in a heterologous system, we can again demonstrate a physical complex in the co-IP assay. Two approaches were taken to establish the portions of each protein required for the physical complex. First, we deleted the C-terminal 7 amino acids of GluA1 (GluA1Δ7). While full-length SAP97 will co-IP full-length GluA1, it will not co-IP GluA1Δ7. Second, the crystal structure of PDZ domains is known and it is possible to introduce mutations such that the PDZ domain becomes incompetent to bind ligands (Morais Cabral et al. 1996). We engineered such mutations into PDZ2 of GluA1 (K323A, K326A) and found that mutant PDZ2 SAP97 did not co-IP full-length GluA1 (Zhou et al. 2008). Thus, GluA1 and SAP97 are part of a physical complex that is likely to be mediated by the binding of the extreme C-terminus of GluA1 to PDZ2 of SAP97.

What biology, if any, is influenced by the GluA1/SAP97 complex? We began by studying the trafficking of GluA1 through the secretory pathway to populate synapses. To address this issue we used a strain of mice in which the WT version of GluA1 has been replaced with a version that is lacking the C-terminal 7 amino acids (Kim et al. 2005). In the homozygous state, this "knock-in" mouse only expresses GluA1Δ7. We find that GluA1Δ7 is synthesized in the endoplasmic reticulum at normal levels, is processed normally in the Golgi apparatus, hetero-oligomerizes normally with other AMPA-R subunits, and inserts into synapses normally (Zhou et al. 2008). Electrophysiological studies of the hippocampus of GluA1Δ7 mice show normal basal synaptic transmission as well as normal LTP/LTP (Kim et al. 2005). Thus, despite the fact that GluA1Δ7 does not physically associate with SAP97, the subunit behaves similar to WT GluA1. In marked contrast, SAP97 does not traffic to synapses in the GluA1Δ7 mice. These observations indicate that GluA1 chaperones SAP97 into synapses.

What is SAP97 doing, in association with GluA1, at synapses? We took two approaches to address this issue. First, we determined the effect on dendrite growth of eliminating SAP97 from neurons. When we knocked down SAP97 with a small hairpin RNA (shRNA), the neuronal dendritic tree is smaller and less branched than WT neurons (Zhou et al. 2008). This implies that endogenous SAP97 is required for normal elaboration of the dendritic tree. We also found that knockdown of SAP97 blocked the dendrite growth-promoting action of GluA1 overexpression. To validate these in vitro observations, we wanted to study mice in which SAP97 is ablated. Unfortunately, SAP97 null mice die at birth owing to cranio-facial abnormalities. To overcome this problem we generated mice in which SAP97 is eliminated specifically in motor neurons. This was achieved using the Hb9-Cre mice mated to mice bearing a floxed allele of SAP97. We found that the dendrites of motor neuron from the *SAP97*^{deltaHb9} mice are smaller and less branched than WT mice (Zhou et al. 2008). Thus, both in vitro and in vivo studies demonstrate that SAP97 plays a key role in the normal development of the neuronal dendritic tree. In addition, all of the dendrite growth-promoting actions of GluA1 are lost in the absence of SAP97. This suggests that SAP97 acts to translate activity from GluA1-containing AMPA-Rs into growth.

Our second approach to understanding what GluA1 and SAP97 are doing at synapses focused on the nature of their physical relationship. Must SAP97 be physically tethered to GluA1 to promote dendrite growth? Or, is colocalization of both proteins to the plasma membrane sufficient for GluA1 to promote dendrite growth? We undertook a series of experiments to explore this issue. First, we found that GluA1, but not GluA1Δ7, overexpressed in neurons in vitro is dendrite growth promoting (Zhou et al. 2008). In addition, we found that coexpression of GluA1 with SAP97 has a synergistic dendrite growth-promoting action, while coexpression of GluA1Δ7 with SAP97 leads to modest dendrite growth (equivalent to the dendrite growth-promoting action of SAP97 itself). So, even though GluA1Δ7 traffics to the cell surface and hetero-oligomerizes normally with other AMPA-R subunits, the lack of physical association with SAP97 blocks the dendrite growth-promoting action of this subunit.

In the next set of experiments we added a palmitoylation sequence to SAP97 (palSAP97) and we show that this leads to membrane targeting of the protein. Both SAP97 and palSAP97 have equivalent dendrite growth-promoting actions when overexpressed in neurons. Armed with this tool we took two approaches to look at the necessity of a physical interaction between GluA1 and SAP97 for the promotion of dendrite growth. First, we asked if coexpression of palSAP97 with GluA1Δ7 rescued the dendrite growth-promoting activity of this version of GluA1. Remarkably, the combination of palSAP97 with GluA1Δ7 promoted dendrite growth to the same degree that the combination of SAP97 + GluA1 did (Zhou et al. 2008).

In our final set of in vitro studies, we palmitoylated the version of SAP97 that contained mutations in PDZ2 that disrupted its physical association with GluA1 (mutPDZ2-palSAP97). In coexpression studies in heterologous cells, we found that GluA1 will co-IP palSAP97, but will not co-IP mutPDZ2-palSAP97 (Zhou et al. 2008). So, even though mutPDZ2-palSAP97 targets to the plasma membrane, this is not sufficient to lead to a physical association with GluA1. We then asked about the dendrite growth-promoting action of mutPDZ2-palSAP97 and we found that when coexpressed with GluA1, both palSAP97 and mutPDZ2-palSAP97 were equally effective in promoting dendrite growth (Zhou et al. 2008). Thus, using two different strategies to disrupt the physical association of SAP97 with GluA1, we come to the same conclusion: coexpression of SAP97 with GluA1 synergistically promoted dendrite growth as long as both proteins are targeted to the plasma membrane. While the native proteins associate as part of a physical complex, experimental manipulations that delink the two proteins demonstrate that colocalization, not physical interaction, are required for dendrite growth.

Potential Implications

Why should we care about this pathway of activity-dependent neuronal plasticity? One reason is that knowledge of this pathway may lead to ways of promoting plasticity in adults. One potential beneficiary might be individuals with spinal cord injury (Kalb 2003). After a thoracic spinal cord lesion, the circuitry in the lumbar spinal cord can be engaged by repetitive activation of selected neuronal pathways (e.g., standing training, ambulation training) which results in remarkable improvement in motor behavior. This is seen both in experimental animals and humans (Barbeau and Rossignol 1987; Dietz et al. 1995; Edgerton et al. 1997, 2004; Fung et al. 1990; Lovely et al. 1990; Rossignol 2000; Wernig et al. 1995, 1998; Wirz et al. 2001). The mechanism for this effect is an use-dependent modification of spinal cord circuitry (Gazula et al. 2004) and so we think that enhancement of activity-dependent plasticity within the spinal cord will have a salubrious effect on functional recovery.

Another reason to study this form of activity-dependent neuronal plasticity relates to developmental disorders of brain. Abnormalities in dendrite structure (i.e., size, branching, and spines) are commonly seen in childhood diseases such as mental retardation, autism, and autism-spectrum disorders (Dierssen and Ramakers 2006;

Kaufmann and Moser 2000). Several lines of evidence indicate that in some forms of these childhood diseases, the primary defect is in activity-dependent development. Many genes linked to familial forms of impaired cognitive and emotional development are involved in activity-dependent synapse formation or stabilization (i.e., actin-related proteins such as cofilin, LIMK, and debrin; Rho-GTPase regulators such as oligophrenin-1 and Kalirin-7; and trophic factors such as BDNF, NRGN-ErbB4; Lin and Koleske 2010). In a study using homozygosity mapping to discover recessive disease genes in autistic patients (Morrow et al. 2008), significant genetic heterogeneity was found. One of the more remarkable findings of this study was that many autism-associated genes are regulated by neuronal activity. For example, the expression of the candidate gene DIA1 is regulated by activity and this transcription factor that controls the expression of other activity-regulated transcripts such as MEF2, NPAS4, CREB, EGR, SRF, and others. If we start with the proposition that perturbation of experience-dependent cortical development underlies some of the defects in autism, then it is critical to understand the varieties of normal activity-dependent development. In this regard, it is perhaps noteworthy that genetic studies link SAP97 to schizophrenia (Sato et al. 2008; Toyooka et al. 2002) and autism (Willatt et al. 2005). It is possible that exploration of motor system development will provide a window onto previously unknown aspects of brain operation.

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