Emerging roles for MAP kinases in agrin signaling

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Abbreviations: AChR, acetylcholine receptor; BDNF, brain-derived neurotrophic factor; CREB, cAMP-response element binding protein; Dok-7, docking protein-7; ECM, extracellular matrix; ERK1/2, extracellular-signal regulated kinases 1 and 2; JNK, c-jun NH2-terminal kinase; Lrp4, LDL receptor related protein 4; MAPK, mitogen-activated protein kinase; MAP2K, MAPK kinase; MAP3K, MAPK kinase kinase; MuSK, muscle-specific kinase; NMJ, neuromuscular junction; PMA, phorbol 12-myristate 13-acetate; Rapsyn, receptor-associated protein at the synapse

Information between neurons and the target cells they innervate passes through sites of functional contact called synapses. How synapses form and are altered by sensory or cognitive experience is central to understand nervous system function. Studies of synapse formation and plasticity have concentrated on a few “model” synapses. The vertebrate neuromuscular junction (NMJ), the synapse between a motoneuron in the spinal cord and a skeletal muscle fiber, is one such model synapse. The extracellular matrix proteoglycan agrin plays an essential organizing role at the NMJ. Agrin is also present at some synapses in the brain and in other organs in the periphery, but its function outside the NMJ is unclear. The core signaling pathway for agrin at the NMJ, which is still incompletely defined, includes molecules specifically involved in this cascade and molecules used in other signaling pathways in many cells. Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved components of intracellular signaling modules that control a myriad of cellular processes. This article reviews emerging evidence that suggests that MAPKs are involved in agrin signaling at the NMJ and in the putative functions of agrin in the formation of a subset of synapses in the brain.

Agrin

All chemical synapses exhibit a high concentration of neurotransmitter receptors in the postsynaptic membrane. This ensures that receptor concentration is not limiting for neurotransmission. Perhaps no other synapse possesses as high a density of receptors as the mature vertebrate neuromuscular junction (NMJ), the synapse between a motoneuron and a skeletal muscle fiber. At the NMJ, acetylcholine receptors (AChRs) are clustered at about 10,000/μm².1 Studies on regeneration of adult neuromuscular synapses suggested that signals in the extracellular matrix (ECM) in the synaptic cleft, the narrow space that separates the nerve terminal from the synaptic sarcolemma, direct the clustering of AChRs at the NMJ.2,3 Agrin was purified from ECM extracts of Torpedo electric organ on the basis of its ability to induce aggregation of AChRs on cultured myotubes.4 Agrin is a large (~400–600 kDa) heparan sulfate proteoglycan that accumulates at the synaptic basal lamina through its binding to laminin.5,6 Agrin is expressed by motoneurons and muscle fibers. However, cell-specific alternative splicing of exons encoding selective amino acid sequences in the C-terminal domain of the protein, generates isoforms with very different AChR clustering activities.7 The isoforms produced by motoneurons, called agrin-z⁺ in mammals,8 are endowed with clustering activity. Isoforms made by muscle cells (agrin-z⁻) essentially lack aggregating activity. Agrin-z⁺ is widely expressed in the periphery, particularly in lung, kidney and immune cells.9 Agrin-z⁻ is also a major structural component of the basement membrane in the blood-brain barrier.10 Agrin-z⁻ is restricted to neurons in the brain and spinal cord.9

Mitogen-activated Protein kinases

Mitogen-activated protein kinases (MAPKs) are components of intracellular signaling modules that control a myriad of cellular processes.11 MAPK modules have three critical protein kinase components. The most downstream component, the actual MAPK, is the S/T kinase that phosphorylates the various cellular proteins, such as transcription factors, cytoskeletal elements or other protein kinases, that are the targets of regulation by signaling cascades initiated at the cell surface. A MAPK is activated by an upstream MAPK kinase (MAP2K). MAP2Ks are unusual in that they phosphorylate both T and Y residues on MAPKs to activate them. A MAP2K is in turn activated by an MAP2K kinase (MAP3K). MAP3Ks are generally at the receiving end of activating signals coming from small, monomeric GTPases such as the Ras family or by other more elaborate mechanisms.12,13 In mammalian cells the prototypical MAPK module consists of the MAPKs ERK1/2 (extracellular-signal regulated kinases 1 and 2), the MAP2Ks MEK1/2 and the MAP3K Raf. Parallel MAPK modules assemble upstream of the MAPKs JNK (c-Jun NH2-terminal kinase) and p38,11,13,14 ERK1/2 is implicated in the regulation of normal cellular responses to multiple growth factors and cytokines during proliferation and differentiation. In general, JNK and p38 play essential roles in the cellular responses
MAPKs in Agrin Signaling at the NMJ

Gain- and loss-of-function manipulations of the agrin gene have established the essential role of agrin-z’ in the formation of the NMJ in vivo.\(^{35,36}\) To induce AChR clusters and other aspects of the postsynaptic apparatus, agrin-z’ binds Lrp4 (LDL receptor related protein 4),\(^{19,20}\) and activates the receptor tyrosine kinase MuSK (muscle specific kinase),\(^{21}\) on the sarcolemma. In addition, Rapsyn (receptor-associated protein at the synapse) and Dok-7 (docking protein-7) are two intracellular proteins that critically mediate agrin’s synaptogenic activity in vitro and in vivo.\(^{22,23}\) Rapsyn is an intracellular peripheral membrane protein that binds to AChRs and links them to the actin cytoskeleton. Dok-7 is a phosphotyrosine-binding protein that binds to activated MuSK. Herbst and Burden\(^{24}\) were the first to report a weak ERK1/2 activation after 30 min of agrin-z’ treatment in cultured myotubes. The ERK activity returned to control levels by 60 min. We recently reported that agrin-z’-induced ERK1/2 activation is quite robust if examined within 10 min after agrin addition, also returning to baseline by 60 min.\(^{25}\) Thus, agrin induces a transient ERK1/2 activation in myotubes. This activation is Lrp4/MuSK-dependent as it was not observed in myotubes deficient for either of these proteins following agrin-z’ treatment.\(^{29}\) Blocking agrin-induced ERK1/2 activation using highly selective chemical MEKI1/2 inhibitors (U0126 and PD0325901,\(^{26}\)) failed to prevent AChR clustering. However, it led to a potentiation of agrin-induced AChR clustering.\(^{25}\) This result suggested that agrin-induced ERK1/2 activation is dispensable for AChR clustering, but is part of a negative feedback loop that regulates the activity of agrin-z’. This conclusion was supported by our finding that PMA (phorbol 12-myristate 13-acetate), a classical inhibitor of agrin-induced AChR clustering in myotubes,\(^{27}\) requires ERK1/2 activity for this effect.\(^{25}\) Thus, PMA appears to co-opt the negative feedback loop mediated by agrin-induced ERK1/2 activation to inhibit AChR clustering. The biological significance of this negative feedback loop remains to be tested in vivo, however, one can speculate that by restraining the clustering activity of agrin, agrin-induced ERK1/2 activation may help localize the synaptogenic signal in space and time to ensure precise apposition of pre- and postsynaptic components, which is critical for synapse structure and function. In the near future it will be important to determine the downstream substrates of agrin-induced ERK1/2 activation. AChRs, Lrp4, MuSK, Rapsyn and Dok-7 should be examined as potential targets of phosphorylation by agrin-activated ERK1/2. Neuregulin-1 and the neurotrophins BDNF (brain-derived neurotrophic factor) and NT-4 can inhibit agrin-induced AChR clustering in cultured myotubes.\(^{28,29}\) All of these factors are known to engage MAPK pathways in many cell types. However, neuregulin-1 does not require ERK1/2 activation to inhibit agrin-induced AChR clustering,\(^{25}\) while it remains to be investigated whether BDNF and NT-4 use MAPKs to exert their effect on AChR clustering.

Transcription of postsynaptic genes such as those encoding AChR subunits and MuSK is highly enriched at synaptic myonuclei. This mechanism supports the high density of postsynaptic proteins at the NMJ. The agrin-Lrp4-MuSK-Dok-7 pathway provides a signal that drives this synapse-specific expression. Prives and colleagues first showed that agrin could activate JNK transiently in cultured myotubes in a Rac/Cdc42-dependent fashion.\(^{30}\) Although the Rho family GTPases Rac and Cdc42 appear to play important roles in agrin-induced AChR clustering in cultured myotubes,\(^{30}\) the JNK activation they stimulate was shown dispensable for it.\(^{31}\) However, Brenner and co-workers showed that JNK activation downstream of agrin-MuSK-Rac/Cdc42 is necessary for transcription of MuSK and AChR encoding genes both in myotubes and adult muscle fibers.\(^{32}\) In this cascade, Rac/Cdc42 seem to activate the JNK-selective MAP2K7.\(^{32}\) Thus, agrin-induced JNK activation plays a role in synapse-specific gene expression.

MAPKs in Agrin Signaling in the Brain

In the central nervous system, MAPKs have been implicated in regulating molecular mechanisms of synaptic plasticity underlying learning and memory.\(^{33}\) The precise function of agrin in the CNS remains elusive. Agrin does not appear as indispensable for general interneuronal synapse formation as it is for neuromuscular synaptogenesis. However, agrin immunostaining co-localizes to a subset of excitatory synapses in the adult mouse brain, and most importantly, there is a selective 30% reduction in the number of excitatory synapses in the adult cortex in mice deficient for agrin in brain and other tissues but not in spinal cord (NMJ-rescued agrin-deficient mice).\(^{34}\) In contrast to standard agrin-deficient mice that are stillborn,\(^{17}\) these mice survive into early adulthood because they express agrin-z’ selectively in motoneurons.\(^{34}\)

The molecular mechanisms underpinning the brain phenotype of the rescued mice are unclear. An increase in the synaptic GTPase-activating protein SynGAP was the most significant change detected in these mice by western blot analysis of various excitatory synaptic components.\(^{34}\) SynGAP is a negative regulator of ERK1/2 activation at excitatory synapses,\(^{35}\) which suggests that synaptic levels of active ERK1/2 may be decreased in NMJ-rescued agrin-deficient mice. In addition, microarray gene expression analysis showed that the JNK-selective MAP2K7 was reduced in NMJ-rescued agrin-deficient brains,\(^{34}\) which might also reduce JNK signaling. Thus, the reduction in synapses in the cortex of NMJ-rescued agrin-deficient mice correlated with a putative decrease in MAPK signaling.

In NMJ-rescued agrin-deficient mice, there were significant reductions in dendrite length, spine number per dendrite and spine density.\(^{36}\) Spines are the sites on dendrites where postsynaptic specializations of most excitatory synapses reside. Filopodia—actin-rich, thin cytoplasmic projections—are thought of as the precursors of spines during excitatory interneuronal synaptogenesis.\(^{36,37}\) A reduction in dendritic filopodia during development would be expected to impact on the number of synapses that form. Agrin promotes formation of filopodia by a mechanism that involves MAPK activation.\(^{38}\) Alternative promoter usage generates two agrin isoforms with
different N-terminal domains encoded by two distinct first exons. One form is secreted and has an N-terminal domain that binds to laminin in the ECM, while the other has a hydrophobic N-terminal domain that makes agrin into a type II transmembrane protein, enriched in dendrites and axons. Antibody-induced clustering or overexpression of transmembrane agrin in neuronal and non-neuronal cells induces filopodia. This activity depends on domains in the N-terminal third of the agrin protein, not overlapping with the C-terminal domains responsible for AChR clustering. ERK1/2 is likely to mediate filopodia induction by transmembrane agrin as PD98059, a MEK1/2 inhibitor, blocked this activity. The ability to induce filopodia on neurons is not limited to transmembrane agrin. Sonderegger and colleagues have reported that secreted agrin bound to the brain ECM can be cleaved by the synaptic serine protease neurotrypsin in an electrical activity-dependent fashion. A neurotrypsin-released, 22-kDa fragment containing the C-terminal domain of agrin induced dendritic filopodia in hippocampal slices from juvenile mice. 22-kDa fragments from both secreted agrin-z+ and agrin-z- have filopodia-inducing activity. Although Lrp4 and MuSK are expressed in the brain, agrin-z+ and agrin-z- have filopodia-inducing activity. The evidence above suggests that these receptors do not mediate the ability of transmembrane or neurotrypsin-cleaved agrin to induce filopodia on dendrites. Smith and colleagues have shown that the α3 subunit of the Na+/K+-ATPase is a good candidate to mediate the filopodia-inducing activity of secreted agrin in neurons. However, this remains to be tested directly. Interestingly, this agrin receptor appears to engage ERK1/2 to induce c-fos expression in cultured cortical neurons.

In cultured hippocampal neurons, agrin-z+ has also been shown to stimulate the phosphorylation of the transcription factor CREB (cAMP-response element binding protein) in an ERK1/2-dependent fashion. Phospho-CREB would then stimulate the expression of downstream genes with direct roles in synaptogenesis and/or genes that regulate dendrite length or branching, which would affect synapse numbers indirectly. Agrin-z+-induced CREB phosphorylation may be Lrp4/MuSK-dependent because knockdown of MuSK expression reduces CREB phosphorylation in hippocampus. However, further tests of this possibility are needed.

Soluble agrin-z+ inhibited axonal elongation in cultured hippocampal neurons via simultaneous activation of ERK1/2 and SREBP. The role of MAPK activation in this process in vivo is unclear, as it appears in conflict with the phenotype of the NMJ-rescued agrin-deficient mice.

In summary, results to date indicate that agrin-z+ activates MAPK signaling through Lrp4/MuSK at the NMJ to negatively regulate its AChR clustering activity via ERK1/2, and to stimulate synapse specific transcription of postsynaptic genes via JNK (Fig. 1A). In neurons, agrin activates ERK1/2 signaling to stimulate filopodial generation and to activate gene expression via CREB (Fig. 1B). A role for agrin-induced JNK activation in the brain is possible but remains undefined. Transmembrane agrin may act as a receptor of a yet unknown ligand to mediate filopodial formation, while secreted agrin may use α3 Na+/K+-ATPase (NKA) to stimulate filopodial formation. The involvement of MAPKs in this pathway is unknown (MAPK?). An unknown ligand may bind to transmembrane agrin (TM agrin), leading to formation of dendritic filopodia via activation of ERK1/2 downstream of the Src-family tyrosine kinase Fyn. These steps have not been confirmed experimentally. For references see text.
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