Agrin in the Developing CNS: New Roles for a Synapse Organizer

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The heparan sulfate proteoglycan agrin is responsible for the formation, maintenance, and regeneration of the neuromuscular junction. In the central nervous system, agrin is widely expressed and concentrated at interneuronal synapses, but its function during synaptogenesis remains controversial. Instead, evidence for additional functions of agrin during axonal growth, establishment of the blood-brain barrier, and Alzheimer’s disease is accumulating.

Fast synaptic transmission requires the precise apposition of pre- as well as postsynaptic specializations. For example, sites of calcium-dependent neurotransmitter release in the presynaptic nerve terminal must be directly apposed to aggregates containing the appropriate neurotransmitter receptor and associated proteins in the plasma membrane of the postsynaptic cell. The coordinate formation of these specializations during development, their maintenance in the adult organism, and their reformation during regeneration or plasticity-related events represent critical steps during synaptogenesis. Most of our knowledge regarding synaptic differentiation is derived from studies of the neuromuscular junction, where the exchange of specific signals between the motoneuron and the muscle fiber directs the coordinate and parallel differentiation of the motoneuron’s axon terminal and the muscle fiber’s postsynaptic apparatus. The key regulator of synaptogenesis at the neuromuscular junction is the extracellular matrix molecule agrin. As originally predicted by the agrin hypothesis (15), agrin is synthesized by motoneurons in the ventral horn of the spinal cord and transported along their axons to the neuromuscular junction, where it is released into the synaptic cleft. In the cleft, agrin becomes stably incorporated into the synaptic portion of the muscle fiber basal lamina and interacts with an unknown receptor in the muscle fiber plasma membrane. The interaction of agrin with its receptor then activates the muscle-specific tyrosine kinase MuSK. MuSK itself does not bind to agrin but most likely represents the signal-transducing part of a heteromeric agrin receptor complex. Activation of MuSK induces a poorly understood intracellular signaling cascade, which involves the phosphorylation of a large number of molecules, including the β-subunit of the acetylcholine receptor (AChR), resulting in the aggregation and stabilization of AChRs, acetylcholinesterase, sodium channels, and numerous other proteins involved in synaptic transmission at the neuromuscular junction. Agrin is also responsible for the formation of most if not all other postsynaptic specializations at the neuromuscular junction, including the aggregation of nuclei in the synaptic region of the muscle fiber, the formation of junctional folds in the muscle fiber plasma membrane, and the expression of synapse-specific proteins. Moreover, agrin directs the embryonic-to-adult switch of the AChR by promoting the expression of the AChR ε-subunit, possibly via a different signaling cascade, which involves neuregulin-mediated activation of tyrosine kinases of the ErbB receptor type. In addition, agrin might also trigger the differentiation of the motoneuron growth cone to a presynaptic terminal capable of calcium-dependent neurotransmitter release (for review, see Ref. 18).

Agrin immunoreactivity is highly concentrated at the adult neuromuscular junction but is also present, albeit in smaller concentrations, in extrasynaptic regions of the muscle fiber, particularly during early embryonic development. When muscle tissue is injured, individual muscle fibers degenerate. After phagocytosis of all cellular elements, agrin immunoreactivity remains associated with the synaptic portion of the muscle fiber basal lamina for several weeks, suggesting that the ability to reform pre- and postsynaptic specializations at the regenerating neuromuscular junction is also mediated by agrin.

Mice with a targeted inactivation of the agrin gene have gross defects in both pre- and postsynaptic structures at their neuromuscular junctions (for review, see Ref. 18). The ingrowing motoneurons branch and continue to grow over the muscle fiber surface and do not differentiate into presynaptic terminals. In addition, no postsynaptic specializations are formed and the few spontaneous AChR aggregates that form in the absence of agrin are not localized at the contact site between motoneuron and muscle fiber. Accordingly, synaptic transmission is severely compromised and homozygous mice die during the last fetal day or immediately after birth due to nonfunctional respiratory musculature. Inactivation of the MuSK gene has a similar phenotype at the neuromuscular junction. Moreover, myotubes taken from MuSK knockout animals are incapable of aggregating AChRs in vitro in response to agrin, demonstrating that downstream activity of MuSK is required for agrin signaling and that agrin and MuSK are necessary for the formation of the neuromuscular junction. Furthermore, ectopic expression of agrin by skeletal muscle fibers induced by injection of agrin-secreting cells or of agrin cDNA into individual myotubes of innervated or denervated muscle results in the synthesis, secretion, and extracellular deposition of exogenous agrin in the muscle fiber basal lam-
ina at extrasynaptic sites. This extrasynaptic agrin induces the formation of postsynaptic specializations, including the formation of activity-resistant aggregates containing the AChR, acetylcholine esterase, and MuSK, aggregation of nuclei, expression of the AChR/G48-subunit, and formation of junctional folds at the site of agrin secretion, demonstrating that agrin is not only necessary but also sufficient for the formation of at least the postsynaptic specializations at the neuromuscular junction (for review, see Ref. 18).

**Structure of the agrin cDNA**

Agrin has been cloned from several species, and the sequences are highly homologous. In all species, the agrin cDNA predicts a number of modules similar to domains found in other extracellular matrix proteins, including domains with similarity to epidermal growth factor, follistatin, and the globular domains of the laminin G44-chain (Fig. 1). After rotary shadowing, agrin appears as an ~95-nm-long particle that consists of a globular, NH2-terminal (NtA) domain, a central rod-like region predominantly formed by the follistatin-like domains, and three globular, COOH-terminal laminin G-like domains. The NtA domain of agrin is responsible for the binding of agrin to basal laminae, most likely by interacting with the γ1-subunit in the long arm of the corresponding laminin heterotrimers. The G1 and G2 domains of agrin are involved in the binding of agrin to dystroglycan, a central component of the dystrophin-glycoprotein complex (2). The COOH-terminal G3-domain (Fig. 1) is necessary and sufficient for the aggregation of AChRs, acetylcholine esterase, and other molecules at the neuromuscular junction. The functions of the other domains are not known, but their presence suggests that agrin might interact with additional, not-yet-identified proteins. The central rod-like domain of agrin contains attachment sites for glycosaminoglycan side chains characteristic of heparan sulfate proteoglycans, resulting in a molecular weight of native agrin of >500 kDa. These glycosaminoglycan side chains of agrin have been implicated in potentiating the hemophilic adhesion of the neural cell adhesion molecule NCAM and the binding of growth factors like heparin-binding growth-associated molecule.

Multiple isoforms of agrin are generated by using alternative splicing at several positions (Fig. 1). These isoforms differ dramatically in their biological activity and in their distribution. The presence of a peptide insert of either 8, 11, or 19 amino acids at a splice site in the COOH-terminal region called “B” in chick and “z” in rodents is necessary for the synaptogenic activity at the neuromuscular junction. Isoforms with a peptide insert at this site (agrin B/z+ isoforms) have specific AChR aggregation activity in the pico- and nanomolar range. Isoforms without a peptide insert at site B/z (agrin B/z0 isoforms) have little or no influence on the distribution of AChRs at the neuromuscular junction in vivo or in vitro. However, these B/z0 isoforms represent the majority of the agrin transcripts in nonneural tissues, including muscle, kidney, blood vessels, and the immune system.

An alternative first exon can be used in the NH2-terminal region to generate agrin isoforms that do not contain the NtA domain and are not secreted but instead immobilized in the plasma membrane with the amino terminus being localized in the cytoplasm and the carboxy terminus facing the extracellular environment (4, 16). Expression of this transmembrane form of agrin is regulated by a distinct promoter, and transcripts coding for transmembrane agrin are enriched in the embryonic central nervous system (CNS), particularly in growing neurons. Since mice in which the secreted form of agrin has been selectively inactivated show the same phenotype at the neuromuscular junction as mice that do not express any agrin at all, the transmembrane form most likely is not directly involved in formation of this particular synapse (4). Although the intracellular sequence of transmembrane agrin is only ~30 amino acids long and does not display significant similarity to...
Expression of agrin in the CNS

Although there is no doubt about agrin’s essential role during formation of the neuromuscular junction, its precise function in other tissues, including kidney and the immune system, remain unresolved. The morphological, biochemical, and functional similarity of the neuromuscular junction with synapses in the CNS together with the widespread expression of all agrin isoforms in the CNS particularly during development suggested that agrin might play a similar role during formation of interneuronal synapses to the role it plays at the neuromuscular junction. Consistent with this hypothesis, agrin has been shown to be concentrated in the synaptic cleft of interneuronal synapses (Fig. 2; Refs. 10 and 13); it has also been shown that agrin is enriched in synaptosomal preparations from CNS (3), that agrin-mediated AChR aggregation activity can be extracted from CNS tissue, and that agrin is secreted in cultures of CNS tissue (13). Transcripts coding for agrin isoforms are not confined to cholinergic neurons but are expressed throughout the CNS by both neurons and glial cells. The levels of agrin transcripts in the developing CNS are highest during the phase of axonal elongation and synaptogenesis but downregulated thereafter, so that only small amounts of agrin are expressed in the adult CNS. However, agrin mRNA expression in specific regions of the adult rat brain is increased upon induction of seizures (17), suggesting that agrin expression can be regulated by neuronal activity. The changes in the agrin expression pattern after seizure induction are rapid and long lasting, consistent with a role of agrin during synaptogenesis or synaptic plasticity in the adult CNS.

Unfortunately, directly examining the function of agrin during CNS synaptogenesis in vivo has proved to be difficult, because agrin knockout mice die at birth and synaptogenesis in most regions of the brain does not start before the first or second week postnatal. Hippocampal tissue from embryonic day 18 (E18) agrin knockout mice show synapses that are indistinguishable in number and morphology from wild-type tissue (19). Similarly, cultures from hippocampus or cortex of agrin knockout mice were indistinguishable from parallel cultures derived from wild-type or heterozygous mice and showed normal distributions of glutamate and GABA receptors and normal function of synaptic transmission, indicating that the absence of agrin does not influence formation of interneuronal synapses. Thus, although agrin is a key regulator during formation of the neuromuscular junction, synaptogenesis of CNS neurons in vivo does not appear to be critically dependent on agrin. This result suggests that neurons in the CNS use different and/or additional mechanisms during synaptogenesis than motoneurons.

In contrast to the results obtained from the agrin knockout mice in vivo, acute inhibition of agrin synthesis in CNS cultures of wild-type animals by antisense oligonucleotides or function-blocking antibodies results in the formation of fewer functional synapses, a decrease in the size, frequency, and probability of evoked and spontaneous miniature excitatory postsynaptic currents between neighboring neurons, an impairment of dendritic development, a selective inhibition of the clustering of the synapse-associated protein synGAP, but not of N-methyl-D-aspartate-sensitive glutamate receptors, and a possibly impaired synaptic vesicle cycling (3). The density and ultrastructural morphology of pre- and postsynaptic specializations, however, appeared to be indistinguishable between cultures from wild-type and agrin knockout mice, suggesting that other molecules are responsible for the morphological differentiation of CNS synapses. Interestingly, these effects could not be evoked by the same oligonucleotides and antibodies in cultures from agrin-deficient mice, indicating that these mice have activated different and possibly agrin-independent mechanisms for synapse formation. Thus one explanation for the apparently normal synaptogenesis in the CNS of agrin-deficient mice might be the activation of compensatory mechanisms for synaptogenesis during early phases of neuronal development. The molecular nature of the compensatory mechanisms is currently unknown, but the results suggest that agrin is at most only one of possibly several regulators involved in the formation of interneuronal synapses.

Nonsynaptogenic roles for agrin in the CNS

Within the CNS, agrin is expressed both by neurons and by glial cells, suggesting that agrin has additional functions not related to synapse formation. Whereas CNS glial cells exclusively express the isoforms inactive in AChR aggregation (B/z0 isoforms), neurons express B/z+ as well as B/z0 isoforms. In fact, individual neurons can express more than one agrin isoform at the same time. The physiological significance of this observation is unknown, but it suggests that agrin expressed by neurons might have more than one function and that different agrin isoforms might be segregated into different neu-

FIGURE 2. Agrin is concentrated in the synaptic cleft of interneuronal synapses. Anti-agrin antiserum was used to label agrin in the retinal inner plexiform layer and was visualized with a peroxidase-conjugated secondary antibody. The peroxidase reaction product was silver intensified and gold toned. Because of the fixation sensitivity of the epitope, only low concentrations of the fixative could be used, resulting in a suboptimal preservation of the synaptic ultrastructure. However, a clear concentration of agrin immunoreactivity can be observed in the synaptic cleft.
Agreement with the cell-specific expression pattern of different agrin transcripts, agrin proteins are also differentially distributed in the CNS. Whereas in the adult CNS the B/z0 isoforms are particularly prominent in the basal laminae of blood vessels and of the pial lining of the brain (1, 12), B/z+ isoforms are enriched in the synapse-containing layer (Fig. 3; Ref. 11). The role of agrin associated with the blood vessel basal lamina is unclear, but agrin immunoreactivity accumulates in this basal lamina at the same time at which blood vessels become impermeable, suggesting that agrin might be involved in the formation of the blood-brain barrier (1). Since endothelial cells as well as perivascular astrocytes have been reported to synthesize agrin, the cellular origin of CNS blood vessel-associated agrin remains to be determined.

Addition of agrin to cultured myotubes induces a redistribution of the AChRs into characteristic aggregates that resemble, by a number of criteria, the postsynaptic apparatus of the neuromuscular junction. Conflicting results were reported regarding the effect of recombinant agrin on synaptogenesis in cultures of CNS neurons. One study reported that addition of recombinant soluble agrin isoforms to hippocampal neurons expressing normal levels of agrin did not affect presynaptic or postsynaptic differentiation and did not induce the formation of aggregates containing neurotransmitter receptors and associated proteins (19). Another study claimed that the number of synapsin-1 and synaptophysin-positive puncta in cultured CNS neurons (corresponding to putative synapses) was increased in the presence of exogenous agrin (14). Thus it remains to be determined whether exogenous agrin is able to induce ectopic accumulation of synaptic molecules on CNS neurons, similar to the formation of synaptic specializations on cultured muscle fibers. However, addition of exogenous agrin to CNS cultures results in a number of additional changes in neurons, particularly in their gene expression pattern. The B/z+ but not the B/z0 agrin isoforms induce the rapid phosphorylation, and thereby activation, of the transcription factor cAMP response element binding protein (CREB) in hippocampal neurons in a calcium- and tyrosine kinase-dependent manner, demonstrating that CNS neurons can respond to exogenous agrin and that this interaction is mediated by the protein backbone and not by the glycosaminoglycan side chains (9). This activation of CREB suggests the possibility that agrin in CNS neurons might have an influence on gene expression, similar to its effect on transcription at the neuromuscular junction. However, the precise intracellular signaling cascade leading to the phosphorylation of CREB and the genes that are activated in response to exogenous agrin are unknown.

The activation of CREB in CNS neurons was isoform specific, and only the B/z+ isoforms of agrin could induce the phosphorylation. In contrast, all agrin isoforms were able to induce the expression of the immediate-early gene c-fos when added to cortical, hippocampal, or cerebellar neurons (7). This suggests that both neuronal responses to agrin are mediated by different receptors, only one of which is isoform specific.
Moreover, induction of c-fos expression could be induced by activation of the nicotinic or glutamate receptors, and the increase in the expression of c-fos was significantly lower in cultured cortical neurons from agrin-deficient mouse embryos compared with wild-type embryos (8). Overactivation of ionotropic glutamate receptors is excitotoxic to cortical neurons. The reduction in glutamate receptor-mediated c-fos expression therefore predicts a concomitant resistance of the agrin-deficient neurons to glutamate-mediated excitotoxicity. Indeed, cultured agrin-deficient neurons are about fourfold less sensitive to excitotoxic damage compared with their wild-type siblings. Agrin-deficient neurons also exhibit reduced levels of c-fos expression following activation of voltage-gated calcium channels. Moreover, in vivo kainate-induced seizures had a much more dramatic effect on wild-type mice compared with heterozygous agrin-deficient animals, demonstrating that the absence of agrin renders mice less sensitive to glutamate-mediated cytotoxicity (8). Although the physiological significance of CREB phosphorylation and c-fos induction remain to be elucidated, the results suggest a role for agrin in the regulation of the response of cortical neurons to excitatory neurotransmitters. The results also demonstrate that agrin, similar to its role in skeletal muscle, is an extracellular and sometimes isomeric-specific initiator of gene transcription in CNS neurons.

In the CNS, agrin (particularly the transmembrane form) is highly expressed on neurons before synapse formation at the time of active axonal growth toward their target cells (6, 10, 16, 19), suggesting that agrin might play a role during neurite elongation and pathfinding. Accordingly, acute inhibition of agrin synthesis or function by antisense oligonucleotides or anti-agrin antibodies, respectively, reduced the rate of axonal arborization, whereas the same treatment did not alter the rate of axonal elongation (3). Addition of recombinant agrin to cultures of hippocampal neurons decreased the rate of elongation of main axons but induced the formation of more axonal branches. Conversely, cultured hippocampal neurons depleted of agrin extended longer, nonbranched axons compared with control cultures with unaltered agrin expression (14). In addition, dendrites grown in the presence of agrin were significantly longer and more branched compared with untreated controls, indicating that agrin might have different effects on axonal and dendritic processes. Agrin itself does not appear to be a suitable substrate for neuronal elongation, since sensory motoneurons grown on Chinese hamster ovary cells transfected with agrin extended shorter processes compared with control cultures growing on untransfected cells. Moreover, purified agrin used as a substrate for retinal ganglion cells did not support axonal growth, and a combination of agrin and laminin-2 appeared to be inhibitory when used as a substrate, whereas laminin-2 alone supported axonal elongation (6).

Agrin function in Alzheimer’s brain

The human agrin gene has been mapped to chromosome 1 region pter-p32, but no disease has so far been associated with this particular chromosomal region, indicating that mutations in the agrin gene might be either lethal or nonsymptomatic for humans. Although mutations in the agrin gene might not cause a particular disease, the agrin protein is associated with pathological events in the CNS. Recent studies suggest the possibility that agrin plays a role in the etiology of Alzheimer’s disease, the most frequent cause of dementia in older persons. Agrin is expressed at high levels in brains of Alzheimer’s patients and represents the major proteoglycan associated with neurofibrillary tangles, cerebral amyloid angiopathy, and diffuse and neuritic plaques, morphological hallmarks characteristic for brains from Alzheimer’s patients (20). Agrin immunoreactivity is colocalized with cerebrovascular deposits of amyloid β-protein (Aβ-protein) in brains from Alzheimer’s patients. In vitro agrin binds to fibrillar but not to nonfibrillar Aβ, and this binding involves the glycosaminoglycan side chains. Binding of agrin to Aβ accelerates Aβ-fibril formation, protects Aβ from proteolysis, and alters the solubility of agrin and Aβ (5), suggesting that agrin might not only be a structural component of senile plaques but might also be an important factor in Aβ-peptide deposition, aggregation, and/or persistence and thus might play a role in the progressive pathogenesis observed in Alzheimer’s patients.

Although a number of questions remain to be answered, agrin’s essential role during formation of the neuromuscular junction is undisputed. In contrast, agrin’s function in the developing and adult CNS is only beginning to emerge. Although an involvement of agrin in CNS synaptogenesis remains possible, other molecules clearly contribute to the differentiation of the pre- and postsynaptic specializations during interneuronal synapse formation. The widespread expression of agrin isoforms in neurons and glial cells, the mosaic structure of the protein enabling agrin to interact with a large number of proteins, as well as the discovery of a transmembrane form of agrin, however, suggest much broader roles for agrin during CNS development, possibly involving functions during axonal growth, establishment of the blood-brain barrier, and in the etiology of Alzheimer’s disease.

We apologize to all colleagues whose work could not be cited due to limitation in available space.

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