

ABSTRACT

KIM, MIN JUNG. Characterization of agrin function in chicken and zebrafish embryogenesis. (Under the direction of Dr. Gregory J.Cole)

Agrin is an extracellular matrix heparan sulfate proteoglycan that plays a key role in the development of the neuromuscular junction (NMJ) by inducing the clustering of acetylcholine receptors at synaptic sites of the NMJ. Although recent studies have extended our understanding of agrin's function in the nervous system, its function in the CNS is not clearly understood.

The present study was undertaken to assess the role of agrin in neurite outgrowth mediated by the basic fibroblast growth factor (FGF-2), using both PC12 cells, and chick retina neuronal cultures. Agrin increases the efficacy of FGF-2 stimulation of neurite outgrowth, as an inhibitor of the FGF receptor abolished neurite outgrowth in the presence of agrin and FGF-2. Agrin augments and sustains a transient early phosphorylation of ERK (extracellular signal-regulated protein kinase) in the presence of FGF-2. Neural agrin contributes to the establishment of axon pathways by modulating the function of neurite promoting molecules such as

FGF-2.

To overcome the lethality of agrin gene disruption and the difficulty of embryonic manipulation of agrin function in mice, a gene encoding zebrafish agrin was identified and characterized. Zebrafish agrin is expressed in the developing CNS, the NMJ, and non-neural structures such as the pronephric duct, and endodermal tissues. A morpholino-based gene targeting against agrin significantly impair development of tail and the NMJ, and cause severe defects in motor neuron axon outgrowth and formation of the midbrain-hindbrain boundary, eye, and otic vesicles. Morphants subsequently develop paralysis, and die at larvae stages.

Knockdown of agrin in zebrafish strikingly resembles phenotypes of zebrafish FGF-related mutants, such as disruption of the MHB, optic and otic vesicles during zebrafish development. Inhibition of FGFR synergizes defects from agrin knockdown resulting in MHB disruption, a shortened tail, small eyes and otic vesicles, which suggest that agrin modulates the activity of FGF signaling pathways.

In conclusion, my studies show that agrin is essential for NMJ formation as well as sensory and motor neuron axonal growth and pathway formation in zebrafish development. Importantly, the HSPG agrin may be involved in regulation of early CNS development via maintenance and regulation of FGF signaling.

**CHARACTERIZATION OF AGRIN FUNCTION IN CHICKEN
AND ZEBRAFISH EMBRYOGENESIS**

by

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DEDICATION

To my parents,
Han-Bae Kim and Soon Jin Oh,
Who made all of this possible,
With their endless encouragement and support,
I could follow my heart's decision and passion.

also to

My lovely husband,
Younghoon Baek,
Who gave me endless love, patience and support.

BIOGRAPHY

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The heart decides, and what it decides is all that really matters.

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INTRODUCTION

Proteoglycans are glycosylated proteins that have covalently linked sulfated glycosaminoglycans, which are attached to serine residues of the protein core (Kjellen and Lindahl, 1991). Glycosaminoglycans (GAGs) are different disaccharide repeated polymers that are generated by the structural modification of deacetylation, sulfation and epimerization that provide diverse biological activities (Lindahl, 1990). These various structural modified GAG chains are critical for developmental processes as well as in pathogenesis. Proteoglycans are present in the extracellular matrix, such as cartilage, basement membrane and connective tissues, as well as at the cell surface. The types of GAGs can be divided into several groups, which are chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, and keratan sulfate (KS) (Figure 1). The structure of GAGs associated with proteoglycans exhibit distinct differences in the amino sugars found in CS-GAGs and HS-GAGs (N-acetylgalactosamine and N-acetylglucosamine, respectively) and in the pattern of sulfation in the individual GAGs. CS-GAGs typically contain higher levels of sulfate residues than HS-GAGs, with one sulfate per repeating disaccharide. HS-GAG contains sulfate on both the uronic acid group and amino sugar group, which gives more variability in sulfation, including the low sulfation regions that are spaced between higher sulfation regions. The variable,

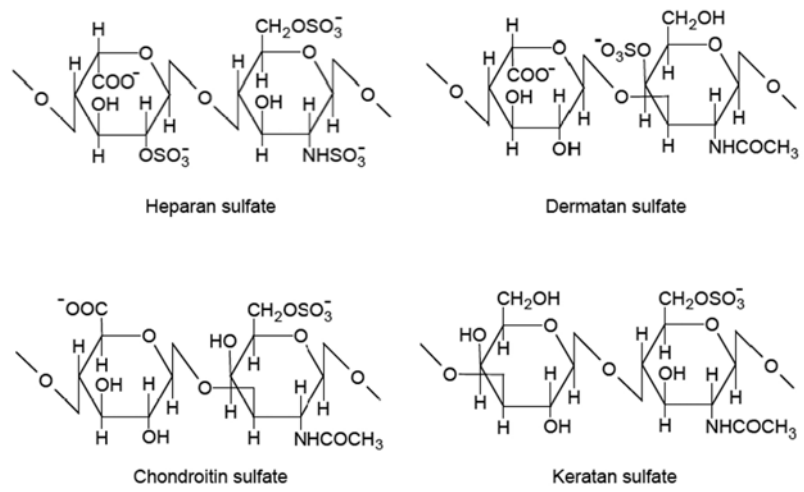


Figure 1. The structure of glycosaminoglycans in proteoglycans.

higher sulfated domains serve as HS-GAG binding domains of HSPGs to heparin-binding proteins to provide discrete functions (Aviezet et al, 1994; Sanderson et al., 1994; Cotman et al., 1999; Herndon et al., 1999; Knox et al., 2002). The change of sulfation pattern of HS-GAGs also has been shown to modulate the binding activity of specific HSPGs (Nurcombe et al., 1993; Brickman et al., 1998).

Among proteoglycans, heparan sulfate proteoglycans show the highest structural variability. Heparan sulfate proteoglycans are extracellular and cell surface macromolecules that consist of a core protein to which heparan sulfate glycosaminoglycan (HS-GAG) chains are attached. The major HSPGs can be divided into families based on structure of core protein, as well as cellular localization. Syndecans and glypicans are cell surface HSPGs (Sanderson and Bernfield., 1988; David et al., 1990; Filmus et al., 1995; Karthikeyan et al., 1992; Litwack et al., 1994; Stipp et al., 1994; Watanabe et al., 1995), and perlecan, agrin and collagen XVII are associated with the extracellular matrix (Lozzo., 1998; Olsen., 1999; Cole and Halfter., 1996; Halfter et al., 1998).

The most important roles for heparan sulfate proteoglycans have been demonstrated in cell adhesion, cell migration, modulation of growth factor function, organization of the extracellular matrix, inflammation, metastasis, and synapse formation. Importantly, recent studies of HSPGs have revealed numerous critical

biological roles in nervous system development. Development of the nervous system can be largely divided into three stages. First, neurons are generated from neural precursors and differentiate into specific cell types, such as neurons or glia, followed by cell migration to their appropriate CNS destination. Second, axons and dendrites extend from neurons toward target cells, and synaptic contacts are formed and mature. Third, a functional CNS requires maintenance and plasticity of synaptic arrangements in order to allow learning, memory, and other important brain functions to occur. During the process of neural development, various types of proteoglycans have been shown to have diverse functions and to interact with molecules essential to development, such as growth factors and morphogens.

In particular, the fibroblast growth factors (FGFs) have well demonstrated interactions with HSPGs in both embryonic development as well as adulthood. FGFs are secreted molecules that are involved in the regulation of cell survival, proliferation, migration and differentiation, as well as the patterning of body axes (Brickman et al., 1998; Vaccarino et al., 2001; Molteni et al., 2001; Perrone-Capano and Di Porzio., 2000; Mufson et al., 1999, Andersn et al., 1993; Grothe and Wewetzer., 1996). At early stages of embryogenesis, FGFs are required to induce mesoderm and establish the anteroposterior and dorsoventral body axis (Draper et al., 2003; Liu et al., 2003; Scholpp et al., 2004; Walshe and Mason, 2003; Wiellette and Sive, 2004; Furthauer et al., 2004). FGFs are also crucial factors for organogenesis,

especially growth and patterning of the brain, the initiation of limb buds, and tooth morphogenesis (Liu et al., 2003, Scholpp et al., 2003; Walshe and Mason, 2003, Wiellette and Sive, 2004).

24 FGF family members and 4 FGF receptors have been identified. The molecular weights of vertebrate FGF proteins are 17 to 34 kDa. FGFs consist of 28 highly conserved domains and six identical amino-acid residues (Otnitz., 2000). The core protein of FGF-1 and FGF-2 is composed of 12 antiparallel β -strands, which serve as the heparin-binding domain (Moy et al., 1996; Li and Seddon., 1994). Most FGFs have N-terminal signal peptides, which allows FGFs to be readily secreted from cells into the extracellular space. However, FGF-1, -2, -9, -16 and -20 lack conventional signal peptides, and rather are secreted molecules which may be released from cells by a mechanism independent of the endoplasmic reticulum-Golgi pathway (Mignatti et al., 1992; Friesel and Maciag.,1999).

Transgenic mice for most FGFs have been generated and display the various phenotypes expected for each FGF mutation, from no phenotypic change to embryonic lethality. Among various FGF family members, FGF-2 deficient mice are viable, but show failure of neural regulation of blood pressure, decrease of vascular smooth muscle contractility, and defects and impairment of the cerebral cortex and cervical spinal cord (Dono et al., 1998; Zhou et al., 1998). FGF-8 deficient mice are lethal at E7

due to gastrulation defects, with these mice displaying defects in cardiac, craniofacial, forebrain, midbrain and cerebellar development (Meyers et al., 1998). Although FGFs are essential molecules in embryonic development as well as adulthood, mild phenotypic changes in several FGF transgenic mice may imply substitution from redundancy of different FGF members. In fact, FGF-8 and FGF-17 can compensate each other with respect to defects of midbrain-hindbrain boundary development (Xu and Ornitz., 2000). Moreover, knock down of different FGF molecules synergize the mild defects of single gene disruption, leading to more severe phenotypic changes. For example, a similar phenotype of smaller otic placodes was shown in FGF-3 knock out mice (Mansour et al., 1994) and the *ace* zebrafish, which is a hypomorphic mutation in the FGF-8 gene (Reifers et al., 1998). Knock down of both genes resulted in a failure of otic placode development in zebrafish embryos (Maroon et al., 2002; Leger and Brand, 2002; Phillips et al., 2004).

FGFs have two different possible receptors: cell-surface bound tyrosine kinase receptors (FGFRs) as high affinity receptors (Coughlin et al., 1988) and heparin-like glycosaminoglycans, such as HSPGs, as low-affinity receptors (Venkatamaran et al., 1999; Stauber et al., 2000). Two FGF molecules can form a complex bound to one FGF receptor, which is connected by a heparan sulfate proteoglycan. The receptor complex can trigger phosphorylation of receptors, causing recruitment and activation of intracellular signaling pathways. Heparan sulfate is required for activation of FGFRs in

vitro and in vivo (Rapraeger et al., 1991; Yayon et al., 1991; Ornitz et al., 1992; Lin et al., 1999). In the absence of HS, FGF cannot bind FGFRs, on the other hand, addition of HS can reconstitute FGF-FGFR complex formation (Yayon et al., 1991). The HS-chains play a key role in orchestrating the formation and stabilization of the FGF:FGFR signaling complex (Guimond and Turnbull., 1999; Guimond et al., 2001).

During embryonic development, HSPGs may play a crucial role to regulate FGF signaling pathways. FGFs are involved in cell proliferation, differentiation and migration during embryonic development and HSPGs can regulate ternary complex formation and FGFR oligomerization to regulate FGF related signal transduction. The relationship between HSPGs and FGFs provides an important clue to their functions during neural development. The central nervous system is generated from neuroectoderm, with neural precursors being localized in the ventricular zone of the neural tube. Neurons and glial cells arise from neural precursor cells in the ventricular layer and the subventricular layer from embryonic stages into adulthood. During neurogenesis, many growth factors and morphogens are required to regulate proliferation and differentiation of neural precursor cells. FGF-2 is a strong candidate for an essential growth factor for neurogenesis in the developing cerebral cortex. The spatio-temporal expression of FGF-2 in the embryonic brain coincides with neurogenesis in mouse (Qian et al., 1997). Moreover, FGF-2 and FGFRs are highly expressed in the cortical ventricular layer. FGF-2 serves as a mitogen for cortical

precursor cells, and promotes self-renewal activity of precursor cells in rat (Qian et al., 1997; Ghosh and Greenberg, 1995; Vicario-Abejón et al., 1995). In addition, low and high concentrations of FGF-2 can regulate cell fate from neuronal to oligodendroglial progeny, respectively (Qian et al., 1997). Since distinct HSPGs have different binding affinities for FGFs in a spatio-temporal manner, differential glycosylation of HSPGs may regulate the specificity of FGF binding during neurogenesis. After neurogenesis occurs, neurons migrate into target regions and extend axons and dendrites to form a network with other neurons. Recent studies have demonstrated the important role of proteoglycans in axon guidance. Wang and Denburg found that exogenously added heparin and heparan sulfates, or elimination of them by heparitinase treatment caused pathfinding errors in pioneer axons of cultured cockroach embryos (Wang and Denburg, 1992). Recent studies have shown that exogenous addition of heparan sulfate resulted in failure of retinal axons to enter the optic tectum in *Xenopus* (Irie et al., 2002). This failure of axon guidance was dependent on FGF-2 binding of HSPGs and only FGF-2-binding HSPGs could cause aberrant axonal targeting. In *Xenopus*, FGF-2 was shown to stimulate axonal outgrowth and also caused a mistargeting of retinal axons following addition of exogenous FGF-2 to optic nerve explants (Walz et al., 1997). Explants treated with a dominant-negative form of FGFR extend axons, but axons failed in target recognition (McFarlane et al., 1996). Since FGF-2 has been suggested to be involved in axonal growth and guidance, HSPGs may therefore modulate FGF:FGFR activation during axonal guidance. Furthermore, in the normal adult brain, FGFs are essential

elements for the process of learning and memory. Addition of FGF-2 can improve long-term potentiation (LTP) after stimulation of subcortical afferents (Abe et al., 1992). Since enzymatic cleavage of HS by heparitinase, as well as addition of soluble heparin-type carbohydrates, prevented expression of LTP in rat hippocampal slices, HSPGs and FGFs may act as important regulators in the modulation of neuronal connectivity (Lauri et al., 1999).

To understand the role of HSPGs in brain development, we must learn how synapse formation occurs in CNS as well as how cell proliferation, differentiation and migration of neurons is regulated during development. A well-known molecule essential to NMJ formation, agrin, is a strong candidate molecule for an important element for neurosynapse formation/maintenance and neurogenesis.

In the 1970s, reformation of synapses after muscle denervation was mediated by extracellular matrix from the synaptic cleft at the neuromuscular junction (Sanes et al., 1978 and Burden et al., 1979). This implied that an essential factor for synapse formation existed in the ECM at the NMJ and could organize the induction of pre- and post-synaptic differentiation. Smith et al purified agrin from basal lamina extracts of the synapse rich electric organ of *Torpedo California* (Smith et al., 1984). They suggested in the “agrin hypothesis” that agrin is the nerve-derived factor required to induce aggregation of acetylcholine receptors and assemble the postsynaptic apparatus.

This hypothesis was confirmed by the following studies. Agrin knock out mice die perinatally from a breathing failure and do not form functional synapses at the NMJ (Gautam et al., 1996). Moreover, overexpression of agrin can restore the reconstruction of a functional mature synaptic apparatus into non-synaptic region of rodent muscle (Meier et al., 1997).

The agrin gene encodes a core protein of over 2,000 amino acids with a predicted molecular mass of approximately 250 kDa. Tsen et al had identified that agrin belongs to the family of heparan sulfate proteoglycans (Tsen et al., 1995). Agrin contains at least three O-linked carbohydrate attachment sites which serve as binding sites for not only heparan sulfate glycosaminoglycan side chains, but also a hybrid HSPG/CSPG (Winzen et al., 2003) and increase the total mass of the protein to over 600 kDa. Agrin is expressed abundantly in the developing brain and the basal laminae of lung and kidney (Tsen et al., 1995, Burgess et al., 2002 and Burg et al., 1995).

Agrin has multiple domains that can interact with a number of molecules. Agrin's primary structure shows a multimodular composition which contains a globular NtA laminin-binding domain, a central rod-like domain with nine follistatin-like protease inhibitor domains, two laminin-like epidermal growth factor (EGF) repeats, two serine/threonine-rich domains, a sea urchin sperm protein enterokinase agrin (SEA) module, four EGF like repeats and three globular, COOH-terminal laminin G

like domains (Figure 2). The globules are connected by flexible rod-like structures. The agrin protein contains three HS favorable attachment sites in the two domains, both located in the central part of the molecule: the first region is located just upstream from the eighth follistatin-like domain and the second, that contains two possible HS attachment sites, directly precedes the SEA module (Winzen et al., 2003).

Alternative splicing is an important mechanism for functional diversity in the molecules controlling neuronal activity (Grabowski et al., 2001). Alternative splicing is also central to the development of the nervous system, the differentiation of neurons and the formation of their connection patterns. Alternative splicing is extremely well suited to produce large numbers of subtly different protein functions. Agrin is alternatively spliced in a highly time and tissue specific manner (Campanelli et al., 1996; O'Connor et al., 1992; Burgess et al., 1999; Kroger et al., 1997; Daggett et al., 1996; Gessmann et al., 1996; Thomas et al., 1993; Stone et al., 1995; McMahan et al., 1992). These multiple isoforms of agrin provide diverse possible functional roles during development (Deyst et al., 1998; Rupp et al., 1991; Rupp et al., 1992; Annies et al., 2002). The best-known function of agrin is the aggregating activity of AChRs at the neuromuscular junction (NMJ), which is dependent on the z insert agrin splicing form in rodent.

Binding activities of agrin are regulated by alternative splicing forms

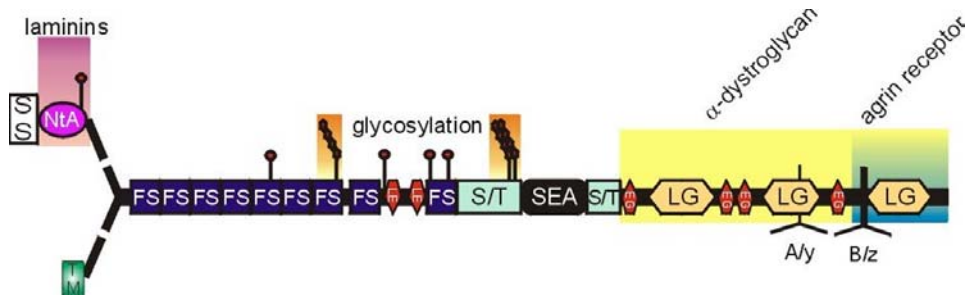


Figure 2. The primary domain structure of agrin (Bezakova, 2002). The signal sequence (SS) determines whether agrin is to be secreted and the NtA domain provides the binding domain to laminin. At the N-terminus, 9 follistatin-like domains (FS), laminin EGF-like domains (LE) and serine/threonine-rich domains (S/T) can be postrtranslationally modified by glycosylation and contains 3 possible GAG attachment sites. At the C-terminus, the sea urchin sperm domain (SEA), EGF-like domains (EG), laminin globular domains (LG) and the A/y and B/z alternative splice sites shows agrin's AChR-aggregating activity at the neuromuscular junction.

at different sites. The COOH-terminal of agrin contains three different splicing sites which are x, y/A, z/B in mammals/chicken, respectively (Ferns et al., 1992). Agrin's C-terminal region plays an essential role in regulating its binding to cell surface proteins and AChR aggregating activity (Rupp et al., 1991; O'Connor et al., 1992; Lakso et al., 1992). Isoforms containing 4 amino acids at y/A and either one or both 8 and 11 amino acids inserts at z/B sites are synthesized by motor neurons and highly active in AChR clustering activity (Ferns et al., 1992). Isoforms without inserts are formed by muscle and Schwann cells and are inactive in clustering. The z exons confer the AChR clustering activity on the protein, whereas the Y exon encodes a heparin-binding site (Campanelli et al., 1996; Gessmann et al., 1996; Bowen et al., 1996; Rossant et al., 1995) (Figure 2). Moreover, z insert agrin is expressed only in neurons, whereas nonneural cells, including glia and myotubes, express only B/z insert-negative forms.

Gautam et al have generated agrin-deficient mutant mice where neuromuscular differentiation was grossly defective in these mice, and no severe phenotypic defect was detected in their brain (Gautam et al., 1996). In addition, the disruption of z inserted isoforms blocked neuromuscular synapse formation and resulted in death after birth. However, the phenotype in the CNS did not show significant change except for a slightly smaller size of brain when compared to wild-type littermates (Serpinskaya et al., 1999). Although y insert of agrin is known for its binding to heparin, the disruption of y inserts did not show any phenotypic change (Burgess et al., 1999). This might

mean a redundant or dispensable function of the γ insert isoforms in mammals. However, α -negative forms were still expressed in these mutants, and compensation of α -negative isoforms cannot be ruled out.

The 5' end of the agrin gene exhibits heterogeneity and gives rise to the diversity of agrin's localization, tissue distribution, and function. Mice express two distinct short NH₂-terminal (SN) and long NH₂-terminal (LN) isoforms of agrin (Neumann et al., 2001; Bixby et al., 2002; Ji et al., 1998; Burgess et al., 2000). The existence of these distinct isoforms explains the previously noted lack of homology between the NH₂ termini of agrin isolated from rats and chicks (Neumann et al., 2001). For chicken, SN-agrin is homologous to the transmembrane (TM) - domain and LN-agrin is equivalent to the secreted signal (SS) -NtA-domain. SN- and LN-agrin are likely to be transcribed from distinct promoters, and they are expressed in different patterns throughout development (Burgess et al., 2000; Shigemoto et al., 2000). SN-agrin is largely confined to the nervous system, whereas LN-agrin is broadly distributed in neuronal and nonneuronal tissues. Moreover, analysis of native and recombinant protein indicate that SN and LN-agrin exhibit distinct subcellular localizations, determined by their NH₂ termini: LN-agrin associates with the ECM while SN-agrin remains attached to cell surfaces (Denzer et al., 1995). LN-agrin isoforms have an NtA domain that is required for binding to basal lamina-associated laminins. SN-agrin is a non-secreted form expressed by neurons as a type II

transmembrane protein. The SN-agrin terminus can mediate externalization and membrane anchoring of heterologous proteins. The perturbation of LN-agrin leads to neonatal lethality caused by a failure of neuromuscular junction formation that is the same phenotype as z insert negative isoforms (Burgess et al., 1999; Burgess et al., 2000). However, both z^{-/-} and LN^{-/-} agrin mice did not show any significant phenotypic change in the CNS. Thus, basal lamina-associated LN-agrin is required for neuromuscular synaptogenesis. Even though agrin mutant analysis proved the importance of agrin in NMJ formation, none of the mutants showed a phenotypic defect during CNS development as a result of loss of agrin.

Besides alternative splicing mechanism contributing to agrin function, different regions of agrin's modular organization provide distinct functional properties, allowing agrin to interact with a number of distinct molecules. The NtA domain is required for binding to laminin, which can localize agrin to the basal lamina (Denzer et al., 1995; Denzer et al., 1997). The laminin-G1 and G2-like domains can bind to α -dystroglycan which can form the syntrophin-associated glycoprotein complex at the muscle cell surface (Gesemann et al., 1996; Hopf and Hoch, 1996). The activity of rod-like central domains has been identified for NCAM, HB-GAM, FGF-2, merosin, thrombospondin, β -amyloid binding (Cole and Halfter, 1996; Cotman et al., 1999; Burg et al., 1995; Daggett et al., 1996). The C-terminus is required for agrin's AChR clustering activity

as well as a binding site for heparin and integrin (Martin and Sanes., 1997; Gesemann et al., 1996; Campanelli et al., 1996).

From studies on agrin's association with multiple molecules, Musk (muscle-specific receptor tyrosine kinase) and α -dystroglycan were identified as strong candidates for agrin's cellular receptor, which mediates signaling for inducing AChR aggregation at the NMJ (Sanes et al., 1998; Ruegg and Bixby., 1998; Montanaro et al., 1998). α -dystroglycan was considered as a direct receptor of agrin due to its activity in AChR aggregation. However, its absence does not interfere with the formation of NMJs and it binds agrin's inactive alternative splicing form B/z-, instead of B/z+ form (Campanelli et al., 1996). Therefore, it is believed dystroglycan might be involved in the consolidation rather than formation of the synaptic apparatus. On the other hand, MuSK can be activated by agrin at the NMJ. MuSK-deficient mice die perinatally and lack postsynaptic specialization at the NMJ much like agrin-deficient mice (Gautam et al., 1999; Sanes., 1997; DeChiara et al., 1996). Absence of MuSK in myotube cultures results in a failure to aggregate AChR in response to agrin, and addition of MuSK allows recovery of the aggregation of AChR (Meier et al., 1997). In addition, the agrin B/z+ form, but not B/z- form, can induce MuSK phosphorylation (Herbst and Burden., 2000). Although MuSK can induce the aggregation of AChRs and post-synaptic apparatus with agrin, agrin does not induce MuSK activation in myotubes or undifferentiated cells (Glass et al., 1996). Therefore undifferentiated muscle cells might

possess another agrin receptor, which may be a co-receptor of MuSK.

Outstanding progress has been made toward understanding synaptogenesis at the NMJ (Bezakove et al., 2001; Terrado et al., 2001; Nitkin et al., 1987; Reist et al., 1992), but synapses in the CNS have been much less accessible to experimentation. For instance, the gap between CNS synapses is much narrower than that of neuromuscular synapses and CNS synaptic clefts do not contain a basal lamina. The diversity of both their functional and molecular properties such as ion channel/receptor complements, (Sharp et al., 1996) also renders characterization difficult. Because of easy access to study mechanisms of synaptogenesis, most studies regarding agrin have focused on NMJ synaptogenesis. However, high expression of agrin in the developing brain implies important roles for agrin in CNS, and the existence of a transmembrane form of agrin could provide a possible explanation that agrin may be expressed at the surface membranes of CNS neurons in the absence of laminin. Even though the role of agrin in synaptogenesis in the CNS is controversial, recent studies have been supportive evidence about this hypothesis (Ma et al., 2000; Borges et al., 2001; Bezakove et al., 2001; Cotman et al., 1999; Halfter et al., 1997; Hering et al., 1999; Ruegg et al., 2001; Uhm et al., 2001; Biroc et al., 1993). Gingras et al showed that synaptogenesis is impaired in neural agrin-deficient superior cervical ganglion (SCG) cultures, with significantly fewer synaptophysin-labeled nerve terminals and synaptic aggregates of the neuronal acetylcholine receptor being formed (Gingras et al., 2002). Also other

studies showed that agrin differentially regulates axonal and dendritic growth which induced both dendritic elongation and dendritic branching (Mantych et al., 2001). Agrin is specifically localized at sympathetic synapses *in vitro*, and is consistent with it playing a role in interneuronal synapse formation (Gingras et al., 2001). The lethality of agrin mutant mice has been limited the study of agrin's role in the CNS. Since neuron-neuron synaptogenesis is progressing even after birth, one cannot rule out the possibility of agrin's function in synaptogenesis in CNS beyond previous studies. Even though the understanding of synaptogenesis in the CNS has been enigmatic, identification of agrin's role in synapse formation, maintenance and maturation will further our understanding of mechanisms of CNS development and agrin's role in CNS development.

Based on evidence that agrin is expressed prior to synapse formation, and its expression is not limited only in neuronal cells, this has suggested a potential role of agrin throughout development and not just in synaptogenesis. These data imply various broader roles of agrin for neurogenesis, axonogenesis, blood-brain barrier formation etc. The generation of distinct classes of neurons at defined positions in the rostro-caudal and dorso-ventral axis of the neural tube is a fundamental step in the establishment of the functional complexity of the vertebrate CNS. CNS neurons are generated through a highly co-coordinated and regulated series of events during which neural stem cells give rise to neuronal progenitors and neuronal progenitors in turn, migrate to targets

and undergo final differentiation. Research over the past two decades has elucidated many of the genetic pathways underlying these biological processes.

Even before the onset of synapse formation, agrin is highly localized in the ventricular and subventricular zone of the developing brain and spinal cord, and declines during development before largely disappearing in the adult brain (Stone et al., 1995; Cohen et al., 1997). Interestingly, the expression pattern of SN-agrin is quite unique in its temporal and spatial manner during development. SN-agrin is highly expressed in postmitotic neurons of the cortical plate, moderate in migrating neuroblasts of the intermediate zone, and lowest in progenitors of the ventricular zone in forebrain. This pattern is opposite that seen for LN-agrin. Also SN-agrin is abundant in midbrain, hindbrain, spinal cord, retina, olfactory epithelium, trigeminal ganglion, and sympathetic ganglia. SN-agrin is selectively expressed by neurons (Neumann et al., 2001; Bixby et al., 2002; Ji et al., 1998; Burgess et al., 2000). Even though the involvement of agrin in neurogenesis is not clear, recent results from agrin null mutant have evoked the question of its role in neurogenesis. Mice lacking α forms of agrin, display a reduced the number of synapses in the CNS (Serpinskaya et al., 1999).

Secreted axon guidance factors play a major role in the formation of neuronal networks in the CNS. Even though in vivo the axon guidance roles of HSPGs are largely unknown, recent studies suggest that heparan sulfate regulates axon guidance.

Agrin is expressed by glial cells and highly localized in axonal pathways like the stratum opticum and the tectobulbar tract in the early developing optic tectum (Cotman et al., 1999), and even olfactory axons in mature adulthood (O'Connor et al., 1994). These expression patterns strongly suggest that agrin could be involved in axonogenesis. Whether agrin could regulate axonogenesis in vivo has not been examined, however, in vitro studies and agrin's binding activity to other axonogenesis-related molecules has been shown. Agrin itself can serve as a transmembrane form and also binds axon growth related molecules like FGF-2, NCAM, HB-GAM, laminin, merosin and thrombospondin (Cotman et al., 1999; Dagget et al., 1996; Halfter et al., 1997; Bixby et al., 2002). Even though it is presently unclear whether agrin inhibits or promotes axon growth, agrin can exert different activity depending on functional domains used and the type of neuronal cell examined. The C-terminus of agrin is known to inhibit axon elongation, but promote dendrite growth in hippocampal neuronal cultures (Mantych and Ferreira., 2001). Using retinal cell cultures agrin, when used as a substratum, is inhibitory for axonal growth (Bixby et al., 2002). In contrast, axonal growth mediated by FGF-2 (Morrison et al., 1986; Walicke et al., 1986) is modulated by agrin in both PC-12 cells and retinal neurons (Kim et al., 2003), with agrin promoting FGF-2 mediated axonal growth.

Recent studies have raised the question of agrin's possible function in non-neuronal phenomena. The resemblance between synaptogenesis and immunological

synapse-like junction formation has been revealed agrin's role in the immune system. Agrin is expressed in lymphocytes and involved in membrane lipid microdomain organization and T-cell signaling (Trautmann and Vivier., 2001; Shaw et al., 2001). Agrin can induce the aggregation of immune-related signaling protein, much like the interaction with AChRs at the NMJ, and can create signaling domains for immune system through a common lipid raft pathway (Khan et al., 2001). Moreover, agrin is a key component for basal lamina formation. Agrin is highly intercalated in the brain microvasculature and the blood brain barrier, where it may provide support as a charge and molecular size filter (Barber and Lieth., 1997; Rascher et al., 2002). Agrin is also present in other microvessels like testis, thymus and kidney glomeruli. Since microvessel maturation for the blood-brain barrier occurs after birth, agrin mutant mice do not allow analysis of agrin's role in processes such as blood-brain barrier formation. Therefore, a detailed analysis of agrin's function in neurogenesis, axonogenesis and non-neuronal basal lamina function is critical, to better understand the contribution of HSPGs such as agrin to development.

CHAPTER I

The Heparan Sulfate Proteoglycan Agrin Modulates Neurite Outgrowth Mediated by FGF-2

ABSTRACT

While the role of agrin in the formation of the neuromuscular junction is well established, other functions for agrin have remained elusive. The present study was undertaken to assess the role of agrin in neurite outgrowth mediated by the heparin-binding growth factor basic fibroblast growth factor (FGF-2), which we have shown previously to bind to agrin with high affinity and that has been shown to mediate neurite outgrowth from a number of neuronal cell types. Using both an established neuronal cell line, PC12 cells, and primary chick retina neuronal cultures, we find that agrin potentiates the ability of FGF-2 to stimulate neurite outgrowth. In PC12 cells and retinal neurons agrin increases the efficacy of FGF-2 stimulation of neurite outgrowth mediated by the FGF receptor, as an inhibitor of the FGF receptor abolished neurite outgrowth in the presence of agrin and FGF-2. We also examined possible mechanisms by which agrin may modulate neurite outgrowth, analyzing ERK phosphorylation and c-fos phosphorylation. These studies indicate that agrin augments a transient early phosphorylation of ERK in the presence of FGF-2, and augments and sustains FGF-2 mediated increases in c-fos phosphorylation. These data are consistent with established mechanisms where heparan sulfate proteoglycans such as agrin may increase the affinity between FGF-2 and the FGF receptor. In summary, our studies suggest that neural agrin contributes to the establishment of axon pathways by modulating the function of neurite promoting molecules such as FGF-2.

Key words: agrin, FGF-2, neurite outgrowth, heparan sulfate proteoglycan

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are extracellular matrix (ECM) and cell surface macromolecules characterized by heparan sulfate glycosaminoglycan (HS-GAG) chains attached to a protein core (Gallagher et al, 1986, Hardingham et al, 1992). It is becoming increasingly apparent that HSPGs mediate a diverse array of biological functions that are usually attributed to their GAG chains (Ruoslahti and Yamaguchi, 1991). Specific examples of important functions carried out by HSPGs include regulating cell adhesion (Cole et al, 1986, Hantaz-Ambroise et al, 1987; Bernfield et al, 1992), cellular growth and differentiation (Castellot et al, 1986, Ruoslahti and Yamaguchi, 1991), wound healing (Andriessen et al, 1997), and tumorigenesis (Tumova et al, 2000, Derksen et al, 2002, Tapanadechopone et al, 2001). The structural heterogeneity of heparan sulfate chains results in specific HSPGs being capable of mediating distinct functions (Aviezer et al, 1994; Herndon et al, 1999; Knox et al, 2002) and modulating binding to specific classes of heparin-binding molecules (Sanderson et al, 1994; Cotman et al, 1999; Knox et al, 2002), resulting in the same HSPG being capable of eliciting different functions in different cell types (Sanderson et al, 1994). In addition, structural modification of a HSPG's heparan sulfate chains during processes such as development can alter the function of the HSPG (Nurcombe et al, 1993; Brickman et al, 1998). It is therefore clear that HSPGs encode a wealth of

information in their HS-GAG chains, which can impinge on a variety of cellular processes particularly during nervous system development.

The importance of HSPGs to nervous system development has been suggested by their abundance in basal laminae, which are known to provide a supportive environment for neurite outgrowth (Halfter et al, 1987; Davies et al, 1987; Condic and Bentley, 1989; Bovolenta and Feraud-Espinosa, 2000; Yamaguchi, 2001). Accordingly, a number of HSPG-binding ECM proteins serve as excellent substrates for neurite outgrowth, including fibronectin (Rogers et al, 1983, 1985), laminin (Rogers et al, 1983, Lander et al, 1985) and thrombospondin (O'Shea et al, 1991). In addition, heparin-binding growth factors such as the fibroblast growth factors (FGFs) are potent neurite-outgrowth promoting molecules (Rydel and Greene, 1987; McFarlane et al, 1995; Chai and Morris, 1999), and the binding of HSPGs to FGFs has been shown to potentiate their neurite-outgrowth promoting activity (Walz et al, 1997). FGF-2 has been shown to be localized to developing axon pathways, and in particular has been shown to play an integral role in the establishment of the retinotectal pathway (McFarlane et al, 1995; Walz et al, 1997). HSPGs have also been shown to co-localize with FGF-2 in the developing retinotectal pathway (Ford et al, 1994; Joseph et al, 1996; Cotman et al, 1999), implicating these HSPGs in FGF-2 function. This is especially noteworthy since FGF activity is dependent on its interaction with HSPGs (Ornitz, 2000), and FGF-2 initiated FGF receptor (FGFR) signaling requires heparan

sulfate as a co-factor that may modulate FGF-FGFR signaling (Kan et al, 1993; Zhang et al, 2001). Thus, identification of the HSPGs that modulate FGF activity would extend our understanding of the role of these molecules in FGF-mediated functions such as axonal growth.

Agrin is a large, ECM HSPG (Tsen et al, 1995) that was originally identified in the electric ray *Torpedo californica* neuromuscular junction based on its ability to promote the aggregation of acetylcholine receptors (Nitkin et al, 1987). Agrin is abundantly expressed in basal laminae in the kidney, lung and brain, and in the kidney glomerular basement membrane agrin has been shown to regulate glomerular filtration (Groffen et al, 1999; Yard et al, 2001). However, in brain the function of agrin has remained elusive. Agrin has been suggested to be required for synaptogenesis during CNS development (Ferreira, 1999; Bose et al, 2000), and recent studies have shown that synapse formation between sympathetic neurons is also impaired in agrin-deficient mice (Gingras et al, 2002). However, *in vitro* studies using CNS neurons from agrin knock-out mice indicates normal synapse formation between neurons (Li et al, 1999). Immunohistochemical studies have shown that agrin expression is developmentally regulated, is strongly correlated with periods of formation of axon pathways (Halfter et al, 1997), and that agrin colocalizes with known neurite-outgrowth promoting molecules, such as merosin and FGF-2, during periods of axonal growth (Halfter et al, 1997; Cotman et al, 1999). In addition, studies from our laboratories have shown that

agrin binds with high affinity to a number of neurite-outgrowth promoting molecules that include laminins, tenascin, thrombospondin, and FGF-2 (Cotman et al, 1999).

Numerous studies have begun to implicate agrin as contributing to cell adhesion processes in the developing nervous system, as agrin has been shown to mediate cell adhesion in vitro (Burg et al, 1995; Martin and Sanes, 1997; Bixby et al, 2002). Extending these observations to ascertain whether agrin's role in cell adhesion may be related to modulating neurite outgrowth have been less informative. Agrin has been shown to promote axonal branching and dendritic branching and elongation in hippocampal neurons, while inhibiting elongation of the main axon in these neurons (Mantych and Ferreira, 2001). Agrin also has been suggested to be inhibitory to neurite outgrowth using other neuronal types (Campagna et al, 1995; Chang et al, 1997; Halfter et al, 1997; Bixby et al, 2002). The ability of agrin to inhibit axonal growth in vitro would seem to run counter to its abundant expression in developing axon pathways such as the optic nerve (Halfter et al, 1997; Cotman et al, 2000), suggesting that agrin may not express inhibitory activity in such developing axon pathways. Accordingly, agrin co-localizes with FGF-2 in the developing optic nerve (Cotman et al, 2000), and FGF-2 has been shown to promote axon growth in the optic tract that is dependent on the presence of HSPGs (McFarlane et al, 1995; Walz et al, 1997), implicating one potential mechanism whereby agrin may positively regulate axon growth.

To test our hypothesis that agrin may act as a co-receptor for FGF-2 and is involved in FGF signaling, we tested whether agrin modulates the ability of a neurite outgrowth-promoting molecule such as FGF-2 to promote neurite outgrowth, using either primary retinal neuronal cultures or the established neuronal PC-12 cell line. Our studies demonstrate that agrin potentiates neurite outgrowth mediated by FGF-2, and that agrin's modulation of FGF-2 mediated neurite outgrowth is dependent on agrin's HS-GAG chains. Our studies therefore suggest that agrin's localization to developing axonal pathways in association with binding ligands such as FGFs is of functional significance, allowing agrin to modulate the function of these neurite outgrowth-promoting molecules and possibly fine tune axonal responses to heparin-binding molecules such as the FGFs.

METHODS

Materials

Fertilized eggs from white horn leg chickens were purchased from the North Carolina State University Department of Poultry Science, and were incubated at 38°C until use. Culture media, sera, and human laminin-1 were purchased from Invitrogen, recombinant human basic FGF (FGF-2) was obtained from Chemicon International. The FGFR-1 inhibitor SU5402 was purchased from Calbiochem. All other chemicals and reagents were purchased from Sigma Aldrich or Bio-Rad. Nitrocellulose membrane was obtained from Schleicher & Schuell. Antibodies were obtained from the following companies: Phospho-ERK kinase (Thr202/Tyr204) antibody, ERK1(K-23) from Santa Cruz Biotechnology, phospho-c-fos (Ab-2) from Oncogene Research Products, and horseradish peroxidase-conjugated secondary antibodies and Supersignal West Pico chemiluminescent substrate kits from Pierce.

Purification of agrin

Agrin was immunopurified from E12 or E14 chick brain or vitreous bodies as previously described (Halfter et al, 1997). To remove HS-GAG chains from agrin,

purified agrin was treated with nitrous acid as previously described (Burg et al, 1995) or treated with heparitinase as previously described (Cotman et al, 2000).

Primary retina neuronal and PC12 cell cultures

E6 chick retinas were isolated, incubated with 0.25% Trypsin-EDTA for 15 minutes, and then triturated with a fire-polished Pasteur pipet as previously described (Burg et al, 1995). Cell suspensions were cultured on a laminin-1 substratum in L15 medium containing 1% BSA and penicillin-streptomycin. The laminin-1 substratum was prepared by coating 35 mm tissue culture plates with nitrocellulose (Lagenaur and Lemmon, 1987) and incubating with 5 µg/ml of laminin-1 (Invitrogen). The substratum was then incubated with 2% BSA in PBS and washed with PBS. Cells (3×10^6) were then plated on the laminin-1 substratum and grown at 37°C in a 5% CO₂ atmosphere.

PC12 rat adrenal pheochromocytoma-derived cells (PC12 cells) were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 10% horse serum and antibiotics at 37°C in a 5% CO₂ atmosphere. Tissue culture plates (6-well) were coated with poly-L-lysine and washed with PBS prior to plating of PC12 cells. Cells were plated at 5×10^5 cells / well to analyze for neurite outgrowth, and 3×10^6 cells / well for immunoblotting experiments. Cells were starved for 24 h in serum free

medium prior to addition of FGF-2 and/or agrin.

Neurite Outgrowth Assays

Retinal neuronal cultures and PC12 cells were treated with FGF-2 (2.5 or 10 ng/ml), agrin(200 ng/ml), HS-GAG free agrin(200 ng/ml) and the FGFR inhibitor SU5402 (20 μ M) for 1 day and 6 days, respectively. Cultures were fixed in 4% paraformaldehyde/PBS and coverslipped in 30% glycerol/PBS prior to quantitation of neurite outgrowth. Cells with neurites of a length at least twice the diameter of the neuronal cell body were scored as positive. The lengths of the longest neurites of 10 cells (10 cells/dish), from random fields, were measured from the photographs of the PC 12 cultures using SPOT software for each individual experiment, and was repeated for 10 fields from each culture dish. For retinal cultures the 25 longest neurites were measured from these cultures, for four independent experiments.

To assess of the role of endogenous HSPGs in FGF-2 mediated neurite outgrowth, freshly plated retinal cell cultures were treated with 1 U/ml heparitinase for 1 h in serum-free medium. Cells were then washed with fresh medium and treated with FGF-2 as described above.

To assess the effects of agrin on neurite outgrowth, agrin from embryonic chick brain or vitreous were employed. In initial studies identical effects were observed for both sources of agrin, and therefore in subsequent experiments only agrin purified from vitreous body was employed.

Immunoblotting analysis

Retinal neuronal and PC12 cells were treated with FGF-2 (2.5 or 10 ng/ml), agrin (200ng/ml), HS-GAG free agrin (200ng/ml) and SU5402 (20 μ M) for 10 minutes, 30 minutes, 1h and 3 h. Cells were then washed with ice-cold PBS at the appropriate time period and incubated in lysis buffer (20mM Tris-HCl(pH 8.0), 137mM NaCl , 0.5mM EDTA, 1% Triton X-100, 10% glycerol, 10mM Na₂P₂O₇, 10mM NaF, 1 μ g/ml aprotinin, 10 μ g /ml leupeptin, 1mM sodium orthovanadate, 1mM PMSF). Cell lysates were centrifuged and protein concentrations were determined by the Bradford assay using the Bio-Rad reagent and immunoglobulin as a standard protein. 30 μ g of supernatant proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunoblots were blocked with 20mM Tris-HCl (pH7.5), 137mM NaCl, 1% BSA, 0.1% Tween-20 and then incubated with polyclonal antibodies specific for either anti-ERK1/2(1:1000), anti-phospho-ERK1/2(1:1000), or anti-phospho-c-fos (1 μ g/ml) at 4°C overnight. Immunoblots were washed with 20 mM Tris-HCl, 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated anti-

rabbit, goat, or mouse antibodies (1:250,000). Membranes were incubated with Supersignal West Pico chemiluminescent Substrate kits (Pierce) and exposed to X-ray film (Kodak). Membranes were stripped with 2% SDS, 62.5mM Tris-Cl (pH6.8), 100mM β -mercaptoethanol at 70°C for 30 minutes prior to reblotting with other antibodies. The density of bands of interest was analyzed using NIH image software.

RESULTS

Agrin specifically stimulates neurite outgrowth mediated by FGF-2 in PC12 cells.

To begin to investigate a role for agrin in neurite outgrowth mediated by heparin-binding neurite-promoting proteins, we used PC12 cells as a model system. PC12 cells are an established cell line that exhibit the properties of neurons in the presence of NGF or FGF-2 (Greene and Tischler, 1976). HS-GAGs have been shown to potentiate the neurite promoting effects of NGF and FGF-2 in PC-12 cells (Damon et al, 1988; Lesma et al, 1996), with greater effects observed when presented in combination with FGF-2 than with NGF (Damon et al, 1988). For our analyses of agrin in modulating FGF-mediated neurite outgrowth, we employed agrin that was immunopurified from either embryonic chick brain or vitreous body. Our initial studies demonstrated identical effects for both brain and vitreous agrin, and therefore the experiments described here employed only vitreous agrin due to the ease of purifying vitreous body agrin.

To examine the role of full-length vitreous agrin on neurite outgrowth, PC12 cells were incubated for 6 days with FGF-2 in the presence or absence of various concentrations of agrin that ranged from 100-250 ng/ml. We found that optimal effects of agrin could be observed at 200 ng/ml, and thus this concentration of agrin was

employed for the experiments described here. Previous studies examining the effect of FGF-2 on neurite outgrowth from PC12 cells have routinely used FGF-2 concentrations ranging from 20-50 ng/ml, so our initial experiments employed 25 or 50 ng/ml of FGF-2 in the absence or presence of agrin. Under these conditions a pronounced neurite growth was elicited from PC-12 cells, but in the presence of agrin there was no difference in the extent of neurite outgrowth (data not shown). In view of recent studies showing that in the developing optic tract FGF-2 mediated axon growth is abolished by elimination of HS-GAGs (Walz et al, 1997), and restored by the addition of exogenous FGF-2, we reasoned that in order to assess the potential role of agrin in modulating neurite outgrowth suboptimal doses of FGF-2 need to be employed in order to mimic the in vivo process.

We therefore carried out studies using 2.5 ng/ml of FGF-2, to determine the efficacy of this concentration of FGF-2 in promoting neurite outgrowth from PC12 cells. PC12 cells remained undifferentiated when untreated or incubated with agrin alone (Figure 3 A,D), or incubated with agrin treated with nitrous acid to remove HS-GAG chains (Figure 3E), indicating that agrin does not promote the differentiation of PC12 cells. We have shown previously that agrin's protein core retains full binding activity (based on ability to bind tenascin) when treated with nitrous acid to remove HS-GAG chains (Cotman et al, 1999). PC12 cell differentiation, as measured

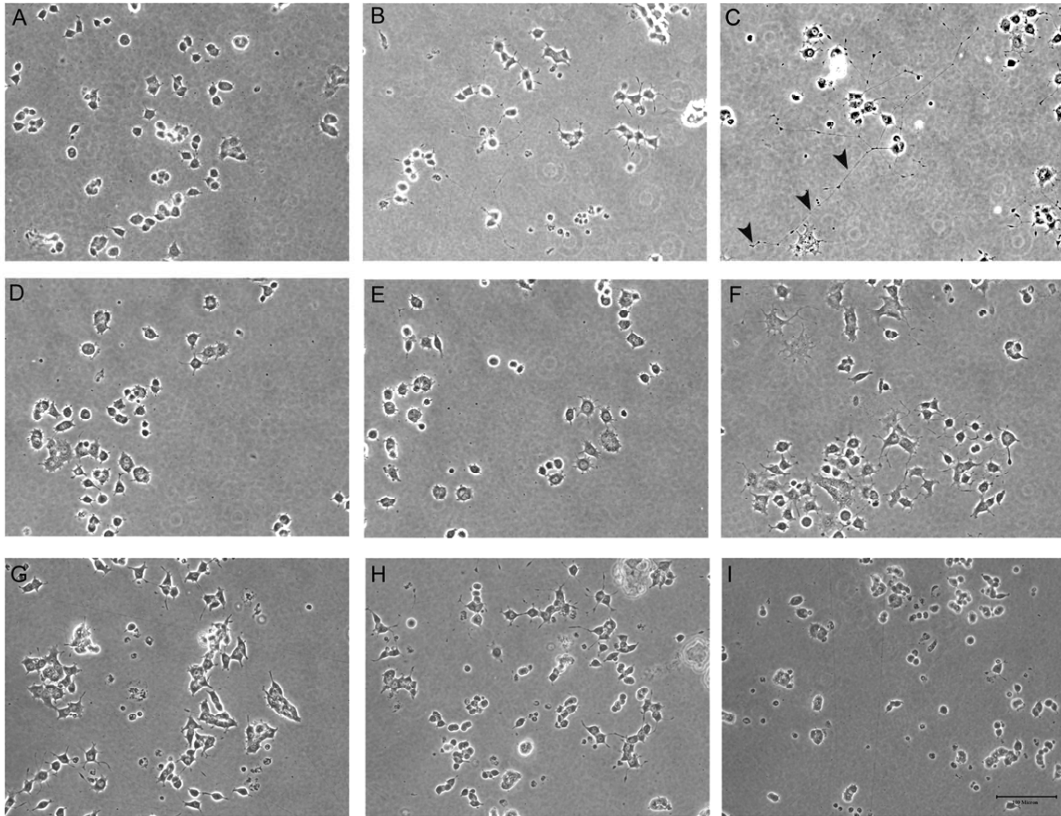


Figure 3. Agrin potentiates the ability of FGF-2 to promote neurite outgrowth from PC12 cells. PC12 cells were maintained in culture as described under Methods, and treated for 6 days with FGF-2 in the presence or absence of agrin immunopurified from E12 chick vitreous body. A, untreated PC12 cells; B, PC12 cells treated with 2.5 ng/ml of FGF-2; C, PC12 cells treated with 2.5 ng/ml FGF-2 and 200 ng/ml of agrin; D, PC12 cells treated with 200 ng/ml of agrin only (Arrowheads indicate the longest neurite from one cell body); E, PC12 cells treated with agrin that had been treated with nitrous acid to remove HS-GAG chains; F, PC12 cells treated with FGF-2 and HS-GAG free agrin; G-I, Treatment of PC12 cells with the FGFR inhibitor SU5402 at the time cells were exposed to FGF-2 (G), FGF-2 and agrin (H), or FGF-2 and HS-GAG free agrin (I). Calibration bar, 100 μ m.

by neurite outgrowth, was observed following treatment of PC12 cells with 2.5 ng/ml of FGF-2 (Figure 3B). The addition of nanomolar concentrations of agrin (200 ng/ml) to FGF-2 treated PC12 cells resulted in a significant enhancement of neurite outgrowth (Figure 3C), which was for the most part eliminated when agrin's HS-GAG chains were removed (Figure 3F).

The extent of neurite outgrowth in response to FGF-2 or FGF-2 and agrin was quantified by measuring the average length of the longest neurites from randomly selected neurons from cultures. For each experiment 10 random microscope fields were selected, and the 10 longest neurites from neurons in each field were measured. As shown in Figure 4A, treatment of PC12 cells with low concentrations of FGF-2 (2.5 ng/ml) resulted in a promotion of neurite outgrowth when compared to untreated PC12 cells. With these lower doses of FGF-2 the extent of neurite outgrowth was significantly less than the neurite outgrowth resulting from exposure of PC12 cells to both FGF-2 and agrin (Figure 4A). For example, the average length of the longest neurites in FGF-2 treated (2.5 ng/ml) PC12 cells is 240.3 μm , while for PC12 cells treated with FGF-2 and agrin the average of the longest neurites is 390.2 μm . At 10 ng/ml doses of FGF-2 a similar pattern is observed, with the average length of these 100 longest neurites being 238.9 μm in FGF-2 treated cells and 476.1 μm in FGF-2/agrin treated cells. As shown in Figure 4A, removal of agrin's HS-GAG chains using nitrous acid for the most part eliminated agrin's ability to augment FGF-2's

effects on neurite outgrowth in PC12 cells, with the average neurite length being 297.5 μm . This remaining neurite outgrowth promoting activity for nitrous acid treated agrin may result from incomplete removal of HS-GAG chains. The distribution of the longest neurites observed in PC12 cells exposed to the various protein treatments was also calculated from 100 neurons, and is shown in Figure 4B. It can be seen that significantly longer neurites are observed in PC12 cells treated with both FGF-2 and agrin, and that a higher percentage of these PC12 cells have longer neurites.

Agrin exerts its effects on FGF-2 mediated neurite outgrowth through the FGF receptor.

To determine the extent to which agrin enhances FGF-2 dependent PC12 cell differentiation through the FGFR and its associated ERK signaling pathway, we tested the effects of the FGFR-1 inhibitor SU5402 on PC12 cell neurite outgrowth. SU5402 interacts with the catalytic domain of the FGFR1 to inhibit its tyrosine kinase activity, and only acts as a weak inhibitor of the PDGF receptor and does not inhibit the insulin and EGF receptors (Mohammadi et al, 1997). PC12 cells were exposed to FGF-2 and/or agrin and SU5402 for six days, and neurite outgrowth was then quantified. As shown in Figure 3 and 4A, the majority of PC12 cells treated with FGF-2 and agrin, and SU5402, did not differentiate, and those cells that did differentiate had minimal

neurite outgrowth. The majority of cells lacked neurites (Figure 3G-I and Figure 4A), indicating the requirement of a functional FGFR for neurite outgrowth in our assays. Thus, these data indicate that agrin's effects on FGF-2 mediated neurite outgrowth in PC12 cells are dependent on a functional FGFR-1.

Agrin exerts its effects on FGF-2 mediated neurite outgrowth by modulating ERK activity in PC12 cells.

Our data showing that the FGFR-1 inhibitor SU5402 eliminates agrin's effects on FGF-2 mediated neurite outgrowth in PC12 cells suggests that agrin must be acting through the FGFR1 and its signaling pathway to modulate FGF-2 activity. To test this hypothesis directly, we have examined the effect of agrin on FGF-2 mediated changes in ERK signaling in PC12 cells. Previous studies have shown that FGFs activate ERK in various cell types, including retinal neurons (Perron and Bixby, 1999), and that sustained activation of ERK was necessary for neurite outgrowth in retinal neurons (Dimitropoulou et al, 2000). Thus, we addressed whether agrin modulates the degree of induction and duration of induction of ERK phosphorylation in PC12 cells. In PC12 cells, FGF-2 stimulated phosphorylation of both the p42 and p44 isoforms of ERK within 10 minutes, ERK phosphorylation continued to increase at 60 min post-exposure, and by 3 h after FGF-2 exposure ERK phosphorylation was substantially

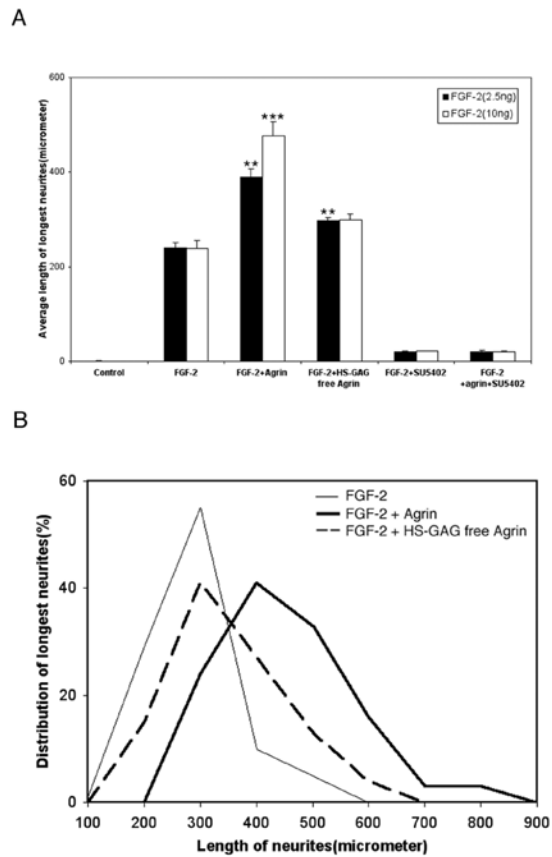


Figure 4. Quantitation of neurite outgrowth from PC12 cultures treated with FGF-2 and/or agrin. A, The average length of the longest neurites on PC12 cells was determined for the treatments shown, using 2.5 or 10 ng/ml of FGF-2 and 200 ng/ml of agrin. Control represents untreated PC12 cells, which exhibited no differentiation. It can be seen that agrin significantly increases neurite outgrowth in the presence of FGF-2, and that treatment of cells with SU5402 reduces growth to untreated levels. ** Statistically different from FGF-2 treated cells, $P < 0.0001$ using ANOVA. *** statistically different from FGF-2 treated cells, $P < 0.0006$. B, Distribution of the 100 longest neurites measured from PC12 cells treated with FGF-2 and/or agrin. Neurites in random fields were measured and the 100 longest neurites from 10 representative experiments were distributed on a plot of % of neurites versus neurite length. The main peak shifts to the right with addition of both FGF-2 and agrin, reflecting a longer length of the 100 longest neurites. There are also more neurites with lengths greater than 500 μm when PC12 cells are exposed to FGF-2 and agrin.

diminished (Figure 5A and 5B). In contrast, PC12 cells exposed to FGF-2 and agrin exhibited stimulation of ERK phosphorylation within 10 minutes, which was still elevated when compared to basal levels at 3 h post-exposure to FGF-2 and agrin (Figure 5A and 5B). Treatment of PC12 cells with agrin alone, or HS-GAG free agrin, did not produce a significant, reproducible elevation of ERK phosphorylation (Figure 5A and 5B), indicating that agrin's effects on ERK phosphorylation are mediated via FGF-2 and the FGFR-1. Densitometric analysis of ERK phosphorylation shows that the level of ERK phosphorylation, when compared to control levels, increased 3.8-fold after 30 minutes, and even increased 4.2-fold after 3 h in the presence of both FGF-2 and agrin, when compared to FGF-2 alone (Figure 5B). In addition, even though activation of ERK by FGF-2 and agrin decreased after 1 h, the level of activation of ERK at 3 h post-exposure was higher than FGF-2 treated cells at 1 h post-exposure. These data provide strong evidence for agrin augmenting the efficacy of FGF-2 activation of the FGFR-1, and maintaining this activation for longer periods than for cells exposed to FGF-2 alone. Interestingly, ERK phosphorylation by FGF-2 and agrin lacking HS-GAG chains was not completely reduced to FGF-2 levels, although the extent of neurite outgrowth under these conditions is similar between the two treatments. Importantly, phosphorylation of ERK was completely blocked by SU 5402, whether agrin or HS-GAG free agrin was used in combination with FGF-2. These data imply that the augmented ERK phosphorylation is mediated by the interaction of agrin with FGF-2 and the FGFR-1. Our results also show that

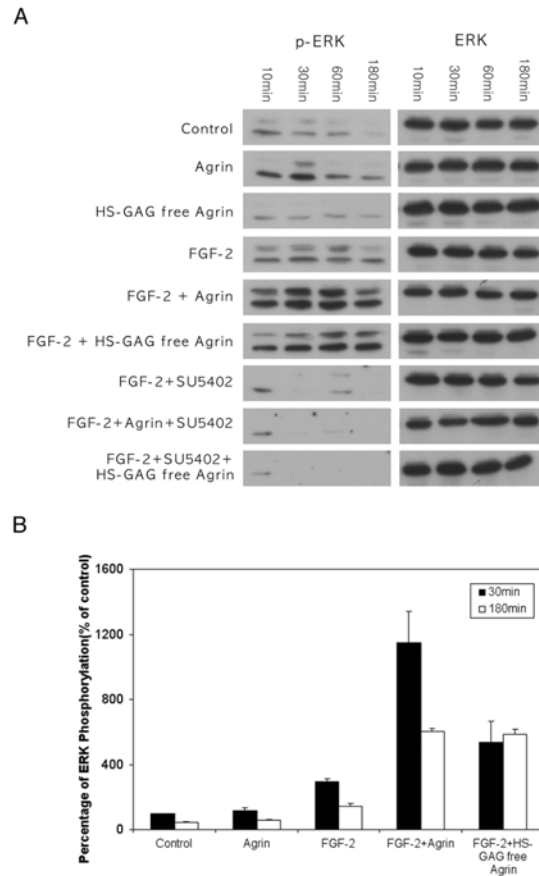


Figure 5. Phosphorylation of p42/44^{ERK} in PC12 cells in response to FGF-2 treatment (2.5 ng/ml) in the presence or absence of 200 ng/ml agrin. A, Immunoblot analysis of p42/44^{ERK} in PC12 cells, showing total ERK (p44) protein in cells for comparison. It can be seen that FGF-2 and agrin treatment leads to a rapid activation of ERK, which is eliminated by inhibition of the FGFR using SU5402, when compared to control (untreated) cells. Interestingly, the removal of agrin's HS-GAG chains does not completely eliminate agrin's augmentation of FGF-2 mediated ERK phosphorylation. B, The effect of FGF-2 and/or agrin on ERK phosphorylation was quantified from densitometric analysis of immunoblots of ERK protein phosphorylation, from three independent experiments. Both P-ERK bands were quantified by densitometry for each treatment.

agrin can significantly augment and maintain for longer periods the high level of ERK phosphorylation in the presence of FGF-2, providing a mechanism for agrin's ability to enhance neurite outgrowth in the presence of concentrations of FGF-2 that are suboptimal for rapid and complete PC12 cell differentiation.

Role of agrin in modulating FGF-2 mediated neurite outgrowth in retinal neuronal cultures.

In light of our demonstration that agrin can potentiate the effects of FGF-2 on PC12 cell neurite outgrowth, we thought it important to analyze the role of agrin on FGF-2 mediated neurite outgrowth in a CNS neuronal cell type known to be regulated by FGF-2. Thus, we selected chick retinal neurons, as it has been demonstrated that retinal neuronal axonal growth is regulated by FGF-2 (McFarlane et al, 1995; Walz et al, 1997; Perron and Bixby, 1999) that is dependent on the presence of HSPGs (Walz et al, 1997). In addition, FGF-2 and HSPGs such as agrin co-localize to the retinotectal pathway during the developmental period associated with establishment of this axon pathway (Ford et al, 1995; Joseph et al, 1996; Cotman et al, 1999). To facilitate survival and neurite outgrowth of primary cultures of retinal neurons these cells were plated on a laminin-1 substratum. Previously we have shown that preparation of a substratum comprised of agrin bound to nitrocellulose, followed by merosin (laminin-

2), did not permit outgrowth of retinal neurites (Halfter et al, 1997). Thus, in the experiments described here we employed a laminin-1 substratum, but added agrin exogenously to the culture medium. Furthermore, the concentrations of agrin added to culture medium (200 ng/ml) were substantially lower than the amounts of agrin (50 μ g/ml) that were adsorbed as a substratum in our previous studies (Halfter et al, 1997). The addition of agrin was in either the presence or absence of FGF-2. Interestingly, when E6 retinal neuronal cultures were exposed to FGF-2 and agrin, sprouting of neurites was observed within 1 h of plating, in contrast to the absence of neuronal sprouting from retinal neurons grown on laminin-1 alone or exposed to FGF-2 alone (Figure 6). Thus, agrin is able to stimulate a rapid neuronal response to FGF-2. Importantly, our data suggest that the ability of ECM proteins and growth factors to modulate neurite outgrowth is highly dependent on the levels of these proteins presented to cells, as previous studies by Perron and Bixby (1999) demonstrated that retinal neurons exhibit rapid neurite outgrowth within 30 minutes on a substratum comprised only of 10 μ g/ml of laminin. Thus, on a substratum of 5 μ g/ml of laminin-1 as used in our experiments, neurons only initiate a rapid neurite sprouting when exposed to both 2.5 ng/ml FGF-2 and 200 ng/ml agrin (Figure 6).

To assess a dose-response relationship for FGF-2 on chick retinal neurons, we exposed retinal neurons to varying concentrations of FGF-2. Previous studies in chick retina have shown that 25 ng/ml of FGF-2 increases the magnitude of neurite

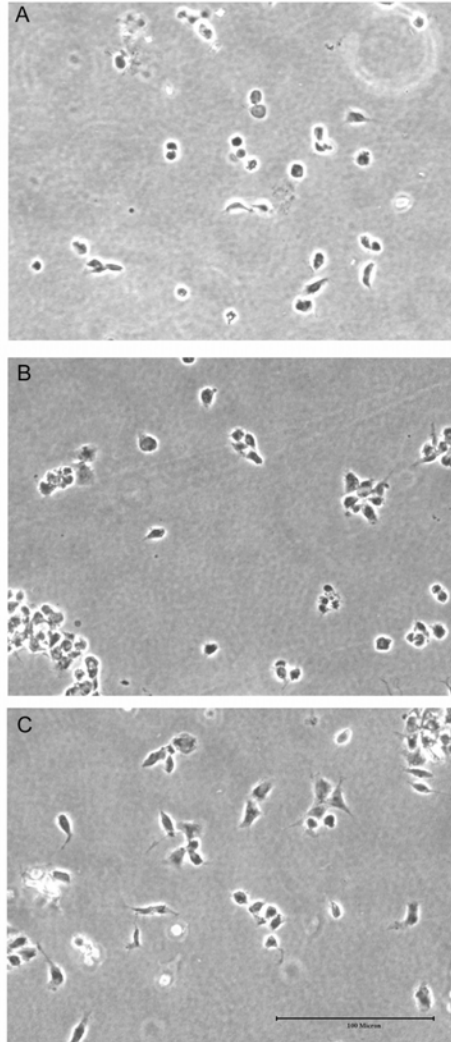


Figure 6. Agrin promotes a rapid initiation of retinal neuronal neurite outgrowth in the presence of FGF-2. A, E6 retinal cultures plated on a laminin-1 substratum, 1 h after plating. B, E6 retinal cultures plated on a laminin-1 substratum (5 $\mu\text{g/ml}$) and exposed to 2.5 ng/ml FGF-2, 1 h after plating. C, E6 retinal cultures plated on a laminin-1 substratum and exposed to FGF-2 and agrin, 1 h after plating. Note that agrin in the presence of FGF-2 leads to detectable neurite outgrowth at 1 h, that is not observed in the presence of laminin-1 only or FGF-2 only. Calibration bar, 100 μm .

outgrowth 2-fold when compared to cells plated on laminin-1 only (Perron and Bixby, 1999). We therefore tested 25 or 50 $\mu\text{g/ml}$ of FGF-2 for its affect on retinal cell neurite outgrowth, and the effect of agrin on this neurite outgrowth. As shown for PC12 cells, we observed extensive neurite outgrowth from chick retinal neurons using 25 or 50 ng/ml of FGF-2, and agrin was unable to augment this neurite outgrowth (data not shown). Thus, retinal cell cultures were exposed to lower concentrations of FGF-2, using 2.5 or 10 ng/ml of FGF-2, to assess the role of agrin in the modulation of FGF-2 mediated neurite outgrowth.

When primary E6 retina cultures are examined at 1 day after treatment with 2.5 ng/ml FGF-2 in the presence or absence of agrin, it can again be seen that agrin modulates the ability of FGF-2 to regulate neurite outgrowth. Compared to retinal neurons grown on a laminin-1 substratum (Figure 7A), the addition of FGF-2 augments neurite outgrowth, and FGF-2 plus agrin further augments outgrowth (Figure 7B and 7C). When 10 ng/ml FGF-2 is employed, the effects of agrin on neurite outgrowth are more pronounced, indicating the potent activity of agrin as a regulator of FGF-2 function (Figure 8A and 8B). When retinal cultures are treated with agrin alone, by adding agrin to the culture medium, we do not observe any change in the extent of neurite outgrowth on laminin-1, indicating that under these conditions agrin does not promote or inhibit neurite outgrowth in response to laminin-1 (Figure 7D).

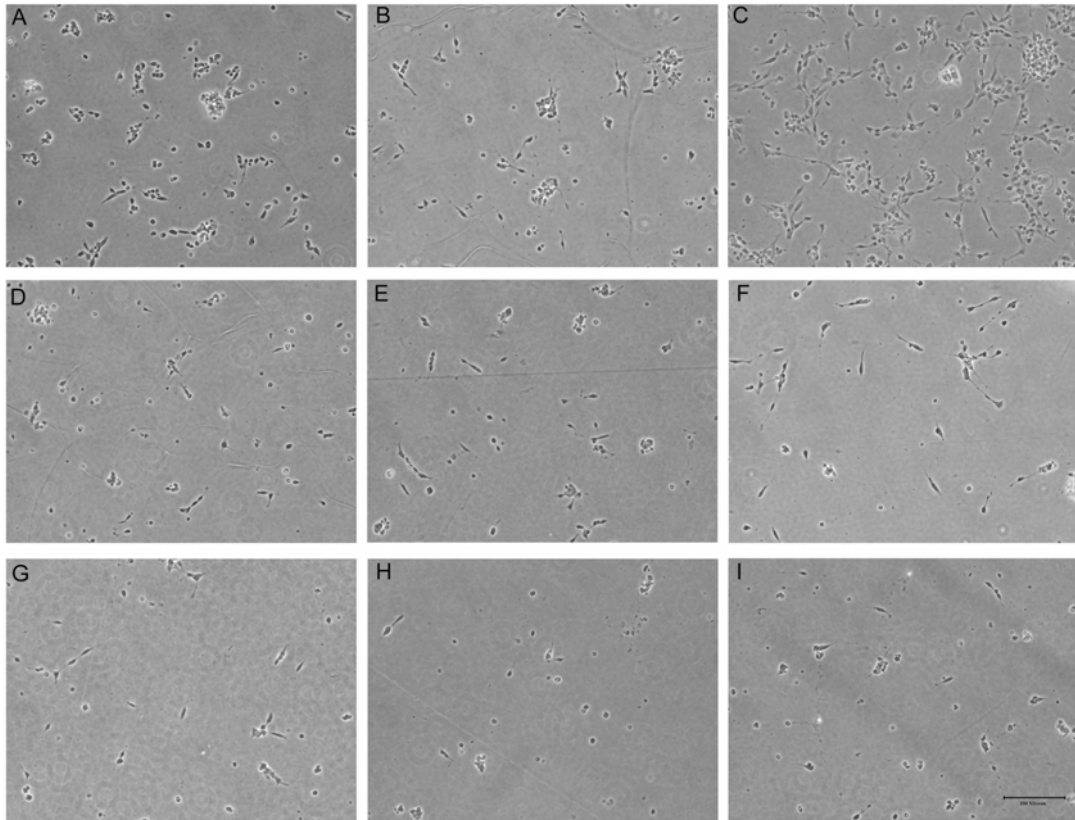


Figure 7. Effect of agrin on FGF-2 mediated neurite outgrowth from retinal neurons. A, untreated retinal neurons; B, Retinal cells treated with 2.5 ng/ml of FGF-2; C, Retinal cells treated with 2.5 ng/ml FGF-2 and 200 ng/ml of agrin; D, Retinal cells treated with 200 ng/ml of agrin only; E, Retinal cells treated with agrin that had been treated with nitrous acid to remove HS-GAG chains; F, Retinal neurons treated with FGF-2 and HS-GAG free agrin; G-I, Treatment of retinal neurons with the FGFR inhibitor SU5402 at the time cells were exposed to FGF-2 (G), FGF-2 and agrin (H), or FGF-2 and HS-GAG free agrin (I). Calibration bar, 100 μ m.

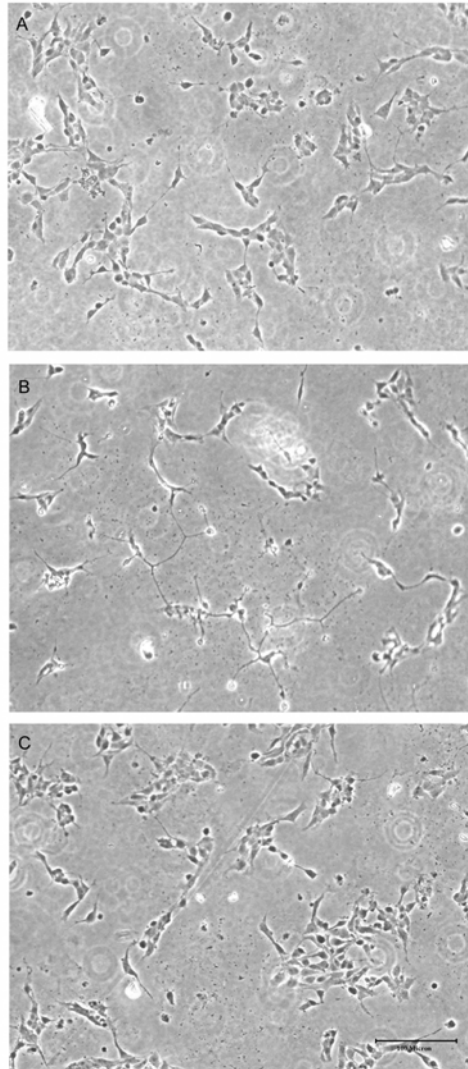


Figure 8. Effect of agrin on FGF-2 mediated retinal neurite outgrowth. E6 retinal cultures were treated with 10 ng/ml of FGF-2, in the presence or absence of agrin. A, Retinal neurite outgrowth in response to 10 ng/ml FGF-2. B, Agrin augments retinal neurite outgrowth in the presence of 10 ng/ml FGF-2. C, HS-GAG free agrin does not augment FGF-2 effects on retinal neurite outgrowth. Calibration bar, 100 μ m.

As shown for PC12 cells, when the 25 longest neurites are quantified in E6 retinal cultures for each experiment, we observe an increase in the average neurite length of these longest neurites for neurons exposed to FGF-2 and agrin (Figure 9A), especially at 10 ng/ml FGF-2. For example, the enhancement of neurite outgrowth by agrin in the presence of 10 ng/ml of FGF-2 is particularly obvious, as these neurons had an average neurite length of 148.1 μm , compared to 46.3 μm for cultures treated with FGF-2 only (Figure 9A). The extent of neurite outgrowth in the presence of 10 ng/ml FGF-2 and agrin was also equivalent to retinal cultures treated with 25 or 50 ng/ml FGF-2 only (data not shown). As shown in Figure 7A, removal of agrin's HS-GAG chains eliminates agrin's enhancement of FGF-2 mediated neurite outgrowth. The increase in the length of the longest retinal neurites is also evident when graphed as a percent distribution of neurites with a particular length (Figure 9B), with a shift in the distribution of neurite length for cultures treated with FGF-2 and agrin (Figure 9B).

Analysis of the quantitative affects of FGF-2 and agrin on retinal neurite outgrowth indicate that the addition of agrin in combination with 2.5 ng/ml FGF-2 results in a 1.8-fold increase in neurite length when compared to FGF-2 alone (Figure 9A), and agrin in combination with 10 ng/ml FGF-2 results in a 3.2-fold increase in neurite length(Figure 9A). Importantly, when compared to retinal cells grown on a laminin-1 substratum only, 10 ng/ml FGF-2 and agrin results in an approximately 7-fold increase in neurite length. This is a dramatic increase in neurite outgrowth, when our

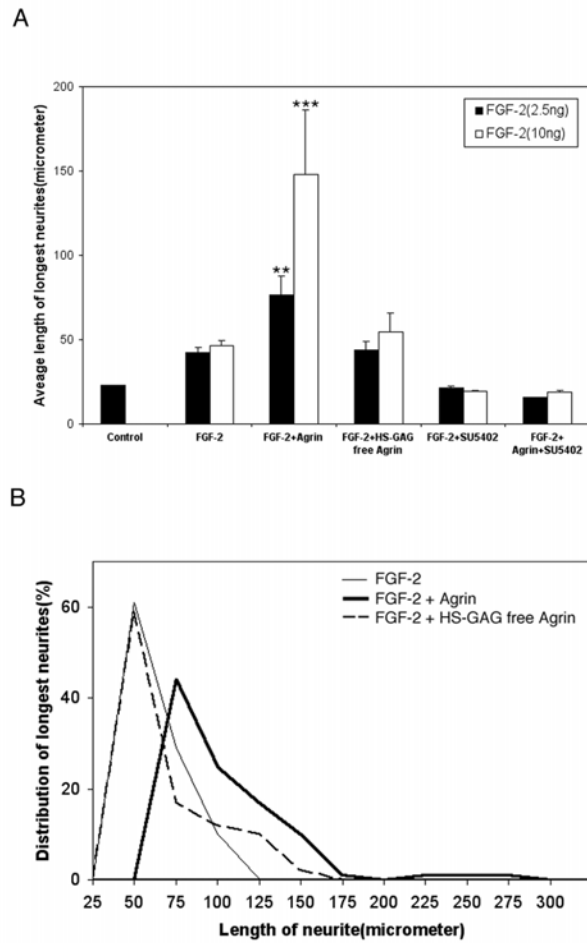


Figure 9. Quantitation of neurite outgrowth from retinal cultures treated with FGF-2 and/or agrin. A, The average length of the longest neurites on retinal neurons was determined for the treatments shown, using 2.5 or 10 ng/ml of FGF-2 and 200 ng/ml of agrin. It can be seen that agrin significantly increases neurite outgrowth in the presence of FGF-2, and that treatment of cells with SU5402 reduces growth to untreated control levels. ** Statistically different from FGF-2 treated cells, $P < 0.02$ using ANOVA. *** Statistically different from FGF-2 treated cells, $P < 0.04$. B, Distribution of the 100 longest neurites measured from retinal neurons treated with FGF-2 and/or agrin. Neurites in random fields were measured and the 100 longest neurites from 4 representative experiments were distributed on a plot of percent of neurites versus neurite length. The main peak shifts to the right with addition of FGF-2 and agrin, reflecting a longer length of the 100 longest neurites. There are also more neurites with lengths greater than 100 μm when retinal neurons are exposed to FGF-2 and agrin.

data are considered in the context of the recent studies of Perron and Bixby (1999) that demonstrated a two-fold increase in neurite outgrowth using 25 ng/ml of FGF-2 in the absence of exogenous HSPGs.

Since FGF-2 treatment alone is capable of promoting neurite outgrowth from retinal neurons, and FGF-2 activity is dependent on binding to HSPGs, we decided to approach whether endogenous HSPGs may be present on the surface of retinal neurons that allow FGF-2 to promote retinal neurite outgrowth in the absence of exogenous HSPGs. We therefore treated retinal cell cultures with heparitinase for 1 h prior to exposure of cells to FGF-2, which resulted in a reduction in the magnitude of neurite outgrowth that approached control levels (Figure 10). In these experiments purified agrin was also treated with heparitinase, to demonstrate that under these conditions heparitinase treatment of agrin abolishes agrin's ability to potentiate FGF-2 mediated neurite outgrowth from retinal neurons (Figure 10). Our results showing that heparitinase treatment of retinal cells does not completely eliminate FGF-s ability to promote neurite outgrowth therefore suggest either the retention of limited neurite outgrowth promoting activity by FGF-2 in the absence of HSPGs, or an inability of heparitinase to completely degrade cell surface HSPGs under these experimental conditions.

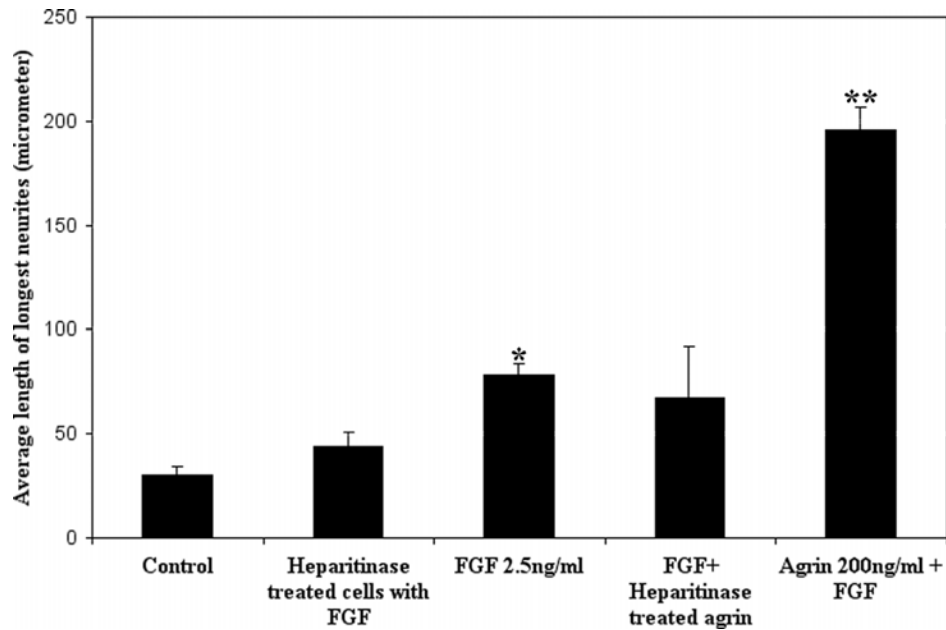


Figure 10. Effect of elimination of cell surface HS-GAGs on retinal neurite outgrowth in response to FGF-2 treatment. E6 retinal cell cultures plated on a laminin-1 substratum were treated with heparitinase as described under “Methods”, and then exposed to 2.5 ng/ml of FGF-2 and neurite outgrowth was quantified after 28 h in culture. For comparison, retinal cells that were not treated with heparitinase were treated with FGF-2 and agrin, or FGF-2 and heparitinase-treated agrin. It can be seen that the majority of FGF-2 mediated neurite outgrowth is eliminated by the removal of cell surface HS-GAGs, and that removal of agrin’s HS-GAGs using heparitinase abolishes agrin’s ability to modulate FGF-2 function. * Significantly different from control cells, $P < 0.003$. ** Significantly different from FGF-agrin treated cells, $P < 0.0001$.

Agrin exerts its effects on retinal neurite outgrowth via an FGF-2 dependent ERK signaling mechanism.

Since agrin was modulating the ability of FGF-2 to stimulate retina neuronal neurite outgrowth, we examined whether these effects of agrin are mediated via the FGFR-1. Therefore, SU5402 was added to retinal cultures at the time FGF-2 and/or agrin was added to the culture. As shown in Figure 7G-H and Figure 9A, SU5402 abolished the stimulatory effect of FGF-2 on retinal neurite outgrowth (Figure 7G, Figure 9A), including in the presence of agrin (Figure 7H,I, Figure 9A). These data indicate that in CNS neurons agrin's ability to modulate FGF-2 mediated neurite outgrowth is dependent on activation of the FGFR.

To confirm that agrin does modulate FGF-mediated signaling, as shown for PC12 cells, we analyzed ERK activation in E6 retina cultures. As shown in Figure 11A,B, FGF-2 stimulates a rapid and transient activation of ERK in retinal neurons, which corresponds to the enhanced neurite outgrowth by these cells. When E6 retinal neurons are exposed to both FGF-2 and agrin the ERK activation is again maintained at elevated levels, when compared to control, at 3 h post-exposure (Figure 11A and 11B). The FGFR-1 inhibitor SU5402 abolishes the activation of ERK by FGF-2 and agrin (Figure 11A), indicating that agrin's effects on ERK activation in retinal neurons are mediated

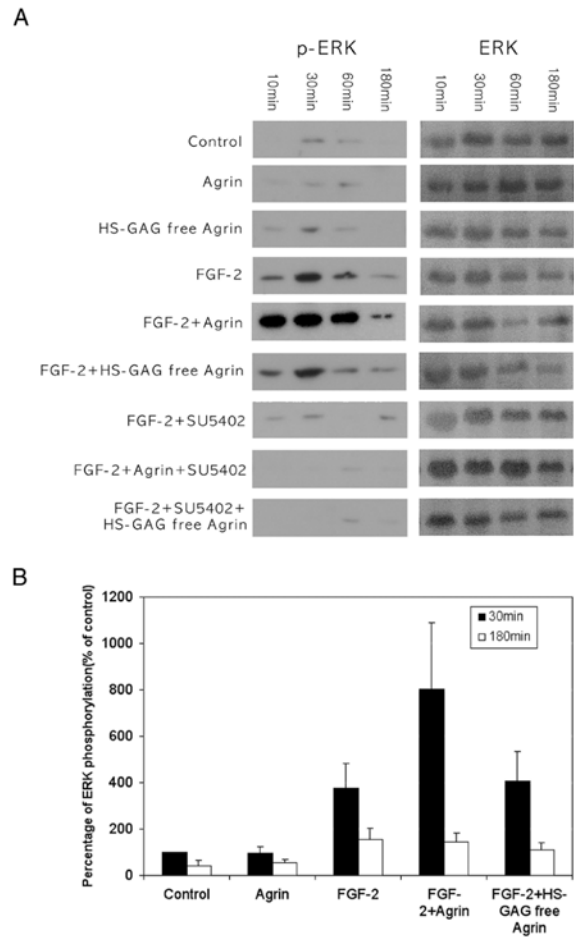


Figure 11. Phosphorylation of P-ERK in retinal neurons in response to treatment with 2.5 ng/ml FGF-2 in the presence or absence of 200 ng/ml agrin. A, Immunoblot analysis of P-ERK in retinal neurons, showing total ERK protein in cells for comparison. Only a single ERK isoform (p43) is expressed in chick retinal neurons as shown. It can be seen that FGF-2 and agrin treatment leads to a rapid activation of ERK, which is eliminated by inhibition of the FGFR using SU5402. B, The effect of FGF-2 and/or agrin on ERK phosphorylation was quantified from densitometric analysis of immunoblots of ERK protein phosphorylation, from three independent experiments.

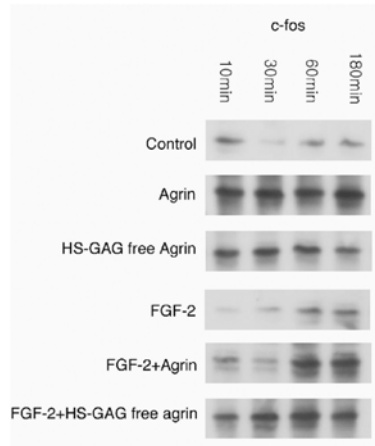
via an enhancement of FGF-2 signaling via the FGFR. The magnitude of the ERK phosphorylation by FGF-2 and agrin, when compared to FGF-2 alone, was also comparable to the extent of increased neurite outgrowth in response to FGF-2 and agrin, as both neurite outgrowth and ERK phosphorylation were increased approximately 2-fold. These data therefore suggest a strong correlation between ERK activation and neurite outgrowth in chick retinal neurons.

Agrin increases and sustains FGF-2 mediated c-fos phosphorylation.

In view of our data showing that agrin can stimulate neurite outgrowth from PC12 cells and E6 retinal neurons in an FGF-2 dependent manner, we thought it of interest to begin to address whether other signaling pathways could also contribute to agrin's effects on neurite outgrowth. This is particularly relevant since recent studies have shown that agrin can regulate activation of the intermediate early gene product c-fos (Hilgenberg et al, 1999), and recent studies have demonstrated that a cell's response to ERK activation is dependent on the length of ERK activation, with c-fos activation acting as a cellular sensor for biological responses mediated by ERK activation (Murphy et al, 2002). Thus, it is of interest to understand whether agrin activates c-fos in PC12 cells, and whether this activation is related to neurite outgrowth effects of agrin. We therefore analyzed c-fos phosphorylation in PC12 cells treated with FGF-2

in the presence or absence of agrin, as well as analyzing cells for c-fos activation in response to agrin alone. As shown previously, we observe a pronounced and rapid phosphorylation of c-fos in response to agrin, which is sustained for 3 h (Figure 12). Activation of c-fos in response to FGF-2 treatment of PC12 cells is similar to untreated cells, suggesting that activation of ERK by FGF-2 does not lead to changes in c-fos activation (Figure 12A and 12B). Interestingly, activation of c-fos in response to FGF-2 and agrin treatment resembles FGF-2 treatment more than agrin treatment of PC12 cells, in that the activation is less rapid and less dramatic than agrin treatment alone (Figure 12A and 12B). Importantly, the activation of c-fos in the presence of FGF-2 and agrin is significantly greater than FGF-2 alone, and the activation is sustained at 3 h (Figure 12B). These data therefore suggest that agrin binds to an unknown cellular receptor to rapidly and robustly activate c-fos, but that when agrin is bound to FGF-2 this receptor is not activated by agrin. Rather, agrin acts to enhance FGF-2 mediated activation of c-fos, which together with the sustained ERK activation may allow agrin to more effectively modulate neuronal responses to FGF-2. Accordingly, HS-GAG free agrin in the presence of FGF-2 leads to c-fos phosphorylation that resembles agrin only treatment, and not FGF-2 treatment (Figure 12A and 12B), suggesting that when presented to PC12 cells alone agrin or FGF-2 bind to different receptors to regulate c-fos phosphorylation.

A



B

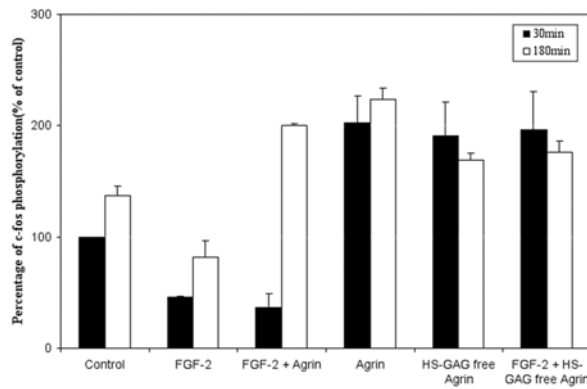


Figure 12. Regulation of c-fos phosphorylation by agrin. A, Agrin activates c-fos phosphorylation in PC12 cells, with activation being rapid, sustained, and not dependent on agrin's HS-GAG chains. Conversely, FGF-2 treatment (2.5 ng/ml) has little effect on c-fos activation, but these effects are augmented and sustained by agrin. B, The effect of FGF and/or agrin on c-fos phosphorylation was quantified from densitometric analysis of immunoblots of c-fos phosphorylation. The densitometric analysis shown is from two independent experiments. A third immunoblot was not quantified due to high film background, but this experiment showed a similar increase and time course in c-fos activation in response to FGF-2 plus agrin.

DISCUSSION

Despite the wealth of information regarding the function of agrin in the development of the neuromuscular junction, the function of agrin in the central nervous system is still poorly understood. It has been proposed that agrin may also regulate synaptogenesis in the developing CNS, participating in neuron-neuron synapse formation by regulating neuronal receptor clustering (Ferreira 1999; Bose et al, 2000). However, conflicting data have been obtained regarding the role of agrin in neuron-neuron synapse formation, with neurons isolated from the CNS of agrin-deficient mice forming phenotypically normal synapses (Li et al, 1999), while peripheral sympathetic neurons exhibit impaired synapse formation in agrin-deficient mice (Gingras et al, 2002). Because agrin is a major basement membrane HSPG in the developing CNS (Halfter 1993; Halfter et al, 1997), and is abundantly expressed in developing axon pathways (Halfter et al, 1997), significant attention has been placed on determining whether agrin may contribute to the establishment of axon pathways. This is especially relevant in view of several recent studies that have shown that agrin interacts with a number of cell adhesion and ECM adhesive proteins that include NCAM (Burg et al, 1995), integrins (Martin and Sanes, 1997), NCAM (Bixby et al, 2002), laminins (Denzer et al, 1997; Cotman et al, 1999), tenascin (Cotman et al, 1999), thrombospondin (Cotman et al, 1999), HB-GAM (Daggett et al, 1996) and FGF-2

(Cotman et al, 1999).

The studies we describe here demonstrate that agrin is capable of acting as a neurite outgrowth promoting factor, and that agrin is not inhibitory to neurite outgrowth when used with adhesive substrates such as laminin-1. We focused on PC12 cells and retinal neurons as models to examine agrin's effects on FGF-mediated neurite outgrowth, since PC12 cells are known to differentiate in response to FGF-2 (Rydel and Greene, 1987) and FGF-2 has been shown to play an integral role in the establishment of the retinotectal pathway (McFarlane et al, 1995; Walz et al, 1997). Importantly, HSPGs have been shown to potentiate the ability of FGF-2 to promote retinal neuronal axonal elongation (Walz et al, 1997), and we have demonstrated that agrin co-localizes with FGF-2 in the developing optic nerve (Cotman et al, 1999). Using both PC12 cells and retinal neuronal cultures, we observe that agrin enhances the efficacy of FGF-2 function, in terms of promoting neurite outgrowth. Agrin's modulation of FGF-2 activity is clearly HS-GAG dependent, and agrin is likely potentiating neurite outgrowth by augmenting FGF-2 binding to the FGFR. Thus, the FGFR-1 specific inhibitor SU5402 abolishes agrin's effects on FGF-2 mediated neurite outgrowth. Interestingly, agrin appears to increase FGF-2 neurite promoting activity by maintaining ERK activation, since with suboptimal doses of FGF-2 the phosphorylation of ERK was transient and not sustained. However, in the presence of agrin, FGF-2 binding to the FGFR leads to a rapid activation of ERK that is maintained

above control levels even 3h post-exposure, which likely is required to initiate the cellular pathways leading to neuronal differentiation. We did observe that the activation of ERK was also augmented to a lesser degree when FGF-2 was presented to cells with HS-GAG free agrin, which may be attributable to either a possible interaction of agrin's core protein with FGF-2 to enhance the efficacy of FGF-2 binding to the FGFR, or incomplete removal of HS-GAGs by nitrous acid treatment. Agrin is a multi-modular HSPG with many distinct functional motifs, resembling other multi-modular HSPGs such as perlecan, and interestingly perlecan's core protein has been shown to bind to FGF-7 (Mongiat et al, 2000).

When comparing our current studies to recent studies that have examined the role of agrin in neurite outgrowth, it is clear that significant discrepancies are apparent. Previous studies have implicated agrin as both a neurite promoting (Mantych and Ferreira, 2001) and inhibitory protein (Campagna et al, 1995; Chang et al, 1997; Halfter et al, 1997; Bixby et al, 2002), although differences in the source of agrin, the region of agrin employed, and neuronal cell types studied, could in part explain these discrepancies. Previous studies have also employed agrin as a substrate in combination with laminin (Halfter et al, 1997; Bixby et al, 2002), or agrin expressed by non-neuronal cells (Campagna et al, 1995; Chang et al, 1997). Based on studies showing that agrin expression during nervous system development is associated with developing axon pathways (Halfter et al, 1997; Kroger 1997; Cotman et al, 2000), and that this

agrin expression is abundant in these pathways, one can speculate that agrin would not likely function as a neurite growth inhibitory molecule *in vivo*, since this would impede rather than promote the formation of these axon pathways. Thus, our studies showing that agrin co-localizes with growth promoting molecules such as FGF-2 in the retinotectal system (Cotman et al, 2000), combined with previous studies demonstrating the importance of FGF-2 and HSPGs in the establishment of the retinotectal pathway (McFarlane et al, 1995; Walz et al, 1997), would lend credence to the proposal that agrin may function as a growth promoting protein, rather than a growth inhibitory protein, in developing axon pathways such as the optic tract. Accordingly, our data reported here support this hypothesis, showing that agrin potentiates the effects of FGF-2 in retinal and PC12 cell cultures. Interestingly, when comparing our findings to studies such as those of Perron and Bixby (1999), the importance of agrin in potentiating the effects of FGF-2 becomes more apparent. Perron and Bixby (1999) showed that FGF-2 is a growth-promoting molecule in chick retinal cultures, and that ERK activation correlates with neurite outgrowth. Most importantly, Perron and Bixby (1999) employed a substratum of 10 $\mu\text{g/ml}$ laminin-1, and 25 ng/ml FGF-2, to examine neurite outgrowth in chick retinal neurons. The average length of neurites in their studies was comparable to that reported in our current studies, using 5 $\mu\text{g/ml}$ laminin-1 and 10 ng/ml FGF-2 in combination with agrin. Furthermore, we observed a greater magnitude of increased neurite outgrowth, relative to neurons grown on a laminin-1 substratum, in our studies using lower concentrations

of laminin-1 and FGF-2, when presented in combination with agrin. These data support our proposal that agrin and other HSPGs may function as crucial regulators of neurite outgrowth mediated by heparin-binding growth factors such as FGF-2.

Recent studies have indicated that the duration of ERK signaling in cells is crucial to biological responses, such as neurite outgrowth, that occur in response to activation of the ERK signaling pathway (Murphy et al, 2002). A prolonged duration of ERK signaling is required to activate other signaling molecules, such as c-fos (Murphy et al, 2002), and thus sustained ERK phosphorylation is essential to activating immediate-early genes that are necessary for biological responses. Interestingly, our studies clearly demonstrate that agrin maintains an elevated ERK activation in response to FGF-2, and additionally we show in the present study that c-fos activation is also augmented and prolonged when agrin is presented to neurons in combination with FGF-2. Agrin has been shown previously to activate c-fos (Hilgenberg et al, 1999), and surprisingly our data show that whereas agrin stimulates a rapid and sustained activation of c-fos, when agrin is presented with FGF-2 the c-fos activation is not as pronounced, although it is sustained. These data therefore suggest that agrin binds to a cellular receptor that rapidly activates c-fos, but when agrin is bound to FGF-2, and presumably the FGFR, the FGFR is activated and agrin acts to sustain both FGFR and c-fos activation. Thus, these data demonstrate one possible mechanism whereby agrin can differentially regulate cell signaling pathways, depending on the presence or

absence of binding ligands for agrin.

Our demonstration that agrin modulates FGF-2 mediated neurite outgrowth is important, especially when placed in the context of putative functions of the FGFR or FGFs in CNS development. Activation of the FGFR has been shown to play a critical role in the modulation of neurite outgrowth by cell adhesion molecules such as N-CAM or N-cadherin (Saffell et al, 1997; Doherty et al, 2000). Since we have shown that agrin binds to both N-CAM and FGF-2 (Burg et al, 1995; Cotman et al, 1999), it is of interest to speculate that a complex of N-CAM, agrin and FGF-2 could serve as an optimal environment for neurite outgrowth in the developing CNS. FGF-2 and agrin expression is also abundant in the ventricular zone of the developing CNS, and neurogenesis has been shown to be regulated by FGF-2 (Ray and Gage, 1994; Ghosh and Greenberg, 1995). Thus, it will be of interest to ascertain whether agrin will modulate the efficacy of FGF-2 regulation of stem cell function and neurogenesis.

It is also worth considering the importance of our demonstration that agrin can increase the effectiveness of FGF-2 as a neurite outgrowth promoting molecule, using concentrations of FGF-2 that are suboptimal for stimulating neurite outgrowth when used alone. These findings suggest a possible mechanism whereby agrin can fine tune axonal responses to FGF-2 as axons extend along developing nerve pathways. For example, one can propose that agrin is important to the promotion of efficient axon

growth in the optic nerve and optic tract, by modulating FGF mediated stimulation of the FGFR and ERK signaling pathway. Accordingly, it has been shown that FGF-2 and agrin are expressed in the developing optic nerve and tract, but are not as abundantly expressed in the optic tectum (McFarlane et al, 1995; Halfter et al, 1997; Cotman et al, 1999). FGF-2 has also been shown to be important for the regulation of retinal ganglion cell axonal growth from the retina to the optic tectum, and high concentrations of exogenous FGF-2 (50-100 ng/ml) disrupt this axon pathfinding to the optic tectum, with retinal ganglion cell axons bypassing the optic tectum (McFarlane et al, 1995). It is therefore tempting to speculate that when axons in the optic tract are exposed to these high exogenous levels of FGF-2, this FGF ameliorates agrin's ability to modulate growth cone responses of optic axons to FGF-2, resulting in the optic axons following aberrant pathways determined by the high FGF-2 levels. Interestingly, the presence of agrin in the optic tectum (Halfter et al, 1997; Cotman et al, 1999), particularly in the target region for retinal ganglion cell axons, could act to modulate FGF-2 mediated growth of retinal ganglion cell axons to their target cells in the tectum, in an environment with lower concentrations of FGF-2. Thus, in such an environment of low FGF-2 (i.e., suboptimal for mediating efficient neurite growth), the presence of agrin could serve to promote neurite growth along these pathways. Under these conditions, differences in FGF-2 concentrations encountered by growing axons in the presence of agrin could serve to guide these axons to their appropriate target cells, such as the stratum opticum in the optic tectum. Further support for the potential

importance of agrin in regulating formation of nerve pathways is derived from studies showing that removal of HS-GAGs from the optic tract, using heparitinase, results in a disruption of axon growth in the tract, that can be overcome by administering high concentrations of FGF-2 (Walz et al, 1997). Thus, HS-GAGs carried by agrin can be postulated to function in the developing optic nerve and tract by enhancing the ability of axons to grow in the presence of concentrations of FGF-2 that are suboptimal for growth in the absence of HSPGs. In addition, agrin levels diminish in the optic nerve and tract coincident with periods of retinal ganglion cell axons reaching the optic tectum (Halfter et al, 1997), and agrin concentrations are high, in the presence of low FGF-2 levels, in the optic tectum at developmental periods when retinal ganglion cell axons invade the optic tectum (Halfter et al, 1997; Cotman et al, 1999). These data therefore suggest that agrin may be a crucial regulatory molecule for FGF-2 mediated axonal growth and target recognition in the retinotectal system.

One caveat in considering our data reported in the present study concerns findings from agrin knock-out mice, since these mice have apparently normal CNS development (Serpinskaya et al, 1999). These data would argue against the importance of agrin in establishing axon pathways such as the retinotectal system. However, one must also consider the possibility of molecular redundancy, with other HSPGs being capable of substituting for agrin in its absence, in order to potentiate the function of molecules such as FGF-2 in developing axon pathways. As one example, it has been reported that

a perlecan-like HSPG is also distributed in the developing retinotectal pathway, and that this HSPG is a modulator of FGF-2 activity (Joseph et al, 1996). Other HSPGs, such as glypican-1, have also been shown to be abundant in axon tracts (Litwack et al, 1998).

In summary, our studies presented here provide evidence that agrin can function as a neurite-promoting molecule when acting in concert with heparin-binding growth factors such as FGF-2. Agrin increases the efficacy of FGF-2 function, acting to maintain activation of the FGFR signaling pathway, and immediate early gene products, that are essential to biological responses mediated by FGFs. In addition, our studies suggest that a crucial function for agrin will be to regulate FGF function in the developing nervous system, thus providing important new insight into the search for functions of agrin in the developing CNS.

CHAPTER II

Agrin is Required for Posterior Development and Axon Pathway Formation in Embryonic Zebrafish.

ABSTRACT

Agrin is an extracellular matrix heparan sulfate proteoglycan that plays a key role in the development of the neuromuscular junction (NMJ) by inducing the clustering of acetylcholine receptors (AChRs) at synaptic sites of the NMJ. Although recent studies have extended our understanding of agrin's function in the nervous system, its function in the CNS is not clearly understood. To overcome the lethality of agrin gene disruption in mice and the difficulty of embryonic manipulation of agrin function in mice, we have identified a gene encoding zebrafish agrin. Zebrafish agrin is expressed in the developing CNS, the NMJ, and non-neural structures such as the pronephric duct. We have applied a morpholino-based gene targeting method to identify the function of agrin in zebrafish development. Targeted morpholinos against agrin significantly inhibit tail development in a dose-dependent manner, and impair development of the NMJ. We also observe severe defects in primary and secondary motor neuron axon outgrowth, and in formation of the midbrain-hindbrain boundary. Morphants subsequently develop paralysis, and die at larvae stages. Moreover, in the CNS agrin morphants show defects in axonal guidance and outgrowth by Rohon-Beard sensory neurons. In conclusion, our results show that zebrafish agrin is essential for NMJ formation, as well as sensory and motor nerve pathway formation in zebrafish.

Keywords: agrin; heparan sulfate proteoglycan; axon guidance; zebrafish

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are a divergent class of macromolecules whose function is classified by the addition of glycosaminoglycan chains (Lindahl and Kjellen., 1991; Yanagishita and Hascall, 1992; David, 1993). The heterogeneity of HSPGs, as a result of modifications of their heparan sulfate glycosaminoglycan chains (HS-GAGs), provides versatility to the biological functional properties of these molecules. Heterogeneity in HS-GAGs provides a diversity of binding activity with other extracellular molecules such as heparin-binding growth factors (Sanderson et al., 1994; Cotman et al., 1999; Matsuda et al., 2001; Knox et al., 2002; Kim et al., 2003), extracellular matrix (ECM) molecules (San Antonio et al., 1993; Herndon et al., 1999), N-CAM (Cole et al., 1986; Kallapur et al., 1995; Prag et al., 2002) and cell surface receptors (Lopez-Casillas et al., 1991; Hayashi et al., 1992; Lowe-Krentz et al., 1992). There is increasing evidence that different proteoglycans act as regulators of cell growth and differentiation (Itoh and Sokol, 1994; Bink et al., 2003), cell adhesion (Cole et al., 1985; Perkins et al., 1989; Reiland et al., 2003), tumorigenesis (Alexander et al., 2000), and wound healing (Andriessen et al., 1997). Inhibition of heparan sulfate synthesis by either antisense technology or gene disruption also provides considerable insight into HSPG function in development (Galli et al., 2003), and demonstrates that elimination of HSPGs is lethal during development (Costell et al., 2002).

In the nervous system recent studies have also contributed to our understanding of the biology of HSPGs, and the importance of this class of macromolecule in nervous system development. It has been suggested that HSPGs may contribute to diverse processes in nervous system development, based on their localization in basal laminae, the extracellular matrix, and the plasma membrane of neural cells. Thus, HSPGs have been suggested to play a regulatory role in spinal neurulation (Yip et al., 2002; Tucklett and Morris-Kay, 1989), cell patterning in the cerebral cortex, midbrain and hindbrain (Inatani et al., 2003), and the regulation of stem cell function (McLaughlin et al., 2003; Park et al., 2003). Numerous studies have provided evidence for a role of HSPGs in the establishment of axon pathways in the developing nervous system. Recent studies have shown HSPGs to modulate axon guidance at the CNS midline (Inatani et al., 2003), which could in part be based on the ability of HSPGs to bind netrins, which are well known regulators of axon guidance (Tessier-Lavigne and Goodman, 1996; Bennett et al., 1997). The exogenous addition of HS-GAGs perturbs pioneer axon guidance in cultured cockroach embryos (Wang and Denburg, 1992), as well as the aberrant targeting of retinal axons to the optic tectum in *Xenopus* (Walz et al., 1997).

Agrin is a 400 kDa HSPG that is localized to the neuromuscular junction as well as axonal pathways of the central nervous system. Agrin is also abundantly expressed in basal laminae in the lung and brain, and in the kidney glomerular basement membrane (Halfter et al., 1997; Groffen et al., 1999; Yard et al., 2001). While agrin's role in the

regulation of synaptogenesis at the NMJ is well established, its function in brain has remained elusive. The lethality of agrin gene disruption in transgenic mice has contributed to our lack of understanding of agrin's potential contribution to CNS development, which is important since recent studies have begun to provide evidence for the role of agrin in some CNS developmental processes. Agrin has been shown to promote axonal branching and dendritic branching and elongation *in vitro* in hippocampal neurons (Mantych and Ferreira, 2001), but also has been suggested to be inhibitory to neurite and axonal outgrowth (Bixby et al., 2002). The ability of agrin to inhibit axonal growth *in vitro* would seem to be inconsistent with its abundant expression in developing axon pathways such as the optic nerve (Halfter et al., 1997). Likewise, our recent studies have shown that agrin modulates the activity of FGF-2 to promote neurite outgrowth (Kim et al., 2003), suggesting that agrin may not possess only an inhibitory activity with regard to the establishment of axonal pathways. Moreover, recent studies have shown that intramuscular axonal branching increases and nerve-evoked skeletal muscle contraction was reduced in agrin mutant mice embryo (E18.5)(Banks et al., 2003). Thus, identification of functions for agrin that are essential for developmental processes would extend our understanding of the role of HSPGs, and in particular agrin, in axon growth and guidance in the developing nervous system.

Zebrafish represents a particularly attractive model system to begin to address *in vivo* agrin's role in developmental processes such as axon guidance and outgrowth, as

the function of specific genes can be perturbed following the microinjection of morpholino antisense oligonucleotides into one-cell embryos. With regard to axon guidance and outgrowth, the simplified CNS of zebrafish and the availability of many cell-type specific antibodies allow one to assess the contribution of specific genes to axon guidance and outgrowth in identified neurons. Our studies reported here describe our initial characterization of the distribution of agrin in the zebrafish embryo, and establish a crucial role for agrin in motor and sensory axon guidance in zebrafish, CNS morphogenesis, and posterior development.

MATERIAL AND METHODS

Fish maintenance

The wild-type adult zebrafish (*AB background) and *islet-1*-GFP transgenic zebrafish (Higashijima et al., 2000) were maintained at 28.5°C under standard laboratory conditions (Westerfield, 2000). Embryos were allowed to develop and staged by hours or days after fertilization at 28.5°C and morphological criteria (Kimmel et al., 1995).

Molecular cloning

To obtain the 5' UTR sequence, 5' RACE was performed using degenerative primer 5'-GGAACGAGTGTGAGCTG-3' and gene specific primer : 5'-CTGGAGTGAAGTGGGTCCCGGGTTTGT -3'. EST clone sequences (AI959096, AI477575, AI477444) were used to obtain sequence near C-terminus region. RT-PCR was performed to connect the gap between EST clones by using primer : zf-agrin-F: 5'-ACCACCACCACCACAATGTCTC -3' and zf agrin-R: 5'-TTTCACAGTGCCTTCACGTC -3'. The 1055 bp of PCR product was cloned and

transformed by TOPO TA cloning kit for sequencing. The clones from 5' RACE and RT-PCR (zf-agrin) were sequenced and analyzed by <http://www.ncbi.nlm.nih.gov/BLAST>.

Antisense Morpholino injection

Antisense morpholino nucleotides (MOs) (Gene Tools, LLC) were designed against 5' sequence near the start site of translation (Nasevicius and Ekker, 2000) and an exon/intron splice donor site (Draper, 2001). The sequences of MOs used were: Agrin-MO1: 5'-CCGCTTTTCTGTCCGCAGAGCCCAT-3', Agrin-MO2: 5'AGAGTTGTACACCTACCAGAGAAAC-3' and Control-MO : 5'-CCTCTTACCTCAGTTACAATTTATA-3'. MOs were solubilized in water at a concentration of 16mg/ml and diluted to working concentrations in water before injection into one- to four-cell stage embryos. Agrin morpholinos at a concentration of 1-9 ng consistently produced reproducible phenotypes with an injection volume around 1-5 nl (Lewis and Eisen, 2003; Maroon et al., 2002) and resulted in a drastic reduction of agrin expression. The control morpholino at the same dose produced no distinguishable effects.

Northern blot analysis

Total RNA was extracted from various stage embryos using Trizol (Invitrogen). 10µg of RNA was loaded into 1% agarose gels and transferred to nitrocellulose membrane (Schleicher and Schuell) using pressure transfer. The antisense probe was synthesized by RT-PCR using 2 days old embryo cDNA as a template (primer set: zf-agrin) and labeled by digoxigenin high prime DNA labeling. The northern blot protocol was according to manufacturer's procedures (DIG high prime DNA labeling and detection kit II, Roche). The density of bands of interest was analyzed using NIH image software.

Whole-mount *in situ* hybridization

Whole-amount *in situ* hybridization was performed as described (Yuan et al., 1997; Thisse et al., 1993). Zebrafish agrin cDNA clone sequences (zf-agrin) were used to make sense and anti-sense digoxigenin-labeled riboprobes of 1055 bp by *in vitro* transcription using T3 and T7 polymerases. Embryos were fixed for overnight at 4°C in 4% paraformaldehyde in PBS and stored at -20°C in 100% methanol. After rehydration, embryos were prehybridized at 65°C in Hybridization mix (HM : 50% formamide, 5X SSC, 50µg/ml of heparin, 50µg/ml of tRNA, 0.1% Tween 20, 9mM citric acid (pH6.0)). *In situ* probes were added in fresh HM and hybridized overnight at 65°C. Embryos

were washed each 15 minutes in the order of HM, 25% 2X SSC/ 75%HM, 50% 2X SSC/50% HM, 75% 2X SSC/25% HM, 2X SSC and twice for 30 minutes in 0.2X SSC. Embryos were equilibrated into PBT by each 5 minutes in 75% 0.2X SSC/ 25% PBT, 50% 0.2XSSC/ 50% PBT, 25% 0.2X SSC/ 75% PBT. Embryos were preblocked in 2% sheep serum, 2mg/ml BSA in PBT for several hours at RT. Embryos were incubated with anti-DIG antibody (1:1000–5000) at 4°C for overnight. Embryos were washed with PBT for 2 hours at room temperature and again in Tris pH 9.5 solution (100mM Tris-Cl (pH 9.5), 50mM MgCl₂, 100mM NaCl, 0.1% Tween 20). Embryos were stained in 4.5µl NBT, 3.5µl BCIP in Tris pH 9.5 solution. Staining reaction was stopped by PBT, then in PBS and embryos were stored in 4% paraformaldehyde/PBS at 4°C.

Antibody production

The zf-agrin clone, which was inserted into TOPO-PCR vector, was digested with EcoRI, ligated with pRSET-B vector and transformed into TOP10F' (Invitrogen). Recombinant agrin protein was expressed under the control of the T7 promoter and extracted by His-bond, Ni²⁺ column chromatography (Novagen). Agrin fusion protein was used for production of polyclonal antisera in rabbits. Anti-agrin antisera were affinity purified by affinity chromatography using agrin fusion protein coupled to Sepharose CL-4B. Specificity of polyclonal antibody was confirmed via Western blot analysis.

Western blot analysis

Embryos were collected and cleaned with Ringer's solution. Embryos were homogenized in protein extraction buffer (10 mM Tris, pH 7.4, 2% Triton-X 100, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM trypsin inhibitor). Lysates were centrifuged and protein concentrations were determined by the Bradford assay using the Bio-Rad reagent and immunoglobulin as a standard protein. To remove HS-GAG chains from agrin, purified agrin was treated with nitrous acid as previously described (Burg et al., 1995). 400 μ g of supernatant protein was separated on 4-20% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblots were blocked with blotto and incubated overnight at 4 °C with a polyclonal antiserum against a zebrafish agrin fusion protein (1:100). Immunoblots were washed and incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (1:250,000). Membranes were incubated with Supersignal West Pico chemiluminescent Substrate kits (Pierce) and exposed to X-ray film (Kodak).

Immunohistochemistry

For whole-mount immunohistochemistry, embryos were fixed with 4% paraformaldehyde/PBS. After washing three times with PBS, embryos were blocked

with 10% goat serum or donkey serum in 0.5% saponin/PBS for 1 hour. The embryos were incubated in the blocking solution containing anti-agrin (1:10), anti-Hu (1:200 from Molecular Probes), mAb anti-znp-1 (1:10 from Developmental Studies Hybridoma Bank), anti-islet (1:10 from Developmental Studies Hybridoma Bank), mAb F59 (1:10 from Dr. Stockdale), mAbF310 (1:10 from Dr. Stockdale) and anti-zn-12 (1:50 from Developmental Studies Hybridoma Bank) overnight at 4°C. The znp-1 (Melancon, 1997) was used to detect the cell bodies and axons of primary motoneurons. The mAb F59 (Crow and Stockdale, 1986) recognizes all slow muscle cells including muscle pioneers (Devoto et al, 1996) and the mAb F310 was used to detect fast muscle cells (Crow and Stockdale, 1986). Embryos were washed several times with PBS and incubated with either secondary antibody (1:800 Cy3 or Cy5 conjugated antibody) or biotinylated secondary antibody (Vectastatin Elite ABC kit) and visualized with Vector Red Substrate.

Analysis of neuromuscular junction development

MO-injected embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature. After 4 hours incubation in water, embryos were incubated with Alexa-594-conjugated α -bungarotoxin (1:2000; Molecular Probes) for 1 hour according to published protocols (Ono et al., 2001).

Bodipy staining

To show morphology of muscle, embryos were incubated in embryonic medium including 100 μ M of Bodipy-ceramide for 1 hour and washed several times. Embryos were allowed to develop until desired stage and fixed in 4% PFA/PBS. Images were taken using a Nikon C-1 confocal microscope.

Analysis of apoptosis in zebrafish embryos

Live zebrafish embryos were incubated with 150 mg/ml acridine orange in PBS for 15 min, and destained for 15 min in PBS according to published protocols (Leger and Brand, 2002).

RESULTS

Cloning and expression pattern of agrin mRNA and protein during embryonic development in zebrafish

The partial sequence of the zebrafish agrin open reading frame was retrieved by 5'-RACE of zebrafish mRNA using a consensus degenerate primer and a gene specific agrin primer. Only one transcript for the NtA domain of the N-terminus of agrin was isolated, and was confirmed by sequencing. The cDNA sequence of the agrin NtA region is ~ 60% identical to the analogous region in chicken, human, rat, and electrical ray agrin (data not shown). Based on the sequence information of agrin EST clones (AI959096, AI477575, AI477444), sequence from the EGF-like and laminin G-like domains was cloned by RT-PCR and used as a probe for *in situ* hybridization and Northern blot analysis. This agrin cDNA was also used to prepare a polyclonal antibody against zebrafish agrin for use in western blotting and immunohistochemical studies.

A transcript of approximately 10 kb, which is similar in size to the agrin transcript from chicken (Tsen et al, 1995), was detected in zebrafish embryos by Northern blot

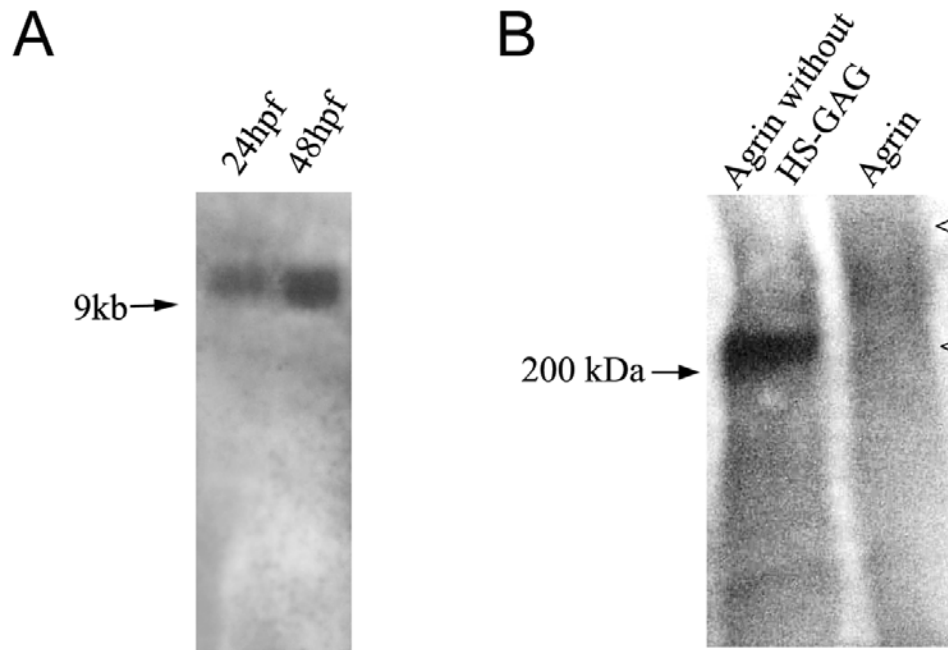
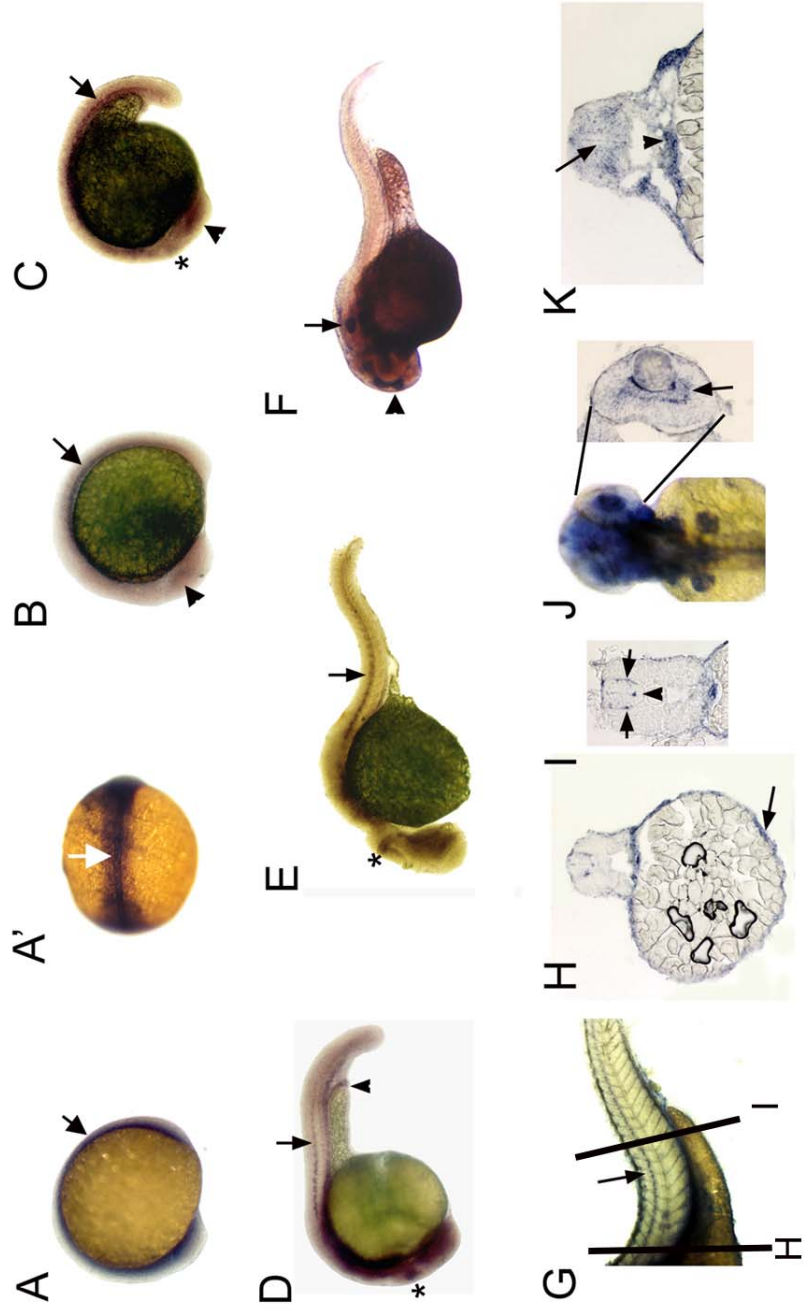


Figure 13. Agrin mRNA and protein are highly expressed in zebrafish embryos. A. Northern blot analysis of total RNA from zebrafish embryos using an agrin antisense probe. A 10 kb transcript is detected, which is differentially regulated with development. B. Western blot analysis demonstrates the typical characteristics of agrin, which appears as a 400-500 kDa smear (arrowhead). The agrin core protein of 200kDa is revealed after removal of HS-GAG chains from agrin by nitrous acid treatment.

analysis (Figure 13). The expression of agrin mRNA exhibits a pronounced increase between 24 hpf and 48 hpf (Figure 13A), and was substantially decreased in adult zebrafish (data not shown). Western blot analysis of zebrafish embryos for expression of agrin protein indicate the characteristic appearance of agrin as a 400-500 kDa smear on SDS-PAGE gels, with an agrin core protein of approximately 200 kDa being detected following treatment of zebrafish protein extracts with nitrous acid to remove HS-GAGs (Figure 13B).

Agrin mRNA expression and distribution in zebrafish embryos was analyzed by *in situ* hybridization. Agrin mRNA expression begins strongly in the notochord (Figure 14A'), while weak and diffuse in the forebrain and eye of tail bud stage embryos (Figure 14A), but was strongly expressed in the ventral region of floor plate and the dorsal part of notochord in early development (Figure 14B and 14C). This weak and diffuse signal remained throughout the brain and somites until 48 hpf (Figure 14B-E). However, strong agrin mRNA expression remained in ventral diencephalon, tectum, eye, hypochord, floor plate, muscle pioneers, notochord and pronephric duct until 48 hpf (Figure 14B-E). At 48 hpf, agrin mRNA expression in tectum disappears and agrin appears in the midbrain and hindbrain boundary (MHB), hindbrain rhombomeres, floor plate, as well as the ventral region of the spinal cord (Figure 14E). The expression of agrin mRNA decreased after 48 hpf, except for weak expression in

Figure 14. Localization of agrin mRNA in the zebrafish embryo. A-F: Whole mount *In situ* hybridization analysis of agrin mRNA expression. **A.** Agrin mRNA is expressed in forebrain and notochord (arrow) in tail bud stages. **A'.** Dorsal view of tail bud stage embryos. White arrow indicates notochord expression. **B.** 6-somite stage embryos. Agrin is weakly expressed in eye (arrowhead) and strongly in notochord (arrow) in early development. **C.** Agrin is expressed in tectum (*), eye (arrowhead) and notochord (arrow) at 16 hpf. **D.** Agrin's expression in CNS became stronger in tectum (*), eye, hindbrain, ventral region of spinal cord (arrow) and pronephric duct (arrowhead) at 22 hpf. **E.** Agrin expression continues at the midbrain-hindbrain boundary (MHB;*) and in the ventral region of spinal cord (arrow) at 48 hpf. Expression of agrin in pronephric duct still remains weakly. **F.** At 48 hpf agrin expression occurs in tectum and ventral diencephalon (arrowhead) and otic vesicle (arrow). **G.** Lateral view of tail. Arrow indicates agrin expression in motor neurons, and H and I show cross sections from G. **H.** Arrow indicates basal laminae. **I.** Arrow indicates motor neurons and arrowhead the ventral region of spinal cord. **J.** Dorsal view of head. Agrin is highly localized in ganglion cell layer of eye (arrow). **K.** Sections of in situ hybridized embryo at 30 hpf. Arrow indicates the midline agrin expression pattern in spinal cord. Agrin expression is also evident in ventral spinal cord, and in endoderm (arrowhead).



the ventricular region and the otic vesicle (Figure 14F). This decrease in agrin mRNA expression was in agreement with Northern analysis data showing low levels of agrin expression in adult zebrafish. Analysis of cryostat sections of embryos revealed a more detailed expression pattern that agrin is specifically expressed in motor neurons in the spinal cord (Figure 14G) basal lamina throughout the entire embryo (Figure 14H and 14I) and the retinal ganglion cell layer of the developing eye (Figure 14J). Agrin mRNA expression is also evident in ventral spinal cord, the spinal cord midline, and endoderm of 30 hpf embryos (Figure 14K).

Agrin protein distribution was analyzed during zebrafish development using an affinity-purified polyclonal antiserum prepared against a zebrafish agrin fusion protein. Most zebrafish agrin protein is strongly localized in the basal laminae, the CNS and in somites in early developmental stages (Figure 15), which is consistent with its distribution during chicken development (Halfter et al., 1997). In the CNS agrin is highly expressed in the basement membrane and forebrain (Figure 15A and 15D), eye and diencephalon (Figure 15B), midbrain-hindbrain boundary (Figure 15A), the ventricular region of hindbrain (Figure 15A and 15D) and notochord (Figure 15E). In the trunk and tail region, the expression pattern of agrin protein does not exactly match that of agrin mRNA in the nervous system and muscle (Figure 15C). Since agrin protein is known to be synthesized by motoneurons and transported along axons to the NMJ (Magill-Solc and McMahan, 1990), this different spatio-temporal expression of

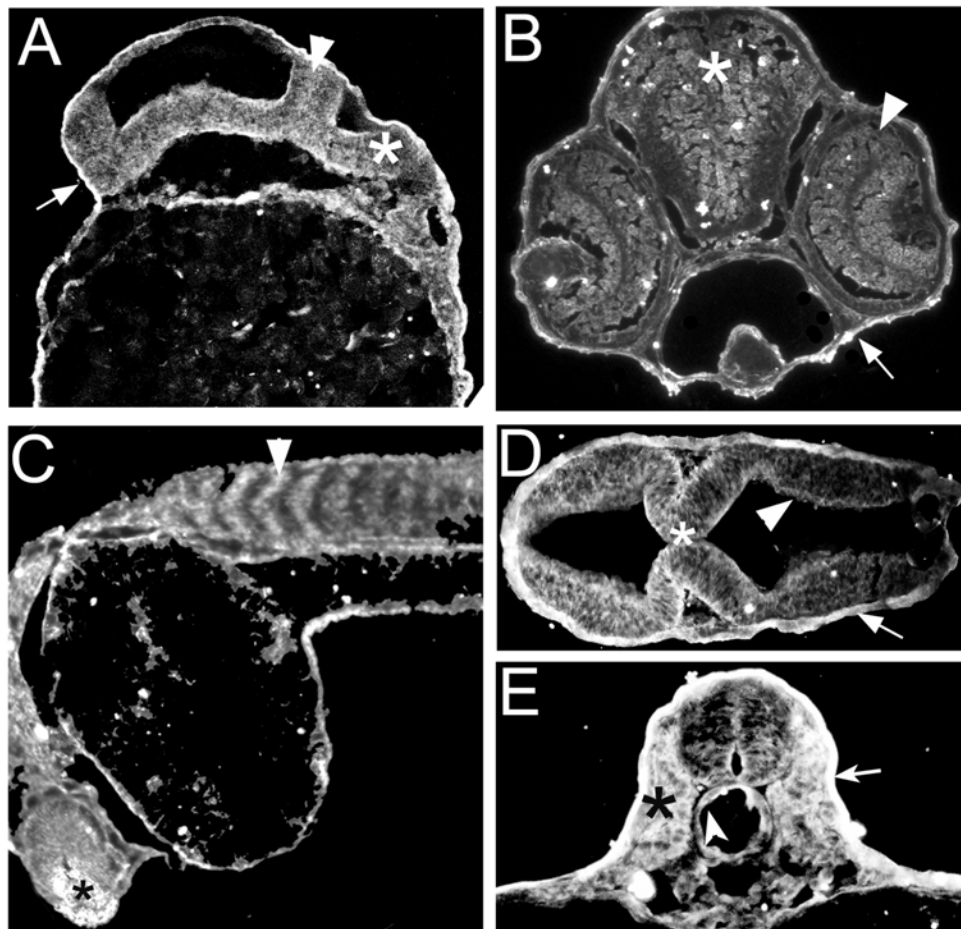


Figure 15. Localization of agrin protein in zebrafish embryo. Immunohistochemical analysis of agrin protein distribution in zebrafish embryos of 26 hpf (A, C, D and E) and 48 hpf (B). Arrow indicates the expression of agrin in the basal laminae. A: Sagittal section of 28 hpf agrin immunolabeled embryo. Agrin is abundantly expressed in the basal laminae (arrow) and brain including forebrain, midbrain (arrowhead), hindbrain (*). B: Cross section of 48 hpf agrin immunolabeled embryo. Agrin is highly expressed in eye (arrowhead) and diencephalon (*). C: Sagittal section of 28 hpf agrin immunolabeled embryo. Agrin appears as a chevron shape in the somites (arrowhead) and is strongly expressed in the forebrain (*). D: Horizontal section of 28 hpf agrin immunolabeled embryos. Agrin is highly expressed in the ventricular region of brain (arrowhead) and midbrain-hindbrain boundary (*). E: Cross section of 28 hpf agrin immunolabeled embryo. Agrin protein appears in the muscle (*) and notochord (arrowhead).

agrin protein and mRNA may reflect a similar mechanism of agrin production and secretion.

Knock-down of agrin expression leads to gross abnormalities in zebrafish posterior development.

To study agrin function during development in zebrafish, morpholino antisense oligonucleotide (MO) knock-down technology was employed to decrease the overall expression of agrin protein in zebrafish. Morpholino antisense oligonucleotides are known to be a powerful inhibitor of translation when targeted to the 5' untranslated region (Ekker, 2000). Recently, MOs synthesized from the sequence at exon-intron splicing sites have also been shown to be effective at blocking gene activity, by preventing the production of alternatively spliced mRNAs (Draper et al., 2001). We confirmed the sequence of the 5'-terminus of zebrafish agrin by 5'-RACE. Agrin-MO1 was generated from the post-spliced mRNA in the region from the 5'-cap to 25 bases 3' to the AUG translational start site. The efficiency of agrin-MO1 to target the translational start region of agrin was examined by immunohistochemistry. Using 5-9ng of agrin-MO1, agrin expression was effectively abolished in zebrafish embryos (Figure 16A-16D). However, since the N-terminus of agrin from chicken, rat and mouse has been shown to have two different alternatively spliced variants (Rupp et al.,

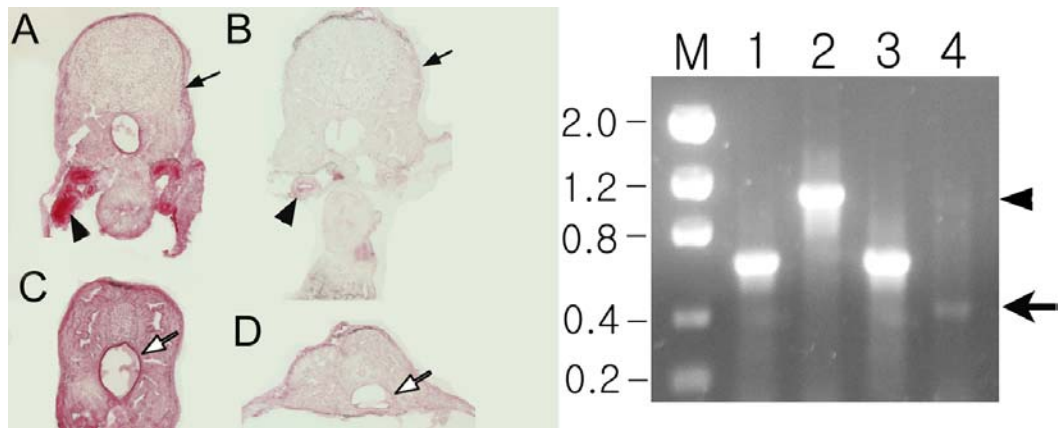


Figure 16. Immunohistochemistry confirms the efficient inhibition of agrin expression and splice-site-targeted agrin morpholino oligonucleotide alters splicing of agrin in zebrafish. A and C: Cross section of control-MO injected embryo. B and D: Cross section of agrin-MO injected embryo shows loss of agrin expression when compared to control-MO injected embryo. A-D. Arrowhead indicates the endodermal tissues, arrow the basal laminae, and open arrow notochord. E: Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of agrin mRNA in wild type and agrin MO-injected embryos. Arrowhead indicates large molecular weight band associated with normal agrin. Arrow indicates smaller molecular weight band corresponding to unspliced agrin detected in MO-injected embryos. RT-PCR of laminin was used as a control. M : Low DNA mass ladder (Invitrogen), 1: zebrafish laminin in control-MO injected embryos, 2 : zebrafish agrin in control-MO injected embryos, 3 : zebrafish laminin in agrin MO-injected embryos, 4 : zebrafish agrin in agrin MO-injected embryos.

1991; Campanelli et al., 1991; Tsim et al., 1992; Denzer et al., 1995; Denzer et al., 1997; Burgess et al., 2000), it remained possible that a complete elimination of agrin expression would not occur in zebrafish using agrin-MO1. Even though only one agrin splice variant was detected by 5'-RACE in zebrafish, to eliminate the possibility of remnants of agrin expression from different N-terminal splice variant we designed another agrin MO (Agrin-MO2). This agrin MO targets an exon-intron junction in the laminin G-like domain of zebrafish agrin. Using RT-PCR, we can show that an aberrant splice variant is created following injection of agrin-MO2 in one-cell zebrafish embryos (Figure 16E), with the normal agrin transcript also significantly decreased. Based on our results of both immunohistochemistry and RT-PCR of agrin-MO injected embryos (Figure 16), we can confirm the specificity of both MOs in inhibiting agrin gene expression. We analyzed the phenotypic change of embryos injected with the two agrin MOs, with both MOs resulting in identical phenotypic changes in zebrafish embryos. First, we compared the effect of different concentrations of MOs on zebrafish development. Injection of agrin MOs generates a dose-dependent knock-down effect and yields more severe defects with increasing concentrations. To avoid toxic effects from high doses of MOs, 9ng of agrin-MO1 or 9ng of agrin-MO2 was injected, and compared to similar concentrations of control MOs. Injection volume ranged between 1nl and 5nl and was also controlled by comparing to control MO injected embryos. With the injection of agrin morpholino, a hypomorphic series of defects was generated and the severeness of defects was increased by morpholino oligonucleotides in a dose-

dependent manner (Table 1). Knock-down of agrin expression results in dramatic changes in the gross morphological appearance of zebrafish, with posterior development in particular affected by agrin MOs (Figure 17). Defects in agrin knock-down embryos included disrupted MHB, smaller eyes, smaller otic vesicle and abnormally shortened posterior development. Agrin MO injected embryos at lower doses (2-3ng) show a mild phenotype with regard to overall body development during tail development before 16 hpf, but at later stages agrin morphants developed accumulated defects such as significantly shorter and curved tails (Figure 17C-D'). Tail development was perturbed further with higher concentrations of agrin MOs (9ng), with tails showing a spiral shape with severe shortened and curved tails (Figure 17D and D'). These defects became further apparent as development proceeded. The number of embryos showing defects was quantified (Table 1). The proportion of severe defects increased and was dependent on increasing agrin morpholino concentration. Even though the morphological phenotype of anterior development was not significantly affected by agrin MO injection, agrin morphants do display anterior phenotypic changes. Embryos exhibited decreases in the size of the eye and otic vesicle (Table 1), and defects in the formation of the MHB (Figure 17E). Other observed effects of agrin MOs included cardiac edema, diminished heart rates and blood flow (data not shown). Agrin knock-down embryos also became paralyzed with development and died at the early larval stage, likely due to an inability to feed.

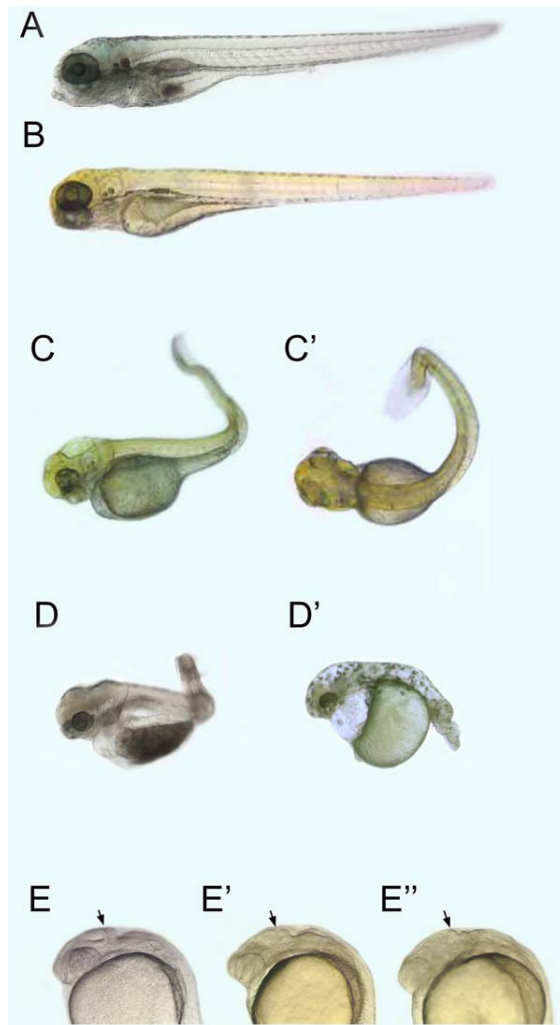


Figure 17. Knock-down of agrin by morpholino oligonucleotide injection leads to gross abnormalities in zebrafish posterior development. A. Lateral view of 9ng control morpholino injected 3 days old embryo (n=50). B. Lateral view of 9ng control morpholino injected 3 days old embryo (n=50). C and C'. Lateral view of 2-3ng agrin 3 days old morphant (n=500). D and D': Lateral view of 5-9ng agrin morpholino injected 3 days old embryo (n=150). Agrin morphants display a curly tail and aberrant border of the somitic boundary. Higher concentrations of agrin MO causes severe abruption of tail development. E-E''. Midbrain-Hindbrain boundary(MHB) defects from agrin inhibition. Arrow indicates the MHB. E. Control morpholino injected embryos. E'. 2-3ng agrin morpholino injected 2 days old embryos. E''. 5-9ng agrin morpholino injected 2 days old embryos.

Table 1. Phenotypes of embryos injected with agrin morpholino oligonucleotides

	N	Normal	I	II	III	Width of eye	Width of otic vesicle
Uninjected	50	100%				27 ± 0.6 μm	17.7 ± 0.4 μm
Control-MO	50	99%	1%				
Agrin-MO (2-3ng)	500	1%	18%	31%	50%	21.1 ± 0.8 μm*	13.3 ± 1.7 μm**
Agrin-MO (5-9ng)	200		3%	15%	82%		

III represents the most severe phenotype such that injected embryos have a shortened and spiral tail, small eyes and otic vesicle, loss of MHB, seriously reduced heart beat and cardiac edema and are immobile.

II displays mild phenotypes : disruption of MHB, short curved and twisted tail, small eyes and otic vesicle and slow heart beat, and gradually losing mobility.

I represents the weak phenotypes : weakly curved tail, but embryos lost mobility after hatching stage.

*: significantly different from uninjected embryos. P<0.006 using ANOVA

** : significantly different from uninjected embryos. P<0.002 using ANOVA

Agrin knock-down embryos exhibit anterior-posterior axis defects of tail elongation.

Since tail malformation was observed in agrin knock down embryos, early anterior-posterior (AP) patterning formation was examined to determine whether these defects occur during very early stages development. At the four-somite stage, elongation of the AP axis occurs such that the head and tail become closer (Figure 18A). The AP axis became shorter in agrin knock-down embryos (Figure 18B and 18C), with the distances between head and tail being especially longer in high-dose agrin morpholino injected embryos (Figure 18C). At the 17-somite stage, the tail extends beyond the yolk tube in wild type embryos (Figure 18D), but in agrin knock-down embryos the tail is shorter than wild type embryos (Figure 18E). The average length between head and tail is shown in Table 2. The width of caudal somites from agrin knock-down embryos was narrower than wild type embryos (Figure 18E). Thus, these experiments show that agrin deficient embryos exhibit a shorter AP axis during early development.

Effect of agrin knock-down on primary motor axon guidance and outgrowth

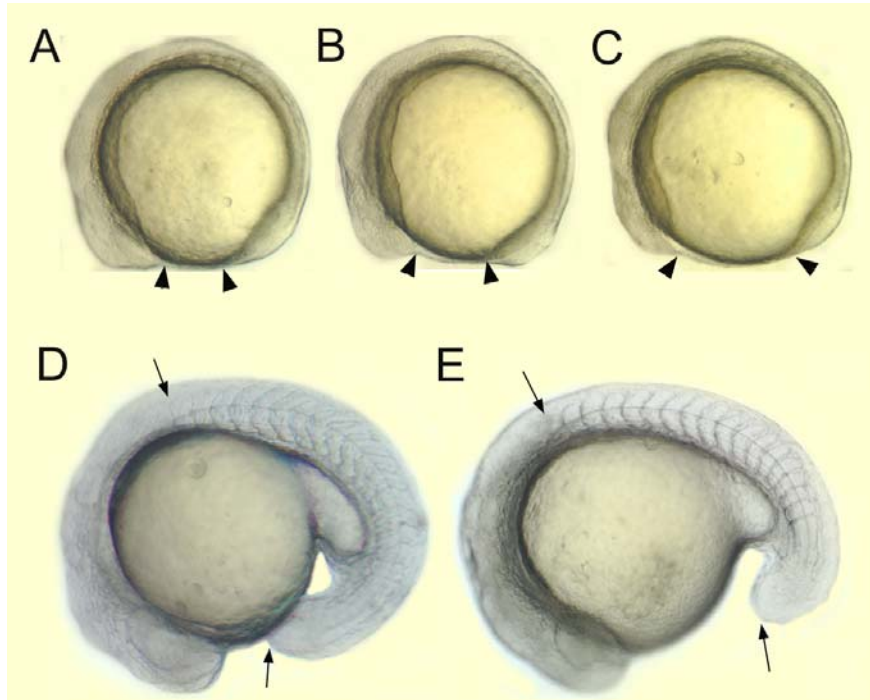


Figure 18. Defects of anterior-posterior development occurred during early development. A-C. 4 somite-stage embryos. Arrowheads indicate the distance between head and tail. A. wild-type embryos show the closest distance between head and tail. B. Low-dose agrin morpholino injected embryos displayed shorter AP axis formation. C. High-dose agrin morpholino injected embryos show severe defects of AP axis elongation. D-E. 17 somite-stage embryos. Arrows indicate the body length. D. Tail of wild type embryos extends beyond the posterior limit of the yolk tube. E. Tail of low-dose agrin morpholino injected embryo is shorter than wild type embryos. The caudal somites are smaller, but not fused. Arrows indicate the beginning of somite and end of tail.

Table 2. The average distance between head and tail of early stage zebrafish embryos.

	2 somites stage	4 somites stage
Uninjected	32.5 ± 0.7 µm	26 ± 2.3 µm
Agrin-MO (1-3ng)	33.5 ± 2.1 µm	24 ± 4.8 µm
Agrin-MO (5-9ng)	43.5 ± 2.1 µm*	38 ± 3.5 µm**

25 embryos were counted for each treatment.

* : significantly different from uninjected embryos. P<0.0004 using ANOVA.

** : significantly different from uninjected embryos. P<0.01 using ANOVA.

Since agrin protein is abundantly expressed in developing axon pathways (Halfter et al., 1997), we reasoned that one function of agrin may be to modulate the formation of axon pathways. To study the effects of agrin knock-down on the formation of axon pathways in zebrafish, we examined axon outgrowth from primary motor neurons. To identify the axons of primary motor neurons we employed the znp-1 mAb, which stains caudal primary (CaP) motor axons and middle primary (MiP) motor axons (Melancon et al., 1997). At 27 hpf, control MO-injected embryos showed stereotypical patterns of outgrowth from CaP motor axons (Figure 19A and 19C). However, agrin MO-injected embryos displayed dramatic changes in this stereotypical pattern, with a more branched axonal projection in a morpholino dose-dependent manner (Figure 19B, 19D and 19E). As development proceeded, the number of branches increased and the CaP axon projection failed to reach its target ventral muscle in agrin MO-treated embryos. The increased axon branching of CaP axons also appeared to impair the formation of fascicles of motor axons in agrin-MO injected embryos. Injection of a higher dose of agrin-MO led to further increases in axonal branching (Figure 19E). In later development, the distribution of motor neuron axons was severely disoriented (Figure 19I and 19J). However, the length of CaP axons was not changed (data not shown). These data imply that axonal guidance of CaP motor axons is dependent on agrin function during zebrafish development.

Dorsal projecting secondary motor nerves are affected by agrin knock-down.

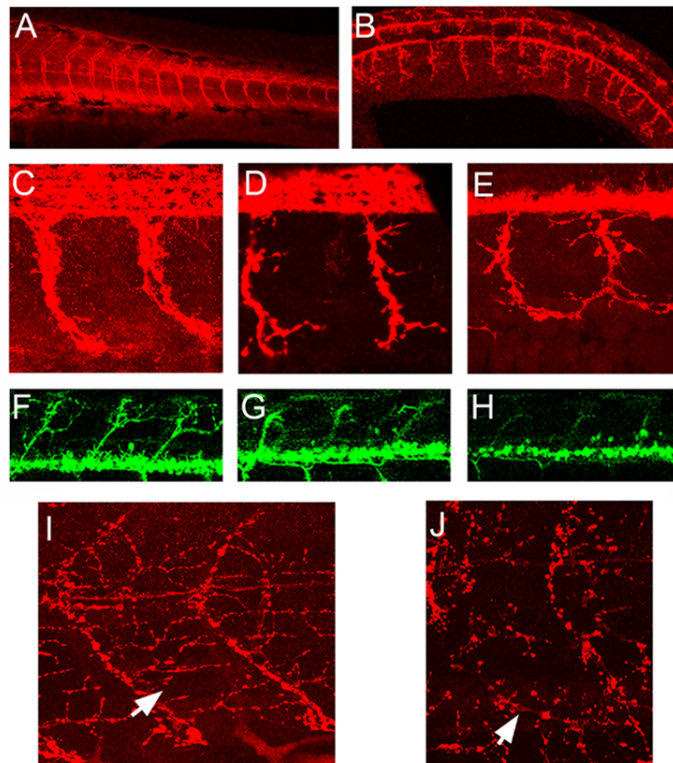


Figure 19. Effects of agrin morpholinos on primary and secondary motoneuron axonal growth. A. Lateral view of confocal image of control MO- injected embryo stained with znp-1 mAb at 27 hpf (n=100/100). B. Lateral view of confocal image of agrin MO-injected embryo stained with znp-1 mAb (n=88/100). C-E. The projection of primary motor neurons is disturbed as a result of defective agrin expression, and is bifurcated in agrin knock-down embryos. High magnification of control morpholino injected embryos (C), 2-3ng morpholino injected embryos (D) and 9ng agrin morpholino injected embryos (E). F-H. Lateral view of confocal images of a transgenic islet-GFP embryo injected with control MO-injected embryo (F; n=50), and agrin-MO injected embryos (G: 3ng; n=42/50 and H: 9ng; n=38/50). The transgenic zebrafish express GFP under the control of the islet-1 promoter, which targets GFP expression to motor neurons. Knock-down of agrin causes diminished axon outgrowth of dorsal projecting motor neurons. The formation of axon pathways of secondary motor neurons was severely disrupted by higher concentrations of agrin morpholino (H). I-J. The formation of motor neuronal pathways was severely disturbed in agrin inhibited 3 day embryo (J: arrow indicates the lumps of motor neurons) while control-MO injected embryo displays a stereotyped pattern of motoneuron arrangement (I).

To determine the effect of agrin knockdown in spinal motor axons, transgenic zebrafish expressing GFP under the control of the islet-1 promoter was analyzed after microinjection of agrin-MOs. Islet-1-GFP transgenic fish express GFP in secondary motoneuron cell bodies and dorsally projected axons from MiP neurons (Higashijima et al., 2000). Agrin or control MO was injected into islet-1-GFP transgenic embryos, and dorsally projecting axons were examined in 3 day-old embryos. Striking effects on secondary motor neuron axon morphology were observed, with dorsally projecting motor axons being truncated, rather than branched, as a result of knock-down of agrin expression (Figure 19F-19H). Agrin-MOs also cause diminished axon outgrowth of dorsal projecting motor neurons. The formation of axon pathways by secondary motor neurons was severely disrupted by higher concentrations of agrin-MO (Figure 19H). More severe truncation and less branching were obvious at higher doses of agrin MOs. These data imply that agrin can differentially regulate the development of axonal pathways during nervous system development.

Agrin knock-down disrupts trigeminal motor neuron axonal projection.

From studies of agrin knock-out mice, the phenotype of these transgenic mice exhibited no pronounced changes in anterior development, except for a slightly smaller

size brain (Gautam et al., 1996). However, our data from MO-based knock-down experiments show disruption in midbrain-hindbrain boundary development, smaller otic vesicle and smaller eye development, the anterior region, with regard to anterior development. Since inhibition of agrin expression produced defects in axon pathway formation throughout the entire trunk region of embryo, we decided to examine whether alterations in axon guidance were occurring in the anterior region of the zebrafish embryo. Therefore, using islet-1-GFP transgenic embryos, we examined the axonal projection of trigeminal (facial) motoneurons. The lateral and anterior extension of trigeminal motor neurons is significantly impaired in agrin knock-down embryos (Figure 20). Agrin knockdown islet-1-GFP transgenic embryos displayed less sprouting, as well as truncation, of trigeminal axons at early stages of development (Figure 20B) and later axonal projections were severely disoriented (Figure 20D). However, arrest of axonal growth of trigeminal motor neurons was overcome and axonal growth was observed, despite being shorter than wild type embryos, after 48 hpf (data not shown). Even though the gross anterior morphology of agrin morphants appears less severe than posterior development, knock-down of agrin expression does result in delayed axonal growth and misdirected pathfinding of at least one population of motor neurons, the trigeminal motor neurons.

Agrin knock-down leads to impaired axon outgrowth by Rohon-Beard sensory neurons.

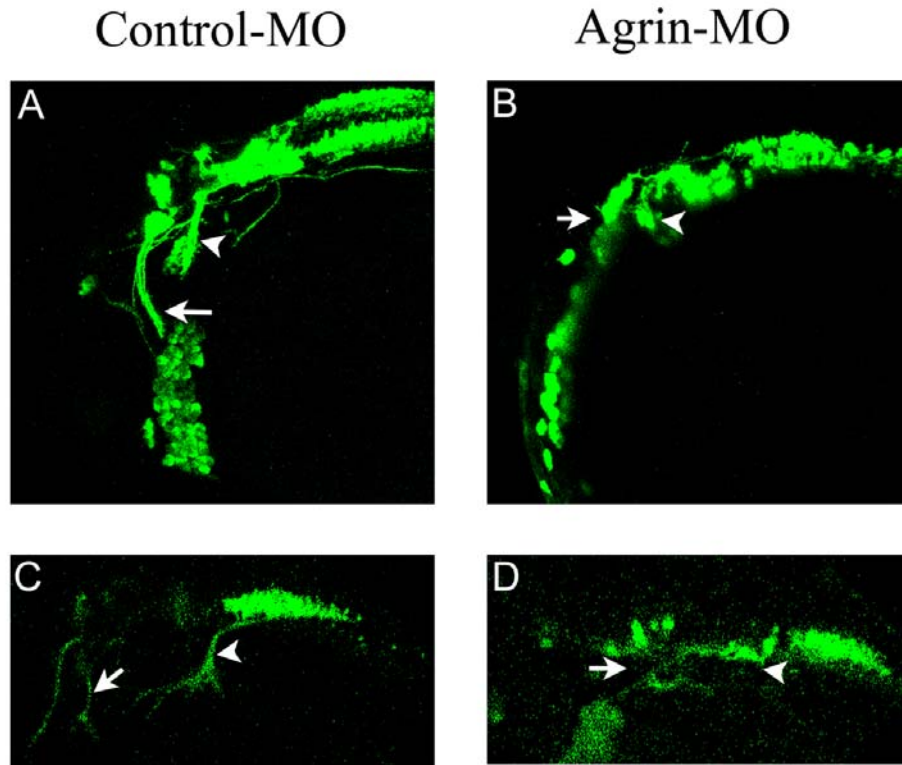


Figure 20. Agrin knock-down disrupts the axonal projection of trigeminal motor neurons throughout development. Confocal image of transgenic islet-GFP 28 hpf and 36 hpf embryos injected with control-MO (A and C; n=50/50) or agrin-MO (B and D; n=38/50). The lateral and anterior extension of trigeminal motor neurons is impaired in agrin knock down embryos. White arrow indicates the peripherally extending axons from the Va cluster of the nV neurons. Arrowhead indicates the main nVII/OLe axons (primarily facial motor axons). Even though the anterior morphology of agrin morphants appears normal, knock-down of agrin causes halted axonal growth and pathfinding of islet-1-positive motor neurons.

Although our studies described above clearly demonstrate a role for agrin in motor neuronal axon outgrowth and guidance, we were interested in determining whether agrin function is also crucial to sensory neuron axon outgrowth and guidance. To demonstrate an effect of agrin knock-down in other neuronal cell types, anti-Hu and anti-zn-12 mAb staining was employed to analyze sensory neuronal development. Anti-Hu mAb can be used to assess neuronal differentiation, with immature neurons staining with anti-Hu (Marusich et al., 1994). Agrin knock-down resulted in a failure of spinal cord sensory neuronal cell bodies to down-regulate Hu antigen, with 48 hpf control zebrafish down-regulating Hu antigen in neurons, but maintaining anti-Hu staining in agrin-MO embryos (Figure 21A and 21B). We used zn-12 mAb for analysis of axonal growth by Rohon-Beard sensory neurons in zebrafish spinal cord (Trevarrow et al., 1990). The projection of axons from Rohon-Beard cells was severely truncated in agrin knock-down embryos, with axon outgrowth being eliminated for the most part by agrin knock-down (Figure 21C and 21D). These data demonstrate that agrin is also critical to axon growth by sensory neurons in the zebrafish spinal cord.

Muscle development appears normal in agrin knockdown embryos.

The most distinct exterior defects of agrin knockdown embryos were seen in tail development. To determine whether abnormal tail development and axonal

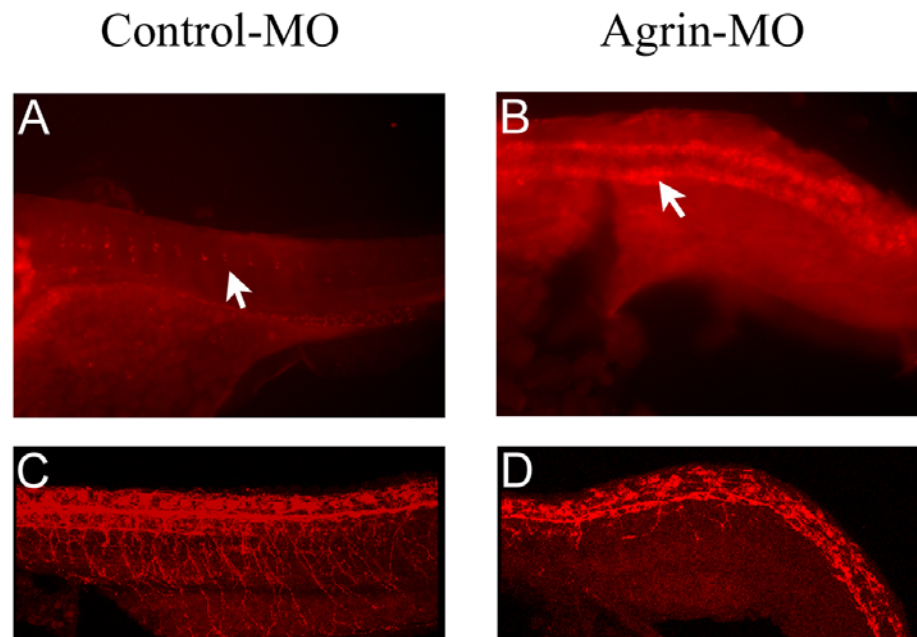


Figure 21. Truncation of axonal outgrowth by spinal cord sensory neurons during zebrafish development. Lateral view of sensory neurons, comprised mainly of Rohon-Beard cells, stained with Hu mAb and zn-12 mAb in 48 hpf control-MO (A and C, respectively) and agrin-MO embryos (B and D, respectively). Prolonged ectopic staining of Hu-positive neurons in spinal cord was detected in agrin-MO injected embryos (arrow). The axonal projection from Rohon-Beard sensory neurons was also truncated in embryos treated with agrin-MO (D).

projection was due to defects in muscle development, we examined muscle development in agrin morphants. Myotome morphology of 48 hpf agrin morphants exhibits a breakdown of somitic boundaries, as control morphants display a chevron shape with clear boundaries in somites (Figure 22A and 22B). As agrin MO embryos display aberrant axon outgrowth and somite boundaries, we analyzed the morphology of AChR clusters in NMJs to address agrin's effect on synaptogenesis in muscle fibers. Using rhodamine-conjugated α -bungarotoxin, the innervation pattern of muscle fibers was assayed in agrin MO embryos. AChR clustering was significantly reduced in agrin MO embryos and the stereotyped pattern of innervation was perturbed and did not evenly distribute in the somite of agrin morphants (Figure 22C and 22D). We next investigated muscle specification and organization by immunohistochemistry using F59 and F310 mAbs in 48 hpf agrin and control MO-injected embryos. This analysis shows that the development of lateral line and muscle pioneer slow muscle cells is unaffected by injection of agrin MOs (Figure 22E and 22F).

In addition, the fast muscle fibers were normally distributed in agrin morphant embryos (Figure 22G and 22H). To determine whether agrin-MO alters cell death during muscle development, we used acridine orange staining, which visualizes apoptotic cells in live zebrafish embryos. Even though the dorsal region of spinal cord in agrin morphants exhibited more apoptotic cell death based on augmented acridine

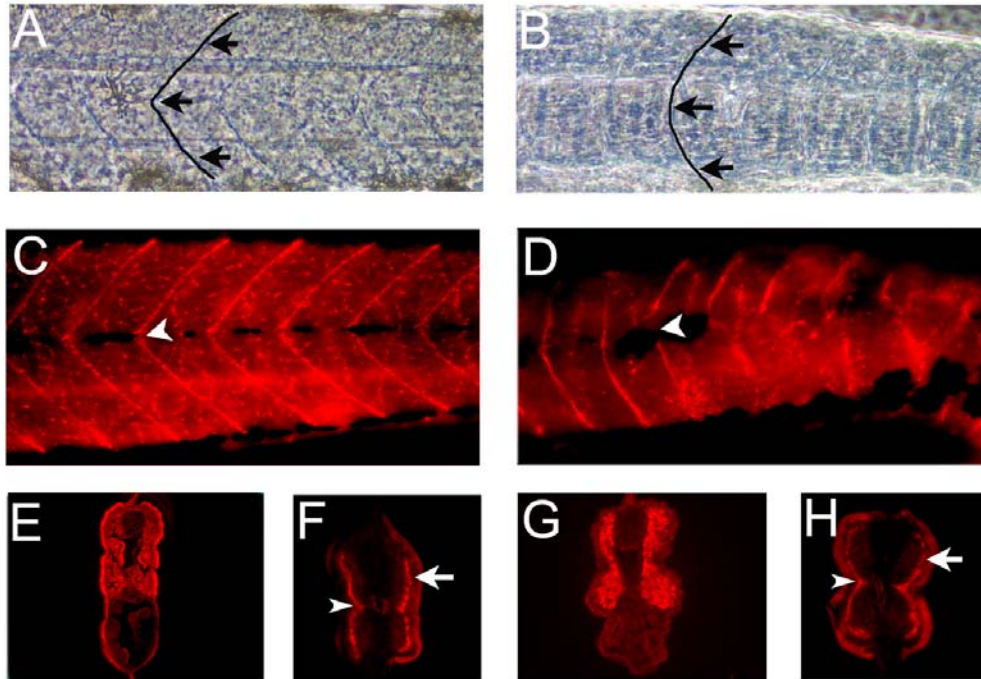


Figure 22. Muscle development was normal in agrin knock down embryos. Nomarski lateral view of mid-trunk muscle from 48hpf embryos injected with control-MO(1mM) (A; n=20) or agrin-MO (1 mM) (B; n=20) is shown. Agrin-MO injected embryos show abnormal somitic boundaries compared to control-MO injected ones as indicated by tracing of a somite boundary in each treatment group. C and D. Lateral view of α -bungarotoxin stained embryos injected with control-MO (C; n=20) and agrin-MO (D; n=20). Agrin knock-down embryos show a decrease in acetylcholine receptor (AChR) clusters in 48 hpf embryos. E and G. Cross section of 26 hpf F310 mAb (fast muscle) stained embryos injected with control-MO (E; n=20) or agrin-MO (G; n=20). Fast muscle development appears normal in agrin knock-down embryos. F and H. Cross section of 26 hpf F59 mAb (slow muscle and muscle pioneers) stained embryos injected with control-MO (F; n=20) or agrin-MO (H; n=20). Slow muscle and muscle pioneers appears normal in agrin-MO injected embryos.

orange staining, significant apoptosis was not detected in developing muscle in agrin knock-down embryos (data not shown).

DISCUSSION

The studies reported here demonstrate that agrin contributes essential functions during zebrafish development, confirming functions previously established in mouse, chicken and electric ray with regard to formation of the NMJ (Nitkin et al., 1986, Gautam et al., 1996), but also establishing new functions for agrin. A rationale for undertaking our current studies was the presence of conflicting data regarding the function of agrin in axonal growth, which was based on the use of various in vitro paradigms to analyze agrin's role in axonal growth (Mantych and Ferreira, 2001; Campagna et al., 1995; Chang et al., 1997; Halfter et al., 1997; Bixby et al., 2002; Kim et al., 2003). In addition, agrin's role in axonal growth was not analyzed, or at least apparent, in agrin knock-out mice (Gautam et al., 1996). Thus, agrin has been reported to inhibit axonal growth when combined with other adhesive proteins (Bixby et al., 2002), agrin promotes dendritic growth but inhibits axonal elongation in hippocampal neurons (Mantych and Ferreira, 2001), and our laboratories have shown that agrin augments FGF-2 mediated axonal growth using both PC12 cells and primary retinal neuronal cultures (Kim et al., 2003). We reasoned that zebrafish would provide an ideal model system to study the role of agrin in the formation of axonal pathways, as the function of genes can readily be examined in vivo during zebrafish development, protein expression can be perturbed using MO antisense oligonucleotides, and recent

studies have provided evidence for the importance of HS-GAGs in zebrafish development (Topczewski et al., 2001; De Cat et al., 2003; Bink et al., 2003).

Our initial studies were intended to confirm the expression of an agrin gene in zebrafish, which was established based on several criteria. We were able to use EST zebrafish cDNAs, as well as cDNAs we isolated from zebrafish cDNA libraries or by 5'-RACE, to establish the presence of a 10 kb zebrafish transcript that hybridizes to our putative zebrafish cDNAs. This transcript is similar in size to agrin transcripts from other species (Tsen et al., 1995; Burgess et al., 2000), is expressed in tissues consistent with agrin expression (Groffen et al., 1999; Yard et al., 2001; Dong et al., 2002), and when used to generate an antiserum to a putative agrin fusion protein this antiserum recognizes a 400-500 kDa HSPG that has a 200 kDa core protein following the removal of HS-GAGs. Thus, these data are consistent with these cDNAs, which exhibit high homology to chicken and rodent agrin, as coding for zebrafish agrin.

Our analysis of the effects of agrin knock-down on zebrafish development yielded a pronounced morphological change in zebrafish embryos, with increasing doses of agrin morpholinos leading to perturbation of anterior as well as posterior development. The most distinct morphological defects are revealed in tail formation. Interestingly, recent studies have shown that morpholino knock-down of heparan sulfate 6-O-sulfotransferase in zebrafish affects tail development, with curved tails

resulting in these zebrafish (Bink et al, 2003). Glypican-3 knock-down or loss of function in zebrafish also impacts tail development, with shorter tails being observed in these zebrafish (Topczewski et al, 2001). Interestingly, recent studies have identified a tail organizer in zebrafish that utilizes nodal, wnt and BMP signaling pathways (Agathon et al, 2003). Perturbation of these signaling pathways results in failures in tail development. In view of evidence that wnt proteins are heparin-binding proteins (Reichsman et al, 1996; De Cat et al, 2003), it is tempting to speculate that agrin knock-down in zebrafish disrupts the function of the wnt signaling pathways that are required for function of the tail organizer in tail development. In addition, the role of BMPs in tail development is worth considering in the context of agrin function, since agrin contains within its protein core numerous follistatin-like domains (Tsen et al, 1995) and it is highly expressed in the notochord during early development. One class of ligands for BMPs are follistatins (Amthor et al, 2002), and if follistatin domains confer upon agrin the ability to bind BMPs, then agrin knock-down in zebrafish could impact two known pathways that are essential to tail development. In addition, the severity of the phenotype with agrin knock-down, when compared to glypican knock-down (Topczewski et al, 2001), may indicate that agrin is a primary secreted HSPG which serve as a mediator to pattern formation molecules for the regulation of tail development in zebrafish.

A more attractive explanation for describing the effects of agrin knock down on zebrafish development may be related to FGF signaling. Expression of dominant-negative FGFRs in zebrafish leads to disruption of tail development (Griffin et al., 1995) and the FGFR inhibitor SU5402 also disrupts tail development (Draper et al., 2003). Knockdown of multiple FGF proteins also disrupts tail development (Draper et al., 2001; Draper et al., 2003), suggesting that agrin is required to maintain FGF function during tail development. Moreover, defects of MHB development, eye and otic vesicle development occur as a result of agrin knock down, again implicating a possible interaction of agrin with FGFs during zebrafish development. Simultaneous knock-down of FGF-8 and FGF-3 results in failure to induce the otic placode and vesicle, and FGF-8 mutant zebrafish do not form a proper MHB during zebrafish development (Reifers et al., 1998; Phillips et al., 2001; Leger et al., 2002 and Liu et al., 2003). Thus, many of the phenotypic changes observed due to agrin knockdown may be the result of perturbation of FGF signaling, and future studies will be needed to test this hypothesis.

The primary objective of our studies was to ascertain whether agrin contributes to the formation of axonal pathways *in vivo* during zebrafish development, and to address this question we employed the use of agrin MOs. We employed two agrin MOs, to the 5'-translation start site or to an exon-intron splice junction, to examine agrin's function during zebrafish development. The fact that we obtained identical phenotypes using

these different agrin MOs provides strong evidence that these MOs are targeting agrin gene expression, which was confirmed by demonstrating that agrin protein expression was for the most part eliminated using these agrin MOs. It should be noted that two different agrin transcripts are expressed in chicken and rodent (Rupp et al., 1991; Campanelli et al., 1991; Tsim et al., 1992; Denzer et al., 1995; Denzer et al., 1997; Burgess et al., 2000), while our 5'-agrin MO would only target one agrin transcript. Since we only isolated one 5'-RACE product, these data may indicate either the absence of 5'-alternatively spliced transcripts, corresponding to SN-agrin, or SN-agrin is not significantly functional during zebrafish development. This would account for a near complete agrin knock-down using the 5'-agrin-MO.

Our analysis of the effect of agrin knock-down on axonal guidance and axonal growth suggests that agrin may contribute distinct functions to different classes of neurons. Accordingly, the most notable effect of agrin knock-down on primary motoneurons in zebrafish appears to be related to axon guidance, with CaP axons showing increases in branching and a loss of turning towards target muscle at somitic boundaries. In contrast, axons from secondary motoneurons exhibit pronounced effects on axonal extension, with these axons being truncated and less branched, and failed to reach targets in agrin-MO treated zebrafish. With regard to motor neurons, perhaps the most marked effects on axon growth, as a result of perturbation of agrin expression, occurs with trigeminal motor neurons. Axonal growth from these motor neurons in

agrin-MO treated embryos is reduced substantially, with the lateral and anterior axonal extension of trigeminal motor neurons being abolished for the most part. However, axonal growth was recovered at later stages of development. This may result from reduction of agrin morpholino activity with time, allowing axonal growth to resume. These combined effects of agrin knockdown on axonal growth, together with agrin's role in the formation of the NMJ, results in substantial loss of motor function in embryonic zebrafish. These likely accounts for the impairment in swimming function for agrin-MO treated zebrafish, difficulties in feeding behavior and ultimately lethality of agrin knock-down in zebrafish embryos.

We also analyzed the effects of perturbation of agrin expression on sensory neuron axonal growth and guidance. Using Rohon-Beard sensory neurons as a neuronal cell type for analysis, we again observe marked effects of agrin knock-down on axonal growth and guidance. Loss of agrin expression results in a near complete loss of axonal outgrowth from Rohon-Beard cells, as well as a continued expression of Hu antigen in these neurons. We suggest that Hu antigen is maintained in these cells since it is typically associated with immature neurons (Marusich et al., 1994), and that failure of Rohon-Beard cells to establish functional connections with target cells may result in these cells failing to fully differentiate by down-regulating Hu expression. Likewise, although the marked inhibition of axonal growth in Rohon-Beard neurons, and other neuronal cell types, may suggest a crucial function for agrin in axonal growth in

zebrafish, it cannot be ruled out that agrin may contribute to processes such as axonal guidance or neuronal differentiation that may also manifest as a loss of axonal growth from these identified neuronal populations.

Our observation of malformation of axonal projection raised the possibility that agrin may function as an axon guidance cue. The ablation of muscle pioneers, which is a subset of adaxial cells, has been shown to not change the target selection of CaP and MiP neurons by perturbing the development of intermediate targets required for axon guidance (Melancon et al., 1997), but does affect axonal extension by these identified motor neurons (Melancon et al., 1997). Moreover, in *diwanka* mutant zebrafish embryos, adaxial cells are present, but fail to produce cues that are essential for primary motor growth cones to pioneer into the somites (Zeller et al., 2002). Myotomal cells produce signals to regulate the projection of spinal motoneurons into somites, and myotome-derived signals are also required for this pathfinding of motor axons. In agrin knock down embryos, slow muscle cells and fast muscle cells appear to develop normally. However, the presence of agrin in proximity to adaxial cells, and aberrant axonal projection in agrin knock-down zebrafish, may imply that agrin may serve as a pathfinding cue for motor growth cones, which is essential to contribute to proper pathway navigation.

Our observation that agrin contributes crucial functions to neuronal differentiation

and axonal outgrowth in multiple neuronal cell types in zebrafish is clearly of interest, although somewhat surprising in view of results from agrin gene disruption in transgenic mice (Gautam et al., 1996). Recent studies using mammalian models have provided evidence for the importance of HSPGs to many developmental processes, which include muscle development (Thomas et al., 2003; Jenniskens et al., 2002) and CNS development (Ford-Perriss et al., 2003). Support for the role of HSPGs in these processes has been provided by disrupting the expression of genes required for HS-GAG biosynthesis (Bullock et al., 1998), as well as impairment of the function of specific HSPGs (Itoh et al., 1994). In zebrafish the role of HSPGs in development is just beginning to be studied, with recent studies showing that HSPGs are required for muscle development (Bink et al, 2003), that glypicans are required for Wnt signaling, cell survival and gastrulation in zebrafish (Topczewski et al, 2001; De Cat et al, 2003), and that syndecan-2 is required for angiogenesis in zebrafish (Chen et al, 2004). Since many of the zebrafish homologs of mammalian HSPGs have not yet been identified in zebrafish, as evidenced by our current studies reporting the characterization of zebrafish agrin, it remains possible that zebrafish will not exhibit as robust a redundancy in HSPG expression as mammalian species. Thus, one explanation for our identification of pronounced effects of agrin perturbation on axonal growth and guidance is that agrin contributes more essential functions in zebrafish, due to an absence of HSPGs that can substitute for agrin function in the absence of agrin. Alternatively, it remains possible that a careful analysis of agrin knock-out mice will

establish similar functions for agrin in the formation of various axonal pathways during mouse development. This may be especially noteworthy since agrin gene disruption in mice is lethal (Gautam et al., 1996), and an impairment of axonal growth and guidance during mouse development, as we have shown in zebrafish, may not manifest as a readily observable phenotype in agrin^{-/-} mice. Thus, we think it is important to extend analyses of agrin function, in both zebrafish and mouse models, in order to better understand how this HSPG may contribute essential functions during development.

CHAPTER III

Agrin regulates the development of CNS structures that require FGF signaling pathways.

ABSTRACT

Early anterior patterning of midbrain and anterior hindbrain is determined by signals from the anterior visceral endoderm and mesendoderm of the anterior neural plate. The embryonic isthmic organizer, which may control the polarized development of neural tube encompassing the prospective forebrain, midbrain, eyes and cerebellum, is established in the neuroepithelium where it is localized at the midbrain-hindbrain boundary. FGFs, such as FGF-8 have the ability of inducing and patterning mesoderm and neural tissues, as well as modulating cell proliferation and neurogenesis in the CNS. FGF-8 is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Agrin is an extracellular matrix heparan sulfate proteoglycan, which is expressed on cell surfaces or in the extracellular matrix of tissues where it is capable of binding FGF. Knockdown of agrin in zebrafish strikingly resembles many aspects observed in zebrafish FGF mutants, such as disruption of the MHB and malformation of optic and otic vesicles during zebrafish development. Inhibition of FGFR synergizes defects from agrin knockdown suggesting modulating activity of agrin with FGFs. In conclusion, agrin knockdown studies suggest that the HSPG agrin may be involved in regulation of early CNS development via maintenance and regulation of FGF signaling.

INTRODUCTION

Discrete regional specification of the early nervous system arises from the neural plate that is subjected to anterior-posterior and dorso-ventral axes patterning. A-P patterning gives rise to the formation of telencephalon, diencephalon, optic vesicles, midbrain, hindbrain and spinal cord (Lumsden and Krumlauf., 1996; Rubenstein and Beachy., 1998). During gastrulation, early anterior patterning of midbrain and anterior hindbrain is determined by signals from the anterior visceral endoderm and mesendoderm of the anterior neural plate (Beddington and Robertson, 1998). The embryonic isthmic organizer, which can influence cell fate, is established in the neuroepithelium where it is localized at the midbrain-hindbrain boundary (Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The isthmic organizer is believed to control the polarized development of neural tube encompassing the prospective forebrain, midbrain, eyes and cerebellum (Joyner, 1996; Puelles et al., 1996; Wassef and Joyner, 1997). Several genes, such as the homeodomain transcription factors OTX1 and OTX2 (Acampora et al., 1997; Suda et al., 1997), Pax2 (Favor et al., 1996; Lun and Brand., 1998) and En1 (Wurst et al., 1991) are required for early development of the neural plate. The secreted protein FGF8 and Wnt1, transcription factors Engrailed (En), Pax2/5/7, Gbx2 and Isl3 and ephrins are expressed in the MHB and are known to be involved in the signaling of midbrain-hindbrain growth and

patterning from the MHB organizer (Hatta et al., 1991; Crossley and Martin., 1995; Joyner, 1996; Crossley et al., 1996; Brand et al., 1996; Favor et al., 1996; Kikuchi et al., 1997; Reifers et al., 1998; Lun and Brand, 1998; Pfeffer et al., 1998; Martinez, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

The fibroblast growth factors (FGFs) play multiple roles in CNS development (Ford-Perriss et al., 2001). FGFs have the ability of inducing and patterning mesoderm and neural tissues (Doniach, 1995), as well as modulation of cell proliferation and neurogenesis. Among over 24 different families of FGFs, FGF-2 and FGF-8 are known to be particularly important to neural patterning, cell proliferation and neurogenesis (Perrone-Capano and Di Porzio., 2000). FGF-8 is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Homozygous acerebellar zebrafish embryos, which were identified as an FGF-8 mutant, lack a cerebellum and the midbrain-hindbrain boundary organizer (Reifers et al., 1998). FGF-8 and FGF-3 are required for zebrafish ear placode induction, maintenance and inner ear patterning as well as otic placode specification (Phillips et al., 2001; Leger et al., 2002; Liu et al., 2003). Moreover, overexpression of a dominant-negative form of an FGFR gene disrupted mesoderm formation, which displayed a lack of posterior development in *Xenopus* embryos (Amaya et al., 1991). The expression patterns of FGFR1 are strikingly similar to FGF-8 and the phenotype of FGFR1 knock-down resembled the FGF-8 mutant acerebellar, suggesting that FGF-8 exerts its function

mainly by binding to FGFR1.

FGF and Wnt signaling is regulated by HSPGs (Lin et al., 2000; Alexander et al., 2000) and HSPGs have a fundamental role in growth factor signaling and morphogenesis for normal development. HSPGs are well known components of the extracellular matrix, such as basement membranes, cartilage and connective tissue. HSPGs are also localized to the cell surface. Inhibition of glycosaminoglycan sulfation, or removal of heparan sulfate from the cell surface, significantly reduces FGF signaling pathways. In addition, cell-associated and membrane HSPGs serve as co-receptors of FGF:FGFR to affect the assembly of a signaling complex on the cell surface (Zhang et al., 2001; Yayon et al., 1991; Steinfeld et al., 1996). HSPGs are involved in the distribution of signaling molecules, such as FGFs, and neural patterning during development (Galli et al., 2003). Recent studies have suggested that proteoglycan synthesis, much like modification of glycosaminoglycan moieties, is critical for the control of signaling pathways during development (Miao et al., 1996; Li et al., 2002).

Agrin is expressed on cell surfaces or in the extracellular matrix of tissues where it is capable of binding FGF or other HS-binding growth factors including Wnts. In this chapter, binding ability of agrin to FGF-8 was demonstrated, and the expression pattern of agrin and FGF were shown to overlap during CNS development. Knock-

down of agrin in zebrafish strikingly phenocopies many aspects observed in zebrafish FGF mutants. Disruption of agrin expression produces disruption of the MHB and malformation of optic and otic vesicles during zebrafish development. Using an FGFR inhibitor, more severe phenotypic changes were observed to FGF knockdown alone, when combined with agrin knockdown suggesting synergistic interactions between agrin and FGFs. Here agrin knock-down studies suggest that the HSPG agrin may be involved in regulation of early CNS development via maintenance and regulation of FGF signaling.

MATERIALS & METHODS

Fish maintenance

The wild-type adult zebrafish (*AB background) and *islet-1*-GFP transgenic zebrafish (Higashijima et al., 2000) were maintained at 28.5°C under standard laboratory conditions (Westerfield, 2000; Brand and Grant, 1999). Embryos were allowed to develop and staged by hours or days after fertilization at 28.5°C and morphological criteria (Kimmel et al., 1995).

Antisense Morpholino injection

Antisense morpholino nucleotides (MOs) (Gene Tools, LLC) were designed against 5' sequence near the start site of translation (Nasevicius and Ekker, 2000) and an exon/intron splice donor site (Draper, 2001). The sequences of MOs used were: Agrin-MO1: 5'-CCGCTTTTCTGTCCGCAGAGCCCAT-3', Agrin-MO2: 5'AGAGTTGTACACCTACCAGAGAAAC-3' and Control-MO : 5'-CCTCTTACCTCAGTTACAATTTATA-3'. MOs were solubilized in water at a concentration of 16mg/ml and diluted to working concentrations in water before

injection into one- to four-cell stage embryos. Agrin morpholino at a concentration of 1-9 ng consistently produced reproducible phenotypes with an injection volume around 1-5 nl (Lewis and Eisen, 2003; Maroon et al., 2002) that resulted in a drastic reduction of agrin expression. The control morpholino at the same dose produced no distinguishable effects.

SU5402 treatment

SU5402 (25mg/ml, Calbiochem) was resuspended in DMSO and diluted before use. Embryos were manually dechorinated and incubated in fish embryo media containing SU5402 of 0.1mg/ml or 0.2mg/ml (dependent on the activity of vial) for 10 minutes at tail bud stage. After several intense washes, embryos were allowed to develop until the desired time point, and then fixed in 4% paraformaldehyde/Phosphate Buffered Saline Buffer (PFA/PBS).

Solid phase binding assay of agrin and FGF-2

5µg/ml of recombinant mouse FGF-8b (R&D systems) was incubated in 100µl coupling buffer (0.2M Carbonate-bicarbonate buffer, pH 9.6) in ELISA wells for overnight at 4°C. 2% bovine serum albumin in coupling buffer was used as a control. After several washes with PBS, agrin (0.1-100 µg/ml) in 2% BSA/PBS was added to wells and allowed to incubate for 3 hours at room temperature. Following several

washes with 1X PBS, 6D2 mouse MAB to agrin (2 μ g/ml) was added and incubated for 1h at room temperature. Biotinylated antibody was then added and incubated for 1 hour. Color development reagent (3,3',5,5'-tetramethyl benzidine (TMB) liquid substrate system for ELISA, Sigma) was added to visualize antibody binding. Coloric reaction was stopped by adding 100 μ l of 1N sulfuric acid to stop color development. Absorbance at 450nm was measured to quantify agrin-FGF8 binding.

RESULTS

Agrin knock down disrupts normal formation of the midbrain-hindbrain boundary, eyes and otic vesicles.

To investigate agrin function in zebrafish embryos, morpholino oligonucleotides were used to inhibit translation of agrin mRNA. Since zebrafish agrin is highly expressed in the CNS during early development, the phenotypic change of knock-down embryos was carefully examined in the brain. With injection of agrin morpholino, a hypomorphic series of defects was generated and the severeness of defects was increased in a dose-dependent manner by agrin morpholino oligonucleotides. Defects in agrin knock-down embryos included disrupted MHB, smaller eyes, smaller otic vesicles and abnormally shortened posterior development (Figure 17). The MHB fold and cerebellar primodium are missing in the agrin knock-down embryos. The MHB can be divided into an anterior portion and a posterior portion. The anterior portion is eliminated and increasing amounts of agrin morpholino induced loss of the posterior portion of MHB (Figure 23A-23F). In later development, MHB malformation became more obvious and folding of the midbrain was wider when compared to control-MO injected embryos (Figure 23K and 23L). Agrin inhibition also leads to perturbed development of eyes and otic vesicles (Figure 23G-23J). These

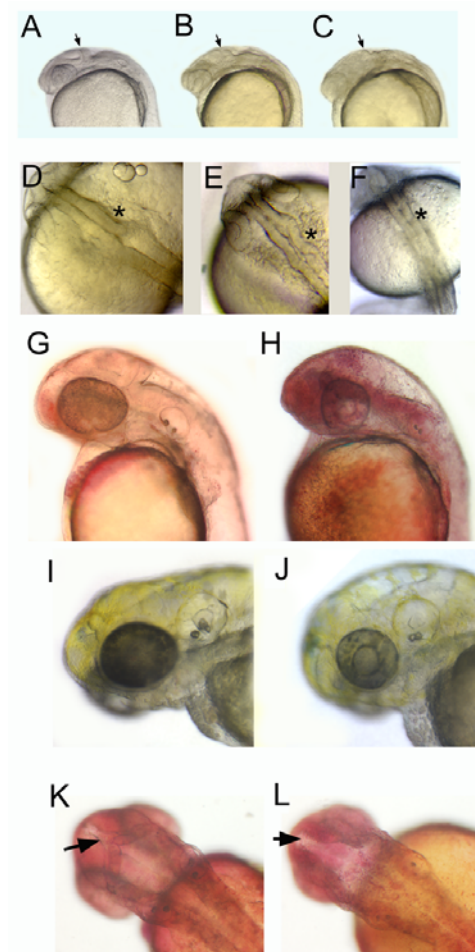


Figure 23. Defects of MHB, eye and otic vesicle in agrin knock-down embryos. A-C: Agrin inhibition leads to disturbed MHB formation in 26 hpf embryos. Arrows indicate the MHB. D-F. Dorsal view of 26 hpf embryos. * indicates MHB. A and D. Uninjected embryos. B and E. 2-3ng agrin morpholino injected embryos. C and F. 9ng agrin morpholino injected embryos. G and I. Lateral view of 3 day uninjected embryos. H and J. Lateral view of agrin morpholino injected 3 day embryos. Note agrin morpholino injected embryos show smaller eye and the otic vesicle. K. Dorsal view of uninjected 3 day embryos. L. Dorsal view of agrin morpholino (2-3ng) injected embryos. Note the wider distance in MHB formation (arrow).

defects of anterior development, as a result of agrin inhibition, are similar to FGF-related mutant phenotypes (Reifers et al., 1998; Shanmugalingam et al., 2000).

Agrin binds to FGF-8 *in vitro*.

Since HSPG is known to bind FGF family members via HS-GAG chains, a solid-phase binding assay was used to determine whether agrin interacts with FGF-8 *in vitro*. In this assay, recombinant FGF-8 protein was immobilized on ELISA plates, purified agrin was added, and agrin binding to FGF8 was visualized using anti-agrin MAB and biotinylated secondary antibodies. Agrin exhibits significantly pronounced binding to FGF-8 in a dose-dependent manner (Figure 24). Above 10 $\mu\text{g/ml}$ of agrin, binding activity was saturated. This experiment confirms that FGF-8 can interact with agrin, and that this interaction may be needed to establish the MHB organizer, as well as otic vesicle and eye development during embryogenesis.

Inhibition of FGF signaling pathways mimics agrin knockdown phenotype in zebrafish embryos.

During *Xenopus* and zebrafish development, the FGF related family was identified as being involved not only in the formation of midbrain and hindbrain, but also mesoderm induction and patterning (Kimelman and Kirschner., 1987; Griffin et

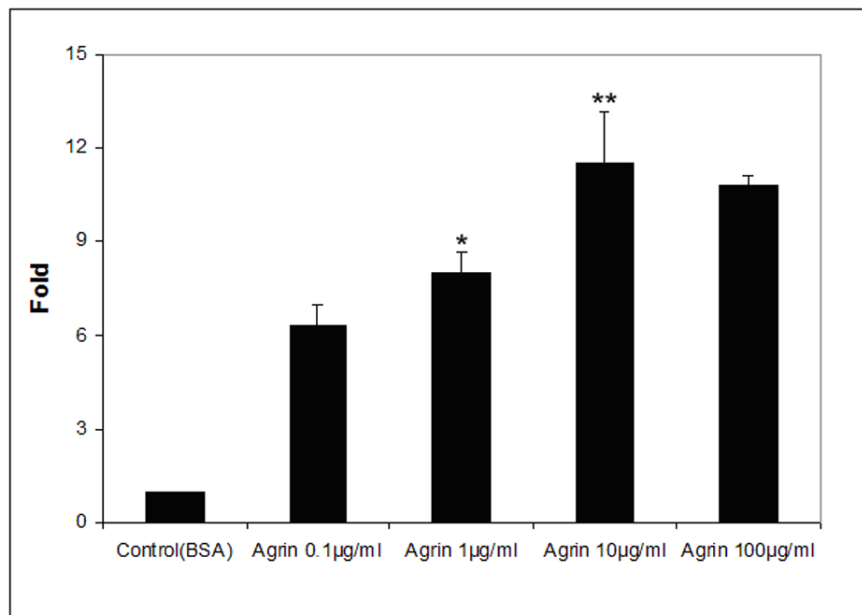


Figure 24. Solid-phase binding analysis of agrin and FGF-8. Mouse-recombinant FGF-8 immobilized on an ELISA plate, and agrin purified from chicken vitreous was added to bind with FGF-8. Binding was quantified by colorimetric reaction *in vitro*. Agrin binds dose-dependently to FGF-8 *in vitro*. (N=3, *, p=0.019 **; p=0.0009)

al., 1995). For example, FGF-3 and FGF-8 are required for mediating rhombomere differentiation and formation of the otic placode and vesicle (Maroon et al., 2002; Maves et al., 2002). The inhibition of FGFR function also resulted in the lack of posterior development (Amaya et al., 1991; Yamaguchi et al., 1994).

From results from agrin knock down studies, loss of agrin expression results in defects in eye, otic vesicle and MHB development, which resemble FGF or FGFR deficient zebrafish. To verify the requirement of agrin in the FGF signaling for zebrafish development, SU5402 was used to block FGFR activation (Mohammadi et al., 1997). SU5402 is a well-known inhibitor of FGF receptors, which blocks kinase activity of all FGF receptors. Embryos were incubated in SU5402 inhibitor for 10 min at tailbud to 3- somite stages, and allowed to develop. To reduce severity of defects from agrin deficiency, and elucidate possible interactions between agrin and FGFs, small amounts (1-2ng) of agrin morpholino were injected in zebrafish embryos. These embryos displayed weak phenotypes with regard to tail development (Figure 25B). The blocking of FGF signaling pathways by inhibitor induced more intermediate tail defects (Figure 25C). Embryos were more severely affected by injection of agrin-MO and SU5402 treatment (Figure 25D), than by injection of agrin MO alone or SU5402 treatment alone (Figure 25B and 25C). Agrin deficiency and inhibition of FGFR activation appear to synergize defects of posterior development (Figure 25), implying that agrin modulates FGF signal transduction pathways during

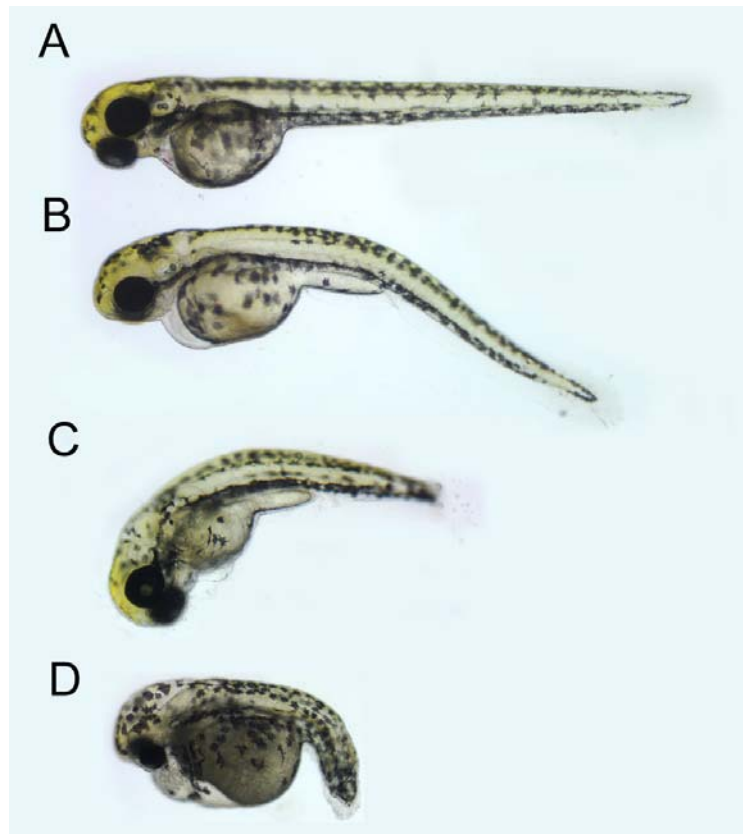


Figure 25. Simultaneous inhibition of FGFR by SU5402 and agrin by agrin-MO causes more severe phenotypic changes in posterior development. A. Wild type embryos were soaked in DMSO (n=20; 20 embryos were normal). B. Embryos injected with agrin morpholino(1-2ng) in DMSO (n=20; 20 embryos showed mild defects of tail development). C. Wild type embryos in 0.1mg/ml of SU5402 (n=20; 20 embryos showed an intermediate shortened tail development). D. Embryos injected with agrin morpholino (1-2ng) in 0.1mg/ml of SU5402 (n=20; 20 embryos showed severely shortened tails compared to agrin treatment alone or SU5402 treatment alone.).

embryogenesis.

Perturbation of CNS development due to agrin knockdown is via disrupted FGF signaling.

The FGF family has been implicated in many aspects of early developmental patterning. *Ace* mutant zebrafish, which were identified as a loss-of-function of FGF-8, have been shown to have aberrant cerebellum formation and disrupted MHB (Reifers et al., 1998). Recent studies have demonstrated an important role for FGFs in the induction and patterning of telencephalon, midbrain and hindbrain (Shinya et al., 2001; Roy and Sagerstrom, 2004). To address whether the observed CNS changes in agrin MO injected embryos were due to disrupted FGF signaling, agrin was simultaneously knockdowned and FGFR function was inhibited. The simultaneous inhibition of agrin and FGFR resulted in smaller eyes and otic vesicles, a lack of MHB formation, and defects in posterior development. The MHB formation was completely perturbed by inactivation of FGFR and knock-down of agrin (Figure 26), compared to either treatment alone. The average size of eyes and otic vesicles of 48hpf embryos was measured, with greater reductions in size by simultaneous agrin-MO and SU5402 treatment (Figure 26 and Table 3). Since FGF-3 and FGF-8 are known to be required for formation of the otic placode and vesicle, this severe defect of otic vesicle development as a result of both agrin knockdown and FGFR inactivation is not

surprising.

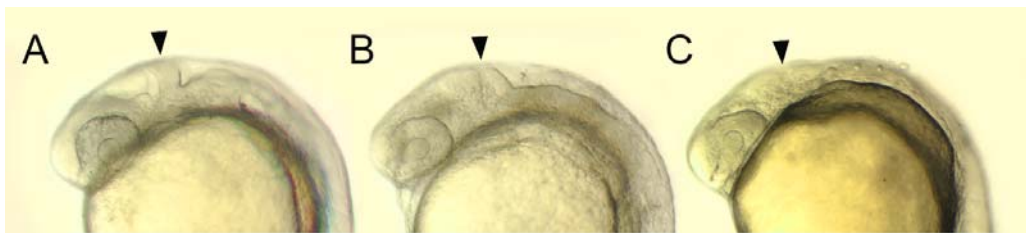


Figure 26. Agrin knock-down and FGFR inactivation exhibit synergistic midbrain-hindbrain boundary defects. Arrowhead indicates the MHB. A. The MHB in 26hpf wild-type of embryo. B. Wild-type embryos were soaked in the 0.1mg/ml of SU5402 and disrupt the MHB. C. Agrin knock down embryos were soaked in the 0.1mg/ml of SU5402 and show lack of MHB formation, and more severe brain defects.

Table 3. Phenotypic change in MHB, otic vesicle and eye in the SU5402-soaked 20 hpf embryos

	N	Defects of MHB	Size of otic vesicle	Size of eye
None in DMSO	20	0	5.87 ± 0.11 μm	9.53 ± 0.23 μm
SU5402 in DMSO	20	18	5.18 ± 0.16 μm	8.78 ± 0.2 μm
Agrin-MO injected embryo in DMSO	20	8	5.65 ± 0.09 μm	8.56 ± 0.45 μm
Agrin-MO injected embryo with SU5402	20	20	3.2 ± 0.72 μm*	5.7 ± 0.26 μm**

*: significantly different from agrin-MO injected embryos. P<0.004.

** : significantly different from agrin-MO injected embryos. P<2.38E-05.

DISCUSSION

The studies in this chapter show that agrin is required for normal development of the otic vesicle and eye, as well as the MHB. Since agrin is not expressed in the eye and otic vesicle during early development, but expressed in this region at later developmental stages, it is likely that agrin is not involved in induction of these tissues, but rather assists in maintenance of otic vesicle and eye development. Even though inhibition of agrin in zebrafish embryos using morpholinos produced a hypomorphic series of phenotypes due to variability of agrin protein loss, a size deduction in these tissues was apparent. Inhibition of agrin generates about 25% reduction in size of otic vesicles and eyes. The most prominent defects as a result of agrin knockdown occurred at the MHB. The disruption of MHB development could be seen during early development, with a complete loss of the MHB at high concentrations of agrin morpholinos.

Since agrin knock-down embryos displayed interesting phenotypes, analogous phenotypes from other gene mutations or knockdowns were explored. FGF-8 mutant embryos (*ace*) show mild defects of dorso-ventral patterning and lack of a cerebellum and the MHB organizer (Reifers et al., 1998). Previously explained, knock down of FGF-8 and FGF-3 also results in failure to induce the otic placode and vesicle (Leger

and Brand, 2002). FGFR1 is also strongly expressed in the otic vesicles, branchial arches and the brain, especially at the midbrain-hindbrain boundary (MHB), and loss of FGFR1 protein produces a phenotype similar to FGF-8/*ace* (Scholpp et al., 2004). FGFR1 conditional knock-out mice display malformations of posterior structures at later stages of embryogenesis, including truncation of embryonic structures, limb bud malformation, partial duplication of the neural tube, tail distortion, and spina bifida caused by the amplification of neural tissue in the posterior portion of the spinal cord (reviewed in Passos-Buenos et al., 1999). To identify agrin's involvement in FGF signaling pathways, agrin binding to FGF-8 was first accessed, showing that agrin binds to FGF-8. Since HSPGs, including agrin, can bind FGFs with high affinity (Cotman et al., 1999), agrin and FGF-8 likely interact via agrin's HS-GAG chains. Next, using the specific FGFR inhibitor, SU5402, inhibition of FGF signaling pathways was examined in zebrafish development, including in agrin knock-down embryos. Even though *ace* mutant zebrafish do not show any severe tail malformation (Reifers et al., 1998), inhibition of both agrin and FGFRs resulted in deteriorated posterior development. At low levels of agrin MOs only modest effects on posterior development are observed, and similar modest inhibition of tail development occurs at low levels of SU5402. Interestingly, when agrin knockdown and FGFR inhibition is combined a severe tail development of phenotype is observed that is similar to the phenotype obtained with high level of agrin MO treatment. This severe phenotype resembles the phenotype obtained when multiple FGFs are knocked down which

suggests that agrin is essential to FGF function in tail development (Draper et al., 2003). It remains possible that the severe tail phenotype from total agrin knockdown suggests that agrin may interact with other early patterning molecules such as BMP, Nodal, or Wnts for tail development (Agathon et al., 2003). Further studies will be necessary to better understand agrin's role in tail development, and to confirm that agrin, via FGF signaling, is necessary for tail development.

My studies on agrin's interactions with FGF signaling pathways in AP axis formation also suggest that agrin may modulate FGF signaling during development. Again lower amounts of agrin MO, as well as lower SU5402 (0.1mg/ml), only produced modest effects on MHB, eye, and otic vesicle development. However, the effects of partial agrin knockdown and partial FGFR inhibition could be amplified when combined, providing support for the proposal that agrin, via FGF signaling, is involved in AP axis patterning. Confirmation of this hypothesis will require the analysis of FGF downstream effectors, as well as genetic markers of AP patterning, as a result of agrin knockdown. Thus, future studies will need to address if agrin knockdown impacts MAP kinase signaling and expression of the erm transcription factor, both of which are downstream effectors of FGF signaling (Tsang et al., 2004; Shinya et al., 2001; Roehl and Nusslein-Volhard., 2001). Analysis of expression of FGF-dependent genes, such as *krox-20*, which are involved in AP patterning will also provide insight into whether agrin directly modulates FGF signaling in CNS

development.

One interesting observation made when agrin and SU5402 treatments were combined was that forebrain morphology appeared to be severely disrupted. While perturbation of FGF-dependent forebrain development may partly explain this phenotype (Scholpp et al., 2003; Walshe and Mason, 2003; Shinya et al., 2001), it will be important to assess if other signaling pathways, such as wnt, BMP, or nodal, are responsible for this dramatic phenotype.

GENERAL SUMMARY AND CONCLUSIONS

Since the discovery of agrin at the neuromuscular junction in the early 1980's, many attempts have been made to characterize agrin's role in biological processes of the central nervous system. It was anticipated that agrin mutant mice would yield this information, but due to perinatal lethality it has not been possible to elucidate agrin's function in the rodent CNS. It was believed that agrin function at the neuromuscular junction would be similar to its function in neuron-neuron synapse formation. However, this has appeared incorrect, as analysis of agrin deficient mice has shown normal synapse formation in the CNS, with impaired synapse formation by peripheral sympathetic neurons (Gingras et al., 2002). However, recent evidence has accumulated to broaden our understanding of agrin's various functions in the CNS. It has been proposed that agrin may contribute to the establishment of CNS neurogenesis, axonal growth and guidance, neuroprotection, learning and memory, and even neuropathological pathways (Reviewed Smith and Hilgenberg., 2002) .

To understand agrin's role in CNS development, there has been a search for new agrin receptors that would mediate neural responses to agrin. HSPGs have been identified as a co-receptor for FGF and FGFR, modulating the localization of FGFs to the ECM and regulating activation of the signal transduction pathway by ligands

(Aviezer et al., 1994). The studies described here demonstrate that agrin, as a FGF co-receptor, is capable of modulating FGF signaling to promote neurite outgrowth from PC12 cells, as well as chicken retinal neurons. FGF-2 has been proposed to play a role in promoting axonal pathway formation in particular the optic pathway in *Xenopus* (Lom et al., 1998; Walz et al., 1997; Mcfarlane et al., 1995). HSPGs have the ability to potentiate FGF-2 signaling for promoting retinal neuronal axonal elongation. In fact, FGF-2 is expressed in the developing axonal tracts of optic nerve and agrin is colocalized with FGF-2 in the developing optic nerve (Halfter et al., 1997; Cotman et al., 1999) and are required for FGF function. The co-localization of FGF-2 and the fact that structural FGF:FGFR:HS complexes form implies diverse functional roles for agrin in development as well as adulthood.

Using both PC12 cells and retinal neuronal cultures, agrin accelerates the ability of FGF-2 to promote neurite outgrowth. This modulating activity of agrin on FGF-2 is clearly HS-GAG dependent, and agrin is likely potentiating neurite outgrowth by augmenting FGF-2 binding to the FGFR. Therefore elimination of HS-GAG or inactivation of FGFR by the inhibitor SU5402 abolishes agrin's effects on FGF-2-mediated neurite outgrowth. Interestingly, examination of downstream effectors of the FGF signaling pathway, after agrin:FGF:FGFR activation, indicates that agrin rapidly increases and sustains ERK activation, which likely is required to initiate the cellular pathways leading to neuronal differentiation in response to FGF-2. Accordingly,

suboptimal doses of FGF-2 lead to only transient and unsustained phosphorylation of ERK. A similar level of ERK activation was obtained when FGF-2 was presented to cells with HS-GAG-free agrin, indicating that agrin's HS-GAGs are required for regulation of FGF signaling. Another early response protein, c-fos, also exhibits augmented and prolonged phosphorylation when agrin is presented to neurons in combination with FGF-2. Surprisingly, whereas agrin alone stimulates a rapid and sustained activation of c-fos, when agrin is presented with FGF-2 the c-fos activation is not as pronounced, although it is sustained. These data therefore suggest that agrin can differentially regulate multiple cell signaling pathways, depending on the presence or absence of binding ligands for agrin, such as FGF-2.

Previous studies provide indirect evidence that agrin can act differentially as both a neurite promoting and inhibitory protein *in vitro* (Mantych and Ferreira, 2001; Campagna et al, 1995; Chang et al, 1997; Halfter et al, 1997; Bixby et al, 2002). Based on studies showing that agrin expression is associated with developing axon pathways during nervous system development, and that this agrin expression is abundant in these pathways (Halfter et al., 1997; Cotman et al., 1999), agrin would not likely function as a neurite growth inhibitory molecule in developing axonal pathways *in vivo*. Moreover, previous studies demonstrated the importance of FGF-2 and HSPGs in the establishment of the retinotectal pathway in *Xenopus* (Walz et al., 1997). Thus, *in vivo* agrin may function as a growth promoting protein, rather than a growth inhibitory

protein, in developing axon pathways such as the optic tract. Accordingly, data reported here support this hypothesis that agrin potentiates the effects of FGF-2 in retinal and PC12 cell cultures, as a crucial regulator of neurite outgrowth mediated by heparin-binding growth factors such as FGF-2.

Zebrafish agrin has been characterized for further investigation of agrin's function(s) *in vivo*. The full length zebrafish agrin transcript is about 10 kb, and the total mass of the agrin protein is about 400-500 kDa with a 200 kDa core protein, in accord with studies in rodent and chicken. Agrin cDNAs exhibit high homology to other species such as chicken, human and rodent. In zebrafish, agrin mRNA is expressed in the basal lamina and in the ventral region of the floor plate, ventral diencephalon, optic tectum, retinal ganglion layer of eye, the midbrain and hindbrain boundary, hindbrain rhombomeres, hypochord, floor plate, muscle pioneers, notochord and pronephric duct. Most zebrafish agrin protein is strongly localized in the basal laminae (basement membrane), forebrain, eye, diencephalon, midbrain-hindbrain boundary, the ventricular region of the developing brain, notochord and somites. This expression pattern in zebrafish resembles agrin's distribution in other species (Groffen et al, 1999; Yard et al, 2001), providing strong support for the conclusion that zebrafish agrin was identical and characterized.

Agrin plays essential functions during zebrafish development, with regard to not only formation of the NMJ, but also contributing to CNS development. The zebrafish model system offers a practical tool to study the function of agrin in axonal growth and guidance, as well as embryogenesis, owing to amenability of zebrafish to gene manipulation during development. The analysis of the effects of agrin knock-down on zebrafish development, by morpholino oligonucleotides, resulted in pronounced morphological changes in zebrafish embryos, such as perturbation of anterior and posterior development, ultimately being lethal due to paralysis at early larval stages. Agrin deficient zebrafish embryos displayed defects of the MHB, eyes and otic vesicles and pronounced defects in posterior development. In the absence of agrin, CaP motor axons exhibited increased branching and a loss of turning towards target muscle at somitic boundaries. In contrast, axons from secondary motoneurons exhibit pronounced effects on axonal extension, with these axons being truncated and less branched in agrin-MO treated zebrafish. Interestingly, these motor neuron phenotypes resemble morphological and growth alterations described for motor neurons as a result of perturbations in posterior and paraxial mesoderm formation (Lewis and Eisen., 2004). These motor neuron defects were attributed to impaired neuronal specification as a result of posterior and paraxial mesoderm defects (Lewis and Eisen., 2004). Since posterior mesoderm development is FGF-dependent (Draper et al., 2003), my data may indicate that the observed changes in motor neuron axonal growth are the result of indirect effects of agrin on paraxial mesoderm development.

The lateral and anterior axonal growth pattern of trigeminal motor neurons was also abolished in agrin-MO injected embryos. These data, together with agrin's role in the formation of the NMJ, results in substantial loss of motor function in embryonic zebrafish. Although other motor nerve pathways were not examined, it is tempting to speculate that multiple motor nerve pathways are dependent on agrin function in zebrafish development. Finally, loss of agrin expression results in a near complete loss of axonal growth from Rohon-Beard cells, which are dorsal spinal cord sensory neurons in zebrafish. In addition, a continued expression of Hu antigen occurs in these neurons, which may be suggestive of a failure of these neurons to complete differentiation. It is therefore tempting to speculate that agrin's effects on both sensory and motor axonal growth are an indirect effect resulting from disruption of neuronal specification due to loss of agrin mediated regulation of FGF signaling pathways.

Besides, axon guidance agrin may be required to maintain CNS patterning. Inhibition of agrin changes the morphology of the CNS, such as the MHB, eye and otic vesicle. The reduction of agrin protein disturbs the normal development and generates a similar phenocopy to FGF mutants. FGF signaling in development has been intensively investigated for early patterning formation in the zebrafish nervous system. Agrin knockdown effects, such as loss of the MHB, small otic vesicle and perturbed eye development closely resemble defects from FGF-8 and FGF-3 mutant embryos (Liu et

al., 2003). Using an inhibitor of FGFRs, combined with agrin knockdown, I observed an enhancement of defects in zebrafish development, providing support for a role for agrin in modulating FGF signaling in CNS patterning. These studies may provide direct in vivo evidence that agrin can activate and accelerate FGF signaling during early neural patterning, and that agrin contributes critical functions to the establishment of a functional CNS.

The most distinct morphological defects of agrin knock-down on zebrafish development are revealed in tail formation. The increasing level of agrin knock down inhibition leads to the more severely shortened tail formation. Interestingly, recent studies have identified a tail organizer of zebrafish that utilizes nodal, wnt and BMP signaling pathways (Agathon et al, 2003). Moreover, inhibition of heparan sulfate 6-O-sulfotransferase in zebrafish affects tail development, with curved tails resulting in these zebrafish (Bink et al, 2003), and another HSPG glypican-3 knock-down or loss of function in zebrafish also disrupts tail development, with shorter tails being observed in these zebrafish (Topczewski et al, 2001). Perturbation of these signaling pathways results in failures of tail development. A more adequate explanation for describing the effects of agrin knock down on zebrafish development may be related to FGF signaling. Inhibition of FGFRs in zebrafish leads to disruption of tail development (Griffin et al., 1995, Draper et al., 2003) and knockdown of multiple FGFs also disrupts posterior

development (Draper et al., 2001; Draper et al., 2003), suggesting that agrin is required to maintain FGF function during posterior development.

Since wnt proteins are heparin-binding proteins (Reichsman et al, 1996; De Cat et al, 2003), follistatins are ligands of BMPs (Amthor et al, 2002), and agrin contains 9 follistatin-like domains (Tsen et al, 1995), it is tempting to speculate that agrin knock-down in zebrafish could also disrupt the function of the wnt and BMP signaling pathways that are required for function of the tail organizer in tail development. Agrin knock-down in zebrafish could therefore impact multiple known signaling pathways that are essential to tail development. In addition, the severity of the phenotype with agrin knock-down, when compared to glypican knock-down (Topczewski et al, 2001), may indicate that agrin is a primary secreted HSPG which serves as a mediator of pattern formation molecules for the regulation of tail development in zebrafish.

In conclusion, my studies provide the first evidence for agrin functions during CNS development *in vivo*. The observed phenotypic changes as a result of agrin knockdown in zebrafish include CNS pattern and developmental defects that also occur as a result of disruption in FGF function. The abnormalities in axonal guidance, both *in vitro* and *in vivo*, are also likely a result of disrupted FGF signaling. Combined with the pronounced disruption in zebrafish tail development with agrin knockdown, which is

also observed in response to perturbation of FGF signaling, it appears that agrin is a key regulator of FGF signaling during vertebrate development.

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