ABSTRACT

LIU, I-HSUAN. Heparan Sulfate Proteoglycan Agrin in CNS Development and Aging. (Under the direction of Dr. Gregory J Cole and Dr. Robert Anholt.)

Agrin is an extracellular matrix heparan sulfate proteoglycan and is best known for its role as the organizer of the neuromuscular junction. Emerging in vitro evidence shows that it plays many possible roles in the central nervous system. The studies presented in Chapter 1 examine the hypothesis that agrin also plays a role in Parkinson’s disease. In support of this hypothesis, this study demonstrates that agrin binds to α-synuclein via its heparan sulfate glycosaminoglycans chains, potentiates conformational changes in α-synuclein into β-sheet structure, and enhances insolubility of α-synuclein. Furthermore, agrin is also found colocalized with α-synuclein in neuronal Lewy bodies in the substantia nigra of Parkinson’s disease human brain. These results suggest that agrin is capable of potentiating the formation of α-synuclein amyloidosis in Parkinson’s disease brain and may indicate shared molecular mechanisms leading to the pathophysiology in Alzheimer’s disease and Parkinson’s disease, the two most common neurodegenerative diseases.

Zebrafish is an attractive animal model for neural development. To begin to address agrin’s function in the central nervous system, a study on agrin knockdown in zebrafish is presented in Chapter 2. Agrin mRNA is detected as a maternal message in embryonic zebrafish, and is expressed in the developing central nervous system and in
nonneural structures such as somites and notochord. Defects in the axon outgrowth by primary motor neurons, subpopulations of branchiomotor neurons, and Rohon–Beard sensory neurons are also observed, which included truncation of axons and increased branching of motor axons, suggesting roles of agrin in both axon outgrowth and guidance. Moreover, agrin morphants exhibit significantly inhibited tail development, as well as defects in the formation of the midbrain–hindbrain boundary and reduced size of eyes and otic vesicles. These results show that agrin plays an important role in the development of both peripheral and central nervous system in zebrafish.

Among the phenotypes that result from agrin knockdown using morpholino antisense oligonucleotides is reduced eye size in agrin morphants. The studies presented in Chapter 3 show that retinal differentiation is impaired in agrin morphants, with retinal lamination being disrupted in a dose-dependent manner following agrin morpholino treatment. \textit{Pax6.1} gene expression, a marker of eye development, is markedly reduced in agrin morphants, providing support for agrin’s role in retinal development. Increased apoptosis is detected suggesting the reason for microphthalmia. Both \textit{pax2a} and \textit{atoh7} gene expression levels are decreased suggesting that both FGF8 and sonic hedgehog signaling are affected. Confocal micrographs of \textit{HuC-GFP} transgenic zebrafish and immunostaining showed impaired differentiation and disorganization in all three cellular layers in the retina in agrin morphants. Midbrain-hindbrain boundary formation, and expression of mRNAs in this organizer region, is disrupted in agrin morphants. The retinotectal topographic projection to the optic tectum is also perturbed in agrin morphants suggesting the role of agrin for FGF8 signaling. Collectively, these
phenotypes in agrin morphants provide support for a crucial role of agrin in retinal development and formation of an ordered retinotectal topographic map in the optic tectum of zebrafish.
HEPARAN SULFATE PROTEOGLYCAN AGRIN IN
CNS DEVELOPMENT AND AGING

by

I-HSUAN LIU

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2007

APPROVED BY:

Dr. Robert R.H. Anholt
Chair of Advisory Committee

Dr. Philip L. Sannes
Minor Representative

Dr. Jane L. Lubischer

Dr. Jeffrey A. Yoder
BIOGRAPHY

- Born: August 24, 1973 in Taipei, Taiwan ROC

Education:

- National Taiwan University, Taipei, Taiwan ROC
  Bachelor in Veterinary Medicine, May 1996

- National Taiwan University, Taipei, Taiwan ROC
  Master in Veterinary Medicine, May 1998

- North Carolina State University, Raleigh, NC
  Ph.D. in Comparative Biomedical Sciences, May 2007

Professional Experience:

- Research Assistant, 2002- present. North Carolina State University, Raleigh, NC


- Research Assistant, 1998-1999. Military Kaohsiung General Hospital, Kaohsiung, Taiwan ROC

- Teaching Assistant, 1996-1998. National Taiwan University, Taipei, Taiwan ROC
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INTRODUCTION

In the course of the search for the key factor for the formation and regeneration of the neuromuscular junction (NMJ), agrin was identified by McMahan’s lab from the electric organ of *Torpedo californica*, based on its activities in aggregation of acetylcholine receptors, acetylcholinesterase, and butyrylcholinesterase (Nitkin et al., 1987). Later, McMahan proposed the “agrin hypothesis” based on several lines of evidence suggesting that agrin is secreted from neurons, transported to nascent synaptic basal lamina, and required for the clustering of AchRs and other postsynaptic molecules (McMahan, 1990). These predictions have been confirmed and proved that agrin is the key organizer for the induction of postsynaptic specializations at the NMJ (Gautam et al., 1996; Magill-Solc and McMahan, 1988; Magill-Solc and McMahan, 1990; Meier et al., 1997).

The mosaic structure of agrin protein

With a molecular weight of ~ 220kDa, the core protein of agrin was predicted according to its cDNA sequence to be conserved in several species and to have a number of domains, as shown in Figure 0-1, that are similar to other extracellular matrix proteins, such as epidermal growth factor (EGF)-like domain, follistatin/Kazal-like domain, and laminin-like globular domain (Campanelli et al., 1991; Rupp et al., 1991; Tsim et al., 1992). Agrin N-terminal (NtA) domain interacts with laminin γ1 chain, and its similar
structure with the metalloproteinase-inhibition domain of metalloproteinases-1 implies potential functions more than just providing the anchor to the basal laminae (Mascarenhas et al., 2003; Stetefeld et al., 2001). A seven amino acid insert at this region produced by alternative splicing is important for the secretion of agrin (Denzer et al., 1995; Tsen et al., 1995b). The function of the follistatin/Kazal-like domains in agrin core protein is unknown, but the homologous sequences imply the potential binding affinity to interacting targets of follistatin and inhibitory activities against proteases such as trypsin, elastase and plasmin (Biroc et al., 1993; Rawlings et al., 2004). An alternative first exon was identified resulting in a transmembrane isoform of agrin (Burgess et al., 2000; Neumann et al., 2001). This isoform is regulated distinctly from the secreted isoform and is enriched in the central nervous system (CNS), especially in growing neurons, implying more potential roles of agrin in neuronal growth, neurite guidance and/or synaptogenesis (Burgess et al., 2000; Neumann et al., 2001).

The C-terminal portion of the agrin core protein contains three laminin globular domains and is responsible for known molecular signaling required for NMJ organization. Multiple isoforms of agrin are generated by alternative splicing at three positions in the C-terminal portion, and biological activities from this region are closely regulated by these isoforms. For example, the third laminin globular domain is sufficient to induce postsynaptic differentiation in muscle fibres when B/z insertion of 8 amino acids is included (Ferns et al., 1992; Ferns et al., 1993; Gesemann et al., 1995; Meier et al., 1998a; Scotton et al., 2006), and the A/y insertion of 4 amino acids at the second laminin globular domain is required for heparin/heparan sulfate binding to agrin
(Campanelli et al., 1996; Gesemann et al., 1996). The first two laminin globular domains are required for the binding of α-dystroglycan, which is part of the agrin receptor serving as a link between the extracellular matrix and cytoskeleton, but the binding activity is negatively regulated by A/y and B/z insertion (Scotton et al., 2006). Although not required for the NMJ clustering, α-dystroglycan is required for the stabilization of the clustered NMJ (Jacobson et al., 2001; Meier et al., 1998a). Agrin also binds to β1 integrins, which facilitate cell adhesion to agrin through the second laminin globular domain and the last EGF-like domain of agrin (Burgess et al., 2002). Integrins, like dystroglycan, are not required for the NMJ clustering, but are important in mediating the adhesion to agrin and modulate agrin signaling (Bezakova and Ruegg, 2003; Martin and Sanes, 1997). Although many agrin isoforms and domains remain functionally unknown, this multiple-domain, mosaic-like structure implies a broad range of activities of agrin in biological processes.

**Agrin is a heparan sulfate proteoglycan**

In searching for the major brain heparan sulfate proteoglycan (HSPG) that interacts with and modulates neural cell adhesion molecule (NCAM), agrin was identified as a member of the HSPG family (Tsen et al., 1995a). Proteoglycans are a class of glycoproteins that consist of a core protein with one or more covalently attached glycosaminoglycan (GAG) chains. GAG chains are long, linear, unbranched repeating disaccharide units that are negatively charged physiologically, due to sulfation and the occurrence of the uronic acid groups (Cole and Liu, 2006). HSPGs are proteoglycans that contain heparan sulfate GAG (HS-GAG) chains, which are composed of, originally,
a UDP-L-glucuronic acid and a UDP-N-acetyl-D-glucosamine in the disaccharide units.

Biosynthesis of HS-GAG is initiated from the core protein region on consensus amino acid attachment sites for HS-GAGs, which usually contain repetitive serine-glycine amino acid sequences flanked by clusters of acidic amino acid residues, primarily aspartic acid and glutamic acid. The HS-GAG disaccharide units are polymerized/elongated after the initial “linkage tetrasaccharide” is synthesized, which is composed of \( \beta \)-xylose, \( \beta 1-4 \)-galactose, \( \beta 1-3 \)-galactose, and \( \beta 1-3 \)-glucuronic acid and is identical for CS-GAG chain synthesis (White lock and Iozzo, 2005). As shown in Figure 0-2, the polymerization/elongation of HS-GAG chains is mediated by the Exostosin (ext) family of heparan sulfate polymerases which confer the UDP-N-acetyglucosamine transferase and UDP-glucuronic acid transferase activities (Lee and Chien, 2004; Lind et al., 1998). As the HS-GAG chain is extended and the proteoglycan is trafficked through the Golgi apparatus, five potential modifications can occur in a specific order, including N-deacetylation and N-sulfation of GlcNAc by N-sulfotransferase, 5-C-epimerization of GlcA by C5-epimerase that converts GlcA into iduronic acid, 2-O-sulfation of GlcA by 2-O-sulfotransferase, 6-O-sulfation of GlcNAc by 6-O-sulfotransferase, and 3-O-sulfation of GlcNAc by 3-O-sulfotransferase (Kusche-Gullberg and Kjellen, 2003; Whitelock and Iozzo, 2005). Various combinations of these modifications result in specific sugar code sequences that may interact with a range of proteins such as cytokines, growth factors, lipase, protease/protease inhibitors, extracellular proteins, axon guidance molecules and morphogens (Lee and Chien, 2004; Whitelock and Iozzo, 2005). Since agrin contains at least three GAG chains and at least two of them are HS-GAG
chains (Winzen et al., 2003), agrin could potentially interact with a broad range of proteins through the attached HS-GAG chains and hence serve diverse functions.

Several laboratories have shown that the same HSPG expressed in different cell types/tissues resulted in different HS-GAG composition, suggesting that the modifications of the HS-GAGs are strictly regulated in biological environments (Kato et al., 1994; Ledin et al., 2004). However, other than the expression of the isoforms of HS modification enzymes and the presence of the types of the UDP-monosaccharides in the lumen of the Golgi apparatus, there is very little known about the regulatory mechanisms underlying the generation of these “sugar codes” during the biosynthesis of the HS-GAG chain (Kreuger et al., 2006; Whitelock and Iozzo, 2005).

In human, there are at least thirteen HSPGs including four transmembrane syndecans, six glycosylphosphatidylinositol (GPI)-anchored glypicans, collagen XVIII, perlecan and agrin. Since all HS-GAGs share the same synthetic and modifying mechanisms (Ledin et al., 2004), the activities mediated by HS-GAGs can be compensated by another HSPG when the core protein of a HSPG is removed. For example, the study on agrin deficient mice demonstrated only the loss of functional NMJs (Gautam et al., 1996), but the abundant expression pattern and the acute inhibition experiments done with antibody and antisense oligonucleotides suggested additional possible functions (Bose et al., 2000; Li et al., 1999).

One way to study the biological activities of HS-GAGs is to interfere with the synthetic/modifying pathways. Studies on Drosophila, mouse and zebrafish suggested
that ext1 and/or ext2 are important for establishing morphogen gradients for embryonic development, especially hedgehog signaling (Bellaiche et al., 1998; Bornemann et al., 2004; Lin et al., 2000; Siekmann and Brand, 2005; The et al., 1999). The N-sulfotransferase homologue in Drosophila, sulfateless, is shown to be important in Wg (Wnt homologue) and fibroblast growth factor (FGF) signaling (Lin et al., 1999; Lin and Perrimon, 1999). Deficiency of glucosaminyl N-deacetylase/N-sulfotransferase (NDST-1) in mice causes some embryonic lethality while survival embryos have phenotypes such as pulmonary hypoplasia, neonatal respiratory distress, and defective skull and eyes (Fan et al., 2000; Ringvall et al., 2000). Since N-deacetylation is required for other sulfation on HS-GAGs, it is reasonable that deficiency in a major N-sulfotransferase gene could cause more severe phenotypes (Bengtsson et al., 2003).

Mutant zebrafish dackel and boxer that have mutations in ext ortholog genes incorrectly sort retinal ganglion cell (RGC) axons in the optic tract, suggesting that HS-GAGs are required for optic tract sorting (Lee et al., 2004). Interestingly, a study in C. elegans demonstrated that lacking either C5-epimerase, 2-O-sulfotransferase or 6-O-sulfotransferase result in axonal and cellular guidance defects, and these phenotypes were linked to neuronal guidance systems (Bulow and Hobert, 2004).

Hs3st1 (homologue of 3-O-sulfotransferase) contributes to the production of anticoagulant heparan sulfate and modulates the binding of antithrombin III in vitro (Uchiyama and Nagasawa, 1991; Zhang et al., 2001). However, mice lacking Hs3st1 demonstrated normal hemostasis and intrauterine growth retardation, while the HS-GAGs and heparin produced by Hs3st1−/− mice have significantly low binding affinity to
antithrombin. These data suggest that Hs3st1/antithrombin may serve alternative biological roles (HajMohammadi et al., 2003; Shworak et al., 2002). Since the binding sequence of the HS sugar code to one protein can be complex and possibly variable, it is possible that the effects of decrease of one modificatory enzyme are compensated by other enzymes. In fact, an in vitro study using cells harvested from mice lacking Hs2st (2-O-sulfotransferase homologue) demonstrated that HS-GAG with elevated 6-O-sulfotransferase activity and no 2-O-sulfotransferase modification lost their high binding affinity to FGF1 and FGF2, while ERK1/2 was phosphorylated in response to the FGF2 signaling (Merry et al., 2001).

Another way to study the biological activities of HS-GAGs is to identify their putative binding ligands. One advantage of this approach is that individual HSPGs from specific tissues can be studied. Many proteins bind directly to agrin through its GAG chains, such as neuregulin, NCAM, pleiotrophin, FGF2, merosin (laminin variants with α2 heavy chain), thrombospondin and β-amyloid (Burg et al., 1995; Cotman et al., 1999; Cotman et al., 2000; Daggett et al., 1996; Meier et al., 1998b). It has been recognized that HSPGs are associated with all types of lesions in Alzheimer’s disease (AD) (Snow et al., 1987), and agrin was identified as the major HSPG accumulated in AD brain and in AD lesions (Cole and Liu, 2006; Cotman et al., 2000; Verbeek et al., 1999).

**Agrin function in neurodegenerative diseases**

AD is a neurodegenerative disease characterized as a protein conformational disorder that involves misfolded pathological proteins/peptides that aggregate and cause neurotoxicity. In AD, the amyloid precursor protein (APP) is cleaved into β-amyloid
peptide, which then aggregates as a result of protein misfolding. Three types of pathological characteristics of this disease have been described: first, senile plaques, which refers to the deposition of the amyloid, mainly composed of β-amyloid peptides, in the gray matter of the brain; second, cerebral amyloid angiopathy (microvascular plaques), which refers to the deposition of the amyloid, mainly composed of β-amyloid peptides, in the wall of the blood vessels of the brain; and third, neurofibrillary tangles, which refers to the aggregation of the pathological protein, mainly composed of hyperphosphorylated microtubule-associated protein tau, within neurons in the brain (Braak and Braak, 1995; Buee et al., 1997; Cole and Liu, 2006; Vinters et al., 1988).

Agrin is associated with AD in various ways: it is the only HSPG that deposits in all three types of pathological lesions in AD brain (Cotman et al., 2000; van Horssen et al., 2001; Verbeek et al., 1999); it binds to β-amyloid and serves as a chaperone that facilitates fibril formation of β-amyloid and hence increases insolubility of β-amyloid (Cotman et al., 2000); its expression level in vascular basement membrane was found altered in association with microvascular damage in AD (Berzin et al., 2000); and it was suggested to upregulate the expression of the microtubule-associated protein tau (Mantych and Ferreira, 2001). Agrin also binds to type four allele of apolipoprotein E (ApoE4) (G.J. Cole, unpublished data), which is a risk factor for AD. ApoE is synthesized by astrocytes, activated microglia, and neurons in the brain, and could potentially increase β-amyloid production, potentiate β-amyloid induced apoptosis, and/or induce neural toxicity in AD brain (Mahley et al., 2006). Cell surface HSPGs serve as receptor or co-receptor with low density lipoprotein (LDL) receptor and LDL receptor-related protein (LRP) and mediate lipid metabolizing activities of ApoE (Ancsin, 2003; Mahley and Ji,
1999; Strittmatter et al., 1993). Furthermore, LRP is suggested to be part of the blood-brain barrier transporting mechanism that is responsible for the clearance of β-amyloid, while agrin has been speculated to play an important role in the formation and maintenance of the blood-brain barrier since it accumulates in the brain microvascular basal laminae during the time the blood vessel becomes impermeable (Barber and Lieth, 1997; Wang et al., 2006).

Like AD, Parkinson’s disease (PD) is also a protein conformational disorder neurodegenerative disease, in which α-synuclein is the fibrillated and aggregated pathological protein (Kruger et al., 1998; Polymeropoulos et al., 1997; Uversky, 2003). A number of factors potentiate the fibril formation and aggregation of α-synuclein, including mutations in the α-synuclein gene (Narhi et al., 1999) and interactions with sulfated macromolecules, especially heparin and HS-GAG (Cohlberg et al., 2002). Furthermore, FGF2 binds to heparinase sensitive sites in Lewy bodies, which are one of the major pathological lesions in Parkinson’s disease, suggesting a possible role of HSPGs in the fibril formation and aggregation of α-synuclein (Perry et al., 1992). In the first chapter of this dissertation, I provide more evidence to support this hypothesis, and further extend the hypothesis that agrin is the HSPG that plays a role in α-synuclein fibril formation and aggregation.

**Agrin function in CNS development**

In addition to binding to morphogens such as Hedgehog, HS-GAGs also bind growth factors such as FGFs, neuregulin-1 and vascular endothelial growth factors (VEGF), indicating that HSPGs play important yet diverse roles during embryonic
development (Whitelock and Iozzo, 2005). HS-GAGs are required for many of FGF’s functions as HSPGs/heparin interact with both FGF ligands and their receptors to form a ternary complex that is necessary to activate downstream signaling pathways (Wu et al., 2003; Yayon et al., 1991). The FGF binding motifs of HS-GAGs have been characterized and N-sulfation, 2-O-sulfation, and 6-O-sulfation in HS-GAGs are important for FGF binding and/or signaling (Ashikari-Hada et al., 2004; Kreuger et al., 2005; Lundin et al., 2000). FGFs are essential molecules for embryonic development. For example, \textit{fgf8} is expressed at the midbrain-hindbrain boundary (MHB) to serve as an organizing factor and, in concert with other molecules, regulate brain patterning, retinotectal axon projections, and formation of the otic vesicle and retina (Leger and Brand, 2002; Liu et al., 1999; Liu et al., 2003; Maroon et al., 2002; Martinez-Morales et al., 2005; Maves et al., 2002; Phillips et al., 2004; Picker and Brand, 2005; Picker et al., 1999; Reifers et al., 1998; Scholpp et al., 2003; Walshe et al., 2002; Walshe and Mason, 2003). Other FGFs, such as FGF2, also contribute to nervous system development, such as promoting neurite outgrowth in cooperation with agrin via agrin’s HS-GAG chains (Kim et al., 2003).

Neuregulin-1 may be concentrated by agrin at the NMJ to serve as a signal for synapse-specific expression of AchR subunits (Rimer, 2003). In other neural development processes, neuregulin-1 has been associated with neuronal proliferation and myelination (Edwards and Bottenstein, 2006; Nave and Salzer, 2006). Interestingly, a recent report of loss-of-function analysis of BACE1, amyloid precursor protein \(\beta\)-secretase, demonstrated hypomyelination and increased levels of full-length neuregulin-1
Microarray analysis also associated the expression of agrin with the maturation of Schwann cells, and α-synuclein with peripheral nerve myelination (D'Antonio et al., 2006). Since HS-GAGs are capable of binding to BACE1, α-synuclein, and neuregulin-1, it is tempting to speculate that agrin may play a role in myelination (Cohlberg et al., 2002; Meier et al., 1998b; Scholefield et al., 2003).

In addition to the HS-GAG bound ligands, many of the agrin core protein bound ligands have been linked to brain development. Mutations in dystroglycans, β1 integrin, or laminin γ1 chain resulted in disorganized cortical layering and misplaced neurons, indicating that these proteins are associated with neuronal migration (Montanaro and Carbonetto, 2003). Dystroglycan null mice demonstrated reduced hippocampal long-term potentiation, indicating its possible role in synaptic transmission (Moore et al., 2002). Knockdown of α-dystroglycan in zebrafish causes increased retinal apoptosis, microphthalmia and retinal delayering (Lunardi et al., 2006). Integrins are part of a receptor complex that laterally transmits force and signals from extracellular matrix, and can serve as a sensor for developing neural cells directing their behavior (Bezakova and Ruegg, 2003; Campos, 2005). Integrins have been reported to be involved in neurite outgrowth, synaptogenesis, neural migration, and blood-brain barrier formation (Marrs et al., 2006; Milner and Campbell, 2002). Interestingly, dystroglycan, integrins, and agrin all are expressed in retina, and both dystroglycan and integrins are associated with lamination/cell migration in retina (Georges-Labouesse et al., 1998; Hálfter et al., 1997; Lunardi et al., 2006). In addition, all three of these proteins are also expressed in blood-brain barrier, although how dystroglycan is associated with the blood-brain barrier still
remains unclear (Barber and Lieth, 1997; Del Zoppo et al., 2006; Milner and Campbell, 2002; Rascher et al., 2002).

Since many of agrin binding ligands are associated with CNS development, and agrin is expressed abundantly in CNS, it is logical to speculate that agrin plays important roles in CNS development. However, studies on agrin deficient mice demonstrated apparent phenotypes only with regard to the formation of functional NMJ (Gautam et al., 1996; Li et al., 1999; Serpinskaya et al., 1999). One possible reason for the lack of obvious CNS phenotypes in agrin null mice is early lethality since development of the CNS continues after birth and agrin deficient mice die perinatally due to breathing failure. Another possible reason for this lack of obvious phenotypes is that the effects of agrin deficiency are compensated by other HSPGs in mouse. This hypothesis is supported by the impairment of synaptogenesis in cultured hippocampal neurons after acute inhibition of agrin by antisense oligonucleotides and antibodies (Bose et al., 2000), and the decreased numbers of synapses and impaired synaptic differentiation in cultured sympathetic neurons from agrin deficient mice (Gingras et al., 2002). In addition, recent in vitro experimental evidence also demonstrated that agrin promotes neurite outgrowth in the CNS (Annies et al., 2006; McCroskery et al., 2006), and HS-GAGs capable of binding to known axon guidance molecules also support a possible role of agrin in axon guidance (Lee and Chien, 2004). However, all these suggestions lack the support of in vivo study.

Zebrafish is an attractive animal model for three major reasons: high fecundity, with a pair of zebrafish laying ~200 eggs every week; manipulability, that manageable size of
the one cell stage embryo with many handy tools available to manipulate gene expression; and transparency, allowing one to observe embryos throughout their development. Zebrafish have a much simpler yet similar nervous system compared to mammals. The duration of their embryonic development is short, as within 3 days the majority of their development is completed. These advantages make zebrafish a very good model for studies on neural development. The second chapter of this dissertation describes the phenotypes observed in agrin knockdown zebrafish embryos, extending our laboratory’s previous studies that provide the rationale for using zebrafish as a new model system to study the biological activities of agrin. The third chapter extends the studies of Chapter 2, to further investigate the role of agrin in zebrafish retinal development. Retinal development requires the concerted activities of FGF signaling and Sonic Hedgehog signaling (Esteve and Bovolenta, 2006). Since both FGFs and sonic hedgehog require HS-GAGs for proper functioning, and agrin is abundantly expressed in the developing retina, I expand the hypothesis that agrin will also play a role in retinal development.
Figure 0-1. The schematic representation of the mosaic structure of agrin. Alternative first exons generate either secreted NtA domain isoform or transmembrane isoform of agrin. Four different insertion sites are also resulted by alternative splicing. The binding capability to various interaction partners at different regions denotes a broad range of potential activities of agrin in biological environments. This protein domain structure was predicted using SMART web-tool (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) with human agrin sequences which were pulled from Ensembl (http://www.ensembl.org/index.html) genome browser as inputs.
Heparan sulfate is a class of glycosaminoglycans comprised of repeating disaccharide units composed of, originally, a UDP-L-glucuronic acid and a UDP-N-acetyl-D-glucosamine. The elongation of HS-GAG chain is mediated by exostosin family heparan sulfate polymerases which confer the D-glucuronyl (GlcA) and N-acetyl-D-glucosaminoglycan (GlcNAc) transferase activities. Five possible modifications can be applied by specific enzymes during the trafficking of the proteoglycan through the Golgi apparatus, including N-deacetylation and N-sulfation of GlcNAc by N-sulfotransferase, 5-C-epimerization of GlcA by C5-epimerase that converts GlcA into iduronic acid, 2-O-sulfation of GlcA by 2-O-sulfotransferase, 6-O-sulfation of GlcNAc by 6-O-sulfotransferase, and 3-O-sulfation of GlcNAc by 3-O-sulfotransferase. Different combinations of modifications can encode specific sugar sequences that might serve various roles in biological environments.
CHAPTER 1

AGRIN BINDS $\alpha$-SYNUCLEIN AND MODULATES

$\alpha$-SYNUCLEIN FIBRIL FORMATION
Abstract

Recent studies have begun to investigate the role of agrin in brain and suggest that agrin’s function likely extends beyond that of a synaptogenic protein. Agrin is associated with the pathological lesions of Alzheimer’s disease (AD) and may contribute to the formation of β-amyloid plaques in AD. I have extended the analysis of agrin’s function in neurodegenerative diseases to investigate its role in Parkinson’s disease (PD). α-Synuclein is a critical molecular determinant in familial and sporadic PD, with the formation of α-synuclein fibrils being enhanced by sulfated macromolecules. In the studies reported here, I show that agrin binds to α-synuclein in a heparan sulfate-dependent (HS-dependent) manner, induces conformational changes in this protein characterized by β-sheet structure, and enhances insolubility of α-synuclein. The association of agrin with PD lesions was also explored in PD human brain, and these studies shown that agrin colocalizes with α-synuclein in neuronal Lewy bodies in the substantia nigra of PD brain. These studies indicate that agrin is capable of accelerating the formation of insoluble protein fibrils in a second common neurodegenerative disease. These findings may indicate shared molecular mechanisms leading to the pathophysiology in these two neurodegenerative disorders.
Introduction

Alzheimer’s disease (AD) and Parkinson’s disease (PD) represent the two most common neurodegenerative disorders in humans. Recent studies demonstrate that these, and many other human diseases, are protein conformational disorders characterized by the formation of unstable protein intermediates that lead to the formation of amyloid protein fibrils and neuronal degeneration (Carrell and Lomas, 1997; Soto, 2003; Uversky and Fink, 2004). In AD, β-amyloid peptide undergoes aggregation to form highly insoluble fibrils that accumulate as extracellular plaques (Glenner and Wong, 1984; Tanzi et al., 1987). A number of different mechanisms contribute to β-amyloid aggregation, including amyloid precursor protein mutations (Cai et al., 1993; Citron et al., 1992; Suzuki et al., 1994) and interaction of β-amyloid with various proteins that include ApoE4 (Strittmatter et al., 1993) and HSPGs (Snow et al., 1994). β-Amyloid fibrils and protofibrils are neurotoxic (Hartley et al., 1999; Lambert et al., 1998; Pike et al., 1991) and are considered an initiating factor in the progression of AD to later pathologies.

PD is a neurodegenerative disease characterized by loss of dopaminergic neurons in the substantia nigra. The aggregation and fibrillar formation of α-synuclein, a 14-kDa presynaptic protein, is a causative factor for PD (Kruger et al., 1998; Polymeropoulos et al., 1997; Uversky, 2003; Uversky and Fink, 2002; Uversky and Fink, 2004). Fibrillar α-synuclein is a major proteinaceous component of Lewy bodies and Lewy neurites (Bayer et al., 1999; Spillantini et al., 1997; Wakabayashi et al., 1998), the primary pathological lesions in PD. The aggregation of α-synuclein is potentiated by a number of different
factors, including α-synuclein mutations (El-Agnaf et al., 1998b; Li et al., 2001; Li et al., 2002; Narhi et al., 1999) and interactions with charged macromolecules, with the extent and rate of fibrillar formation being increased by sulfated molecules, such as heparin (Cohlberg et al., 2002; Uversky, 2003; Uversky and Fink, 2002; Uversky and Fink, in press).

Agrin is an extracellular matrix and transmembrane HSPG in the CNS (Cohen et al., 1997; Halfter et al., 1997; Tsen et al., 1995a). Recent studies have shown that agrin localizes to all lesion types in AD (Cotman et al., 2000; Donahue et al., 1999; Verbeek et al., 1999) and potentiates β-amyloid fibril formation (Cotman et al., 2000). In light of evidence that HS may also contribute to the pathophysiology of PD, it is important to examine agrin’s role in regulating the aggregation state of α-synuclein. An ability of agrin to modulate fibrillar formation of both α-synuclein and β-amyloid would raise the possibility that these two neurodegenerative diseases might share similar molecular mechanisms that contribute to their respective protein aggregations.

In this study, I examined the interaction of agrin with α-synuclein and demonstrated that agrin binds to and promotes α-synuclein insolubility and accelerates the conformational change of α-synuclein towards β-sheet structure. I also show that agrin and α-synuclein colocalize in Lewy bodies in pigmented neurons in the substantia nigra of PD brain. These data indicate that agrin may contribute to the etiology of PD by modulating the aggregation state of α-synuclein in dopaminergic neurons.
Materials and Methods

Analysis of agrin–α-synuclein binding

Agrin binding to α-synuclein was assessed using an ELISA, according to previously published protocols (Cotman et al., 2000). Agrin MAbs (6D2 and 3A12, 50 μg/mL) were adsorbed to an ELISA well, and chick vitreous body agrin (1 μg) was bound to the well. Chick vitreous body agrin was purified from E14 chick vitreous bodies, as previously described (Cotman et al., 1999). Recombinant human α-synuclein was purified, as previously described (Uversky et al., 2001), and 10, 20, or 40 μg in 100 μL of phosphatebuffered saline (PBS)–2% bovine serum albumin (BSA) was added to the well and incubated with the agrin for 2 h. Wells were washed three times with PBS between incubations. Binding of α-synuclein was visualized by incubating wells with polyclonal goat antisera to α-synuclein (Biodesign International, Saco, ME), followed by biotinylated anti-goat antiserum and avidin–biotin reagent (Vector Laboratories, Burlingame, CA). Wells were then incubated with 3,3′,S,S′-tetramethylbenzidine peroxidase substrate (Sigma, St. Louis, MO) and read at 450 nm using an ELISA plate reader.

Agrin binding to α-synuclein was also assessed by incubating chick vitreous agrin (5 μg) with 50 μg α-synuclein at 37°C without agitation for 7 days. Aliquots of this reaction mixture diluted in 100 μL of PBS–2% BSA, corresponding to either 9 or 15 μg of α-synuclein and 0.9 or 1.5 μg agrin, were then added to 96-well plates coated with agrin MAb, as described above. Following a 2-h incubation, α-synuclein polyclonal
antiserum was added to visualize α-synuclein “captured” by binding of agrin to the agrin antibody coated well. α-Synuclein bound to the agrin was then quantified, as described above. α-Synuclein in the absence of agrin was not observed to bind to the agrin MAb in this assay.

**Analysis of agrin’s effects on α-synuclein solubility**

An alternative approach to assess the ability of agrin to bind to α-synuclein was to incubate agrin with α-synuclein at 37°C without agitation for 7 days, to determine whether agrin could regulate α-synuclein solubility. These studies were carried out, as previously described, in our laboratory using β-amyloid peptide (Cotman et al., 2000). Briefly, α-synuclein (100 μg; 1 mg/mL) was incubated with chick agrin (5 μg; 50 μg/mL) for 7 days at 37°C without agitation. Reaction mixtures were then centrifuged at 13,000 × g for 30 min, supernatants were saved (soluble fraction), and pellets (insoluble fraction) were washed once with PBS. Aliquots of soluble and insoluble fractions were then analyzed by SDS–PAGE for the presence of agrin or α-synuclein, using either a 4% gel (agrin) or 15% gel (α-synuclein), followed by western blotting using a mouse MAb to agrin or a goat antiserum to α-synuclein. Because of the small amounts of agrin that became associated with the insoluble fraction, significantly smaller amounts of insoluble α-synuclein, when compared with soluble α-synuclein, were analyzed by SDS–PAGE.

**Analysis of α-synuclein conformation by circular dichroism**

Circular dichroism (CD) is a type of spectroscopy that can determine the secondary structure of organic molecules based on the absorption of the left and right circularly polarized light of different wavelengths. CD analysis of α-synuclein as a result of agrin
binding was assessed, according to published methods (McLaurin et al., 1999). α-Synuclein (100 μM) was incubated with or without agrin (0.5 μM; 300 ng/μL) in 50 mM PBS at 37°C for up to 6 days without agitation. Samples were 10 times diluted immediately before CD spectra being recorded on a Jasco Circular Dichroism Spectrometer (Model J-600; Tokyo, Japan) at room temperature. Spectra were scanned from 200 to 260 nm, with 1 mm cell pathlength, 1 nm bandwidth, 0.4 nm steps, and 100 nm/min speed. Every sample was scanned eight times to eliminate the background to noise signal.

**Immunohistochemical localization of agrin in PD brain**

Human brain tissue from PD patients and age-matched controls were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA). Paraffin sections were deparaffinized in xylene and rehydrated through an ethanol to water series. Sections were then treated with 88% formic acid for 5 min, rinsed in water, and then processed for immunohistochemistry, as previously described (Cotman et al., 2000). For confocal microscopy, paraffin sections of human brain that had been deparaffinized and rehydrated were blocked with 5% donkey serum in PBS, and then incubated overnight at 4°C with primary antibodies to agrin. The polyclonal antiserum against agrin was prepared to a fusion protein of recombinant agrin (Cotman et al., 2000) and was immunopurified in an affinity column containing immobilized human agrin protein. This antiserum has been shown to only react with agrin in human brain (Cotman et al., 2000). Following the 2-h incubation with Cy3-conjugated anti-rabbit IgG (Jackson Immunobiologicals, West Grove, PA), sections were incubated overnight at 4°C with
anti-α-synuclein (Biodesign International) in PBS–2% BSA. Sections were then incubated for 2 h with Cy2-conjugated anti-goat IgG (Jackson Immunobiologicals). Fluorescence was visualized using either Nikon C1 confocal microscopy imaging system or Nikon Diaphot-inverted microscope (Nikon, Melville, NY).

**Analysis of α-synuclein neuronal cytotoxicity**

To assess a potential role for agrin in α-synuclein cytotoxicity, I followed previously published protocols that analyzed the effect of extracellular administration of α-synuclein on neuronal cytotoxicity (El-Agnaf et al., 1998a). α-Synuclein (110 μM) was incubated in the presence or absence of agrin (440 nM) for 0–6 days, to generate α-synuclein with differing fibrillar formation states. Cultures of SH-SY5Y cells (7500 cells/well) were assayed for cytotoxicity in 96-well plates, by adding aliquots of α-synuclein or α-synuclein–agrin (final concentration of 10 μM α-synuclein/well) and incubating for 48 hours. To quantify cell death, MTT assayed was performed. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St.Louis, MO) was dissolved in PBS to make 10mg/ml (10x) MTT reagent. MTT reagent was added into the culture wells 4 hours before the end of the culture incubation. Medium in the culture wells are removed and 100 μL DMSO was added and the plate was shaked at 60rpm for 5 minutes to dissolve the crystal. The relative cell number was determined by reading the plate at the wavelength of 570nm. Triplicate wells were used per treatment per experiment, and the experiment was repeated for three times.
Results

Agrin binds to α-synuclein

In view of recent studies that have shown the capability of sulfated macromolecules, including heparin, to regulate fibrillar formation of α-synuclein (Cohlberg et al., 2002), I initiated experiments to assess whether agrin would be one CNS HSPG capable of interacting with α-synuclein. Our rationale for these studies was that significant heterogeneity in binding has been demonstrated for proteoglycans, with the same proteoglycan exhibiting differences in binding specificity (Cotman et al., 1999; Knox et al., 2002; Sanderson et al., 1994) or changes in proteoglycan glycosylation modifying its binding specificity for the same heparin-binding proteins (Herndon et al., 1999; Sanderson et al., 1994). Thus, it is conceivable that despite fibrillar formation of α-synuclein being regulated by sulfated macromolecules, agrin may not be the sulfated macromolecule responsible for, or capable of, modulating fibrillar formation of α-synuclein.

Agrin binding to α-synuclein was assessed using an enzyme-linked immunosorbent assay (ELISA), following protocols used previously in our laboratory to examine agrin binding to β-amyloid (Cotman et al., 2000). Agrin monoclonal antibodies (MAbs) were adsorbed to an ELISA well, and purified chick vitreous body agrin was bound to the antibody-coated well. Recombinant α-synuclein (10, 20, or 40 µg) was then added to the immobilized agrin, and binding was quantified using a goat antiserum to α-synuclein. As shown in Figure 1-1A, α-synuclein does bind to immobilized agrin in a dose-dependent
As an alternative approach to assess agrin binding to α-synuclein, agrin was incubated with α-synuclein, and an anti-agrin MAb was employed to capture agrin and any associated α-synuclein (Figure 1-1B). These studies also demonstrated that α-synuclein binds agrin, because the captured agrin contained associated α-synuclein. When α-synuclein was incubated alone with the agrin antibody-coated wells, binding of α-synuclein to agrin MAb was not observed (data not shown).

**Agrin binding to α-synuclein alters α-synuclein solubility**

Previously we have shown that when agrin is incubated in solution with β-amyloid peptide, we can detect binding of agrin to β-amyloid, with a concomitant decrease in β-amyloid and agrin solubility (Cotman et al., 2000). We therefore reasoned that if agrin is also capable of modulating fibrillar formation of α-synuclein as a result of binding to α-synuclein, then it should also affect α-synuclein solubility. To address this question, agrin (50 μg/mL) was incubated with α-synuclein (1 mg/ml) without agitation for 7 days, followed by centrifugation to assess changes in agrin and α-synuclein solubility. These experiments demonstrated that although α-synuclein incubated alone did not form significant insoluble aggregates, the incubation of α-synuclein with agrin did result in significant insoluble aggregates. When the soluble (supernatant) and insoluble (pellet) fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting, we observed a pronounced decrease in solubility of α-synuclein when coincubated with agrin (Figure 1-2). We also observed a shift to higher molecular weight of the α-synuclein incubated with agrin, suggesting enhanced
aggregation of the α-synuclein, which is even resistant to dissociation under reducing conditions. Similar shifts in the molecular mass of α-synuclein have been observed previously with aggregation and fibrillar formation (Gosavi et al., 2002). When agrin was treated with nitrous acid or heparitinase, resulting in the degradation of HS chains, and then incubated with α-synuclein, we did not observe a decreased solubility of α-synuclein (data not shown). This indicates that agrin’s ability to regulate α-synuclein solubility is dependent on its HS chains. Interestingly, we also observed that agrin incubated with α-synuclein becomes insoluble as a result of binding to α-synuclein, and only a subset of agrin that is more highly glycosylated is associated with the insoluble fraction (Figure 1-2). Thus, these data suggest that a subset of agrin molecules, which are more heavily glycosylated, bind to α-synuclein and promote its aggregation.

**Agrin binding induced conformational changes in α-synuclein**

Numerous studies have provided evidence for the interaction of HSPGs with amyloid proteins, with HSPGs being capable of inducing conformational changes in proteins. This conformational change is characterized by augmented β-sheet structure that leads to the formation of amyloid fibrils. In neurodegenerative diseases, McLaurin et al. (1999) used CD and a marine sponge HSPG to show that HS-GAGs induce a conformational change in β-amyloid peptide, changing β-amyloid from a peptide with random structure to primarily β-sheet structure. I have documented using CD that agrin induces a conformational change with β-sheet structure in β-amyloid (Cole and Liu, 2006), indicating that agrin may be a physiologically relevant HSPG capable of modulating the structure of amyloid-associated proteins. To determine whether agrin
binding to α-synuclein may also lead to alteration in α-synuclein conformation, we analyzed α-synuclein structure by CD in the presence and absence of agrin. We employed α-synuclein samples incubated in the absence or presence of agrin without agitation, because agitation is known to accelerate the formation of amyloid fibrils (Cohlberg et al., 2002). We observed marked changes in α-synuclein conformation as the result of binding to agrin, which could be detected by CD immediately following the addition of agrin (Figure 1-3A). Agrin structure as elucidated by CD is primarily α-helical in conformation (data not shown), and agrin present at the molar concentration (40 nM) used for the analysis of α-synuclein conformation was shown to lack β-sheet structure (Figure 1-3A). CD spectra of α-synuclein in the presence of agrin exhibited a shift in wavelength that is characteristic of β-sheet structure in proteins (Figure 1-3B). With increasing incubation times in the absence of agitation, it could be observed that agrin continued to induce a conformational change in α-synuclein, with α-synuclein adopting β-sheet structure in the absence of agrin by 5 days of incubation (Figure 1-3D). Thus, it is apparent that agrin acts as a catalyst to accelerate conformational changes in α-synuclein.

**Immunohistochemical analysis of agrin distribution in PD brain**

The ability of agrin to bind α-synuclein and accelerate its fibrillar formation raises the crucial question of whether agrin is associated with α-synuclein in the pathological lesions of PD. This would suggest the interesting possibility that agrin might contribute to the pathophysiology of this neurodegenerative disease.

To address the question of whether agrin becomes associated with α-synuclein in
PD brain, I analyzed agrin and α-synuclein distribution in PD and age-matched control human brain tissue, using double labeling of tissue sections. The antisera I used in these experiments were a rabbit antiserum to recombinant human agrin that had been immunopurified using a human agrin affinity column and shown to react only with agrin in human brain (Cotman et al., 2000) and a goat antiserum to an α-synuclein synthetic peptide. When the substantia nigra of PD patients is analyzed using these polyclonal antisera to either agrin or α-synuclein, we observe localization of agrin to α-synuclein-positive Lewy bodies in dopaminergic neurons in the substantia nigra (Figure 1-4). Only a subset of neurons was found to be immunopositive for α-synuclein (Figure 1-4A and D), and these neurons were also stained using anti-agrin antiserum (Figure 1-4B and D). Staining with 4′-6-diamidino-2-phenyindole (DAPI) showed that only a small subset of neurons contain Lewy bodies immunoreactive for agrin and α-synuclein (Figure 1-4C). Importantly, the immunopositive neurons, when analyzed by phase contrast, were identified as pigmented neurons (Figure 1-4E), the target of PD lesions in the substantia nigra. Similar labeling of pigmented neurons, with either α-synuclein antiserum or agrin antiserum, was observed when sister sections were labeled individually, indicating that the detection of agrin immunoreactivity in α-synuclein-positive neurons was specific (data not shown). Structures with an appearance similar to Lewy neurites are also stained by both α-synuclein and agrin antisera (Figure 1-4A–D). Analysis of a second PD brain sample is shown in Figure 1-4H–J and again shows at higher magnification that α-synuclein and agrin colocalize in Lewy bodies in substantia nigral neurons. In age-matched control patients, we did not observe detectable levels of agrin in neurons and primarily observed agrin associated with the microvasculature (Cotman et al., 2000) (data
not shown). These data raise the question of how agrin becomes localized intracellularly in neuronal lesions, such as neurofibrillary tangles in AD and Lewy bodies and neurites in PD, and suggests possible alterations in agrin trafficking or endocytosis.

Modulation of α-synuclein cytotoxicity by agrin

The precise role of α-synuclein in the pathogenesis of PD and other neurodegenerative diseases characterized by α-synuclein aggregation is unknown, although α-synuclein is cytotoxic toward neurons. Overexpression of wild-type and mutant α-synuclein causes dopaminergic neuronal cell death in both primary and established neuronal cultures (Zhou et al., 2000; Zhou et al., 2002). Extracellular administration of α-synuclein induces neuronal cell death, with fibrillar α-synuclein exhibiting greater cytotoxicity (El-Agnaf et al., 1998a), although the physiological significance of this cytotoxicity may be questioned because α-synuclein is an intracellular protein. To ascertain whether agrin may modulate the ability of α-synuclein to induce cell death, as a result of modulating fibrillar formation α-synuclein, I examined the effect of agrin on α-synuclein-mediated cytotoxicity. In these experiments, α-synuclein was incubated for 0–6 days with or without agrin. α-Synuclein preparations were then added to the medium of human neuronal SH-SY5Y cell cultures, and after 48 h exposure to α-synuclein or α-synuclein-agrin fibrils, cytotoxicity was quantified by MTT assay. MTT is originally yellow and can be reduced to purple crystal of formazan in the mitochondria of living cells. In MTT assay, the amount of produced formazan is proportional to the number of cells incubated with MTT. I found that agrin had instant effects on α-synuclein cytotoxicity, augmenting α-synuclein cytotoxicity in the presence of freshly
added agrin while the toxicity declined along with the increased time period of incubation of the samples (Figure 1-5). In addition, both fresh α-synuclein preparations without agrin and fibrillar α-synuclein-agrin mixtures showed lower cytotoxicity (Figure 1-5). These data were consistent with the current suggestion that the protofibril α-synuclein is the cause of the cytotoxicity while the aggregated fibrils are cytoprotective (Tanaka et al., 2004; Volles and Lansbury, 2002; Volles et al., 2001).
Discussion

This study was undertaken to ascertain the role of the HSPG agrin in a second common neurodegenerative disease characterized by protein misfolding, PD. Recent studies have shown that agrin is associated with all lesion types found in AD (Cotman et al., 2000; Donahue et al., 1999; Verbeek et al., 1999), which include β-amyloid senile plaques, β-amyloid deposits in the microvasculature, and neurofibrillary tangles. The presence of agrin in β-amyloid deposits in the microvasculature has been suggested to lead to increased blood brain barrier permeability (Berzin et al., 2000), which may contribute to the etiology of AD. Agrin binds to β-amyloid and accelerates fibril formation by β-amyloid, suggesting a possible role for agrin in the formation of AD pathologies (Cotman et al., 2000). HSPGs, or at least heparin or HS-GAGs, may also contribute to the etiology of other protein conformational disorder diseases. HSPGs have been localized to the early stages of plaque formation in mouse and hamster models of scrapie prion disease (McBride et al., 1998; Snow et al., 1990), are associated with prion protein in human prion diseases (Snow et al., 1989; Snow et al., 1990), bind islet amyloid polypeptide and localize to amyloid plaques in type 2 diabetes (Castillo et al., 1998; Young et al., 1992), and are associated with inflammation-associated amyloid deposits (Snow et al., 1991; Snow et al., 1987). HSPGs accelerate the formation of amyloid plaques in these protein conformational diseases, and HSPG binding to amyloid-associated proteins leads to conformational changes in these proteins that ultimately result in amyloid fibril formation (Cole and Liu, 2006; McCubbin et al., 1988; McLaurin
et al., 1999). Sulfated macromolecules, such as heparin and HS, also bind to α-synuclein and accelerate α-synuclein fibril formation (Cohlberg et al., 2002). The heparin GAG chains become incorporated into the α-synuclein fibrils (Cohlberg et al., 2002), indicating the importance of heparin-like molecules in regulating fibrillar formation of α-synuclein. The presence of HSPGs has also been suggested in the amyloid lesions of PD, as FGF2 binding to Lewy bodies is eliminated by heparinase treatment (Perry et al., 1992).

Based on these experimental observations, I carried out studies to evaluate the role agrin may play in fibrillar formation of α-synuclein and the etiology of PD. Using a variety of experimental approaches, I demonstrate that agrin binds to α-synuclein. Of particular interest is our demonstration that incubation of α-synuclein with agrin leads to enhanced insolubility of α-synuclein as a result of fibril formation, with agrin becoming incorporated into the fibrils based on the ability to sediment agrin with α-synuclein fibrils. In addition, we observe that only a subset of agrin is associated with the α-synuclein fibrils when analyzed by SDS–PAGE and immunoblotting, with this agrin being higher in molecular weight. Although not confirmed in these studies, it is likely that this high molecular weight fraction of agrin contains either longer HSGAG chains or larger numbers of HSGAG chains. The agrin used in these studies was purified from embryonic chick vitreous bodies, and chicken agrin contains three consensus GAG attachment sites (Denzer et al., 1995; Tsen et al., 1995a). Our recent studies have shown that agrin contains two clusters of potential GAG attachment sites, with the one cluster containing multiple sites for the addition of HS (Winzen et al., 2003), indicating that subsets of glycosylated agrin are likely to exist in brain, with these subsets of agrin
possessing distinct functions that can include binding to α-synuclein. It is unknown whether agrin HS-GAG structure is altered in neurodegenerative diseases, such as AD or PD, or whether a more glycosylated form of agrin possesses higher affinity for proteins, such as α-synuclein. Previous analyses of HS-GAG structure in AD have been inconclusive. An analysis of brain HS-GAG structure indicated no change in HSGAGs in AD brain (Lindahl et al., 1995), although AD fibroblasts did exhibit changes in HSGAG structure (Zebrower et al., 1992). In addition, HS-GAG sulfation is altered in inflammation associated amyloidosis (Lindahl and Lindahl, 1997). Thus, it will be of interest to analyze agrin’s HSGAG structure in diseases, such as PD, to ascertain whether changes in agrin HSGAG structure may contribute to α-synuclein binding and the formation of amyloid fibrils.

Because I was able to demonstrate that agrin is capable of binding α-synuclein, I investigated whether agrin would modulate α-synuclein fibril formation, as has been shown for β-amyloid in AD (Cotman et al., 2000), and whether agrin would be capable of inducing conformational changes in α-synuclein, as has been shown for β-amyloid (Cole and Liu, 2006). These studies followed established protocols to quantify fibrillar formation of α-synuclein and to measure the kinetics of α-synuclein fibril formation (Cohlberg et al., 2002). My data show that agrin can induce rapid alterations in α-synuclein structure, with β-sheet structure becoming pronounced as a result of agrin binding. Agrin accelerates fibrillar formation of α-synuclein by decreasing the lag time and half-time of α-synuclein fibril formation, which is suggestive of agrin regulating the nucleation of α-synuclein fibrils (Liu et al., 2005). Fibrillar formation of α-synuclein is a
nucleation-dependent process (Uversky et al., 2001; Wood et al., 1999), with fibrillar formation in progressive steps. Thus, small punctate aggregates of α-synuclein are first observed (Gosavi et al., 2002), which are likely the cellular equivalent of protofibrils (Lee and Lee, 2002). The protofibrils are intermediates in the process of fibrillar formation of α-synuclein (Goldberg and Lansbury, 2000), with large fibrillar inclusions of α-synuclein subsequently forming in cells (Gosavi et al., 2002). EM analysis (Liu et al., 2005) of α-synuclein fibril formation shows that the presence of agrin accelerates the formation of small punctate aggregates of α-synuclein when analyzed at 4 h of incubation, as well as more readily discernible protofibrils when analyzed at 12–18 h of incubation. Formation of fibrillar aggregates was also enhanced in the presence of agrin when α-synuclein samples were analyzed for 65 h of incubation by EM. Thus, agrin is capable of playing a critical role in the regulation of fibrillar formation of α-synuclein, much as has been shown for β-amyloid peptide.

The experiments conducted here were extended to examine the distribution of agrin in PD brain, which is important in view of our demonstration that agrin can bind α-synuclein and regulate its fibrillar formation. Analysis of the substantia nigra of PD patients using confocal fluorescence microscopy indicates colocalization of agrin and α-synuclein in Lewy bodies, the pathological lesion of PD. Importantly, we observed the expression of agrin and α-synuclein in pigmented neurons of the substantia nigra, providing additional support for the conclusion that agrin is associated with Lewy body lesions in dopaminergic neurons of the substantia nigra. These results provide support for the previous suggestion that HSPGs are a component of Lewy bodies, because FGF
binding sites in Lewy bodies are heparinase sensitive (Perry et al., 1992). However, although previous studies suggested that heparinase-sensitive FGF2-binding sites were present in only a fraction of Lewy bodies in PD brain (Perry et al., 1992), our studies suggest that agrin may be associated with a majority of α-synuclein-positive Lewy bodies, at least in the four patient samples analyzed in our study. It should be noted, however, that we did not observe all Lewy bodies as being agrin positive, and we did analyze one PD patient with only sparse agrin staining (data not shown). It therefore remains possible that there is a variable expression of agrin in the lesions of PD, with some patients exhibiting a disease state that is characterized by only a subset of lesions containing agrin. Accordingly, although agrin is associated with all pathological lesions in AD (Cotman et al., 2000; Donahue et al., 1999; Verbeek et al., 1999), only a subset of lesions are agrin positive in hereditary cerebral hemorrhage with amyloidosis (Dutch type) (van Horssen et al., 2001).

It is also important to note that recent studies by van Horssen et al. (2004) analyzed the distribution of HSPGs in PD brain and demonstrated an absence of agrin immunoreactivity, as well as other HSPG core proteins, in Lewy bodies in PD brain. This led to the conclusion that HSPGs are not localized to Lewy bodies in PD and will not play a significant role in the pathophysiology of PD (van Horssen et al., 2004). However, the agrin antibody used in these studies was a MAb, compared with a polyclonal antiserum generated against human agrin used in our studies described here. In light of the demonstration that heparinase-sensitive FGF2-binding sites are present in Lewy bodies (Perry et al., 1992), the wealth of evidence that HSPGs are a shared
component of amyloid deposits in protein conformational disorders (Ancsin, 2003; Cole and Liu, 2006), and that our agrin antiserum specifically stains Lewy bodies in PD, it is clear that additional studies are warranted to further characterize the potential role of agrin, or other HSPGs, in a protein conformation disorder, such as PD.

I think it is also important to consider the physiological significance of HSPGs, such as agrin, being localized to intracellular lesions, such as Lewy bodies, because HSPGs are typically thought of as cell surface and extracellular matrix molecules. However, there is a wealth of evidence that demonstrates an intracellular localization of HSPGs, especially in disease states (Cotman et al., 2000; Donahue et al., 1999; Lundqvist and Schmidtchen, 2001; Odawara et al., 1998; Perry et al., 1992; Roskams et al., 1998; Su et al., 1992; Verbeek et al., 1999). The demonstration of the intracellular localization of HSPGs includes numerous neurodegenerative diseases, which include AD (Cotman et al., 2000; Donahue et al., 1999; Su et al., 1992; Verbeek et al., 1999) and PD (Perry et al., 1992). In particular, our laboratory and others have shown that HSPGs, such as agrin, are associated with neurofibrillary tangles in AD (Verbeek et al., 1999), and HSPGs associate with Pick bodies in Pick disease (Odawara et al., 1998). Furthermore, HSPGs colocalize intracellularly in neurons with β-amyloid cleavage enzyme 1 (Scholefield et al., 2003). It is therefore conceivable that in pathological conditions associated with a disease, such as PD, agrin becomes localized intracellularly, where it could function as a pathological chaperone to affect α-synuclein aggregation. It has recently been documented that proteins, such as torsinA, which are molecular chaperones that are not normally cytosolic, can contribute to α-synuclein aggregation (Shashidharan et al., 2000),
and thus it is tempting to speculate that an HSPG, such as agrin, could have a similar function when subjected to an abnormal subcellular localization. Alternative mechanisms by which agrin could modulate α-synuclein aggregation can be considered in light of recent evidence that α-synuclein is localized intracellularly in secretory vesicles and is secreted from neuronal cells in an endoplasmic reticulum-/Golgi-independent pathway (Lee et al., 2005). It is therefore tempting to speculate that agrin could become associated with α-synuclein in intracellular secretory vesicles, or once secreted from cells, α-synuclein could bind agrin to form a α-synuclein/agrin complex which subsequently being endocytosed in PD.

In view of evidence that overexpression of either wildtype or mutant α-synuclein in neurons leads to cell death (Zhou et al., 2000; Zhou et al., 2002) and that extracellular exposure of cells to fibrillar α-synuclein promotes cell death (El-Agnaf et al., 1998a), I examined the effect of agrin on α-synuclein mediated neuronal toxicity. I expected to observe a noticeable enhancement of α-synuclein cytotoxicity by agrin as a result of augmented formation of α-synuclein protofibrils and fibrils in the presence of agrin, because α-synuclein cytotoxicity is fibril-dependent, at least when α-synuclein is exposed to cells in the extracellular environment (El-Agnaf et al., 1998a), and oligomers of α-synuclein may mediate neuronal cytotoxicity (Volles et al., 2001). An important caveat to these studies, however, is that exposure of cells to α-synuclein in the extracellular environment may not be physiologically relevant, because α-synuclein is an intracellular protein. As shown with the CD data that agrin has an instant effect in promoting the fibrillar formation of α-synuclein, this effect also can be observed in cytotoxicity assays
in that the maximum cytotoxicity from the α-synuclein/agrin mixtures was from the non-incubated preparation, while the 1-day-old α-synuclein alone preparations exhibited the maximum cytotoxicity. However, since the accumulation of protofibrils of α-synuclein could be the cause of the cytotoxicity to dopaminergic neurons (Cappai et al., 2005; Conway et al., 2001), whether this promotion of fibrillar formation of α-synuclein by agrin serves cytotoxic effects or cytoprotective effects in Parkinson’s patients still requires further studies. Furthermore, it remains to be determined whether agrin’s binding to intracellular α-synuclein will modulate α-synuclein cytotoxicity.

It is of interest that both AD and PD are members of a family of neurodegenerative diseases referred to as protein conformational disorders (Carrell and Lomas, 1997; Soto, 2003), as these diseases are characterized by the formation of unstable protein intermediates that form amyloid fibrils, and that ultimately lead to neuronal cell death (Carrell and Lomas, 1997; Uversky and Fink, in press). Our data showing that agrin can regulate the formation of amyloid fibrils by both α-synuclein and β-amyloid raise the interesting possibility that agrin may function as a pathological chaperone capable of binding to the unstable protein intermediates in these diseases, driving the reaction toward the formation of aggregates, including protofibrils and amyloid fibrils. The demonstration that HSPGs are present in immature prion protein plaques in scrapie mice (McBride et al., 1998) and hamsters (Snow et al., 1990) and in Creutzfeld–Jakob disease (Snow et al., 1989; Snow et al., 1990) lends credence to this hypothesis, as prion diseases are also protein conformational disorders (Carrell and Lomas, 1997; Soto, 2003). However, it remains to be determined whether agrin is present in prion plaques and is
capable of binding prion protein.
Figure 1-1. Analysis of agrin binding to α-synuclein. A, Agrin binding to α-synuclein was assessed using an enzyme-linked immunosorbent assay (ELISA), as described in Materials and methods. Agrin monoclonal antibodies (MAbs) were adsorbed to an ELISA well, and chick vitreous body agrin (1 μg) was bound to the immobilized MAbs. Recombinant α-synuclein (10, 20, or 40 μg) was then added to the well, and binding of α-synuclein was quantified using a goat antiserum to α-synuclein. Background binding, determined using binding of α-synuclein to bovine serum albumin (BSA)/MAB-coated wells, is also shown. The mean ± SEM for three experiments is shown, with each experiment conducted using duplicate wells. *, Statistically significant compared with BSA, by Student’s t-test, p < 0.01. B, Agrin binding to α-synuclein was assessed by incubating agrin with α-synuclein, followed by absorption to ELISA plates coated with agrin MAb. It can be seen that with increasing amounts of agrin incubated with α-synuclein, that increasing amounts of α-synuclein are captured by the agrin MAb-coated wells.
Figure 1-2. Agrin binding to α-synuclein alters the solubility of α-synuclein. In these experiments, α-synuclein (1 mg/mL) was incubated with chick agrin (50 μg/mL) for 7 days at 37°C without agitation. Reaction mixtures were then centrifuged, and supernatants (soluble fraction) and pellets (insoluble fraction) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with antibodies to α-synuclein or agrin. These data show that α-synuclein incubated in the absence of agrin remains soluble with no formation of insoluble aggregates. Conversely, in the presence of agrin α-synuclein becomes mostly insoluble, exhibited by an increase in molecular weight that likely results from enhanced aggregation and hydrophobicity of the α-synuclein, with little α-synuclein present in the soluble fraction. Agrin also becomes insoluble, with a subset of higher molecular weight, more heavily glycosylated agrin binding to α-synuclein and becoming insoluble as part of an agrin–α-synuclein complex. Agrin incubated in the absence of α-synuclein remains soluble and is not detected as an insoluble pellet (30; data not shown).
Figure 1-3. Analysis of effect of agrin on α-synuclein conformation using circular dichroism (CD). A, α-Synuclein (10 μM) was incubated in the presence or absence of agrin (40 nM), as described in Materials and methods, and structure was compared with agrin alone (40 nM). B, Agrin added directly to α-synuclein induces a rapid conformational change in α-synuclein; C, agrin and α-synuclein incubated for 2 days; D, agrin and α-synuclein incubated for 5 days. In all cases, it can be seen that 40 nM agrin, which lacks β-sheet structure, induces a conformational change in α-synuclein. α-Synuclein incubated in the absence of agrin acquires β-sheet structure by 5 days.
Figure 1-4. Analysis of agrin and α-synuclein distribution in Parkinson's disease (PD) brain. Paraffin sections were incubated with antisera to agrin and α-synuclein, and antibody binding was visualized using Cy3-conjugated anti-rabbit antibody (agrin) or Cy2-conjugated anti-goat antibody (α-synuclein). In the absence of primary antisera, with incubation with secondary antiserum, only background fluorescence was observed (F), with no staining of neurons or Lewy bodies and neurites. A, α-Synuclein distribution in the substantia nigra of a PD patient. Arrows denote immunoreactive, pigmented neurons. Immunopositive staining of apparent Lewy neurites is also indicated (arrowhead). B, Agrin distribution in the same brain section. C, DAPI staining of the same section to identify neuronal nuclei, thereby showing that only a subset of neurons contain Lewy bodies. D, Merged view, showing colocalization of agrin and α-synuclein in Lewy bodies in pigmented neurons in the substantia nigra. E, Bright-field image showing that only pigmented neurons are immunopositive for agrin and α-synuclein. F, A section from a PD patient in the absence of primary antisera, showing background fluorescence. G, Higher magnification analysis of α-synuclein immunoreactivity in the substantia nigra of a PD patient. H, Agrin immunoreactivity in the same section. I, DAPI staining of the same section. J, Merged image of agrin and α-synuclein immunoreactivity. Calibration bars: A–F, 10 μm; G–J, 20 μm.
Figure 1-5. Analysis of α-synuclein neuronal cytotoxicity. After incubation for 0-6 days in the presence or absence of agrin, α-synuclein (10 μM) was added into cultured SH-SY5Y cells and assayed for cytotoxicity as described in Materials and methods. α-Synuclein showed an instant cytotoxic effect immediately after agrin was added, while the maximum cytotoxicity from α-Synuclein alone appeared from 1-day-old samples.
CHAPTER 2

AGRIN IS REQUIRED FOR AXONAL OUTGROWTH AND BRANCHING IN EMBRYONIC ZEBRAFISH
Abstract

Although recent studies have extended our understanding of agrin’s function during development, its function in the CNS is not clearly understood. To address this question, zebrafish agrin was identified and characterized. Zebrafish agrin is expressed in the developing CNS and in nonneural structures such as somites and notochord. Defects in the axon outgrowth by primary motor neurons, subpopulations of branchiomotor neurons, and Rohon–Beard sensory neurons were also observed, which included truncation of axons and increased branching of motor axons. Moreover, agrin morphants exhibit significantly inhibited tail development in a dose-dependent manner, as well as defects in the formation of the midbrain–hindbrain boundary and reduced size of eyes and otic vesicles. Together these results show that agrin plays an important role in both peripheral and CNS development in zebrafish.
Introduction

HSPGs are a diverse class of macromolecules with equally diverse functions, having documented roles as regulators of cell growth and differentiation (Bink et al., 2003; Itoh and Sokol, 1994), cell adhesion (Cole et al., 1986; Perkins et al., 1989; Reiland et al., 2004), tumorigenesis (Alexander et al., 2000), and wound healing (Andriessen et al., 1997). Inhibition of heparan sulfate synthesis by either antisense or gene disruption has also provided insight into HSPG function in the development (Galli et al., 2003), and the elimination of HSPGs during embryonic life is lethal (Costell et al., 2002).

Agrin is an HSPG that is localized to the neuromuscular junction as well as axon pathways of the CNS. Agrin is also abundantly expressed in the lung and brain basal laminae and in the kidney glomerular basement membrane (Groffen et al., 1999; Halfter et al., 1997; Yard et al., 2001). Although agrin’s role in neuromuscular synaptogenesis (Banks et al., 2003) is well established, its function in the brain has remained elusive. Agrin has been shown to promote axon and dendritic branching and elongation in hippocampal neurons in vitro (Mantych and Ferreira, 2001), but also has been suggested to inhibit neurite and axon outgrowth (Baerwald-De La Torre et al., 2004; Bixby et al., 2002). The ability of agrin to inhibit axon growth in vitro would seem to be inconsistent with its abundant expression in the developing axon pathways such as the optic nerve (Halfter et al., 1997). Moreover, our recent studies have shown that agrin modulates the activity of FGF2 to promote neurite outgrowth (Kim et al., 2003), suggesting that agrin
may play several roles in the establishment of axon pathways. Recently, clustering of transmembrane agrin has been shown to induce formation of filopodia-like processes in association with cytoskeletal changes in neuronal processes, suggesting that agrin may regulate cytoskeletal dynamics during neurite outgrowth (Annies et al., 2006). Overexpression of transmembrane agrin also increases filopodia on hippocampal neurites, and siRNA inhibition of agrin expression reduces filopodia on hippocampal neuritis (McCroskery et al., 2006). Interestingly, the N-terminal half of agrin, which contains the GAG chain attachment sites, was required for these effects. Thus, it appears that agrin may play critical roles in the mediation of axonal growth and pathfinding during the development, by modulating the formation and stabilization of filopodia on axons.

Zebrafish are a particularly attractive model system to begin to address agrin’s role in developmental processes such as axon guidance and outgrowth in vivo. The simplified CNS and PNS of zebrafish, morpholinos to knockdown gene expression, and the availability of many cell-type specific antibodies allow the contribution of specific genes to axon guidance, outgrowth, and synapse formation in identified neurons to be assessed. Here, I describe the distribution of agrin in the zebrafish embryo and establish an important role for agrin in motor and sensory axon growth and CNS morphogenesis.
Materials And Methods

Fish maintenance

Wild-type adult zebrafish (*AB or Ekkwill 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 using morphological criteria (Kimmel et al., 1995).

Molecular cloning

EST clone sequences (AI959096, AI477575, and AI477444) were used to obtain sequence near the C-terminal region. RT–PCR was performed to connect the gap between EST clones by using the following primers: zf-agrin-F (5’-ACCACCACCACCACAATGTCTC-3’) and zf-agrin-R (5’-TTTCACAGTGCCCTCAGTC-3’). The 1055-bp PCR product was cloned and transformed using a TOPO TA cloning kit (Invitrogen) for sequencing. The clones from 5’ RACE and RT–PCR (zf-agrin) were sequenced and analyzed as described at http://www.ncbi.nlm.nih.gov/BLAST.

Antisense morpholino injection

Antisense morpholino nucleotides (MOs) (Gene Tools) were designed against two exon–intron splice donor sites (Draper et al., 2001). The sequences of MOs used, which target two different intron–exon splicing sites, were zfAgrinLG1-MO (5’-AGAGTTGTACACCTACCAGAGAAAC-3’), zfAgrinLG2-MO (5’-CCTCTCCTTTACGCTGTGAAGACAA-3’), and control MO (5’-
CCTCTACACCTCAGTTACAATTTATA-3’). MOs were solubilized in water at a concentration of 2mM and diluted in injection buffer before injection into one- to four-cell stage embryos. Agrin MOs (1–9 ng) consistently produced reproducible phenotypes with an injection volume around 1–5 nL (Lewis and Eisen, 2003; Maroon et al., 2002) and robustly reduced agrin expression. The control MO at the same concentration and volume produced no detectable effects.

**Whole-mount in situ hybridization**

Zebrafish agrin cDNA clone sequences (zf-agrin) were used to make sense and antisense digoxigenin-labeled riboprobes of 1055 bp by *in vitro* transcription, using T7 polymerases, as described previously. Whole-mount in situ hybridization was performed as previously described, with probe hybridization at 55 °C (Thisse et al., 1993; Yuan et al., 1997).

**Immunohistochemistry**

For whole-mount immunohistochemistry, embryos were fixed with 4% paraformaldehyde/PBS. After washing with PBS, embryos were blocked with 10% goat serum or donkey serum in 0.5% saponin/PBS for 1 h. The embryos were incubated in the blocking solution plus MAb zn-1 (1:100 from Developmental Studies Hybridoma Bank, DSHB), MAb zn-12 (1:100 from DSHB) or MAb zn-5 (1:500 from DSHB) overnight at 4 °C. The anti-zn-1 antibody (Melancon et al., 1997) was used to detect the cell bodies and axons of primary motor neurons, zn-12 and zn-5 to detect neuron cell bodies and axons. Embryos were washed several times with PBS and incubated with secondary antibody (1:800 Cy3- or Cy5-conjugated antibody).
Results

Expression pattern of agrin mRNA during embryonic development in zebrafish

The expression of agrin mRNA was first analyzed by RT–PCR to identify the developmental stages where agrin transcripts are expressed. A semi-quantitative approach was used, with equivalent β-actin levels at each developmental age compared with agrin RT–PCR product. Agrin PCR products were detected at low levels at 2 hpf (Walshe and Mason, 2003)(Figure 2-1), indicating that maternal agrin transcripts are initially present early in zebrafish development. This low level of maternal agrin mRNA expression was detected up to 7 hpf, and by 24 hpf, a significant increase in agrin mRNA expression was apparent (Figure 2-1). Agrin mRNA was detected at these levels up to 72 hpf and maintained until 6 dpf. These data are in accord with other species where agrin is abundantly expressed during development and is down-regulated during later embryogenesis (Tsen et al., 1995a).

The pattern of agrin mRNA expression during early zebrafish embryogenesis was then analyzed. Agrin mRNA expression was readily detected in tailbud stage embryos (Figure 2-2A), and by the eight-somite stage, agrin mRNA was strongly expressed in the ventral region of the spinal cord and in notochord, with weak expression in the developing eye (Figure 2-2B). At 16 hpf, low levels of agrin mRNA expression were detected throughout the brain, including the midbrain–hindbrain boundary (MHB), and in somites (Figure 2-2C). At 22 hpf, strong agrin mRNA expression was detected in the ventral diencephalon, tectum, the MHB, eye, otic vesicle, spinal cord, and pronephric
duct (Figure 2-2D). Analysis of agrin mRNA expression at higher magnification is shown for 25-hpf embryos in Figure 2-2E–H and J. Dorsal views show pronounced agrin mRNA in the developing forebrain and strong expression in the MHB (Figure 2-2E). Weaker expression in hindbrain and rostral spinal cord was detected (Figure 2-2F), and agrin mRNA localization to ventral spinal cord consistent with the position of spinal cord motor neuron cell bodies was detected (Figure 2-2G). Agrin expression in ventral spinal cord was confirmed by analysis of cryostat sections of zebrafish embryos (Figure 2-2H). Agrin mRNA was also detected in the tail region of 25-hpf embryos (Figure 2-2I). Agrin expression in the developing CNS was maintained from 36 to 60 hpf (Figure 2-2J–L), although agrin mRNA expression in spinal cord was no longer detectable in 60-hpf embryos (Figure 2-2L). Agrin has also been shown to be abundantly expressed in the developing eye, in particular, in retinal ganglion cells (Halfter et al., 1997), and accordingly zebrafish agrin mRNA was expressed by retinal ganglion cells of 48-hpf embryos (Figure 2-2M). Collectively, these in situ hybridization studies indicate that zebrafish agrin is expressed in tissues in patterns similar to agrin expression in other species.

**Knockdown of agrin expression leads to morphological changes in zebrafish embryos that correlate with regions of agrin mRNA expression**

To study agrin function during development in zebrafish, antisense morpholino oligonucleotides (MOs) were used to decrease the overall expression of agrin protein. Since the N-terminus of agrin from chicken, rat, and mouse has two different alternatively spliced variants (Burgess et al., 2000; Campanelli et al., 1991; Denzer et al., 1997; Denzer et al., 1995; Neumann et al., 2001; Rupp et al., 1991; Tsim et al., 1992), it
remained possible that a complete elimination of agrin expression would not occur in zebrafish, using an MO to the agrin mRNA translation start site. In support of this suggestion, an agrin MO to the translation start site produced only mild phenotypes, although the phenotypes were representative of those obtained using splice site-targeted MOs (data not shown). Thus, two different agrin MOs were designed to target two exon–intron junctions in order to perturb mRNA splicing: ZfAgrinLG1-MO targets an exon–intron junction in the laminin G-like domain, and ZfAgrinLG2-MO targets a different splice site in this laminin G-like domain. Using RT–PCR, I showed that splicing was perturbed following injection of either MO into one-cell zebrafish embryos (Figure 2-3A).

With the injection of agrin MO, dramatic changes in the gross morphological appearance of agrin morphants were observed from 20 to 72 hpf. Agrin morphants injected with lower doses of MO (2–3 ng) showed a mild phenotype with regard to overall body development before 16 hpf, but at later stages, agrin morphants displayed defects such as shortened and curved tails, compacted and rounded somites (Figure 2-3B–J), impaired MHB development (Figure 2-3K–P), small eyes (Figure 2-3Q–S), and small otic vesicles (Figure 2-3T–V). Tail development was perturbed further with higher concentrations of agrin MOs (9 ng), with tails showing a spiral shape with severely shortening and curved tails, as well as compaction of somites (Figure 2-3J). These defects became more apparent as development proceeded.

Even though morphological aspects of anterior development were not significantly affected by agrin MO injection, agrin morphants did display anterior phenotypic changes.
Morphants exhibited impaired formation of the MHB (Figure 2-3K–P), with a defined MHB being absent in severe agrin morphants (Figure 2-3M and P). Analysis of marker genes for MHB development, such as Pax-2a, efna5a, and efna5b, showed diminished or absent expression of these mRNAs in agrin morphant MHB, providing additional evidence for a role for agrin in MHB development (Figure 2-4). In addition, expression of Pax-2a mRNA in 28-hpf agrin morphants is strongly suggestive of a critical role for agrin in other aspects of anterior–posterior (AP) CNS patterning besides MHB patterning, as Pax-2a gene expression was markedly reduced in the optic stalk, MHB, and otic vesicle of 28-hpf morphants (Figure 2-4C and D).

A dose-dependent reduction in eye size was also apparent in agrin morphants (Figure 2-3Q–S). Otic vesicles were likewise reduced in size in agrin morphants (Figure 2-3T–V). Other observed effects in agrin morphants included cardiac edema and diminished heart rate and blood flow (data not shown). Agrin morphants died at the early larval stage when treated with higher doses of MO, likely due to an inability to feed. However, all of the representative morphants shown in Figure 2-3C–J were viable at the time of analysis and displayed abnormal swimming patterns likely due to the severe morphological defects in the tail.

**Effect of agrin knockdown on primary motor axon outgrowth and branching**

Since agrin protein is abundantly expressed in the developing axon pathways (Halfter et al., 1997) and recent studies indicate that agrin may play a critical role in filopodia formation on growing axons (Annies et al., 2006; McCroskery et al., 2006), I examined axon outgrowth from primary motor neurons, using the znp-1 monoclonal
antibody (mAb), which stains caudal primary (CaP) motor axons and middle primary (MiP) motor axons (Melancon et al., 1997). Outgrowth of CaP motor axons was analyzed from 19 to 55 hpf, with CaP outgrowth appearing first from rostral motor neurons at 19 hpf and then extending in a rostral-to-caudal temporal gradient as development proceeded (Figure 2-5). At 19 hpf, control embryos showed stereotyped patterns of outgrowth from CaP motor axons, with more pronounced axonal outgrowth along the spinal cord observed in 25-hpf control embryos (Figure 2-5A and C). In contrast, agrin morphants displayed clear differences in the morphology of CaP axonal trajectories, with truncated axonal arbors being observed in 19-hpf morphants and heterogeneous axons with erratic trajectories being observed in 25-hpf morphants (Figure 2-5B and D). Similar patterns of CaP axonal outgrowth were observed at 31 and 48 hpf, with poorly organized axon fascicles observed in agrin morphants (Figure 2-5E–H). In older embryos at 55 hpf, the distribution of motor neuron axons in myotomes was severely disrupted (Figure 2-5I and J), with less branching of axons in muscle from agrin morphants and poorly defined axon projections along somite borders. These data imply that axon guidance of CaP motor axons is dependent on agrin function during zebrafish development and is important for presynaptic patterning in skeletal muscle.

**Agrin knockdown disrupts branchiomotor neuron axonal projections**

Agrin knockout mice do not display obvious morphological phenotypes in the CNS, an exception being slightly smaller brain size (Gautam et al., 1996). However, unlike agrin knockout mice, zebrafish agrin morphants showed impairments in MHB development, smaller otic vesicles, and smaller eyes. Since inhibition of agrin expression
produced defects in axon outgrowth and branching throughout the entire trunk region of the embryo, I decided to examine whether axon pathway defects were also apparent in anterior regions of agrin morphants. Using transgenic zebrafish embryos that express EGFP under control of the islet-1 promoter (Higashijima et al., 2000) and zn-5 antibody staining of axonal processes, the axonal projection of branchiomotor neuron axons was examined. Islet-1-EGFP expression in agrin morphants was first examined to assess whether agrin morphants display abnormalities in branchiomotor neuron differentiation or cell migration. In 22- and 30-hpf agrin morphants, the pattern of EGFP expression in branchiomotor neuron cell bodies in cranial nuclei was for the most part normal, with the most obvious difference in EGFP distribution being in trigeminal motor neurons, with a slight reduction in the size of the trigeminal nucleus (Figure 2-6A–D). Thus, differentiation and/or migration of trigeminal motor neurons appears to be modulated by agrin. EGFP expression also allows branchiomotor neuron axons to be visualized, and in agrin morphants, several types of branchiomotor neuron axons exhibited altered trajectories. In severe agrin morphants at 22 hpf, when viewed dorsally, an absence of branchiomotor axon growth was observed (Figure 2-6A and B). However, by 30 hpf, axons were observed to be extending from the trigeminal, facial, and glossopharyngeal nuclei (Figure 2-6C and D). When viewed laterally, the effects of agrin MOs on branchiomotor neuron axon growth were particularly evident. In mild agrin morphants, trigeminal and facial axon outgrowth appears fairly normal at 30 hpf (Figure 2-6E and F), but the facial sensory ganglion was markedly reduced in size and glossopharyngeal and vagal axons were not observed by EGFP expression (Figure 2-6E and F). In severe morphants at 30 hpf, diminished trigeminal and facial axon outgrowth was observed, in
addition to glossopharyngeal and vagal axon growth (Figure 2-6G and H). Interestingly, in these agrin morphants, growth of the posterior–lateral line axons was similar to that in control embryos, indicating growth of these axons was unaffected by agrin knockdown (Figures 2-7F and H and 2-8). Importantly, branchiomotor axon outgrowth was normal from 22 to 30 hpf, and the magnitude of disruption of axon outgrowth was dose-dependent (Figure 2-6A–H). Furthermore, after 48 hpf, fairly normal patterns of branchiomotor axon outgrowth were for the most part observed, although axons were shorter than in control MO-injected embryos (see Figure 2-6K and L for trigeminal axon growth at 48 hpf).

Projections of branchiomotor neuron axons were also examined using zn-5 immunostaining. Zn-5 is a general axon marker, although the antibody also stains some nonneuronal tissues. In 31- and 48-hpf agrin morphants, zn-5 staining identified several marked differences in morphant hindbrain development (Figure 2-6I–L). At 31 hpf, axonal outgrowth from the abducens nucleus was markedly inhibited in agrin morphants (Figure 2-6I and J), and endodermal pouches that form between the pharyngeal arches were abnormal in morphology in morphants (Figure 2-6I and J). At 48 hpf, axon outgrowth from the abducens nucleus was apparent in morphants, although the magnitude of outgrowth was severely truncated when compared with control embryos (Figure 2-6K and L). Trigeminal axon outgrowth was also evident with zn-5 staining in agrin morphants, although these axons were defasciculated when compared with control embryos (Figure 2-6K and L). Zn-5 staining of the tectal wall was also apparent in 48-hpf control embryos, with this staining absent in agrin morphants, likely due to agrin’s
role in MHB development. Interestingly, in 48-hpf morphants, the morphology of endodermal pouches appeared normal in morphants (Figure 2-6K and L).

**Agrin knockdown leads to impaired axon outgrowth by Rohon–Beard sensory neurons and lateral line axons**

Although our studies described earlier clearly demonstrate a role for agrin in motor neuron axon outgrowth, I was also interested in determining whether agrin function is critical to sensory neuron axon outgrowth and guidance. To determine the effect of agrin knockdown in other neuronal cell types, zn-12 mAb staining was employed to analyze sensory neuron development. Zn-12 mAb has been used to analyze axon growth by Rohon–Beard sensory neurons in zebrafish spinal cord (Trevarrow et al., 1990) and as a general neuronal marker. In the head region, zn-12 staining was similar between 19-hpf control embryos and agrin morphants, but augmented defasciculation of axons bundles was apparent in agrin morphants (Figure 2-7A and C). In the trunk region of 19-hpf morphants, the number of zn-12-positive axons projecting from the spinal cord was markedly reduced, and these axonal extensions exhibited defasciculation (Figure 2-7B and D). In accord with this observation, in 25-, 32-, and 48-hpf agrin morphant embryos, there was a reduction in the density of zn-12-positive Rohon–Beard axons (Figure 2-7E–N). This may result from decreased numbers of Rohon–Beard neurons in agrin morphants, based on HuC-GFP expression in agrin morphants (Figure 2-8H–J). Furthermore, in some agrin morphants, a truncation of Rohon–Beard axons was also observed (Figure 2-7E–H).

We also observed other differences in zn-12 immunostaining between control and
agrin morphant zebrafish. Lateral line axons were truncated in agrin morphants, with AP growth being reduced in 25-hpf morphants, when compared with control embryos (Figure 2-7E–H). This may be due to delayed development of this axonal projection, since lateral line axons remained zn-12-positive in 48-hpf agrin morphants, when the lateral line nerve was no longer zn-12-positive in control embryos (Figure 2-7J and N). Zn-12 immunostaining, which was detected in axons in 25-hpf control embryo skeletal muscle, was not detected in 25-hpf agrin morphants (Figure 2-7F and H), but was detected in 31-hpf agrin morphants (Figure 2-7L). Finally, as observed with zn-5 staining of branchiomotor neurons in Figure 7, zn-12 staining of branchiomotor neuron axons indicated a reduction and altered pattern of trigeminal axon outgrowth, as shown for 31-hpf morphants (Figure 2-7I and K).

**Neuronal differentiation may be impaired in agrin morphants**

Analysis of axonal growth, using zn-12 antibody, suggested that some of the effects of agrin on axonal growth may be due to delays in development, as evident with zn-12 staining of lateral-line axons and other axon populations in control and agrin morphant embryos. Hu protein expression has been used as a marker of neuronal differentiation (Marusich et al., 1994), and therefore, HuC-GFP transgenic zebrafish were employed to analyze agrin function during zebrafish nervous system development. Consistent with zn-12 immunostaining of control and morphant embryos, the phenotypes of HuC-GFP transgenic zebrafish suggest that agrin may play a role in neuronal differentiation during zebrafish nervous system development. In 20-hpf embryos, agrin morphants displayed a reduction in GFP-positive neuronal cell bodies in hindbrain, in association with neuron
clusters in the developing rhombomeres (Figure 2-8A and B). In 28-hpf embryos, the number of GFP-positive neurons was reduced in the hindbrain neuron clusters of agrin morphants (Figure 2-8C–G), although GFP expression in the trigeminal ganglion of HuC-GFP morphant embryos appeared normal (Figure 2-8C–G). In spinal cord of 28-hpf HuC-GFP embryos, agrin morphants also displayed significant reduction of GFP-positive neurons (Figure 2-8H–J), especially in embryos injected with higher amounts of agrin MO (Figure 2-8J). The loss of dorsal sensory neurons expressing GFP was particularly evident, suggesting loss of Rohon–Beard neurons. These data would explain the lower density of Rohon–Beard axons detected with zn-12 antibody in agrin morphants (Figure 2-7E–N).
Discussion

The studies reported here demonstrate that agrin plays essential roles during zebrafish development. Our studies revealed new functions for agrin \textit{in vivo}, such as roles in axon outgrowth, eye development, and MHB development. With regard to the role of agrin in axon outgrowth, conflicting data exist based on \textit{in vitro} paradigms (Bixby et al., 2002; Campagna et al., 1995; Chang et al., 1997; Halfter et al., 1997; Kim et al., 2003; Mantych and Ferreira, 2001). \textit{In vivo}, agrin’s role in axon outgrowth has not been analyzed in detail, except for an analysis of intramuscular axon branching, which was aberrant in agrin knockout mice (Gautam et al., 1996). Thus, agrin has been reported to inhibit axon outgrowth when combined with other adhesive proteins (Baerwald-De La Torre et al., 2004; Bixby et al., 2002) and to promote dendritic growth, but inhibits axon elongation in hippocampal neurons (Mantych and Ferreira, 2001). We have previously shown that agrin augments FGF2-mediated axonal outgrowth, using both PC12 cells and primary retinal neuronal cultures (Kim et al., 2003). In addition, recent \textit{in vitro} studies have shown that either agrin clustering in axonal membranes or overexpression of agrin promotes filopodia formation in axons, suggesting positive roles for agrin with regard to axon outgrowth. It is therefore apparent that conflicting roles for agrin have been obtained using \textit{in vitro} assays and that zebrafish offer the opportunity to assess agrin function \textit{in vivo}.

Zebrafish EST cDNAs and cDNAs isolated from zebrafish cDNA libraries or by 5’ RACE were used to establish the presence of a 10-kb zebrafish transcript that hybridizes
to our putative zebrafish cDNAs (Kim et al., 2007). This transcript is similar in size to agrin transcripts from other species (Burgess et al., 2000; Tsen et al., 1995a), is expressed in tissues consistent with agrin expression (Dong et al., 2002; Groffen et al., 1999; Yard et al., 2001), 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 (Kim et al., 2007). Thus, these data are consistent with these cDNAs, which exhibit high homology to chicken and rodent agrin, as coding for zebrafish agrin.

The knockdown effects from agrin morpholinos can be specifically attributed to the disruption of agrin mRNA splicing, as shown by two distinct agrin morpholinos that attack different mRNA splicing sites, and generate identical yet different severity phenotypes, while the same volume and concentration of control morpholino produces no apparent phenotypes in zebrafish embryos (Kim et al., 2007). Therefore, the phenotypes observed in agrin morphants are the result of diminished agrin mRNA. Rescue experiments are the best way to demonstrate one specific gene was knocked down. However, in vitro mRNA synthesis is difficult for agrin’s size. Ectopic expression of chicken agrin gene driven by an ubiquitous promoter was attempted, but caused more severe phenotypes (data not shown). Because of the diversed interaction partners, the expression of agrin may require fine regulation spatially and temporally. In addition, overexpression of agrin can also cause developmental abnormalities (Fuerst et al., 2006).

Analysis of the effect of agrin knockdown on axonal growth suggests that agrin may contribute distinct functions to different classes of neurons. For example, the most
notable effect of agrin knockdown on ventrally projecting primary motor neurons (CaP) in zebrafish appears to be related to axon outgrowth manifested by a modest decrease in axonal length, but significantly increased axonal branching and significant heterogeneity with regard to the extent of CaP axon outgrowth in agrin morphants. In agrin morphants, CaP axons showed increases in branching and impaired turning toward target muscle at somitic boundaries, and at late developmental stages, poorly defined axonal endings were present in somites and along somite borders. Interestingly, these motor neuron phenotypes resemble morphological and outgrowth alterations described for motor neurons as a result of perturbations in posterior and paraxial mesoderm formation (Lewis and Eisen, 2003). These motor neuron defects were attributed to impaired neuronal specification as a result of posterior and paraxial mesoderm defects (Lewis and Eisen, 2003). Interestingly, recent studies have reported that the “unplugged” phenotype in zebrafish is the result of targeting of a MuSK zebrafish gene, with defects including disruption of primary motoneuron axonal pathway choice (Zhang et al., 2004). Since this isoform of MuSK lacks the extracellular domains that are required for agrin responsiveness, these data provide support for the hypothesis that agrin’s effects on zebrafish development are mediated by MuSK-independent pathways.

I also analyzed the effects of perturbation of agrin expression on axonal outgrowth of other axon pathways, using mAbs that are general markers for growing axons (zn-12 and zn-5), as well as islet-1-EGFP and HuC-GFP transgenic zebrafish. These studies support a role for agrin in the establishment of distinct axon pathways. In islet-1-EGFP transgenic zebrafish, it was demonstrated that agrin is important in the initial outgrowth
of branchiomotor neuron axons, which was supported by zn-5 staining of agrin morphants. In HuC-GFP transgenic zebrafish injected with agrin MOs and in agrin morphants stained with zn-12 mAb, impaired axon outgrowth of specific axons, such as Rohon–Beard and lateral-line axons, was also observed. Our data suggest that some of the effects of agrin MOs on axon outgrowth may be due at least in part to impaired and/or delayed neuronal differentiation in agrin morphants, as evidenced by zn-12 expression in spinal cord neurons in agrin morphants. For example, the pattern and intensity of zn-12 staining in agrin morphants suggest that differences in axon outgrowth between control and morphant zebrafish may occur as a result of delayed development of sensory spinal cord neurons in agrin morphants. Our data also suggest that agrin’s effects on branchiomotor axon outgrowth could be due in part to delayed outgrowth of these axons, as in later stage, agrin morphants’ branchiomotor neuron axon outgrowth was present, although at diminished levels, and this outgrowth was in the stereotypical pattern expected for the respective branchiomotor axons. However, it is important to note that even in these axon populations that exhibited delayed outgrowth, the axon outgrowth pattern at later ages remained abnormal, with increased branching or other morphological differences observed.

Our observation that agrin regulates development of specific neuronal structures, such as the MHB, and axonal outgrowth from multiple neuronal cell types in zebrafish is also of interest though 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 in many developmental processes, which
include muscle development (Jenniskens et al., 2002; Thomas et al., 2003) 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 and Sokol, 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 (De Cat et al., 2003; Topczewski et al., 2001), 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, it remains possible that zebrafish will not exhibit a redundancy in HSPG expression as in mammalian species. Thus, one explanation for pronounced effects of agrin on several developmental processes is that agrin contributes more essential functions in zebrafish because of an absence of other HSPGs that can compensate for agrin function. Alternatively, it remains possible that a careful analysis of agrin knockout mice will establish similar functions for agrin in the formation of various axonal pathways, or CNS processes such as MHB and otic vesicle formation during mouse development. This may be especially noteworthy since agrin gene disruption in mice is lethal (Gautam et al., 1996), and defects in axon outgrowth and branching have not been carefully examined in agrin−/− mice. Thus, it will be 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.
The mechanisms underlying agrin modulation of CNS development in zebrafish are unknown. It is also unclear whether the disparate phenotypes observed are mechanistically linked or are independent. It is possible that these different agrin morphant phenotypes are indeed linked via a common signaling pathway, such as FGF signaling. FGFs have been shown to be crucial for telencephalic development, with disruption of \textit{fgf3} and \textit{fgf8} resulting in abnormalities in optic stalk development, telencephalic commissure formation, and forebrain patterning (Shinya et al., 2001; Walshe and Mason, 2003). The diencephalon–midbrain boundary (DMB) is also dependent on FGF function, with FGF8 helping to position the DMB (Scholpp et al., 2003). \textit{Fgf}s (\textit{fgf8}, \textit{fgf17}, and \textit{fgf18}) are also expressed in the MHB (MHB in zebrafish; isthmus in chicken and mouse), with these FGFs being essential to midbrain and hindbrain patterning (Draper et al., 2001; Draper et al., 2003; Irving and Mason, 2000; Liu et al., 1999; Liu et al., 2003; Reifers et al., 1998; Wiellette and Sive, 2004). Ectopic expression of these \textit{fgf}s can result in changing midbrain cell fate to cerebellar cell fate (Liu et al., 2003; Matsumoto et al., 2004), indicating the importance of controlled expression of \textit{fgf}s in the MHB for midbrain and hindbrain development. \textit{Fgf3} and \textit{fgf8} also play a crucial role in hindbrain patterning, as demonstrated by gene expression in the developing rhombomeres (Walshe and Mason, 2003; Wiellette and Sive, 2004), and in otic vesicle formation (Leger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2004). Thus, a majority of the CNS phenotypes observed in agrin morphants may arise due to disrupted FGF signaling. In support of this proposal, as shown in the present studies, known FGF-dependent genes exhibit perturbed expression in agrin morphants. Specifically, \textit{pax2a} gene expression was markedly reduced in the optic stalk, MHB, and
otic vesicles of agrin morphants, in a pattern similar to \textit{fgf} knockdown in zebrafish (Reifers et al., 1998; Walshe and Mason, 2003). In addition, in previous studies, we have shown that FGF2 mediated axon outgrowth is modulated by agrin (Kim et al., 2003). It is therefore tempting to speculate that agrin is an important regulator of FGF signaling during zebrafish CNS development.
Figure 2-1. RT–PCR analysis of agrin mRNA expression during zebrafish development. Equivalent amounts of cDNA were analyzed on the basis of b-actin amplification, and these data show that agrin mRNA is present as maternal mRNA in early embryos and then increases in abundance between 7 and 48 hpf.
Figure 2-2. Analysis of agrin mRNA localization by in situ hybridization. [(A)–(D)] Whole-mount in situ hybridization analysis of agrin mRNA expression. (A) Agrin mRNA is expressed in forebrain and notochord (arrow) in tail bud stage. (B) Eight-somite stage embryos. Agrin is expressed weakly in the eye (arrowhead) and strongly in notochord (arrow) in early development. (C) Agrin is expressed in MHB (*), the eye (arrowhead), and posterior mesenchyme (arrow) at 16 hpf. (D) At 22 hpf, agrin mRNA expression in CNS is stronger and is expressed in the MHB (*), the eye, hindbrain, ventral region of spinal cord, and pronephric duct. [(E)–(J)] Analysis of agrin mRNA expression in 25-hpf embryos at higher magnification. (E) Agrin mRNA is localized to forebrain and is expressed strongly in MHB. (F) Agrin mRNA expression in hindbrain and rostral spinal cord. (G) Agrin mRNA localization in spinal cord, dorsal view. (H) Agrin mRNA localization to ventral spinal cord, consistent with expression in spinal cord motor neurons, observed using cryostat sections of 36-hpf embryo. (I) Agrin mRNA localization in tail region of 25-hpf embryo. (J) Agrin mRNA expression in 36-hpf embryo. (K) Agrin mRNA localization in 48-hpf embryo. Arrow shows expression in spinal cord at this stage of development. (L) Agrin mRNA localization in brain at 60 hpf, with mRNA expression no longer being detected in the trunk region at this stage of development (arrow). (M) Agrin mRNA is localized to the retinal ganglion cells in the developing retina.
Figure 2-3. Agrin knockdown with antisense morpholinos leads to distinct phenotypes associated with nervous system and posterior development. (A) RT–PCR (26 cycles) analysis of agrin mRNA in wild-type, zfAgrinLG1-MO, and zfAgrinLG2 injected embryos. RT–PCR of b-actin was used as a control. [(B)–(J)] Distinct morphological changes occur in zebrafish embryos microinjected with agrin MO; (B) control 20-hpf embryo; (C) 20-hpf mild agrin morphant injected with 2–3 ng of agrin MO, showing type II mild phenotype characterized by shortened tail and smaller eyes; (D) 36-hpf control embryo; (E) 36-hpf morphant showing mild type II phenotype, especially shortened and curved tails, rounded and compacted somites, and smaller eyes; (F) 55-hpf control embryo; (G) 55-hpf agrin morphant showing mild type II phenotype; (H) control MO-injected 72-hpf embryo; (I) 72-hpf morphant displays a curved tail and aberrant somite borders (type II phenotype). (J) Lateral view of 5–9-ng agrin morpholino-injected 72-hpf embryo. Higher concentrations of agrin MO cause severe disruption of tail development, small eyes and otic vesicles, and cardiac edema (type III phenotype). [(K)–(P)] Analysis of 25-hpf MHB defects, with the arrow denoting the location of the MHB. (K) Lateral view of MHB in control embryo. (N) Dorsal view of MHB in control embryo. [(L) and (N)] Lateral and dorsal views of MHB in mild agrin morphants. [(M) and (P)] Lateral and dorsal views of MHB in severe agrin morphants. [(Q)–(S)] Eye development in control (Q), mild morphant (R), and severe morphant (S) embryos. [(T)–(V)] Otic vesicle development in control (T), mild morphant (U), and severe morphant (V) embryos.
Figure 2-4. MHB marker gene expression is reduced or absent in agrin morphants. Expression of Pax-2a [(A)–(D)], efna5a [(E) and (F)], and efna5b [(G) and (H)] mRNAs was analyzed by in situ hybridization in control [(A), (C), (E), (G)] and agrin morphant embryos [(B), (D), (F), (H)]. Pax-2a gene expression in 11-hpf embryos [(A) and (B)] indicated that MHB expression (arrow) of Pax-2a was markedly reduced in agrin morphants. In 28-hpf embryos [(C) and (D)], Pax-2a expression in agrin morphants was markedly reduced in the optic stalk (arrow), MHB (arrowhead), and otic vesicle (asterisk). [(E)–(H)] efna5a and efna5b gene expression was analyzed in 25-hpf embryos, and showed diminished mRNA expression of these MHB markers in the MHB of agrin morphants (arrow).
Figure 2-5. Effects of agrin MOs on primary motor neuron axonal outgrowth and branching. Axon outgrowth from CaP primary motor neurons was analyzed at 19–55 hpf, using zn3p-1 mAb to visualize CaP axons. [(A), (C), (E), (G), (I)] Control embryos. [(B), (D), (F), (H), (J)] Agrin morphants. [(A) and (B)] Primary motor neuron axon growth is partially disrupted in 19-hpf agrin morphants. [(C) and (D)] In 25-hpf embryos, agrin morphants display varying degrees of heterogeneous axon growth that includes partially truncated axons. [(E) and (F)] In 31-hpf embryos, CaP axon outgrowth in agrin morphants indicates defasciculation of axon bundles and absence of axon turning toward target muscle. [(G) and (H)] In 48-hpf embryos, CaP axons in agrin morphants continue to show impaired guidance toward muscle. [(I) and (J)] In 55-hpf embryos, CaP axons in control embryos exhibit defined pathways along somite boundaries and formation of axon endings in skeletal muscle (I). In agrin morphants, CaP axon growth along somite boundaries indicates disorganized somite boundaries, and a diminished number of axon endings are apparent in skeletal muscle (J).
Figure 2-6. Branchiomotor neuron axon projections are abnormal in agrin morphants. [(A)–(F)] Branchiomotor neuron axon outgrowth in islet-1-EGFP transgenic zebrafish; 22-hpf embryos showing branchiomotor nuclei in control (A) and agrin morphant (B) embryos. V, trigeminal nucleus; VII, facial nucleus; X, vagal nucleus; 30-hpf embryos showing branchiomotor nuclei and axon growth in control (C) and morphant (D) embryos. [(E)–(H)] Lateral view of branchiomotor neuron axon growth in 30-hpf control [(E) and (G)], mild agrin morphant (F), and severe morphant (H) embryos. Yellow arrow shows trigeminal axons; red arrow the facial axons, white arrow the glossopharyngeal axons, and blue arrow the vagal axons. The asterisks denote the facial sensory ganglion, and arrowheads the posterior lateral line axons. [(I)–(L)] Zn-5 immunostaining of zebrafish hindbrain; [(I) and (J)] 31-hpf control (I) and morphant (J) embryos showing impaired abducens axon growth (arrows), abnormal endodermal pouches (arrowheads), and tectal wall (asterisk) in morphants; [(K) and (L)] 48-hpf control (K) and morphant (L) embryos. At this stage, trigeminal axonal growth (arrowheads) appears normal in morphants, but axon bundles are defasciculated and exhibit branching. The tectal wall is zn5-negative in morphants (asterisk), and abducens axons are truncated (arrows). It can be seen that endodermal pouches in morphant embryos exhibit a normal morphology at this stage. V, trigeminal nucleus; Va, anterior trigeminal; Vp, posterior trigeminal; VII, facial nucleus; IX, glossopharyngeal nucleus; OV, otic vesicle.
Figure 2-7. Sensory neuronal development and axon outgrowth are partially disrupted in agrin morphants. Embryos were stained with zn-12 mAb to visualize sensory neurons. [(A), (B), (E), (F), (I), (J), (M)] Control embryos. [(C), (D), (G), (H), (K), (L), (N)] Agrin morphant embryos. [(A)–(D)] zn-12 staining in 19-hpf embryos. Agrin morphant axons display increased defasciculation in the head region, denoted by arrowheads (C), and truncated, defasciculated axons in the trunk region [(D), arrow]. [(E)–(H)] zn-12 staining of 25-hpf embryos. Rohon-Beard axons exhibit reduced growth in agrin morphants [(G) and (H), arrows], and lateral line axon terminals are located more anteriorly in agrin morphants [(G) and (H), arrowhead]. [(I)–(L)] zn-12 staining of 31-hpf embryos. Significantly reduced zn-12 staining of axons is apparent in morphant brain (K) when compared with control brain (I). In the trunk region of mild morphants, zn-12 staining of 31-hpf embryos (L) is remarkably similar to 25-hpf control embryos (F). [(M) and (N)] zn-12 staining of 48-hpf embryos. It can be seen that while a zn-12-positive lateral line nerve is absent in control embryos (M), the lateral line nerve remains zn-12-positive in morphants (N, arrowheads).
Figure 2-8. Agrin knockdown impairs neuronal development in HuC-GFP transgenic zebrafish. HuC-GFP transgenic zebrafish were injected with agrin LG2 MO and assessed for GFP expression in nervous tissue at 20 or 28 hpf. [(A), (C), (E), (H)] Control embryos. [(B), (D), (F), (G), (I), (J)] Agrin morphants. At 20 hpf, reduced GFP expression is observed in morphant hindbrain in early differentiating neuron clusters in the developing rhombomeres [(B), asterisks], compared with control embryos (A). In 28-hpf embryos, decreased number of GFP-expressing neurons are present in the neuron clusters in rhombomeres of morphant hindbrain [(D), asterisks]. [(E)–(G)] In lateral views, a reduced number of GFP-expressing neurons in the neuron clusters are observed in hindbrain rhombomeres of agrin morphants [(F) and (G), asterisks], with severe phenotype morphants exhibiting more marked reduction in GFP-positive neurons (G) when compared with control (E) or mild morphant (F) hindbrain. [(H)–(L)] In lateral views of spinal cord, mild phenotype morphants exhibit a reduction in density of GFP-positive neurons in spinal cord (I), with more marked decreases in GFP-positive neurons observed in severe phenotype morphants (J). Reduction in GFP-positive neurons is more pronounced along dorsal spinal cord, suggestive of loss of Rohon–Beard cells.
CHAPTER 3

AGRIN MODULATES RETINAL DEVELOPMENT IN ZEBRAFISH
Abstract

Recent studies from our laboratory have elucidated the role of agrin in zebrafish development. Among the phenotypes that result from agrin knockdown using morpholino antisense oligonucleotides is reduced eye size in agrin morphants. To begin to understand the mechanisms underlying agrin’s role in eye development, I have analyzed retinal development in agrin morphants. These studies show that retinal differentiation is impaired in agrin morphants, with retinal lamination being disrupted in a dose-dependent manner following agrin morpholino treatment. Pax 6.1 gene expression, a marker of eye development, is markedly reduced in agrin morphants, providing support for agrin’s role in retinal development. Formation of the optic fiber layer of the zebrafish retina is also impaired, exhibited as both reduced size of the optic fiber layer and severe disruption of retinal ganglion cell axon growth to the optic tectum. The retinotectal topographic projection to the optic tectum is also perturbed in agrin morphants. MHB formation, and expression of mRNAs in this organizer region, is disrupted in agrin morphants. Expression of Pax2a mRNA in the optic stalk and MHB is also disrupted in agrin morphants. Collectively, these phenotypes in agrin morphants provide support for a crucial role of agrin in retinal development and formation of an ordered retinotectal topographic map in the optic tectum of zebrafish.
Introduction

HSPGs are cell surface and extracellular matrix proteins that mediate a diverse range of crucial functions during vertebrate and invertebrate development. These functions include regulation of cell growth and differentiation (Bink et al., 2003; Itoh and Sokol, 1994) and modulation of axon outgrowth in the developing nervous system (Bovolenta and Fernaud-Espinoza, 2000; Chai and Morris, 1999; Walz et al., 1997). HSPGs are capable of modulating FGF-mediated axon growth in the retinotectal system (McFarlane et al., 1995; Walz et al., 1997), and axon guidance molecules such as netrins, slit, and semaphorins and/or their receptors interact with HSPGs (Ethell et al., 2001; Hu, 2001; Kantor et al., 2004; Liang et al., 1999). The importance of HSPGs in development has also been illustrated by the effects of mutations of HSPG core protein genes, or genes for heparan sulfate synthesis, on key developmental processes (Kirkpatrick et al., 2004; Lee et al., 2004; Norton et al., 2005). In addition, the elimination of HSPGs during embryonic life is lethal (Costell et al., 2002), and agrin knock-out mice die at birth (Serpinskaya et al., 1999).

Agrin is a HSPG (Denzer et al., 1995; Tsen et al., 1995a) that was initially discovered and characterized functionally based on its essential role in neuromuscular synaptogenesis (Banks et al., 2003; Nitkin et al., 1987). Agrin is expressed by spinal cord motor neurons and skeletal muscle, as well as basal laminae in lung, kidney, brain and the eye (Groffen et al., 1999; Halfter et al., 1997; Yard et al., 2001). Recent studies have begun to characterize CNS functions of agrin, which include regulation of axon
outgrowth and axon branching (Baerwald-De La Torre et al., 2004; Bixby et al., 2002; Mantych and Ferreira, 2001), and filopodia formation of growing axons (Annies et al., 2006; McCroskery et al., 2006). In zebrafish, our laboratory has shown that agrin contributes essential functions during development, as agrin knockdown leads to CNS phenotypes that include impaired MHB formation, decreased axon outgrowth and increased axon branching of motor neuron axons, impaired neuromuscular synaptogenesis, and perturbed posterior development (Kim et al., 2007).

One of the phenotypes observed in agrin morphant zebrafish is reduced eye size. Since earlier studies from our laboratory demonstrated that agrin modulates FGF2-mediated axon outgrowth from chicken retinal ganglion cell (RGC) axons (Kim et al., 2003), and FGF2-mediated formation of the *Xenopus* retinotectal pathway is also HSPG-dependent, this raised the possibility that one effect of agrin knockdown on eye development may relate to disrupted optic nerve formation. Likewise, retinal development is dependent on FGF function (Martinez-Morales et al., 2005; Picker and Brand, 2005), with FGFs requiring HSPG binding for activity (Ornitz, 2000). Thus, agrin may contribute to eye and retina development via modulation of FGF function. I therefore initiated studies to characterize the role of agrin in zebrafish retina development, focusing on retina differentiation and formation of the retinotectal projection. Our studies show that agrin is essential to retinal development, as agrin morphants exhibit impaired retinal differentiation, altered expression of genes that regulate eye development, and disruption of the retinotectal projection.
Materials and Methods

Fish maintenance

Wild-type adult zebrafish (*AB or Ekkwill background), HuC-GFP transgenic zebrafish (Park et al., 2000) and Brn3c:GAP43-GFP\textsubscript{p356t} transgenic Zebrafish (Xiao et al., 2005) 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 using morphological criteria (Kimmel et al., 1995).

Antisense Morpholino injection

Antisense morpholino nucleotides (MOs) (Gene Tools, Philomath, OR) were designed against two exon/intron splice sites (Draper et al., 2001). The sequences of MOs used, which target two different intron-exon splicing sites were: zfAgrinLG1-MO: 5’AGAGTTGTACACCTACCAGAGAAAC-3’, zfAgrinLG2-MO: 5’-CCTCTCCTTTACGCTGTGAAGACAA-3’, and Control-MO : 5’-CCTCTTACCTCAGTTACAATTATA-3’. MOs were solubilized in water at a concentration of 2mM and diluted to 1mM in injection buffer before injection into one- to four-cell stage embryos. Agrin MOs (1-5 pmole) consistently produced reproducible phenotypes with an injection volume around 1-5 nL (Lewis and Eisen, 2003; Maroon et al., 2002) and robustly reduced agrin expression. The control MO at the same concentration and volume produced no detectable effects.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described, with
probe hybridization at 55°C (Kim et al., 2007; Thisse et al., 1993). Digoxigenin-labeled riboprobes were transcribed from cDNAs encoding Pax 6.1, Pax2a, efnA5A, or efnA5b.

**Immunohistochemistry**

For whole-mount immunohistochemistry, embryos were fixed with 4% paraformaldehyde/PBS. After washing with PBS, embryos were blocked with donkey serum in PBS-0.2% Triton X-100 for 2 hours (Kim et al., 2007; Thisse et al., 1993). The embryos were incubated in the blocking solution plus MAb Zn-5 (1:500, from Developmental Studies Hybridoma Bank) or zpr-1 (1:400, from Zebrafish International Resource Center) overnight at 4°C or 2 hours at room temperature. Embryos were washed three times with PBS-0.2% Triton X-100 and incubated with secondary antibody (1:800 Cy3 conjugated antibody) in blocking solution.

**Bodipy FL-ceramide staining of zebrafish embryos**

Stock solution of BODIPY FL C5-ceramide complexed to BSA (Molecular Probes) was prepared according to the manual. Living embryos were dechorinated and soaked in 50-100μM BODIPY FL C5-ceramide in embryo medium with 10mM HEPES for more than 2 hours in the dark. The embryos were then washed with embryo medium three times for five minutes and confocal images were taken with a Zeiss LSM-510 confocal microscope.

**Analysis of apoptosis by acridine orange staining**

Living embryos were dechorinated and soaked in 5μg/ml acridine orange (Molecular Probes) in embryo medium for 2 minutes, followed by three washes with embryo medium. Fluorescent images were acquired using a Nikon Diaphot inverted
microscope and SPOT RT camera (Diagnostic Instruments) for analyzing apoptosis.

**DiI and DiO labeling of RGC axons**

Anterograde RGC axon labeling was performed as described (Picker et al., 1999). Briefly, Zebrafish larvae were fixed in 4% paraformaldehyde in PBS over night at 4 °C and briefly washed with PBS before being labeled by pressure injection with DiI or DiO (Molecular Probes). The labeled larvae were then transferred back into PBS and stored over 1 day at 4 °C before the confocal micrographs were taken.

**FGF beads implantation**

Heparin-coated acrylic beads (Sigma) were incubated in 250 μg/ml mouse FGF8b (Sigma) in PBS (Picker and Brand, 2005). The beads were washed 3 times for 10 minutes with PBS before being implanted. Five-somite stage (11.5-12.5hpf) larvae were dechorinated and transferred into 3% methylcellulose in 1x modified Barth’s solution (MBS) in a small petri dish while implantation was performed. After the implantation was done, embryos were incubated in 1x MBS at 28.5°C.
Results

Agrin mRNA is expressed in developing retina and agrin morphants exhibit impaired eye development phenotypes

Previous studies demonstrated that agrin is expressed in the developing zebrafish CNS, including retina (Kim et al., 2007). Injection of agrin morpholinos into one-cell embryos resulted in decreased eye size, suggesting a role for agrin in zebrafish eye development. I extended these studies to analyze in more detail agrin’s function during zebrafish eye development.

As shown in Figure 3-1 and Figure 2-2, agrin mRNA is expressed in the developing zebrafish eye as early as 8-somite stage (~13hpf), and can be visualized throughout the developing retina as early as 19 hours post-fertilization (hpf) (Figure 3-1 A-B). As retinal differentiation starts around 24hpf, the expression level was slightly decreased especially in the outer edge (Figure 3-1 C-D). This expression pattern grows more obvious at later stages (Figure 3-1 E-H) with agrin mRNA signal concentrated in the retinal ganglion cell (RGC) layer and sparsely distributed at the inner nuclear layer (INL) while missing in the outer nuclear layer (ONL).

Two distinct morpholino antisense oligonucleotides were used to disrupt agrin protein expression in zebrafish embryos as described in chapter 2 (Figure 2-3A). Knockdown of agrin protein in zebrafish embryos leads to a smaller eye phenotype (Figure 2-3 Q-S). Agrin knockdown also impairs development of the MHB (Figure 2-3 K-M), which is noteworthy since this organizer region may play an essential role in
regulating formation of the retinotectal topographic axon map (Picker et al., 1999).

Since the transcription factor Pax6 is a known regulator of eye development, with mutations in this gene leading to the small-eye phenotype (Glaser et al., 1992), I examined whether Pax6 gene expression is perturbed in agrin morphants. Zebrafish possess two Pax6 genes, Pax6.1 and Pax6.2, with Pax6.1 exhibiting a broader expression pattern characterized by expression in eye, diencephalon, and hindbrain (Krauss et al., 1991a; Krauss et al., 1991b), and Pax6.2 expression in eye and spinal cord (Nornes et al., 1998). In agrin morphants Pax6.1 expression was reduced in 54 hpf morphant eye, but was unaffected in diencephalon and hindbrain (Figure 3-2) nor in the eye of 27hpf morphants (data not shown). Pax6.2 expression exhibited a similar pattern in agrin morphants, being markedly reduced in morphant eyes (data not shown). Importantly, Figure 3-2 shows that Pax6.1 mRNA expression occurs in the retina and lens in the developing zebrafish eye (Figure 3-2C), and is primarily reduced in the retina of agrin morphants (Figure 3-2D).

**Apoptosis caused the microphthalmia phenotype in agrin morphants**

Since agrin knockdown led to an obvious reduction in eye size (Kim et al., 2007), it is of interest to ascertain whether it was caused by increased cell death or decreased cell proliferation in morphant embryos. To analyze cell proliferation, 27hpf embryos were soaked in BrdU labeling medium for 1 hour and mitotic nuclei were labeled by anti-BrdU antibody after fixation with 4% paraformaldehyde. Divided cells were counted in confocal micrographs, and the data were statistically analyzed using student’s *t*-test. The agrin morphants showed no statistically significant difference (*n*=7, *p*>0.05) compared to
uninjected embryos (n=9) (Figure 3-3). To assess apoptosis in control (n=11) and agrin morphant (n=10) embryos, I used the vital stain acridine orange which binds to condensed chromatin in dead cells. Statistical analysis using student’s \(t\)-test shows a significant increase (\(p<0.01\)) in the number of acridine orange stained cells in agrin morphants (Figure 3-4), suggesting that agrin knockdown augments apoptosis in the developing retina of zebrafish.

**Retinal differentiation is perturbed in agrin morphants**

Since the eyes of agrin morphants were reduced in size, in association with reduced expression of \(Pax6.1\) mRNA, I decided to ascertain whether normal retinal differentiation occurred in agrin morphants. To address this question, I first used the vital stain Bodipy FL C5-Ceramide to examine the cytoarchitecture of the developing retina. This vital stain inserts into the plasma membrane, allowing the cellular and axon layers of the retina to be observed. In control embryos a well-laminated retina was observed at 48 and 81 hpf (Figure 3-5 A,D). In contrast, in both mild and severe agrin morphants lamination of the retina was not observed (Figure 3-5 B,C,E,F). In addition, while fiber layers of the retina, including the optic fiber layer, were observable (Figure 3-5 B,C), a complete absence of lamination and differentiation of the retina was observed in severe agrin morphants (Figure 3-5 E,F).

In order to further assess the perturbation of differentiation in agrin morphants, several molecular markers that specify distinct compartments and stages of retina development were analyzed. For early differentiation, \(pax2a\) and \(atoh7\) RNA probes were used for ISH. \(Atoh7\) is a downstream gene of \(sonic hedgehog\) and is expressed in a
wave-like pattern that marks the initial differentiation of the first-born neurons in vertebrate retina (Kay et al., 2005; Neumann and Nuesslein-Volhard, 2000). In 25hpf zebrafish embryos (Figure 3-6A), atoh7 mRNA starts to be expressed in the ventral-nasal corner of the retina and expands throughout the whole retina in the next few hours. In agrin morphants (Figure 3-6B), not only the confined initial expression spot was missing, but morphants also showed a dispersed ubiquitous background expression, implying the disturbance of Sonic Hedgehog signaling. Pax2a is a characterized marker for the formation of CNS structures, such as the MHB and optic stalk, and its expression is regulated by FGF8 (Lun and Brand, 1998; Macdonald et al., 1997; Walshe and Mason, 2003). The MHB is the signaling center for zebrafish retinal patterning (Picker and Brand, 2005). As shown in Figure 3-6C, pax2a mRNA is expressed in the optic stalk and MHB in 25hpf zebrafish embryos, and the expression level in both regions is markedly reduced in agrin morphants (Figure 3-6C), in a manner similar to fgf8 knockdown (Walshe and Mason, 2003).

For later stages of retinal differentiation, I performed immunohistochemical staining with zn-5 and zpr-1 monoclonal antibodies and examined expression of HuC RNA binding protein. Zn-5 antibody transiently labels a subset of neurons, including RGC, during the outgrowth of their axons to reach their targets (Fashena and Westerfield, 1999). Zpr-1 antibody labels red/green cone cells and hence is a marker for photoreceptor cell differentiation. HuC is a differentiation marker for RGC and amacrine cells in the INL (Ekstrom and Johansson, 2003; Marusich et al., 1994), and HuC-GFP transgenic zebrafish that I used in this study express GFP under the control of the HuC
promoter (Park et al., 2000). Zn-5 immunostaining of 54hpf (Figure 3-7 A and B) and 70hpf (Figure 3-7 C and D) mild agrin morphants showed a pronounced reduction in the extent of axon labeling in both the retina and optic nerve of agrin morphants (Figure 3-7 B and D) when compared to uninjected embryos (Figure 3-7 A and B). In addition, we visualized an impairment of retinal development by virtue of a reduction in the size of the optic fiber layer in the retina of agrin morphants, both in terms of the thickness of the optic fiber layer and the peripheral expansion of this layer in agrin morphants (Figure 3-7 A-D). Photoreceptors are normally differentiated by 3 dpf in zebrafish embryos as zpr-1 immunostaining showed positive signal in the outer segment (Figure 3-7 E), while there is no zpr-1 staining observed in agrin morphants (Figure 3-7 F), indicating the impairment of photoreceptor differentiation. In 4dpf HuC-GFP agrin morphant embryos, I observed a disruption of normal retinal development and differentiation as shown by reduced HuC expression in the cell bodies and processes of retinal neurons (Figure 3-7 G-I).

**Formation of the retinotectal topographic map is disrupted in agrin morphants**

Previous studies have shown that HSPGs are required for the growth of retinal ganglion cell axons to the optic tectum in *Xenopus* (Walz et al., 1997), and for formation of the appropriate retinotectal topographic map in the optic tectum of zebrafish (Lee et al., 2004). Since our data not only demonstrate marked impairment of retinal differentiation in the absence of agrin expression but also show diminished optic nerve, this may provide one possible mechanism for the crucial role of HSPGs in the establishment of the retinotectal map. To assess whether the retinal ganglion cell axons
that were present in mild agrin morphants would project normally to the optic tectum, I assessed the formation of an appropriate retinotectal map in mild agrin morphants using DiI and DiO labeling of retinal ganglion cell axons. These studies were restricted to mild agrin morphants, since our DiI whole eye fill experiments showed no optic nerve outgrowth in many of the severe agrin morphants (data not shown). DiI and DiO were injected into specific quadrants of the zebrafish embryonic eye cup from 5 dpf embryos, and projection of retinal ganglion cell axons to the optic tectum was visualized by confocal microscopy. As shown in Figure 3-8, retinal ganglion cell axons from the dorsal-temporal or ventral-nasal quadrants of the retina project to specific tectal regions. Injection of DiO into dorsal-temporal eye cup results in the labeling of retinal ganglion cell axons that project to the lateral-posterior tectum (Figure 3-8A). Injection of DiI into the ventral-nasal eye cup results in labeling of axons that project to the medial-anterior tectum (Figure 3-8A). In agrin morphant embryos, three abnormal retinotectal sorting patterns were observed (Figure 3-8 B-D). Figure 3-8B shows the first abnormal pattern that axons projecting from ventral-nasal retina had projections that detour to medial side of the tectum to reach the posterior part of tectum, and the labeling pattern indicated partial delocalization of tectal termination field similar to the phenotype in fgf8 mutant Zebrafish (Picker et al., 1999). The second abnormal pattern is shown in Figure 3-8C and is characterized by significantly diminished numbers of axons that cross the chiasm, but failed to enter the tectum. The ipsilateral projection, as the third abnormal pattern, is characterized by few axons projected to the same side of the tectum is shown in Figure 3-8D.
Expression patterns of axon guidance cues are perturbed in agrin morphants

To begin to understand possible molecular mechanisms by which agrin may regulate retinal development in zebrafish, I examined the expression of mRNAs that have been proposed to contribute to development of the retinotectal system. Perturbed expression of pax2a in the MHB, as I have shown in Figure 3-6D, is of interest since the MHB may contribute an important role in midbrain polarization and formation of the retinotectal map (Picker et al., 1999). I therefore examined other molecular markers of the MHB that may play a role in midbrain polarization and retinotectal map formation. Two members of the ephrin ligand family, efna5a and efna5b, are expressed in the MHB and exhibit disrupted expression in the acerebellar fgf8 mutant zebrafish (Picker and Brand, 2005; Picker et al., 1999). Efna5a is expressed in a gradient from anterior optic tectum to the posterior tectal wall in the MHB (Figure 3-9A), and in agrin morphants exhibited a pronounced down-regulation (Figure 3-9B). Efna5b is also expressed in the MHB and in agrin morphants displayed a marked down-regulation in expression (Figure 3-9 C, D). Efna5b is also expressed in the nasal half of the developing retina, with this expression pattern perturbed in agrin morphants. Thus, multiple molecular markers of the MHB, as well as pax2a in the optic stalk (Figure 3-6 C and D), were perturbed in terms of gene expression in agrin morphants, providing insight into potential molecular mechanisms for agrin function.

Agrin morphant phenotypes may occur due to disrupted FGF signaling

The expression of pax2a and pax6.1 transcription factor genes, as well as the expression of ephrin ligand genes efna5a and efna5b, are regulated by FGF signaling
pathways, as perturbed $fgf$ expression leads to perturbation of expression of these genes (Picker et al., 1999; Walshe and Mason, 2003). The disruption of expression of these mRNAs in agrin morphants raises the possibility that agrin’s effects on retinal development may at least in part be due to perturbed FGF signaling. Since FGFs require heparin binding for activity (Kan et al., 1993; Yayon et al., 1991), and agrin is a HSPG (Denzer et al., 1995; Tsen et al., 1995a), these data are consistent with a mechanism whereby agrin regulation of FGF signaling contributes to retinal development. To test this possibility, I first carried out studies where FGF8-coated beads were implanted in the vicinity of the developing eye cup of 5-somite stage (11.5-12.5hpf) zebrafish embryos, in order to assess whether exogenous FGF8 could rescue the agrin morphant phenotype. To strengthen the rescue effect, I reduced the morpholino to a marginal dose of 0.5mM, compared to 1mM I normally used, while the volume injected remained the same. Analysis of retinal development of the bead implanted eye (5/5), when compared to the untreated embryo (4/7), shows that FGF8 overexpression is capable of partial rescue of the disrupted retinal development observed in agrin morphants (Figure 3-10).
Discussion

HSPGs are involved in various neural developmental activities (Hantaz-Ambroise et al., 1987; Moody et al., 1989; Nitkin et al., 1987; Yamaguchi, 2001). As a member of the HSPG family, agrin may play roles in CNS development that include synaptogenesis (Bose et al., 2000), axon guidance (Escher et al., 1996), and axon outgrowth (McCroskery et al., 2006). I have previously showed that knockdown of embryonic zebrafish agrin caused various neural and non-neural developmental defects, including axon outgrowth, posterior patterning, MHB formation and eye development (Kim et al., 2007). In addition to our previous report that described the augmentation of FGF2-mediated neurite outgrowth by agrin using PC12 cells and primary retinal neuronal cultures (Kim et al., 2003), other reports also demonstrated essential roles of HSPGs in modulating FGF signaling (Lin X et al, 1999; Rapraeger AC et al, 1991). In this chapter, I demonstrate additional phenotypes in zebrafish retinal development following agrin knockdown in zebrafish embryos, and propose a possible mechanism that involves the modulation of zebrafish retinal development by agrin through FGF signaling pathways.

We have previously reported that agrin is a maternal mRNA in zebrafish and can be detected in the forebrain before the formation of the optic vesicle (Kim et al., 2007), and similar reports that agrin exist in developing retina can also be found in rat, mouse and chicken (Biroc et al., 1993; Halfter et al., 1997). In this report, I documented agrin mRNA expression in earlier stages (19, 25hpf) before and during early retinal differentiation, then a confined expression pattern in the differentiated RGC layer and
inner part of undifferentiated retina at 36hpf, and a confined higher expression pattern in the RGC layer and inner plexiform layer (IPL) and a dispersed lower expression pattern in INL when the three-layer structure is formed and inter-layer connections are being established at later development stages. These observations are consistent with previous observations in other species and correlate with the suggested involvement of agrin in retinal synaptogenesis in the IPL/RGC (Halfter et al., 1997; Hering and Kroger, 1999; Ma et al., 1994; Mann and Kroger, 1996).

The knockdown effects from agrin morpholinos can be specifically attributed to the disruption of agrin mRNA splicing, as shown by two distinct agrin morpholinos that attack different mRNA splicing sites, and generate identical yet different severity phenotypes, while the same volume and concentration of control morpholino produces no apparent phenotypes in zebrafish embryos (Kim et al., 2007). Therefore, the phenotypes observed in agrin morphants are the result of diminished agrin mRNA. In support of this conclusion, a recent study in transgenic mice that overexpressed agrin also produced eye defects that included alterations in Pax6 expression (Fuerst et al., 2006). However, in the case of agrin overexpression, there was an expansion of the Pax6 expression domain that correlated with misdifferentiation of the optic stalk (Fuerst et al., 2006). This result correlates well with our data regarding Pax6 expression being regulated by agrin.

Agrin interacts through its core protein and the three attached GAG chains with many proteins including extracellular matrix proteins, such as laminin, membrane bound proteins, such as dystroglycan and integrin, and signaling molecules, such as FGF2 (Burgess et al., 2002; Cotman et al., 1999). Among the core protein bound agrin ligands,
several members are involved in visual development. Knockdown of dystroglycan in *Xenopus* caused microphthalmia, increased cell death and layers intermingling (Lunardi et al., 2006). Laminin alpha 1 mutation in zebrafish causes defects in RGC axonal projection and anterior segment of the eye, such as disorganized iridocorneal angle and corneal dysplasia (Semina et al., 2006). β1-Integrin stabilizes dendritic arbors in cultured developing chicken RGC (Marrs et al., 2006), which may implicate this protein in the process of synaptogenesis. Noticeably, the core protein of the transmembrane isoform of agrin promotes filopodial branching of axons in RGC and hippocampal neuron cultures (Annies et al., 2006; McCroskery et al., 2006).

Agrin is a major HSPG in the retinal inner limiting membrane (ILM), and the integrity of the ILM is essential for RGC survival and axon outgrowth (Dong et al., 2002; Halfter et al., 2001; Halfter et al., 1997; Halfter et al., 2005). Knockdown of agrin mRNA in developing embryos could compromise the integrity of the ILM and hence cause increased cell death and abnormalities in RGC axon outgrowth. Studies in agrin knockout mice did not reveal an obvious eye phenotype, suggesting redundancy in agrin’s role in mouse visual system development; however, the data I show in this chapter suggest that agrin’s roles, at least part of them, in zebrafish visual development are not compensated for by other HSPGs, indicating no redundancy for agrin function.

Many experimental data perturbing the synthesis and modification of HS-GAG chains have suggested that HSPGs play important roles in the retinotectal projection. Mice lacking different HS sulfotransferases (*Hs2st*, and *Hs6st1*) produce distinct retinal axon guidance phenotypes (Pratt et al., 2006). Mutant zebrafish that lack one of two
different HS synthesis enzymes (ext2, ext3) showed retinal axon mis-sorting and ipsilateral RGC axon projection in double mutant fish (Lee et al., 2004). I also observed this phenotype. Mice with mutation of the HS modification enzyme GlcNAc N-deacetylase/N-sulfotransferase 1 (Ndst1) have defects in their eyes, consistent with impaired Sonic Hedgehog and FGF signaling (Grobe et al., 2005; Pan et al., 2006). Interestingly, retinal development might be modulated by the concerted actions of FGF and Sonic Hedgehog signaling (Martinez-Morales et al., 2005; Neumann and Nuesslein-Volhard, 2000), and HSPGs may mediate morphogen signaling and interact with axon guidance cues through their HS-GAG chains (Fujise et al., 2003; Han et al., 2004; Kirkpatrick et al., 2004; Lee and Chien, 2004; Takei et al., 2004).

FGF8, as the signaling center for retinal development, triggers initial retinal differentiation and restores Sonic Hedgehog signaling in differentiated RGC in the differentiation front (Martinez-Morales et al., 2005). Sonic Hedgehog signaling originates from the midline and is triggered by FGF8 which regulates the expression of vax1/2 genes which then regulate pax2/6 expression which is necessary for regional differentiation of the optic stalk and optic cup (Kim and Lemke, 2006; Take-uchi et al., 2003). The gradient expression of fgf8 in retina and MHB patterns the expression of axon guidance molecules, and hence shapes the retinotectal topography (Picker and Brand, 2005; Picker et al., 1999). Since HSPGs could modulate the distribution of Sonic Hedgehog and binding affinity of FGF/FGFR, it is likely that knockdown of agrin affects at least one of these signaling pathways (Figure 3-11). My data support the hypothesis that agrin mediates the distribution of Sonic Hedgehog by showing diminished
expression level of the Sonic Hedgehog downstream gene atoh7 in developing zebrafish eye. Furthermore, my data strongly support the hypothesis that agrin mediates FGF8 signaling first by showing the duplication of FGF8 mutant phenotypes in the retinotectal map in agrin morphants, and secondly by the rescue of agrin morphants phenotypes by implantation of FGF8 soaked beads into zebrafish embryos.

In conclusion, the studies in this chapter extend our understanding of agrin function during development, by showing that agrin contributes crucial functions during eye development. My data support the hypothesis that agrin is a critical modulator of FGF signaling, and also suggest that agrin may modulate, either directly or indirectly, the function of other molecules, such as Sonic Hedgehog and Pax6, that are essential to eye development.
Figure 3-1. Agrin mRNA is expressed in the central nervous system during zebrafish embryonic development. A and B, Agrin mRNA expression in 19 hours post-fertilization (hpf) embryos. C and D, 25hpf embryos. Note that agrin mRNA is expressed in the optic vesicle (OV), MHB and optic tectum (ot). At 25hpf, when retinal differentiation is initiated, agrin mRNA is expressed in a higher level around the primordial lens, where the retina ganglion cells begin to differentiate, and the posterior tectum, where Fgf8 and guidance molecules of the ephrin family are expressed. E, F and G, 36hpf embryos. H, I and J, 48hpf embryos. Along with the differentiation wave in the retina, agrin mRNA expression in the retina is also expanded.
Figure 3-2. *Pax6.1* mRNA expression in retina is reduced in 54hpf agrin morphants. Pax 6.1 is a known regulator of retinal and eye development and was therefore examined in agrin morphants. At 25hpf (A and B), *pax6.1* is expressed in telencephalon (t), diencephalons (d), pretectum (pt), retina and lens (green arrow head). The expression pattern in agrin morphants (B) is the same as in uninjected control (A). At 50hpf (C and D), the uninjected (C) and agrin morphant (D) embryos show different Pax6.1 mRNA expression patterns in the mesencephalon (m), as well as markedly reduced Pax 6.1 mRNA expression in agrin morphants, while the expression pattern in telencephalon and diencephalons remains the same in the agrin morphants when compared to the uninjected control.
Figure 3-3. Agrin morphants show no significant difference in cell proliferation in developing eyes. 26hpf embryos were incubated with BrdU in DMSO for 2 hours, and proliferated cells were labeled with BrdU antibody. Proliferated cells were counted from the confocal micrographs and analyzed with student’s t-test. Agrin morphants (B, n=5; p>0.1) show no significant difference in proliferation in the developing eyes compared to uninjected embryos (A, n=6).
Figure 3-4. Agrin morphants show increased apoptosis in developing eyes. 28hpf embryos were stained with the vital stain acridine orange in order to visualize cells undergoing apoptosis. Agrin morphants (C and D, n=10; p<0.01) show significantly greater numbers of apoptotic cells in the developing eyes than uninjected embryos (A and B, n=11).
Figure 3-5. Agrin morphants fail to laminate the layer structure in the retina. Confocal micrographs were taken from the Bodipy FL5-ceramide stained embryos. A, B and C, 48hpf. D, E and F, 81hpf. Wild-type embryos (A and D) demonstrate complete layer structures in the retina, including retina ganglion cell (RGC) layer, inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL). In mild phenotype embryos (B and E), cell differentiate (arrows in E) but the formation of layer structure is not complete. Severe phenotype morphants show no differentiation and no layer structure was formed. Note that the all the micrographs are 200x, except for C (400x).
Figure 3-6. Atoh7 (A and B) and Pax2a (C and D) mRNA expression as early retinal differentiation markers are disrupted in 25hpf agrin morphants (B and D). A and B, atoh7 mRNA starts its expression in the ventral-nasal corner of the retina around 25hpf (A) and expands in a wave-like pattern in next few hours, whereas the confined expression pattern is missing in agrin morphants. C and D, Pax2a mRNA expression is markedly reduced in optic stalk, MHB and otic vesicle (not shown in the picture) in 25hpf agrin morphants (D), compared to uninjected zebrafish embryos (C).
Figure 3-7. Molecular markers demonstrate the disruption of retinal differentiation. A-D, confocal micrographs of zn-5 monoclonal antibody staining demonstrate thinner RGC layers (arrows) and optic nerves (arrowheads) in 54hpf (A and B) and 70hpf (C and D) mild phenotype agrin morphants (B and D), compared to uninjected embryos (A and C). Note that the distance from zn-5 positive cells to the edge of the retina is longer in agrin morphants, suggesting that the retinal ganglion cell differentiation wave in the retina is impaired (arrows). E and F, confocal micrographs of zpr-1 antibody labeling indicate no red/green cones were differentiated in 72hpf agrin morphants (F) compared to uninjected embryos (E). G and I, confocal micrographs taken from uninjected 4dpf HuC:GFP transgenic zebrafish (G) are characterized by green fluorescent signal in the RGC, and amacrine cells in INL, compared to thinner and pattern disrupted HuC:GFP positive areas in agrin morphants (H and I). Arrows in G-I also indicate further distance from the edge of the retina to RGC positive area in agrin morphants, as shown in A-D.
Figure 3-8. Disruption of formation of retinotectal topographic map in agrin morphants. Dil and DiO were injected into ventral-temporal and dorsal-nasal areas of the left eye, respectively, in 5 dpf uninjected embryos (A) and agrin morphants (B to D), in order to study the retinotectal topographic map in the optic tectum. Disruption of the topography of the axon projection can be observed in mild phenotype morphants, as shown by overlap between projections (arrows in B). In more severe phenotype agrin morphants, an ipsilateral projection (arrows in D) or failure of projection (arrow in C) is also observed.
Figure 3-9. EphrinA5a and ephrinA5b mRNA expression in posterior tectum is altered in agrin morphants. The MHB serves as an organizer for polarization of retinotectal projections to the optic tectum. The expression of MHB marker genes, ephrinA5a (A and B) and ephrinA5b (C and D), was therefore analyzed in agrin morphants. In situ hybridization with 25hpf embryos shows that ephrinA5a mRNA expression is markedly reduced in agrin morphants (B). The expression level of ephrinA5b is likewise reduced in the MHB of agrin morphants (D), and sporadic expression was also detected.
Figure 3-10. Fgf8 rescue of agrin morphant phenotype in developing retina. Under marginal doses (0.5mM) of agrin morpholino injection, some unimplanted 2dpf embryos failed to delaminate their retina into three layer structures (normal: 4/7), and the retinal layer structures in all agrin morphants are rescued by implantation of Fgf8b-soaked beads (normal: 5/5).
Figure 3-11. The schematic representation of the roles of agrin in zebrafish retinal development.

Two isoforms of agrin have been characterized in mouse, rat, chicken and human. Transmembrane agrin (tm-agrin) is mainly expressed by neurons, whereas secreted form of agrin (NtA-agrin) is mainly seen in the extracellular matrix, including inner limiting membrane (ILM) in the retina. HS-GAGs are involved in sonic hedgehog (Shh) distribution and a downstream gene of sonic hedgehog, atoh7, is diminished in agrin morphants suggesting that agrin is involved in sonic hedgehog signaling pathway in zebrafish retinal development. Rescue experiment demonstrated that agrin modulates retinal development, at least in part, via Fgf signaling pathway. In addition, many downstream genes of Fgf signaling pathway are involved in retinal development, and are affected by agrin knockdown. For example, pax2a is related to the formation of optic stalk and MHB, and ephrin ligands (efn) are involved in RGC projections. Zebrafish agrin may contribute to the retinal development via both sonic hedgehog and Fgf signaling pathways.
SUMMARY AND PERSPECTIVE

Agrin was discovered and characterized as an NMJ organizer more than 20 years ago. It is also highly expressed in the CNS, but its role in the CNS is just beginning clear. Experimental data, which are reviewed in the Introduction, suggest that agrin is associated with numerous CNS activities, including distribution and signal transduction of morphogens and growth factors during embryonic development, axon outgrowth and guidance, synaptogenesis, myelination, formation and maintenance of the blood-brain barrier, and neurodegenerative diseases.

In this dissertation, I demonstrate that agrin binds to α-synuclein, promotes the conformational change of α-synuclein that leads to amyloid formation, and colocalizes with α-synuclein in the lesions of PD brain. However, agrin in the CNS is primarily a transmembrane protein with a small intracellular domain, and therefore the mechanism by which agrin, or other HSPGs, might interact with α-synuclein becomes important for development of therapeutic approaches. In AD, an HS-GAG mimetic has been under clinical trial as a new drug to prevent the progress of AD by interacting with soluble β-amyloid to reduce fibrillar formation of β-amyloid (Aisen, 2005). Evidence shows that cell surface HSPGs can bind to extracellular molecules such as FGFs and ApoE and mediate the internalization of these molecules (Belting, 2003). α-Synuclein traffickes in secretory vesicles suggesting that secretion is part of the normal α-synuclein life cycle (Lee et al., 2005). Hence, putative HS-GAG mimetics that prevent either the soluble α-synuclein from fibrillar formation or the protofibrillar α-synuclein from translocating into
the cytosol may be applied as therapeutic approaches to PD, if agrin, or other HSPGs, interact with α-synuclein extracellularly and then subsequently are internalized into neurons. Alternatively, it is possible that agrin is internalized prior to interaction with α-synuclein, and therefore alternate strategies would need to be employed to prevent internalization of agrin.

Another interesting observation is that the maximum cytotoxicity of α-synuclein appears earlier in the presence of agrin. Experimental evidence suggests that protofibrillar α-synuclein mediates neuronal cytotoxicity, and that fibrillar amyloid α-synuclein is not the cytotoxic species (Volles and Lansbury, 2002; Volles et al., 2001). In addition, the protofibrils were enhanced and stabilized by dopamine quinines, the oxidation product of dopamine, suggesting the mechanism of dopaminergic-specific toxicity in substantia nigra from protofibrillar α-synuclein (Conway et al., 2001). Hence, it is possible that the effect of agrin promoting the fibrillar formation of α-synuclein serves as a cytoprotective role. In support of this hypothesis, a transgenic Drosophila study suggested that inclusion bodies resulted from α-synuclein aggregation may protect neurons from α-synuclein toxicity (Chen and Feany, 2005). Hence, putative HS-GAG mimetics that facilitate fibrillar formation of α-synuclein may also serve as a possible therapeutic strategy for PD.

To understand the role of agrin in CNS development, its expression pattern and knockdown effects by morpholinos are characterized in Chapter 2. The expression pattern and predicted protein sequence of zebrafish agrin are consistent with other species. Consistent with studies in other vertebrate species, agrin in zebrafish is also
crucial to NMJ formation. Interestingly, zebrafish allowed elucidation of other agrin functions during development. Confocal micrographs of immunostained and GFP-expressing transgenic fish demonstrated over-branching, shortened, defasciculated and heterogeneous axonal growth by multiple axon populations, signifying a role for agrin in axon outgrowth and guidance. Decreased numbers of GFP positive cells, including sensory Rohon-Beard cells, in HuC-GFP transgenic zebrafish indicate that agrin may be involved in regulating neuronal proliferation or apoptosis. Agrin knockdown perturbed the development of tail, MHB, otic vesicle and eye, and the detailed mechanisms in respective regions are of interests for further investigation. And the further investigation for the role of agrin in retinal development is reported in Chapter 3.

In agrin morphants, retinal lamination and differentiation is disrupted, which is similar to phenotypes in sonic hedgehog zebrafish mutants (Shkumatava et al., 2004). The retinotectal projections also are not patterned correctly, which is similar to the phenotypes in fgf8, ext2, and extl3 zebrafish mutants (Lee et al., 2004; Picker and Brand, 2005; Picker et al., 1999). These results support the suggestions that HS-GAGs are required for proper functions of Hedgehog and FGF signaling. Accordingly, the signaling markers for Sonic Hedgehog and FGF, such as atoh7, pax2a, and Sef (data not shown), are affected in agrin morphants. As a HSPG, agrin may affect various signaling pathways, and thus it is of interest to analyze gene expression profiles in agrin knockdown embryos. With tools like laser microdissection, individual tissue/regions can be dissected, mRNA extracted, and the gene profiling can be analyzed more accurately. This may provide further insight into specific molecular pathways by which agrin
regulates CNS development in zebrafish.

Recent studies revealed common mechanisms that regulate vascular and neuronal outgrowth and guidance (Carmeliet and Tessier-Lavigne, 2005). Zebrafish has been a convenient model to study neural development, and fli1:EGFP transgenic zebrafish, in which the blood vessels are marked with EGFP, makes zebrafish a great model to study angiogenesis (Lawson and Weinstein, 2002). Since agrin mediates axonal outgrowth and guidance in many regions during zebrafish development, it is of interest to investigate angiogenesis in agrin morphants. Furthermore, this study might be helpful to understand the role of agrin in blood-brain barrier formation and maintenance, especially since agrin levels are altered in blood-brain barrier in neurodegenerative diseases such as AD (Berzin et al., 2000; Donahue et al., 1999).

In conclusion, my studies provide evidence that agrin potentiates the aggregation of the PD pathological protein \( \alpha \)-synuclein, and hence provides a potential strategy for drug development for PD. Characterization of the agrin knockdown effect during zebrafish development provides an initial step for using zebrafish as a model system to study agrin function. My also support the hypothesis that agrin is involved in axon outgrowth and guidance and may be important for neuron cell survival. Further investigation in retina provides insight into the importance of agrin in Sonic Hedgehog and FGF signaling, indicating that agrin is an important co-factor for retina development.
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