Agrin is required for posterior development and motor axon outgrowth and branching in embryonic zebrafish

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Although recent studies have extended our understanding of agrin’s function during development, its function in the central nervous system (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. In agrin morphant embryos, acetylcholine receptor (AChR) cluster number and size on muscle fibers at the choice point were unaffected, whereas AChR clusters on muscle fibers in the dorsal and ventral regions of the myotome were reduced or absent. 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 and also modulates posterior development in zebrafish.

Key words: agrin/heparan sulfate proteoglycan/axon outgrowth/zebrafish/posterior development/Fgt/neuromuscular synaptogenesis

Introduction

Heparan sulfate proteoglycans (HSPGs) are a diverse class of macromolecules with equally diverse functions, having documented roles as regulators of cell growth and differentiation (Itoh and Sokol, 1994; Bink et al. 2003), cell adhesion (Cole et al. 1986; Perkins et al. 1989; Reiland et al. 2003), tumorigenesis (Alexander et al. 2000), and wound healing (Andriessen et al. 1997). Inhibition of heparan sulfate synthesis by either antisense 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 central nervous system (CNS). Agrin is also abundantly expressed in the lung and brain basal laminae and in the kidney glomerular basement membrane (Halfter et al. 1997; Groffen et al. 1999; 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 (Bixby et al. 2002; Baerwald-De La Torre et al. 2004). 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 Fgft2 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 (Annie et al. 2006). Overexpression of transmembrane agrin also increases filopodia on hippocampal neurites, and siRNA inhibition of agrin expression reduces filopodia on hippocampal neurites (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 peripheral nervous system 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, we describe the distribution of agrin in the zebrafish embryo and establish an important role for agrin in acetylcholine receptor (AChR) clustering, motor and sensory axon growth, CNS morphogenesis, and posterior development.
Results

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

The partial sequence of the zebrafish agrin open-reading frame was retrieved by 5'-RACE of zebrafish mRNA using a consensus degenerate primer and a gene-specific agrin primer. Although agrin mRNAs have been shown to contain two alternatively spliced exons encoding either a secreted or a transmembrane form of agrin (Burgess et al. 2000), only one transcript for the NtA domain of the N-terminus of agrin was isolated in our studies and was confirmed by sequencing. The cDNA sequence of the agrin NtA region is approximately 60% identical to the analogous region in chicken, human, rat, and electric ray agrin (data not shown). On the basis of the sequence information of agrin EST clones, sequences from the epidermal growth factor (EGF)-like and laminin G-like domains were cloned by reverse transcriptase–polymerase chain reaction (RT–PCR) and used as a probe for in situ hybridization and northern blot analysis. This agrin cDNA was also used to prepare a polyclonal antibody against zebrafish agrin, which was suitable for use in western blotting, but not in immunohistochemical studies.

A transcript of approximately 10 kb, which is similar in size to the agrin transcript from chicken (Tsen et al. 1995), was detected by northern blot analysis in zebrafish embryos at 24 and 48 hpf (Figure 1A). Western blot analysis of zebrafish embryos for the expression of agrin protein indicated the characteristic appearance of agrin as a 400–500-kDa smear on SDS–PAGE gels, with an agrin core protein of approximately 200 kDa detected following treatment of zebrafish protein extracts with nitric acid to remove HS-GAGs (Figure 1B).

The expression of agrin mRNA was first analyzed by RT–PCR to identify the developmental stages where agrin transcripts are expressed. A semiquantitative 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 (Figure 1C), 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 1C). 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 downregulated during later embryogenesis (Tsen et al. 1995; Halfter et al. 1997).

We next conducted an analysis of the pattern of agrin mRNA expression during early zebrafish embryogenesis. Agrin mRNA expression was readily detected in tailbud stage embryos (Figure 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 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 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 2D). Analysis of agrin mRNA expression at higher magnification is shown for 25-hpf embryos in Figure 2E–H and J. Dorsal views show pronounced agrin mRNA in the developing forebrain and strong expression in the MHB (Figure 2E). Weaker expression in hindbrain and rostral spinal cord was detected (Figure 2F), and agrin mRNA localization to ventral spinal cord consistent with the position of spinal cord motor neuron cell bodies was detected (Figure 2G). Agrin expression in ventral spinal cord was confirmed by analysis of cryostat sections of zebrafish embryos (Figure 2H). Agrin mRNA was also detected in the tail region of 25-hpf embryos (Figure 2I). Agrin expression in the developing CNS was maintained from 36 to 60 hpf (Figure 2J–L), although agrin mRNA expression in spinal cord was no longer detectable in 60-hpf embryos (Figure 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 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 been shown to have two different alternatively spliced variants (Campanelli et al. 1991; Rupp et al. 1991; Tsim et al. 1992; Denzer et al. 1995, 1997; Burgess et al. 2000; Neumann et al. 2001), 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, we showed that splicing was perturbed following injection of either MO into one-cell zebrafish embryos (Figure 3A).

We first analyzed the external phenotypes of zfAgrinLG1-MO and zfAgrinLG2-MO morphants. Both MOs resulted in similar phenotypic changes in zebrafish embryos, although zfAgrinLG1-MO produced more severe phenotypes on the basis of the proportion of morphants demonstrating severe, moderate, or mild morphological phenotypes (Table I). Interestingly, the regions of agrin mRNA expression correlate strongly with the gross morphological phenotypes observed in agrin morphants. To analyze the effects of agrin knockdown on zebrafish development, we first compared the effect of different concentrations of agrin MOs on zebrafish development. The number of morphants showing mild, moderate, and severe defects was quantified for each MO concentration injected (Table I). The proportion of severe defects increased and was dependent on increasing agrin MO concentration (Table I). To avoid toxic effects observed with high doses of MOs, 9 ng of zfAgrinLG1-MO was injected and compared with similar concentrations of control MO, which was a random sequence control MO obtained from Gene Tools Inc. (Philomath, OR). Injection volume ranged between 1 and 5 nL and was also controlled by comparing with control MO-injected embryos.

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 3B–J), impaired MHB development (Figure 3K–P), small eyes (Figure 3Q–S), and small otic vesicles (Figure 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 3I). These defects became more apparent as development proceeded.
Fig. 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 β-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.

Table I. Phenotypes of embryos injected with agrin MOs

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Normal (%)</th>
<th>I (%)</th>
<th>II (%)</th>
<th>III (%)</th>
<th>Size of the eye (μm)</th>
<th>Size of the otic vesicle (μm)</th>
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<tbody>
<tr>
<td>Uninjected</td>
<td>50</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td>27 ± 0.6</td>
<td>17.7 ± 0.4</td>
</tr>
<tr>
<td>Control MO</td>
<td>50</td>
<td>98</td>
<td>2</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>zfAgrinLG1-MO (2–3 ng)</td>
<td>500</td>
<td>1</td>
<td>18</td>
<td>31</td>
<td>50</td>
<td>21.1 ± 0.8*</td>
<td>13.3 ± 1.7**</td>
</tr>
<tr>
<td>zfAgrinLG1-MO (5–9 ng)</td>
<td>200</td>
<td>3</td>
<td>15</td>
<td>82</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>zfAgrinLG2-MO (3–5 ng)</td>
<td>111</td>
<td>13</td>
<td>13</td>
<td>63</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

Data shown as mean ± SEM; ND, not determined; I represents weak phenotypes: slightly curved tail, but embryos had reduced mobility after hatching stage; II represents mild phenotypes: partial disruption of MHB, short curved tail, smaller eyes and otic vesicles; III represents the most severe phenotypes: shortened and spiral tail, small eyes and otic vesicles, loss of MHB, reduced heart rate, and cardiac edema, and are immobile. Length of the eye was measured along the longest axis, and otic vesicle size was measured along the AP axis.

*Significantly different from uninjected embryos. P < 0.006 using ANOVA.
**Significantly different from uninjected embryos. P < 0.002 using ANOVA.
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 3K–P; Table I), with a defined MHB being absent in severe agrin morphants (Figure 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 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 4C and D).

A dose-dependent reduction in eye size was also apparent in agrin morphants (Figure 3Q–S; Table I). Otic vesicles were likewise reduced in size in agrin morphants (Figure 3T–V; Table I). 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 3C–J were viable at the time of analysis and displayed abnormal swimming patterns likely due to the severe morphological defects in the tail.

**Agrin knockdown embryos exhibit AP axis defects**

Since tail malformation was observed in agrin morphants, early AP pattern formation was examined to determine whether these defects occur during very early stages of development. At the four-somite stage, elongation of the AP axis occurs such that the head and tail become closer (Figure 5A). The AP axis became shorter in agrin morphants (Figure 5B and C), with the distances between head and tail being longer in high-dose agrin morphants (Figure 5C). At the 17-somite stage, the tail extends beyond the yolk tube in control embryos (Figure 5D), but in agrin morphants, the tail is shorter than control embryos (Figure 5E). The average length

![Fig. 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).](https://academic.oup.com/glycob/article-abstract/17/2/231/653057)

![Fig. 5. Defects of AP development occur during early development. [(A)–(C)] Four-somite-stage embryos. Arrowheads indicate the distance between head and tail. (A) Control embryos display a short distance between head and tail. (B) Low-dose agrin MO-injected embryos displayed shorter AP axis formation. (C) High-dose agrin MO-injected embryos show severe defects of AP axis elongation. [(D)–(E)] Seventeen-somite-stage embryos. (D) Tail of control embryos extends beyond the posterior limit of the yolk tube. (E) Tail of low-dose agrin MO-injected embryo is shorter than wild-type embryos. The caudal somites are smaller, but not fused. Arrows indicate the beginning of somite and end of tail.](https://academic.oup.com/glycob/article-abstract/17/2/231/653057)
between head and tail is shown in Table II. The width of caudal somites from agrin morphants was narrower than control embryos (Figure 5E). Thus, these experiments show that agrin-deficient embryos exhibit a shorter AP axis during early development. Importantly, since somite number was the same in these embryos at the same developmental age, these data suggest that while AP axis development is being impaired, other developmental processes such as somite development are progressing normally. This indicates that rather than a general slowing effect on development, agrin MOs result in specific effects on the formation of the AP axis in early development (Figure 5), as well as effects on AP pattern in the CNS, as shown by altered gene expression at later stages of development (Figure 4).

**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), we examined axon outgrowth from primary motor neurons, using the zn-5 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 6). 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 6A 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 6B 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 6E–H). In older embryos at 55 hpf, the distribution of motor neuron axons in myotomes was severely disrupted (Figure 6I 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.

**Table II.** The average distance between head and tail of early-stage zebrafish embryos

<table>
<thead>
<tr>
<th></th>
<th>Two-somite stage (μm)</th>
<th>Four-somite stage (μm)</th>
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<tbody>
<tr>
<td>Uninjected</td>
<td>32.5 ± 0.7</td>
<td>26 ± 2.3</td>
</tr>
<tr>
<td>Agrin-MO (1–3 ng)</td>
<td>33.5 ± 2.1</td>
<td>24 ± 4.8</td>
</tr>
<tr>
<td>Agrin-MO (5–9 ng)</td>
<td>43.5 ± 2.1*</td>
<td>38 ± 3.5**</td>
</tr>
</tbody>
</table>

For each treatment, 25 embryos were counted. Data are shown as mean ± SEM.

*Significantly different from uninjected embryos. \( P < 0.0004 \) using ANOVA.

**Significantly different from uninjected embryos. \( P < 0.01 \) using ANOVA.

Axonal outgrowth from dorsally projecting spinal cord motor neurons was also examined in agrin morphants, using islet1-GFP transgenic zebrafish. Dorsally projecting motor axons extend from the spinal cord and branch in the dorsal myotome (Figure 6K). In agrin morphants, a pronounced reduction in both the extent and pattern of axon outgrowth and branching in the myotome was observed (Figure 6L). The diameter of axon bundles of the dorsally projecting motor axons also appeared to be diminished in agrin morphants (Figure 6 K and L).

**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, we 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 7A–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 7A and B). However, by 30 hpf, axons were observed to be extending from the trigeminal, facial, and glossohypoglossal nuclei (Figure 7C 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 7E and F), but the facial sensory ganglion was markedly reduced in size and glossohypoglossal and vagal axons were not observed by EGFP expression (Figure 7E and F). In severe morphants at 30 hpf, diminished trigeminal and facial axon outgrowth was observed, in addition to glossohypoglossal and vagal axon growth (Figure 7G 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 7F and H and 8). Importantly, branchiomotor axon outgrowth was normal from 22 to 30 hpf, and the magnitude of disruption of axon outgrowth was dose-dependent (Figure 7A–H). Furthermore, after 48 hpf, fairly normal patterns of branchiomotor axon...
Fig. 6. 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 znp-1 mAb to visualize CaP axons. [(A), (C), (E), (G), (I), (K)] Control embryos. [(B), (D), (F), (H), (J), (L)] 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). [(K) and (L)] Outgrowth of dorsally projecting spinal cord motor neuron axons was also disrupted in agrin morphants.
outgrowth were for the most part observed, although axons were shorter than in control MO-injected embryos (see Figure 7K 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 7I–L). At 31 hpf, axonal outgrowth from the abducens nucleus was markedly inhibited in agrin morphants (Figure 7I and J), and endodermal pouches that form between the pharyngeal arches were abnormal in morphology in morphants (Figure 7I 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 7K 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 7K and L). Zn-5 staining of the tectal wall was also apparent in 48-hpf control embryos. V, trigeminal nucleus; Va, anterior trigeminal; Vp, posterior trigeminal; VII, facial nucleus; IX, glossopharyngeal nucleus; OV, otic vesicle.

Fig. 7. Branchiomotor neuron axon projections are abnormal in agrin morphants. 
[(A)–(D)] 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.
Agrin regulates axon growth and tail development in zebrafish

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 7K and L).

Agmin 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, we were 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 8A 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 8B 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 8E–N). This may result from decreased numbers of Rohon–Beard neurons in agrin morphants, based on HuC-GFP expression in agrin morphants (Figure 9H–J). Furthermore, in some agrin morphants, a truncation of Rohon–Beard axons was also observed (Figure 8E–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 8E–H). This may be due to delayed

Fig. 8. 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 (D). [(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).
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 8J 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 8F and H), but was detected in 31-hpf agrin morphants (Figure 8L). 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 8I 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 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]–[J]) In lateral views of spinal cord, mild phenotype morphants exhibit a reduction in density of GFP-positive neurons in spinal cord ([J]), 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.

Fig. 9. 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]–[J]) In lateral views of spinal cord, mild phenotype morphants exhibit a reduction in density of GFP-positive neurons in spinal cord ([J]), 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.
especially in embryos injected with higher amounts of agrin MO (Figure 9J). 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 8E–N).

**Agrin MO treatment leads to disruption of AchR clustering**

Agrin’s discovery was based on its crucial role in AchR aggregation during neuromuscular synaptogenesis (Nitkin et al. 1986). Its function in neuromuscular synaptogenesis has been confirmed by studies showing that gene disruption in mouse leads to failure to form functional synapses (Gautam et al. 1996). Thus, it was important to confirm that agrin knockdown disrupted AchR aggregation in zebrafish embryos. Analysis of early motor axon outgrowth in 24-hpf embryos, using antibodies to SV2, combined with α-bungarotoxin (BTX) staining to localize AchRs, showed that the pre-patterning of AchR clusters was not affected and that the dispersion of prepatterned AchR clusters was initiated normally (Figure 10). Moreover, the formation of AchR clusters and presynaptic terminals on muscle fibers at the choice point were unaffected. However, dorsal and ventral to the choice point, AchR clusters were reduced or absent (Figure 10). This indicates that post-prepattern AchR clustering is agrin-dependent, as has been shown in mice (Burgess et al. 1999; Liu et al. 2001). AchR clusters remained aberrant in 48-hpf agrin morphants, as visualized by α-BTX staining (Figure 11C and D).

**Muscle development appears normal in agrin knockdown embryos**

The most prominent external defects in agrin morphants were observed in tail development. To determine whether abnormal tail development and axon projections were due to defects in muscle development, myotome morphology was examined in 48-hpf agrin morphants and control MO-injected embryos. In agrin morphants, disruption of somite boundaries was observed, whereas in control morphants, a characteristic chevron-shaped somite with clear boundaries was observed (Figure 11A and B). We next investigated muscle development, using immunostaining with F59 and F310 mAbs in 48-hpf agrin and control MO-injected embryos. This analysis showed that the development of lateral line and muscle pioneer slow muscle cells was unaffected by injection of agrin MOs that produced mild agrin phenotypes (Figure 11F and H). In addition, fast muscle fibers were normally distributed in agrin morphant embryos with mild phenotypes (Figure 11E and G). However, in severe agrin morphant embryos, muscle morphology was abnormal (data not shown).

**Discussion**

The studies reported here demonstrate that agrin plays essential roles during zebrafish development, confirming observations in mouse, chicken, and electric ray with regard to formation of neuromuscular synapses (Nitkin et al. 1986; Gautam et al. 1996). Our studies also revealed new functions for agrin in vivo, such as roles in axon outgrowth, eye

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**Fig. 10.** Aberrant AchR clustering in agrin morphants. Motor axons and terminals were labeled with an antibody against SV2 (green), and postsynaptic AchR clusters were labeled with rhodamine α-BTX (red) in 24-hpf wild-type [(A)–(A')] and agrin morphant [(B)–(B')] embryos. In rostral segments [(A)–(A')] and (B)–(B'), primary motor axons have grown past the choice point and have begun to extend along the medial surface of the myotome. In wild-type embryos, AchR clusters are present along the entire axon and colocalize with presynaptic SV2 clusters [(A), arrow, (A')]. In agrin morphants, AchR clusters on muscle fibers at the choice point (asterisk) are unaffected and are apposed by SV2 clusters as in wild-type embryos. However, AchR clusters dorsal and ventral to the choice point are small and diffuse or are absent (arrow). In middle segments near the end of the yolk extension [(A')–(B')] primary motor axons have reached the choice point, with some clusters remaining visible throughout the dorso-ventral extent of the myotome. Scale bar: 10 μm.
development, and MHB development. With regard to the role of agrin in axon outgrowth, conflicting data exist based on in vitro paradigms (Campagna et al. 1995; Chang et al. 1997; Halfter et al. 1997; Mantych and Ferreira 2001; Bixby et al. 2002; Kim et al. 2003). 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 (Bixby et al. 2002; Baerwald-De La Torre et al. 2004) 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, 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 (Bixby et al. 2002; Baerwald-De La Torre et al. 2004) 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 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 in vitro assays and that zebrafish offer the opportunity to assess agrin function 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. This transcript is similar in size to agrin transcripts from other species (Tseng et al. 1995; Burgess et al. 2000), is expressed in tissues consistent with agrin expression (Groffen et al. 1999; Yard et al. 2001; Dong et al. 2002), and when used to generate an antiserum to a putative agrin fusion protein, this antiserum recognizes a 400–500-kDa HSPG that has a 200-kDa core protein, following the removal of HS-GAGs. Thus, these data are consistent with these cDNAs, which exhibit high homology to chicken and rodent agrin, as coding for zebrafish agrin.

Our analysis of the effect of agrin MOs on zebrafish development is consistent with the identification of zebrafish agrin, as the MOs disrupt AChR clustering in muscle from 24 to 48 hpf. This phenotype is similar to that observed with agrin knockdown in mice (Gautam et al. 1996), providing support that agrin was being specifically targeted by agrin MO. Our studies also show that agrin does not play a role in the initial clustering of AchRs, which occurs prior to motor innervation (prepatterned AChRs; Panzer et al. 2005, 2006), as these receptor clusters are unaffected by agrin MO treatment. This is in agreement with previous studies in mice (Lin et al. 2001; Yang et al. 2000, 2001). Although AChR clustering at the choice point is agrin-independent, as is the rest of the AChR prepattern, agrin is required for AChR clustering at later times in development, as axons extend beyond the dorsal–ventral edges of the myotome and branch into lateral muscle layers. At these later times, agrin may be important for stabilizing AChR clusters that would otherwise disperse. This possibility will be addressed in future studies.

Our analysis of the effects of agrin knockdown on zebrafish development yielded a pronounced morphological

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**Fig. 11.** Muscle development appears normal in agrin morphant embryos. [(A) and (B)] Nomarski lateral view of mid-trunk muscle from 48-hpf embryos injected with control-MO (2–3 ng) [(A) \( n = 20 \)] or agrin-MO (2–3 ng) [(B) \( n = 20 \)] is shown. Agrin morphants show abnormal somite boundaries compared with control-MO-injected embryos as indicated by tracing of a somite boundary in each treatment group. Lateral view of \( \alpha \)-BTX-stained embryos (48 hpf) injected with control-MO [(C) \( n = 20 \)] and agrin-MO [(D) \( n = 20 \)]. Agrin morphants show a decrease in AChR clusters in 48-hpf embryos. Arrowhead denotes somite boundary. Arrows show aggregates of AChRs that are abnormal in agrin morphants [(D) compared with control embryos (C)]. [(E) and (G)] Cross section of 26-hpf F310 mAb (fast muscle)-stained embryos injected with control-MO [(E) \( n = 20 \)] or agrin-MO [(G) \( n = 20 \)]. Fast muscle development appears normal in agrin knockdown embryos. [(F) and (H)] Cross section of 26-hpf F59 mAb (slow muscle and muscle pioneers)-stained embryos injected with control-MO [(F) \( n = 20 \)] or agrin-MO [(H) \( n = 20 \)]. Slow muscle and muscle pioneers largely appear normal in agrin morphants.
change in zebrafish embryos, with increasing doses of agrin morpholinos leading to perturbation of anterior as well as posterior development. The most distinct morphological defects are revealed in tail formation. Interestingly, recent studies have shown that morpholino knockdown of heparan sulfate 6-O-sulfotransferase in zebrafish results in curved tails and impaired tail development (Bink et al. 2003). Glypican-3 knockdown or loss of function in zebrafish also impacts tail development, with shorter tails being observed in these zebrafish (Topczewski et al. 2001). Recent studies on the Ext1 and Ext2 genes, which encode heparan sulfate synthetic enzymes, also demonstrate that these enzymes are abundantly expressed in the tail region, as well as in the CNS (Siekmann and Brand 2005). These data collectively provide support for our data demonstrating a role for HSPGs in tail development.

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 2004). These motor neuron defects were attributed to impaired neuronal specification as a result of posterior and paraxial mesoderm defects (Lewis and Eisen 2004).

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.

We 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 (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 and posterior 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. A wealth of data in the developing mouse, chicken, and zebrafish CNS implicate Fgfs as crucial for the patterning of the AP axis during CNS development. Fgfs have been shown to
be crucial for telencephalic development, with disruption of Fgf3 and Fgf8 function resulting in abnormalities in optic stalk development, telencephalic commissure formation, and forebrain patterning (Shinya et al. 2001; Walsh and Mason 2003). The diencephalon–midbrain boundary (DMB) is also dependent on Fgf function, with Fgf8 helping to position the DMB (Schlopp et al. 2003). Fgfs (Fgf8, Fgf17, and 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 (Reifers et al. 1998; Liu et al. 1999, Liu, Li et al. 2003; Irving and Mason 2000; Draper et al. 2001, 2003; Wiellette and Sive 2004). Ectopic expression of these Fgfs can result in changing midbrain cell fate to cerebellar cell fate (Liu, Chu et al. 2003; Matsumoto et al. 2004), indicating the importance of controlled expression of Fgfs in the MHB for midbrain and hindbrain development. Fgf3 and Fgf8 also play a crucial role in hindbrain patterning, as demonstrated by gene expression in the developing rhombomeres (Walsh 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 mutants 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 mutants. Specifically, Pax-2a gene expression was markedly reduced in the optic stalk, MHB, and otic vesicles of agrin mutants, in a pattern similar to Fgf knockdown in zebrafish (Reifers et al. 1998; Walsh and Mason 2003). In addition, in previous studies, we have shown that Fgf-2 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.

Fgfs also play a critical role in posterior development, particularly posterior mesoderm formation. The “no tail” (ntl) and “spade tail” (spt) zebrafish mutants are hypersensitive to disruption of Fgf signaling, indicating the importance of Fgf signaling in posterior development (Draper et al. 2003). Fgf8 mutant zebrafish exhibit normal posterior/tail development (Reifers et al. 1998), but further knockdown of Fgf8 in these mutant zebrafish results in severe perturbation of tail development and reduced posterior mesoderm (Draper et al. 2001). Further disruption of tail development occurs when additional Fgfs are knocked down, such as Fgf24 and Fgf3. The combined Fgf knockdown phenotype is similar (although not as severe) to a phenotype observed when dominant-negative Fgf receptors (FGFRs) are expressed in zebrafish (Griffin et al. 1995), when FGFR function is inhibited using pharmacologic methods (Draper et al. 2003), and when agrin protein expression is blocked using high doses of agrin MOs. Thus, it is evident that Fgf signaling is required to modulate posterior development in zebrafish, and agrin is a candidate HSPG that may be essential to Fgf signaling in these processes.

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

To obtain the 5′ UTR sequence of the agrin gene, 5′ RACE was performed using a degenerative primer 5′-GGAACGAGTG TGAAGCTG-3′ and a gene-specific primer 5′-CTGGAGTAGA G TGGGTCCCGGGTTTGT-3′. Expressed sequence tag (EST) clone sequences (AI959096, AI477575, and AI477444) were used to obtain sequence near the C-terminal region. Reverse transcriptase-polymerase chain reaction (RT–PCR) was performed to connect the gap between EST clones by using the following primers: zf-agrin-F (5′-ACCACCACCACCAAA TGTCTC-3′) and zf agrin-R (5′-TTTCAGTGCTACTTA CGTC-3′). The 1055-bp PCR product was cloned and transformed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) 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 morpholinol injection

Antisense morpholino nucleotides (MOs) (Gene Tools, Philomath, OR) were designed against 5′ sequence near the start site of translation (Nasevicius and Ekker 2000) and 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′-AGAGTTTGA CACCTACCAGAAAC-3′), zfAgrinLG2-MO (5′-CCTCT CTTTTAGCTGTGAAGCA-3′), and control MO (5′-CCTCTTACCTGTTAATTATAA-3′). MOs were solubilized in water at a concentration of 16 μg/mL and diluted in water 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 (Maroon et al. 2002; Lewis and Eisen 2003) and robustly reduced agrin expression. The control MO at the same concentration and volume produced no detectable effects.

Northern blot analysis

Total RNA was extracted from various stage embryos, using Trizol (Invitrogen), 10 μg of RNA was separated on a 1% agarose gel, and probed using digoxigenin-labeled antisense riboprobe. Northern blots were performed according to the manufacturer’s procedures (digoxigenin high prime DNA labeling and detection kit II, Roche, Indianapolis, IN). The density of bands of interest was analyzed using NIH Image software.

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 T3 and T7 polymerases, as described previously. Whole-mount in situ hybridization was performed according to published protocols, with probe hybridization at 55°C (Thissen et al. 1993; Yuan et al. 1997).

Antibody production

The zf-agrin clone, which was inserted into TOPO-PCR vector, was digested with EcoRI, ligated with pRSET-B
vector, and transformed into TOP10F<sup>®</sup> (Invitrogen). Recombinant agrin protein was expressed under the control of the T7 promoter and extracted by His-bond, Ni<sup>2+</sup> column chromatography (Novagen, San Diego, CA). Agrin fusion protein was used for the production of polyclonal antisera in rabbits. Antiagrin antisera were affinity-purified by affinity chromatography using agrin fusion protein coupled to Sepharose CL-4B. Antibody specificity was confirmed via western blot analysis.

**Western blot analysis**

Embryos were collected, washed in the Ringer solution, and homogenized in protein extraction buffer (10 mM Tris, pH 7.4, 2% Triton-X 100, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, and 1 mM trypsin inhibitor). Lysates were centrifuged and protein concentrations were determined by the Bradford assay, using the Bio-Rad reagent and immunoglobulin as a standard protein. To remove HS-GAG chains from agrin, purified agrin was treated with nitrous acid, as previously described (Burg et al. 1995). On 4–20% gradient SDS–PAGE gels, 400 µg of supernatant protein was separated and transferred to nitrocellulose membranes. Immunoblots were blocked with 5% nonfat dry milk suspended in Tris-bufffered saline and incubated overnight at 4 °C with a polyclonal antiserum against a zebrafish agrin fusion protein (1:100). Immunoblots were washed and incubated with horseradish peroxidase-conjugated antirabbit antibodies (1:25 000). Membranes were incubated with Supersignal West Pico chemiluminescent Substrate kits (Pierce, Rockford, IL) and exposed to X-ray film (Kodak, Rochester, NY).

**Immunohistochemistry**

For whole-mount immunohistochemistry, embryos were fixed with 4% paraformaldehyde/phosphate-buffered saline (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 znp-1 (1:100 from Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA), MAb zn-12 (1:100 from DSHB), MAb zn-5 (1:500 from DSHB), MAb anti-SV2 (1:100 from DSHB), anti-islet (1:10 from DSHB), MAb F59 (1:10 from Dr Stockdale), or MAb F310 (1:10 from Dr Stockdale) overnight at 4 °C. The anti-znp-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, MAb F59 (Crow and Stockdale 1986) to detect all slow muscle cells including muscle pioneers (Devoto et al. 1996), and MAb F310 to detect fast muscle cells (Crow and Stockdale 1986). Embryos were washed several times with PBS and incubated with secondary antibody (1:800 Cy3- or Cy5-conjugated antibody).

**Analysis of neuromuscular junction development**

MO-injected embryos were fixed at 4% paraformaldehyde for 2 h at room temperature. After 4-h incubation in water, embryos were incubated with Alexa-594-conjugated α-BTX (1:2000; Molecular Probes, Eugene, OR) for 1 h according to published protocols (Ono et al. 2001). Alternatively, embryos were incubated in 1 mg/mL collagenase (Sigma, Louis, MO) in PBS for 8 min (depending on embryo age, 8 min for 24 hpf). After collagenase treatment, embryos were rinsed in PBS and incubated in 10 µg/mL fluorescently-conjugated-BTX (Molecular Probes) diluted in blocking solution (0.1% sodium azide, 2% bovine serum albumin, 0.5% Triton X-100 in PBS). After rinsing, embryos were incubated overnight at 4 °C in primary antibody diluted in blocking solution [SV2 (1:50 to 1:100, DSHB)].

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**Conflict of interest statement**

None declared.

**Abbreviations**

AchR, acetylcholine receptor; AP, anterior–posterior; BTX, bungarotoxin; CaP, caudal primary; CNS, central nervous system; DMB, diencephalon–midbrain boundary; DSHB, Developmental Studies Hybridoma Bank; FGFRs, FGF receptors; HSPGs, heparan sulfate proteoglycans; MHB, midbrain–hindbrain boundary; MiP, middle primary; MO, morpholinol oligonucleotides; RT–PCR, reverse transcriptase–polymerase chain reaction.

**References**


proteoglycan that interacts with the neural cell adhesion molecule. J Neurosci Res. 41:49–64.


Agrin regulates axon growth and tail development in zebrafish


