Tissue- and Age-Specific Expression Patterns of Alternatively Spliced *Agrin* mRNA Transcripts in Embryonic Rat Suggest Novel Developmental Roles

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Agrin is an extracellular matrix protein that mediates the nerve-induced clustering of nicotinic acetylcholine receptors on target muscle cells, and thus plays a key role in development of the neuromuscular synapse. Alternative exon usage within the rat *agrin* gene predicts numerous protein isoforms, which differ by the inclusion or exclusion of small inserts at three sites in the C-terminal half of the molecule; the insert status at two of these sites, termed Y and Z, profoundly influences the acetylcholine receptor clustering activity. We have examined the cellular expression patterns of *agrin* messenger RNA transcripts during rat embryogenesis by *in situ* hybridization with isoform-specific probes. Six 36-mer oligonucleotide probes were designed to distinguish between mRNA isoforms at either the Y site: the encoded protein contains either no insert (Y0) or a 4-amino acid insert (Y4), or the Z site: the encoded protein contains either no insert or one of 8 (Z8), 11 (Z11), or 19 (Z19) amino acids. Strikingly different expression patterns were observed for the individual Y- and Z-site encoding messages. While optional exon usage predicts the possibility of eight different *agrin* isoforms at the two splice sites, we detected only four isoforms *in vivo*: Y4Z0, Y0Z0, Y4Z8, and Y4Z19. The Y4Z0 transcript, which comprised the majority of the *agrin* expressed, was localized exclusively to nervous tissue and exhibited a distribution profile suggestive of a potential role in neurogenesis and/or neural differentiation. From embryonic day 13 to birth, Y4Z0 was found in mitotic ventricular zones, spinal, cranial, and sympathetic ganglia, and diffusely throughout the brain. In contrast, Y0Z0 was not expressed in neurons, but specifically labeled capillary endothelial cells within the developing nervous system. Y4Z8 and Y4Z19, the forms most active in acetylcholine receptor aggregation, were expressed at low levels only in spinal and brainstem motor neurons; Z19 expression declined from embryonic day 15 to adulthood, whereas Z8 expression increased slightly during this period. Transcripts encoding the Z11 insert could not be detected. These data suggest potential novel

biological roles for *agrin* beyond that originally proposed in synapse formation.

[Key words: agrin, *in situ* hybridization, alternatively spliced, neuronal-specific, protein isoforms, extracellular matrix]

Alternative splicing of nuclear precursor RNAs (pre-mRNAs) represents an important control point in the regulation of eukaryotic gene expression. Differential splice site selection within a primary RNA transcript can yield multiple protein isoforms, often with differing activities or functions. The expression patterns of these isoforms can vary within different cell types and at different stages of development (see Breitbart et al., 1987; Smith et al., 1989, for review). While the factors involved in regulation of splice site selection are poorly understood, alternative exon usage appears to be a common mechanism for generating protein isoform diversity from genes coding for extracellular matrix-associated proteins (Boyd et al., 1993).

Agrin, which encodes several isoforms of a large multidomain protein localized to extracellular matrix, represents one example of a gene subject to complex regulated alternative splicing. *Agrin* was initially isolated and purified from the electric organ of the marine ray *Torpedo californica* based on its ability to induce aggregation of nicotinic acetylcholine receptors (AChR) in muscle cell membranes (Nitkin et al., 1987); as such, it was hypothesized to represent the motor neuron-derived signal that initiates neuromuscular synapse formation (McMahan, 1990; Hall and Sanes, 1993). Consistent with this interpretation, cells transfected with *agrin* cDNA express *agrin* on their extracellular surface and, when cocultured with primary muscle fibers, induce clustering of AChRs at sites of cell contact (Campanelli et al., 1994). *Agrin* is synthesized within spinal cord motor neurons at the time of initial synapse formation (Rupp et al., 1991; Smith et al., 1992; Tsim et al., 1992) and transported down the motor neuron axon (Magill-Solc and McMahan, 1990); moreover, anti-*agrin* antibodies can inhibit the formation of AChR clusters at developing neuromuscular contacts in culture (Reist et al., 1992). Subsequent to receptor clustering, *agrin* induces at this site the accumulation of several other molecules important in the multistep process of synapse maturation, such as acetylcholinesterase, laminin, and heparan sulfate proteoglycan (Wallace, 1989; Nitkin and Rothschild, 1990).

Cloning of *agrin* cDNAs from rat (Rupp et al., 1991) and chick (Tsim et al., 1992) and a homology search of the predicted amino acid sequences, revealed *agrin* to be a complex protein with a predicted molecular weight of ~205 kDa, containing several sequence motifs common to other extracellular matrix and

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Figure 1. Modular organization of the rat *agrin* protein and location of oligonucleotide probes for Y- and Z-site isoforms. Protein modules of *agrin* are represented by boxes; accepted amino acid inserts are shown below or above corresponding Y or Z alternative splice sites, respectively. Probes were complementary to messenger RNA sequences encoding the indicated amino acid stretches (solid lines). Dashed lines represent a direct connection between the 5' and 3' encompassing probe sequences. SS, signal sequence.

secreted proteins. Thus, *agrin* can be divided roughly into three regions: the N-terminal portion, which contains nine follistatin-like modules preceded by a putative secretory signal sequence and interrupted by a laminin B-homologous domain between the eighth and ninth follistatin repeats; the middle portion, rich in serine, threonine, and proline; and the C-terminal portion containing four EGF domains interspersed with three laminin A-homologous modules (Rupp et al., 1992; Patthy and Nikolics, 1993, 1994). Based on cDNA sequence analysis, several protein isoforms of *agrin* are predicted; isoform diversity results from the inclusion or exclusion by alternative splicing of multiple small exons at the 3' end of the mRNA coding region. In the chick protein, these sites have been termed the A and B sites, and reside at amino acid positions 1610 and 1746 (Tsim et al., 1992), respectively. In rat *agrin*, the corresponding sites (termed Y and Z) are located, respectively, at amino acid positions 1710 and 1779; an additional insert site (the X site) was identified at position 1144 (Rupp et al., 1991; Ferns et al., 1992; Hoch et al., 1993). The X site can contain either no insert or a 3- or 12-amino acid insert, the Y site either no insert or a 4-amino acid insert, and the Z site either no insert, or one of 8-, 11-, or 19 (8+11) amino acids; each insert is encoded on a separate exon in the corresponding mouse gene (Rupp et al., 1992).

Recent experiments with recombinant *agrin* have shown that not all isoforms induce AChR clustering to a similar extent (Ferns et al., 1992, 1993; Ruegg et al., 1992; Daggett et al., 1994). Thus, striking differences were observed in the clustering activity mediated by the different Z-site isoforms on muscle cells *in vitro*, lesser differences were found for Y-site isoforms, and no differences in activity were observed for X-site variants (Ferns et al., 1993). Whereas all isoforms were active on C2 mouse myotubes, those containing inserts at the Y- and Z-splice sites were more active than the form lacking inserts; Y4Z8 was the most active isoform.

In initial attempts to understand the regulation of *agrin* during development, pan-specific *agrin* probes were used to examine

the distribution of *agrin* mRNA, both by Northern analysis and by *in situ* hybridization (Tsim et al., 1992; Biroc et al., 1993; Hoch et al., 1993; Thomas et al., 1993; Ma et al., 1994; O'Connor et al., 1994). Additionally, PCR analyses defined tissue- and age-specific expression patterns of differentially spliced *agrin* mRNA transcripts (Ruegg et al., 1992; Hoch et al., 1993; Thomas et al., 1993). However, these studies have not allowed for the direct localization of individual mRNA variants at the cellular level. By *in situ* hybridization with isoform-specific probes we have previously characterized the cell-specific distribution of chick *agrin* mRNAs in developing spinal cord (Honig et al., 1993). In the present study we have focused on mammalian development, and demonstrate a more comprehensive analysis of the cell type- and age-specific expression patterns of alternatively spliced *agrin* transcripts in the developing rat nervous system. The observed unique and distinct distribution patterns of mRNA transcripts suggest potential novel developmental roles for *agrin* variants, beyond that previously recognized in synaptogenesis.

Materials and Methods

In situ hybridization. Timed pregnant female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). To obtain fetal tissues prior to embryonic day 20 (E20), mothers were killed by CO₂ inhalation followed by decapitation, and embryos were removed and placed in 4% paraformaldehyde (PF) in 0.1 M phosphate buffer, pH 7.2, at 4°C from 4 hr over night. To obtain tissues from developmentally older animals, rats were anesthetized on ice (postnatal day 1; P1) or with sodium pentobarbital (P7-adult), and perfused transcardially with 4% PF in phosphate buffer. Brains and spinal cords were removed and postfixed in perfusate for 1–2 hr. Tissues were washed in phosphate-buffered saline (PBS), cryoprotected in 20% sucrose in PBS at 4°C for 24–48 hr, embedded in Tissue Tech O.C.T compound (Miles Inc., Elkhart, IN) and frozen at –70°C. Sections 16 µm thick were cut on a cryostat, thaw mounted onto Superfrost Plus glass slides (Baxter, Deerfield, IL), and allowed to dry for 30 min at room temperature, then at 42°C for 20 min. Slide-mounted sections were placed in 4% PF in phosphate buffer for 5 min at 4°C, washed twice in PBS, then de-

Figure 2. Specificity of rat *agrin* isoform probes. Eight identical Southern blots, each containing four rat *agrin* cDNA variants, were hybridized separately to the eight rat *agrin* oligonucleotide probes. Probes were tailed with α - 32 P-dATP, and hybridization and washing conditions were similar to those used for *in situ* hybridization. From left to right, the four lanes in each blot contain size-separated EcoRV-Hind III-digested fragments of Y4Z0 (0), Y4Z8 (8), Y4Z11 (11), and Y4Z19 (19) cDNAs, respectively. The individual probes are shown above the blots to which they were hybridized. The 1.4 kb fragment represents bases #4446–5878 of the rat *agrin* cDNA.

hydrated in 70% and 95% ethanol, sequentially. Slides were stored at 4°C in 95% ethanol until required.

Six 36-mer oligonucleotides were designed to recognize specifically the six different splice variants at the Y and Z sites. Additionally, one pan-specific 36-mer oligonucleotide, designated “antisense,” was designed to hybridize to all splice variants, and its reverse complement, “sense,” was designed as a negative control probe. Probes are identified by a letter followed by a number, representing the splice site and the encoded amino acid insert size, respectively. The probe sequences were: Y0 (beginning at nucleotide 5110; Rupp et al., 1991), 5′-GAGCATG-GTGTGCGGAACCGGAGATTCCCTAGCAC-3′; Y4, 5′-GGTGTG-CGGAACCTTGC GGAGATTCCCG-3′; Z0 (beginning at nucleotide 5520), 5′-CTCTGCAGTGCCTTCTCAGTCTCAATCAC-AGCATTG-3′; Z8, 5′-CTCTGCAGTGCCTTCTCGGCTGGGATCTC-ATTGGTC-3′; Z11, 5′-GAATCCAGAGTTTCAGGACTCTCAATCA-CAGCATTG-3′; Z19, 5′-GGAATCCAGAGTTTCAGGGGCTGGGA-TCTCATTTGGT-3′; antisense (nucleotides 4895–4390), 5′-CATCTGT-CTTCTGCCCCATTGTAGAGGAGTAAGCCAC-3′; sense, 5′-GTGG-CTTACTCTCTACAATGGGCGAGAAGACAGATG-3′. Oligonucleotides were labeled with α - 32 S-dATP (> 1000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and terminal transferase (Boehringer-Mannheim, Indianapolis, IN), to a specific activity of 1×10^7 cpm/ μ g. Probes were purified on Bio-Spin 6 columns (Bio-Rad Laboratories, Richmond, CA).

The *in situ* hybridization protocol was carried out essentially as described by Wisden and Morris (1994). Immediately prior to hybridization, slides were removed from ethanol and air dried. Labeled probe was added in hybridization buffer ($\sim 3 \times 10^5$ cpm/100 μ l hybridization buffer per slide), sections were covered with parafilm coverslips, and slides incubated horizontally in a humid chamber (saturated with 50% formamide, 4 $\times$ SSC) over night at 52°C (for Y0 and Y4 probes) or 42°C (all other probes). Hybridization buffer was 50% formamide, 4 $\times$ SSC, 10% dextran sulfate, 5 $\times$ Denhardt’s (50 $\times$ Denhardt’s is: 1% each (w/v) Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 200 μ g/ml sonicated salmon sperm DNA, 100 μ g/ml polyadenylic acid (Sigma Chemical Co.), 25 mM sodium phosphate, pH 7.0, 1 mM sodium pyrophosphate, and 50 mM dithiothreitol. After hybridization, coverslips

Figure 3. *Agrin* mRNA in E13 rat embryo. Film images of sagittal embryo sections (A–C) show identical hybridization patterns for anti-sense (A) and Z0 (B) probes. In a slightly more lateral section, Y4 probe (C) hybridized specifically to neural tissue but did not label other regions. Panels D–F show higher magnification, dark-field images of an emulsion-dipped section adjacent to that shown in panel C, hybridized to the Y4 probe. Very dense labeling with the Y4 probe was seen in dorsal root ganglia and spinal cord ventricular zone; sympathetic ganglia were also prominently labeled (D). In the brain (E), Y4 hybridized to germinal cells immediately surrounding the lateral forebrain ventricle, and cells in the outlying neuroepithelial mantle. F and G show higher power dark-field and bright-field images, respectively, of region indicated by an asterisk in E. *aq*, aqueduct; *drg*, dorsal root ganglia; *lv*, lateral ventricle; *sc*, spinal cord; *sg*, sympathetic ganglia; *vz*, ventricular zone; *4v*, fourth ventricle. Scale bar: 1 mm (A–C) or 100 μ m (D–G).

were removed and slides were washed 10 min in 1 $\times$ SSC at room temperature, 40 min in 1 $\times$ SSC at 55°C, dehydrated in ethanol (70%, 95%), air dried, and apposed to β -max film (Amersham Corp.) for 5 d. Slides were subsequently dipped in LM-1 emulsion (diluted 1:1 with ddH₂O; Amersham Corp.), and exposed at 4°C for 6–8 weeks. Following autoradiographic development, sections were counterstained with hematoxylin, dehydrated through graded ethanols and coverslipped with Pro-Texx mounting medium (Baxter Scientific, McGaw Park, IL). Controls for hybridization specificity included either hybridization with a labeled “sense” oligonucleotide or, on some sections, inclusion of 100-fold excess unlabeled oligonucleotide. Both methods yielded very low or absent “background” staining with no evidence of specific cellular labeling.

Southern blot. Full-length rat *agrin* cDNA isoforms Y4Z0, Y4Z8, Y4Z11, and Y4Z19 (obtained from R. H. Scheller, Stanford University) were digested with EcoRV and HindIII (New England Biolabs, Beverly, MA) resulting in the liberation of a 1.4 kilobase (kb) fragment (base #4446–5878) containing both the Y- and Z-splice sites, and aliquots of 200 μ g per lane were electrophoresed on a 1% agarose gel. Eight identical gels of the four isoforms were prepared, and the DNA was denatured and transferred to Gene Screen Plus hybridization membrane (Du Pont NEN, Boston, MA) by alkaline capillary transfer according to the manufacturer’s protocol. DNA was fixed to the membrane by UV cross-linking, and the eight blots hybridized separately to the eight rat *agrin* oligonucleotide probes. Probes were tailed with α - 32 P-dATP (> 3000 Ci/mmol, Amersham Corp.) by terminal transferase to a specific

Figure 4. Hybridization of Y-isoform probes in rat E15 embryo. Film images of sagittal embryo sections hybridized with Y0 (A) or Y4 (B) probes revealed strikingly different expression patterns. Panels C–E depict higher power, dark-field images of E15 spinal cord regions (arrowhead marks approximate location) from emulsion-dipped sections hybridized with Y0 (C), Y4 (D), or sense (E) probes. *aq*, aqueduct; *d*, dorsal; *lv*, lateral ventricle; *mc*, motor column; *sc*, spinal cord; *v*, ventral; *vz*, ventricular zone; *4v*, fourth ventricle. Scale bar: 1 mm (A and B) or 300 μ m (C–E).

activity of 1×10^7 cpm/ μ g, and used at 4×10^5 cpm/ml hybridization buffer. Hybridization buffer was: 50% formamide, $6 \times$ SSC, $5 \times$ Denhardt's, 50 mM sodium phosphate, pH 7.0, 100 μ g/ml polyadenylic acid, and 0.5% SDS; hybridization was carried out overnight at 52°C for Y-site probes and 42°C for all others. Blots were washed to a stringency of $1 \times$ SSC at 55°C for 30 min, and apposed to Kodak XAR-5 film for 8 hr.

Immunohistochemistry. Slide-mounted tissue sections (see above) were allowed to air dry for 30 min at room temperature, then placed in 4% PF in phosphate buffer for 5 min at room temperature. Slides were subsequently washed $2 \times$ in PBS (5 min each), then placed in 1% NP-40 in PBS for 5 min and in blocking buffer (10% fetal calf serum in PBS) for 10 min at room temperature. A rabbit polyclonal antibody to CD34 (anti-mCD34; Baumhueter et al., 1993) was applied in blocking buffer at a dilution of 1:100 for 24 hr at 4°C. Slides were then washed $3 \times$ (4 min each) in PBS, and biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) was applied as secondary antibody at a 1:100 dilution in blocking buffer. After a 1 hr incubation, slides were washed in PBS ($3 \times$ 4 min) and incubated with FITC-labeled avidin D (Vector Labs), 1:100 in blocking buffer for 1 hr. Slides were washed in PBS and coverslipped with Vectashield mounting medium (Vector Labs).

Results

In situ hybridization, under stringency conditions favoring destabilization of short duplexes (< 20 nucleotides; Wisden and Morris, 1994), was carried out on sections of rat tissue from developmental ages E13–adult. Six 36-mer oligonucleotides were synthesized as probes, designed to distinguish the six splice variants of *agrin* at the Y and Z sites. Additionally, two 36-mer oligonucleotides, designated antisense (recognizes all variants) and sense (reverse complement of antisense) were generated as positive and negative controls, respectively. Figure 1 diagrams the location of the splice sites in the encoded rat *agrin* protein, and the strategy used for designing the isoform-specific probes.

To demonstrate probe specificity, rat *agrin* cDNAs encoding the four different Z-site isoforms were subjected to Southern blotting. Under stringency conditions similar to those used for *in situ* hybridization, eight identical blots were hybridized individually with the eight labeled *agrin* oligonucleotide probes. As

Figure 5. Hybridization of Z-isoform probes in rat E15 embryo. Film images are shown of sagittal embryo sections hybridized with Z0 (A) or Z19 (B) probes. Panels C–F are dark-field images of E15 spinal cord regions (arrowhead in A marks approximate location) from Z0- (C), Z8 (D), Z11 (E), or Z19 (F)-labeled sections. Note specific hybridization of Z8 and Z19 only to ventral spinal cord (arrows in D and F). Abbreviations and scale bars are as in Figure 3.

shown in Figure 2, each of the four Z-site probes hybridized specifically to its corresponding cDNA, with little or no crosshybridization to other Z-site variants (Fig. 2, upper panels). All four cDNAs tested contained the Y4 insert; thus, the Y4 probe hybridized to all, and the Y0 probe to none, of the cDNA variants (Fig. 2, lower panels). Additionally, all four cDNAs hybridized to both the antisense and the sense probes, due to the presence of both DNA strands. We did detect a faint crosshybridization of the Z8 probe to Z19 cDNA; therefore, we cannot rule out the possibility that a minor fraction of the Z8-specific signal *in vivo* could be attributable to Z19 mRNA. However, we believe that the intensity of the Z8 signal *in vivo*, and its presence at later developmental stages when Z19 was not observed, suggest that the majority of this signal was specific to Z8 mRNA.

All eight labeled probes were hybridized individually to adjacent tissue sections at selected developmental ages. At E13, the earliest gestational age examined, *agrin* was strongly expressed in the developing nervous system (Fig. 3). Hybridization with the antisense probe revealed abundant message in mitotic ventricular zones of the spinal cord and brain; additionally, a widespread, above-background signal was detected in peripheral tissues (Fig. 3A). Hybridization with isoform-specific probes revealed that the Y4Z0 variant accounted for all the neurally expressed *agrin* observed at this age (Fig. 3B,C). Under dark-field optics, emulsion-dipped sections exhibited pronounced Y4 expression in developing spinal and sympathetic ganglia (Fig. 3D). In brain ventricular zones, Y4 message was predominantly localized to cells in the neuroepithelial mantle layer (Fig. 3E-G). The Z0 variant exhibited an expression pattern identical to that of Y4, with the addition of diffuse low-level expression in peripheral tissues; only the Y0 probe correspondingly localized to peripheral tissues (data not shown, but see Fig. 4A for a later developmental age), suggesting a predominantly non-neuronal distribution of Y0Z0 expression. No signal was detected for Z8 or Z11 probes, whereas the Z19 probe faintly hybridized to ventral lumbar spinal cord (data not shown).

At gestational age E15, strikingly different expression patterns were exhibited by the two Y-site splice variants (Fig. 4). Whereas the Y0 variant was expressed at a low-level throughout many peripheral tissues, and was only barely detectable in spinal cord and brain (Fig. 4A), Y4 was expressed abundantly and specifically within neural tissue: predominantly in mitotic ventricular zones and to a lesser extent throughout the entire spinal column and developing brain (Fig. 4B). Dark-field illumination of emulsion-dipped sections revealed the faint neural expression of the Y0 variant to be confined to capillaries and meninges (Fig. 4C, see also Fig. 6C,E); conversely, the Y4 variant diffusely labeled both gray and white matter of spinal cord (Fig. 4D), but was not expressed in vascular tissue. Figure 4E depicts the low level of background labeling seen with the sense probe.

Of the Z-site variants, Z0 was the most abundantly expressed at this age (Fig. 5A), exhibiting a labeling pattern nearly identical to that of the Y0 and Y4 probes combined (compare Fig. 5A to Fig. 4A,B). Thus, the major *agrin* isoforms expressed at E15 were Y4Z0 within the nervous system and Y0Z0 in non-neural tissues. Z19 specifically labeled ventral spinal cord (Fig. 5B), the region containing presumptive motor neurons. Z8 and Z11 hybridization could not be detected on x-ray film after a 5 d exposure (data not shown). At a higher magnification, dark-field images of lumbar spinal cord regions revealed that the Z0 variant was expressed in nearly all the regions in which the two Y-site

Figure 6. *Agrin* isoform mRNA in transverse sections of E15 lumbar spinal cord. Hybridization pattern seen with the antisense probe (A) was a composite of the labeling patterns of Y0 (C), Y4 (D), and Z0 (E) probes combined. Note the diffuse, yet above-background signal, exhibited by the antisense, Y0, and Z0 probes in peripheral tissues. The sense probe (B) showed a very low level of background labeling. Hybridization of Z8, Z11, and Z19 probes are shown in panels F, G, and H, respectively. Only Z8 and Z19 specifically hybridized to ventral spinal cord, the region containing presumptive motor neurons. *drg*, dorsal root ganglia; *m*, motor column; *vz*, ventricular zone. Scale bar: 300 μ m.

variants were detected: in germinal ventricular zones, spinal cord capillaries, and dura mater surrounding the spinal cord (Fig. 5C). Z8 and Z19 were expressed prominently in ventral motor columns, with a lesser diffuse labeling throughout spinal cord tissue (Fig. 5D,F); message for the Z11 variant could not be detected (Fig. 5E).

Transverse sections of E15 lumbar spinal cord are shown in Figure 6. Intense labeling with the antisense probe was detected in mitotic ventricular zones, dorsal root ganglia (DRG), ventral motor columns, and spinal cord meninges (Fig. 6A). Less abundant, but pronounced hybridization was detected throughout spinal cord tissue, within both gray and white matter, and a weaker diffuse hybridization signal was observed over several peripheral tissues (including muscle, skin, kidney and lung; data not shown, but see Fig. 13). Labeling patterns exhibited by individ-

Figure 7. *Agrin* mRNA in developing spinal and cranial ganglia. *A*, Film image of antisense probe hybridized to an E15 sagittal embryo section, more lateral than those shown in Figures 3 and 4. *B* and *C* show higher power dark-field images of dorsal root ganglia from emulsion-dipped sections labeled with the Y0 (*B*) or Y4 (*C*) probes, respectively. The region indicated by an asterisk in (*C*) is shown at higher power in dark-field (*D*) and bright-field (*E*). *cg*, cranial ganglia; *drg*, dorsal root ganglia; *tg*, trigeminal ganglia. Scale bar: 1 mm (*A*) or 100 μ m (*B–E*).

ual isoform-specific probes revealed the antisense pattern to be a composite of the component isoform variants. Thus, the major *agrin* isoform expressed in ventricular zone, DRGs, and diffusely throughout spinal cord tissue was Y4Z0 (Fig. 6*D,E*); that expressed in ventral motor columns was predominantly Y4Z19 and, to a lesser extent, Y4Z8 (Fig. 6*D,F,H*). Y0Z0 was expressed diffusely in peripheral tissues, and abundantly in both spinal cord capillaries and surrounding dura mater (Fig. 6*C,E*).

In addition to its prominent expression in the CNS, *agrin* was abundantly expressed in peripheral sensory ganglia. Figure 7*A* depicts hybridization of the antisense probe to the trigeminal and other cranial ganglia; the isoform expressed in cranial ganglia was Y4Z0 (data not shown). Y4 (Fig. 7*C*) and Z0 (data not shown, but see Fig. 3*B*) variants were also colocalized in developing DRGs, whereas the Y0 probe (Fig. 7*B*) exhibited a much weaker, more diffuse hybridization signal, primarily concentrated over what appeared to be intervening blood vessels.

Figure 8. *Agrin* mRNA in developing brain. Film images of horizontal E15 brain show widespread expression of Y0 (*A*), Y4 (*B*), and Z0 (*C*) isoforms. Note ventricular labeling by Y4 and Z0 isoforms (arrows). Y0- and Z0-labeled brain meninges (arrowheads). *D*, Antisense probe hybridization. *aq*, aqueduct; *lv*, lateral ventricle; *3v*, third ventricle. Scale bar: 1 mm.

At higher power, the Y4 hybridization signal could not be localized to any specific cellular subset within DRGs, due to the close packing of cells within the ganglion at this developmental age (Fig. 7*D,E*).

Agrin message was abundantly expressed in developing (E15) brain (Fig. 8), the major transcripts being Y4Z0 and Y0Z0 (Fig. 8*A–C*). Z8, Z11, and Z19 probes yielded a hybridization signal slightly above that of background, but the labeling appeared diffuse and was not localized to any specific brain region (data not shown). At a higher magnification and under dark-field optics, sagittal sections of E15 brainstem revealed several interesting features (Fig. 9*B–F*). Y4Z0 labeled mitotic zones surrounding the brain ventricles: most prominently, those surrounding the aqueduct, third, and fourth ventricles, and less prominently the forebrain ventricular zones (Fig. 9*C,D*). As in the spinal cord (see Fig. 6*C,E*), Y0Z0 message was expressed specifically within brain capillaries, and in the surrounding dura mater (Fig. 9*B,D*). In an attempt to define the cellular localization of the Y0Z0 isoform, adjacent brain sections were labeled with a polyclonal antibody to CD34 (Fig. 9*H–I*), a specific cell-surface marker for vascular endothelial cells (Fina et al., 1990). Under fluorescence optics, the immunohistochemical staining pattern of CD34 corresponded closely to the Y0 hybridization pattern, suggesting localization of the Y0Z0 message to capillary endothelial cells. Z8 and Z19 probe variants specifically labeled a brainstem motor nucleus, that of the facial nerve (Fig. 9*E,F*). Y4 (Fig. 9*C*) but not Y0 (Fig. 9*B*), message was also expressed in the facial motor nucleus, suggesting that both Y4Z8 and Y4Z19 isoforms are synthesized in brainstem motor neurons.

Figure 9. Higher power magnification of *agr* isoform expression in E15 brainstem. Photomicrographs depict boxed region in A. Dark-field images of sagittal sections reveal hybridization of Y0 (B), Y4 (C), Z0 (D), Z8 (E), Z19 (F), and sense (G) probes. Y4, Z8, and Z19 labeled specifically the motor nucleus of the seventh nerve (arrows in C, E, and F). H, Immunohistochemical localization of CD34, detected with an FITC-labeled secondary antibody and viewed under fluorescence optics. I and J show higher power views of CD34 staining and Y0 hybridization, respectively. *aq*, aqueduct; *cp*, choroid plexus; *cv*, cephalic flexure; *4v*, fourth ventricle. Scale bars: 300 μ m.

At a later stage in brain development (E19–P1) Y4Z0 was clearly the most abundant *agr* variant, showing specific expression in developing cortex, corpus striatum, hippocampus, and cerebellum (Fig. 10A–C,E–G). Intense expression of Y4Z0 was also seen in diminishing mitotic ventricular zones surrounding the aqueduct and third ventricles (Fig. 10B,C). Whereas Y0Z0 expression could still be detected within brain capillaries at P1 (Fig. 10D), the signal appeared less intense than at earlier developmental ages.

In general, *agr* expression appeared to decline throughout development, with the most intense hybridization signals appearing at the earliest ages examined. Figure 11 depicts a developmental profile of Z19 mRNA expression within spinal cord motor neurons. Hybridization signal intensity appeared strongest from ages E15–E18 (Fig. 11A–C), and thereafter declined slightly to P1 (Fig. 11D–E). Only very faint labeling was observed at P7 (Fig. 11F) and no Z19 hybridization could be detected in adult spinal cord (shown in Fig. 12G). Of the remaining Z isoforms, only Z0 and Z8 exhibited specific hybridization to adult

spinal cord (Fig. 12D,E), the former as Y4Z0 within individual cells scattered throughout spinal cord gray matter (Fig. 12C,D), and the latter as Y4Z8 in motor neurons of the ventral spinal cord (Fig. 12C,E). Expression of the Y0Z0 isoform was not evident in capillaries in adult spinal cord sections (Fig. 12B).

In peripheral tissues, prominent expression of the Y0Z0 isoform was observed in developing lung and kidney (Fig. 13A–D). Here, the label was associated with epithelial cells lining the developing lung bronchioles and kidney tubules (Fig. 13B,D). Interestingly, punctate *agr* expression was seen in the liver, in which a small subset of scattered cells expressed Y0Z19 and, to a lesser extent, Y0Z8 isoforms (Fig. 13E,F).

Table 1 presents a summary of the developmental expression patterns of *agr* isoforms from ages E13 to adult. With the exception of motor neurons, in which a developmentally regulated shift in isoform expression was observed, most other tissues maintained the expression of specific isoforms throughout development.

Discussion

In situ hybridization with isoform-specific oligonucleotide probes has allowed us to directly visualize and compare the developmental expression patterns of alternatively spliced *agr* mRNAs. We show here unique and distinct tissue- and cell type-specific distributions of different Y- and Z-site *agr* mRNA transcripts. Hybridization patterns of the six Y- and Z-site-specific oligonucleotide probes, when taken in sum, corresponded closely to the labeling pattern detected with a pan-specific antisense probe. Additionally, our antisense probe demonstrated a developmental hybridization profile that closely matched that of another pan-specific probe derived from a different region of the rat *agr* cDNA (Biroc et al., 1993).

At E13, the earliest gestational age examined, *agr* was very strongly expressed in mitotic ventricular zones and developing sensory and sympathetic ganglia (see Fig. 3); the predominant isoform was Y4Z0. Expression of Y4Z0 declined with developing age, correlating primarily with the birth and migration of neurons away from mitotic ventricular zones. By analogy to its proposed function in neuromuscular synapse formation, *agr* has previously been hypothesized to play a role in synaptogenesis in the brain (Thomas et al., 1993; O'Connor et al., 1994). However, the pattern and time course of *agr* expression seen here: i.e., in newly born neurons prior to their migration to outlying brain areas and long before they begin to send out axons to establish synaptic connections, suggest an additional role for *agr* much earlier in neuronal development. One intriguing hypothesis derives from the presence of multiple follistatin-homologous regions in the N-terminus of *agr*, and the recently proposed role for follistatin as a neural inducer (Hemmati-Briantou et al., 1994). In *Xenopus* embryogenesis, follistatin is able to promote neuralization by binding directly to activin, a member of the transforming growth factor β (TGF- β) superfamily, thus blocking the normal morphogenic activity of activin and allowing the default neural pathway to ensue. The multiple follistatin domains in *agr* (Patthy and Nikolics, 1993) could serve a potentially analogous role in mammalian neurogenesis. Although *agr* does not appear to bind activin (D. Stone and K. Nikolics, unpublished results), the follistatin-like repeats in *agr* may bind to some other TGF- β or related cysteine-knot superfamily member (McDonald and Hendrickson, 1993), which might be locally produced in or near *agr*-expressing cells. At its location within extracellular matrix surrounding the cells in

Figure 10. *Agrin* mRNA variants in E19–P1 brain. *A–C* depict film images of horizontal E19 brain sections, hybridized to antisense (*A*), Y4 (*B*), or Z0 (*C*) probes. Ventricular labeling is still evident at this age surrounding the aqueduct and third ventricle. Higher power dark-field images of emulsion-dipped sections are shown in panels *D–H*. The region depicted is the rostral edge of the frontal cortex in horizontal sections from P1 brain. Hybridization patterns of Y0 (*D*), Y4 (*E*), Z0 (*F*), antisense (*G*), and sense (*H*) probes are shown. *aq*, aqueduct; *3v*, third ventricle; *cc*, corpus callosum; *str*, neostriatum; *lv*, lateral ventricle. Scale bars: 1 mm.

which it is produced, *agrin* could act to bind, and thus regulate the function and availability of a growth factor involved in the mitogenesis or differentiation of *agrin*-expressing cells (Patthy and Nikolics, 1993, 1994).

Distribution patterns of the mRNA transcripts for Y-site variants differed strikingly (see Fig. 4). The alternative splicing event leading to insertion of a 12-nucleotide sequence encoding the amino acids KSRK, corresponding to exon 28 of the mouse gene (Y4; Rupp et al., 1992), occurred only within nervous tissue, including spinal cord, brain, and cranial, spinal and sympathetic ganglia. In contrast, the mRNA transcript encoding no insert at this site (Y0) was detected only in non-neural tissues, including skin, muscle, kidney, lung, and vascular tissues. Our results are in agreement with a prior study using PCR analysis to examine expression patterns of *agrin* variants (Hoch et al., 1993). These investigators reported a tissue-specific distribution of alternatively spliced Y- and Z-site isoforms: whereas the Y4 variant was expressed primarily in neural tissues, the Y0 variant was expressed in all tissues examined. However, unlike the previous study, which reported a minor (5–20%) expression of the Y4 variant in heart, muscle, and other non-neural tissues, we did not detect any non-neural expression of the Y4 variant by *in situ* hybridization.

Of the Z-site variants, Z0 was the most widely expressed transcript, encoding the majority of both neurally (in the form of Y4Z0) and non-neurally expressed (Y0Z0) *agrin* protein isoforms. Tissue hybridization patterns of the Z0 probe appeared nearly identical to those of the antisense probe, yet were often

of a slightly lower signal intensity (see, for example, Fig. 3*A,B*). Transcripts encoding the Z8 and Z19-containing isoforms were expressed diffusely at a very low level in brain tissue and spinal cord; specific localized expression was detected only within motor neuron cell bodies, in both ventral spinal cord and brainstem. Additionally, expression of the Z19 transcript was most pronounced at early developmental ages (E15–E18), the period corresponding to peripheral synapse formation. The Z8 variant was detected in all regions that expressed Z19, yet at a slightly lower concentration; a notable exception was observed in adult spinal cord, in which only Z8, but not Z19, was detected in motor neuron cell bodies. Our data agree with those of Hoch and co-workers (1993), suggesting that a developmentally regulated shift in *agrin* splicing patterns occurs within motor neurons. The exclusive expression of the Z8 and Z19 isoforms in motor neurons is compatible with a role for these insert-containing isoforms as the neuron-derived inducers of muscle fiber AChR aggregation, since only those *agrin* variants most active in receptor clustering assays *in vitro* (Fern et al., 1993; Daggett et al., 1994) were expressed in the appropriate cells at the time of initial synapse formation. Additionally, in agreement with Ruegg et al. (1992), we found splicing of inserts at the Y- and Z-sites to be coordinately regulated, since only the Y4, but not the Y0 transcript, was colocalized with Z-insert containing forms in presumptive motor neurons. An unusual variation, however, was observed in liver, in which scattered cells appeared to express either Y0Z8 or Y0Z19 isoforms. We currently have no explanation for this finding. We did not observe any specific hybrid-

Figure 11. Developmental decline of *agrin* Z19 expression in spinal cord motor neurons. Dark-field images of transverse sections through lower thoracic spinal cord hybridized to Z19 probe are shown at ages E15 (A), E16 (B), E18 (C), E19 (D), P1 (E), and P7 (F). Peak expression occurred from E16–E18. Scale bar: 300 μ m.

ization with the Z11 probe variant, suggesting that expression of this isoform was either absent or below the level of detectability under our assay conditions. In this regard, our data conflict with those of Hoch and co-workers (1993), who found by PCR significant levels of Z11 mRNA in brain and spinal cord at comparable developmental stages.

The Y and Z sites of the *agrin* protein are located, respectively, within the second and third of three laminin A modules in the C-terminal portion of the protein; these modules are hypothesized to specify independent globular folding units, similar to the G-domain of laminin A (Beck et al., 1991; Pathy and Nikolics, 1994). In the case of laminin, this domain binds to proteoglycans within extracellular matrix (Ott et al., 1982) and to a dystroglycan-related protein from embryonic brain (Gee et al., 1993). In *agrin*, these modules are important for binding to α -dystroglycan (Hoch et al., 1994), a potential *agrin* receptor (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). It seems reasonable to predict that the insert status at the Y and Z sites may influence protein activity by altering the structural folding of this region of the molecule and, hence, interaction of *agrin* with its receptor. Inserts at the Z site have been shown to induce an apparent conformational change in protein structure, detected by the appearance of a new monoclonal antibody epitope (Hoch et al., 1994). However, no differences in binding affinity of the different *agrin* isoforms for α -dystroglycan have yet been detected (Sugiyama et al., 1994).

The regulatory factors involved in *agrin* splice site selection remain currently unexplored. Several examples of neuron-spe-

Figure 12. *Agrin* isoform mRNA in adult spinal cord. Dark-field images of transverse sections through thoracic spinal cord hybridized with antisense (A), Y0 (B), Y4 (C), Z0 (D), Z8 (E), Z11 (F), or Z19 (G) probes. Only the antisense, Y4, and Z8 probes specifically labeled ventral horn motor neurons (arrows). Scattered neurons elsewhere within gray matter were labeled with the Y4 and Z0 probes, and the Y0 probe exhibited a diffuse, above-background signal over both gray and white matter. Scale bar: 300 μ m.

cific exon splicing have been described previously (Stamm et al., 1992; Had et al., 1993; Schell et al., 1993; Yamada et al., 1993). One well-studied example is that of the *c-src* oncogene, in which insertion of an 18-nucleotide exon, alone or in conjunction with a 33-nucleotide exon, occurs specifically in neurons; these isoforms are excluded from other tissues in which *c-src* is expressed (Levy et al., 1987; Martinez et al., 1987). In this and other studies, the *cis*-acting sequence elements responsible for the neuron-specific splicing event were localized to the optional exons and their flanking intronic sequences (Black, 1992; Stamm et al., 1993; Yamada et al., 1993). An emerging view from these studies is that cell type- and age-specific differences in the relative concentrations or activities of *trans*-act-

Figure 13. Specific expression of *agrin* mRNA in non-neuronal tissues. Under dark-field optics, dense hybridization of the Y0 probe was observed in E15 lung (A) and kidney (C). Higher power, bright-field images of regions indicated by asterisks in A and C, respectively, show label to be concentrated over epithelial cells lining the developing lung bronchioles (B), and kidney tubules (D). Panels E–F depict the punctate hybridization pattern seen in embryonic liver with Y0 (E) and Z19 (F) probes. Scale bars: 100 μ m.

ing factors, i.e., both small nuclear ribonucleoproteins (snRNPs) and non-snRNP RNA-binding proteins that function as essential elements of the constitutive splicing apparatus, may serve to regulate splice site selection (see Rio, 1993; Norton, 1994, for review).

A novel finding from the present study was the specific expression of the Y0Z0 *agrin* variant in brain and spinal cord capillary endothelial cells. Immunohistochemical staining with anti-*agrin* antibodies have previously localized *agrin* protein to capillaries throughout the CNS of chick (Magill-Solc and McMahan, 1988; Godfrey, 1991), rat (Rupp et al., 1991), frog, and *Torpedo* (Magill-Solc and McMahan, 1988), as well as the surface of the brain and spinal cord (Magill-Solc and McMahan, 1988). Electron microscopy revealed that the label associated with capillaries was concentrated in the basal lamina lying be-

tween the capillaries and the astrocyte end-foot processes (Magill-Solc and McMahan, 1988). Our data suggest endothelial cells to be the source of the capillary-associated *agrin*, since immunohistochemical staining with a specific marker for endothelial cells showed an identical distribution pattern; presumably, *agrin* is synthesized in the endothelial cell and secreted from the baso-lateral side, becoming subsequently incorporated into the underlying extracellular matrix. The presence of *agrin* message in vascular endothelial cells at early developmental ages suggest a potential novel role for *agrin* in angiogenesis. One intriguing possibility is a role for *agrin* in blood–brain barrier formation or maintenance, since *agrin* appeared to be expressed specifically in brain and spinal cord capillary endothelium, but not in non-neural vasculature (however, Y0Z0 expression in peripheral capillaries could have been masked by the low level, diffuse hybridization signal exhibited throughout the periphery). The recent identification of α -dystroglycan as an *agrin*-binding protein (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), and the previously characterized association between α -dystroglycan, the dystrophin-glycoprotein complex (an oligomeric complex that spans the plasma membrane; Ervasti and Campbell, 1991), and actin (Ervasti and Campbell, 1993) suggest that a primary function of *agrin* may be to act as an extracellular matrix–cytoskeletal link. Such a link could serve to organize local cell surface specializations during development, including not only the clustering of neurotransmitter receptors in postsynaptic membranes, but the establishment of domains involved in cell polarization or intercellular contact, such as the capillary endothelial cell tight junctions that underly the structural basis of the blood–brain barrier (Bradbury, 1993). One specific type of membrane specialization, the orthogonal intramembraneous particle assemblies that are found highly concentrated in perivascular astroglial end feet, can be induced by coculture of astrocytes with either brain endothelial or meningeal cells (Tao-Cheng et al., 1990). Additionally, these assembly patches correlate with the presence of basal lamina, suggesting that the inducing factor(s) is concentrated in the basal lamina lying between brain endothelial and astroglial cells.

The Y0Z0 *agrin* isoform was also expressed prominently in epithelial cells lining the developing lung bronchioles and kidney tubules; the latter could represent the source of *agrin* immunoreactivity identified in kidney extracellular matrix (Godfrey, 1991). The early (E15) expression of *agrin* in these epithelial cell populations is consistent with a role for this isoform in the organization or establishment of cell polarization, particularly in light of the three laminin G domain homologous regions in the C-terminal third of *agrin*, a domain hypothesized to mediate the epithelial cell polarity induced both by laminin

Table 1. *Agrin* isoforms expressed in various tissues during development

	E15–E19	P1	Adult
CNS ventricular zones, spinal and cranial ganglia	Y4Z0 ^a	Y4Z0	n.d.
Brain	Y4Z0 ^a	Y4Z0	Y4Z0
Motor neurons	Y4Z19 > Z8	Y4Z8 > Z19	Y4Z8
CNS capillary endothelial cells	Y0Z0	Y0Z0	—
Muscle, lung, kidney	Y0Z0	Y0Z0	n.d.

n.d., not determined; —, undetectable.

^a Detected as early as E13.

and the *Drosophila* protein crumbs (Klein et al., 1988; Patthy, 1991).

In conclusion, mRNA transcripts for differentially spliced *agrin* variants were shown to have unique and distinct cell type-specific distribution patterns, suggesting that *agrin* protein expression is subject to complex regulation during mammalian development. While optional exon usage predicts the possibility of eight different *agrin* isoforms at the Y and Z sites, we detected only four predominant mRNA transcripts *in vivo*: Y4Z0, Y0Z0, Y4Z8, and Y4Z19. In agreement with PCR and Northern analyses, forms containing inserts at both the Y and Z sites were expressed specifically within neural tissue, whereas the insertless isoform was localized only to peripheral tissues. The expression of the two Z-insert-containing isoforms in spinal motor neurons correlated with the proposed role of neural *agrin* in regulating AChR aggregation. However, the remaining two isoforms, which comprised the majority of the *agrin* expressed, exhibited distribution profiles suggestive of biological functions for *agrin* beyond its originally proposed activity, including potential roles in neurogenesis (Y4Z0) and nervous system endothelial cell differentiation (Y0Z0). With the availability of specific *agrin* isoforms produced by recombinant methods *in vitro*, we can begin to explore some of the potential novel developmental roles for *agrin* isoforms presented here.

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