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AGRIN GENE EXPRESSION IN MOUSE SOMATOSENSORY CORTICAL NEURONS DURING DEVELOPMENT *IN VIVO* AND IN CELL CULTURE

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Abstract—Agrin is an extracellular matrix protein involved in the formation of the postsynaptic apparatus of the neuromuscular junction. In addition to spinal motor neurons, agrin is expressed by many other neuronal populations throughout the nervous system. Agrin's role outside of the neuromuscular junction, however, is poorly understood. Here we use the polymerase chain reaction to examine expression and alternative splicing of agrin in mouse somatosensory cortex during early postnatal development *in vivo* and in dissociated cell culture. Peak levels of agrin gene expression in developing cortex coincide with ingrowth of thalamic afferent fibres and formation of thalamocortical and intracortical synapses. Analysis of alternatively spliced agrin messenger RNA variants shows that greater than 95% of all agrin in developing and adult somatosensory cortex originates in neurons, including isoforms that have little or no activity in acetylcholine receptor aggregation assays. The levels of expression of "active" and "inactive" isoforms, however, are regulated during development. A similar pattern of agrin gene expression is also observed during a period when new synapses are being formed between somatosensory neurons growing in dissociated cell culture. Changes in agrin gene expression, observed both *in vivo* and *in vitro*, are consistent with a role for agrin in synapse formation in the central nervous system. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: agrin, somatosensory cortex, barrel cortex, synapse formation.

Fast synaptic transmission between neurons, mediated by chemical synapses, is critically important for nervous system function. Understanding the mechanisms that direct synapse formation during development and their maintenance or reorganization in mature nervous systems are, therefore, fundamental questions in neurobiology. Much of what we know about this process comes from studies of the neuromuscular junction, where signals exchanged between motor neurons and the muscle fibres they innervate co-ordinate differentiation of the motor nerve terminal and formation of a specialized postsynaptic apparatus in the muscle fibre. Biochemical and molecular genetic studies have shown that one such regulatory agent is agrin, an extracellular matrix protein synthesized by motor neurons that triggers the accumulation of acetylcholine receptors (AChRs) and other components of the postsynaptic apparatus at the site of nerve–muscle contact.[8,20] In comparison to the neuromuscular junction, relatively little is known about the formation and maintenance of chemical synapses between neurons. Structural similarity to the neuromuscular junction however, suggests that similar mechanisms may direct the organization of neuron–neuron synapses. Like AChR at the neuromuscular junction, nicotinic AChR[31] and receptors for other chemical neurotransmitters[40,44,45,50,54,58] are concentrated in the postsynaptic membranes of many classes of neuronal synapses. Similarly, gephyrin, a protein that appears to be a functional homologue of the 43,000 mol. wt protein that plays a role in anchoring AChR at the neuromuscular junction,[19] is localized to the postsynaptic membranes of inhibitory synapses where it appears to function as a cytoskeletal anchor for glycine and GABA receptors.[9,13,39,58] The observation that brain extracts contain agrin[27,2837] and that agrin mRNA can be detected in virtually all neuronal populations in the developing and adult CNS[35,41,55] has, therefore, raised the possibility that agrin might play a role in the formation and maintenance of synaptic contacts between neurons. Recent immunohistochemical studies showing that

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Abbreviations: 4-AP, 4-aminopyridine; AChR, acetylcholine receptor; APV, D-2-amino-5-phosphonovaleric acid; BMC, bicuculline methchloride; BSS, balanced salt solution; CaM IIK, calcium calmodulin-dependent type II protein kinase; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DM, defined medium; EGTA, ethyleneglycolbis-(aminoethylether)tetra-acetate; FBS-MEM, minimal essential medium supplemented with 10% fetal bovine serum; GAD, glutamate decarboxylase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HSPG, heparan sulfate proteoglycan; NCAM, neural cell adhesion molecule; NMDA, *N*-methyl-D-aspartate; P, postnatal day; PCR, polymerase chain reaction; PSP, postsynaptic potential; RT-PCR, reverse transcription-PCR.

extracellular agrin first appears as punctate deposits within the synapse-rich plexiform layers of the retina at about the time that synapses form[34] provides further support for this hypothesis.

Native agrin is a heparan sulphate proteoglycan (HSPG) with an apparent mol. wt of 400–600,000.[59] Alternative splicing of agrin pre-mRNA gives rise to a family of protein isoforms that differ in their AChR aggregating activity.[17,18,47,60] Two locations, designated y and z in rodent[17,18] or A and B, respectively, in chick,[47,60] in which optional inclusion of a four amino acid exon at the y/A site and eight and 11 amino acid exons at the z/B site, appear to be particularly important. In rat, protein isoforms y_0z_0 or y_4z_0 have little or no AChR aggregating activity whereas y_4z_8, y_4z_{11}, or y_4z_{19} molecules have high levels of activity.[17,18] Similar differences in AChR aggregating activity are also apparent between the homologous agrin isoforms in chick.[23,47,60] Agrin is a complex multidomain protein with regions of similarity to other proteins including epidermal growth factor, laminin, and follistatin/protease inhibitor-like regions.[43] However, all of the structural determinants necessary for agrin induced aggregation of AChR on cultured muscle fibres are located within a carboxy-terminal fragment that represents only about 10% of agrin's predicted amino acid sequence and contains the alternatively spliced z region and one laminin G-domain.[23,24] These findings suggest that different agrin isoforms or regions on agrin might serve additional or alternate functions to those known at the neuromuscular junction. Further support for this hypothesis comes from the demonstration that agrin is identical to a neural cell adhesion molecule (NCAM) binding HSPG[59] and may act as differentiation-inducing stop signal for certain populations of neurons.[10]

Several studies have surveyed agrin gene expression in the rodent central nervous system and established that many neuronal populations in developing and adult brains express agrin.[30,41,55] Moreover, all four agrin mRNAs resulting from alternative splicing in the z region are present in the brain although their relative abundance varies during development and by region.[30,41,55] Progress towards understanding agrin's role in the mammalian CNS however, has been hampered by the lack of a well-characterized and accessible system in which to pursue these studies further. The functional neuroanatomy of rodent somatosensory cortex has been studied extensively in adult and during development (reviewed in Ref. 43) and the time-course of arrival of thalamic fibres and formation of thalamocortical and intracortical synaptic contacts during development well documented.[1–4,33] This system has also proven useful in investigating the role of neural activity[49] and mechanisms underlying experience-dependent changes in neuronal connectivity.[15] Therefore, to explore agrin's role in the CNS, we have examined agrin gene expression in developing mouse somato-sensory cortical neurons during postnatal development *in vivo* and in cell culture.

EXPERIMENTAL PROCEDURES

Tissue and RNA preparation

Tissue for the study was obtained from mice (ICR, Harlan Sprague Dawley, San Diego) between 0 and 21 days postnatal (P0–P21, P0 being the first 24 h after birth) or adult ($\geq$P60). Pregnant females were monitored at 12 h intervals to determine time of birth. Pups from P0 to P5 were anaesthetized by cooling on ice; older pups were anaesthetized by halothane inhalation. Anaesthetized animals were decapitated and the brains rapidly removed, placed in ice-cold saline and sliced at a thickness of 400–600 μm on a Vibroslicer (Campden Instruments). Somatosensory cortex was visually identified in transilluminated slices and dissected free from the surrounding tissue. Total RNA was isolated by the single-step method of Chomczynski and Sacchi[12] from somatosensory cortices or cultured neurons obtained from one to seven mice. First strand cDNA was synthesized by reverse transcription of 100–250 ng total RNA as previously described.[41]

Polymerase chain reactions

The concentration of agrin mRNA/μg total RNA isolated from somatosensory cortex was determined by competitive polymerase chain reaction (PCR).[25] A competing template, pMA$_{Bam}$, representing a PCR fragment of mouse agrin homologous to 4469–4882 base pairs of the rat agrin cDNA clone[48] was constructed in the plasmid pGEM-T (Promega). pMA$_{Bam}$ contains a fortuitous PCR induced mutation that created a unique BamHI restriction site used to distinguish PCR products amplified from competing versus native agrin templates. Competitive PCR was performed as previously described.[56,57] For each determination, cDNA from 250 ng of total RNA was co-amplified for 40 cycles (94°C, 0.5 min; 57°C, 1 min; 72°C, 1.5 min) with 1–300 fg of pMA$_{Bam}$ using the mouse agrin primer pair F7/B7 derived from sequence analysis of pMA$_{Bam}$ and corresponding to base pairs 4469–4486 and base pairs 4865–4882 of rat agrin, a region common to all alternatively spliced agrin mRNAs. PCR products were labelled by addition of approximately 2×10^5 c.p.m. of ^{32}P-labelled backward primer. Two microlitres of the amplified mixture were subject to a 10-fold overdigestion with BamHI followed by electrophoresis through a 6% nondenaturing polyacrylamide gel. The relative abundance of the labelled fragments amplified from the competing and native agrin templates was determined by analysis on a phosphorimager (Molecular Dynamics, CA) and levels of agrin mRNA expression calculated by linear estimation based on the amount of competing template added to each reaction.

The relative abundance of agrin mRNAs, alternatively spliced at either the y or z site, was determined by reverse transcription-PCR (RT-PCR) using primers flanking each site. For y site transcripts, first strand cDNA synthesized from 100 ng total RNA was amplified for 35 cycles (94°C, 0.5 min; 55°C, 1 min; 72°C, 1.5 min) using the F111/B112 primer pair, corresponding to or the reverse complement of nucleotides 5044–5064 and 5143–5162, respectively, in rat agrin. Specificity of the F111/B112 primers was confirmed based on molecular weight and sequence analysis of the amplified products. Analysis of the z region transcripts was performed using the rat primer pair F24/B2 described previously.[41] Amplified products, labelled by inclusion of ^{32}P-labelled forward primer in the PCR reaction, were separated by electrophoresis on 8% polyacrylamide gels and the relative abundance determined by analysis on a phosphorimager.

In some experiments multiplex RT-PCR using two rounds of amplification and nested primers was used to

identify mRNAs encoding agrin, calcium calmodulin-dependent type II protein kinase (CaM IIK) and GAD_{67}, the high molecular weight form of glutamic acid decarboxylase, co-expressed within a single RNA sample isolated from neurons cultured from somatosensory cortex. For the first round of amplification, first strand cDNA synthesized from 100 ng total RNA was co-amplified for 35 cycles (94°C, 0.5 min; 58°C, 1 min; 72°C, 1.5 min) using the rat agrin primer pair F13/B6,[41] and F72/B73 and F96/B97 derived from the published sequences of mouse CaM IIK (GenBank accession number X14836) and GAD_{67},[32] respectively. Aliquots of the first PCR reaction were subsequently diluted 1000-fold and re-amplified in separate PCR reactions using internal primers F24/B2 for agrin, F65/B66 for CaM IIK, and F98/B99 for GAD_{67}. Second round PCRs were performed for 35 cycles at an annealing temperature of 55°C. PCR products were labelled by inclusion of ^{32}P-labelled forward primer in the PCR reaction and analysed by polyacrylamide gel electrophoresis and autoradiography.

Tissue culture

Somatosensory cortical neurons were grown in defined medium, on glass coverslips placed on top of a confluent layer of non-neuronal cells growing in 35 mm tissue culture dishes using a procedure similar to that described by Baughman et al.[5] Briefly, small pieces (≈ 1 mm^2) of somatosensory cortices from P0 mice were incubated for 30 min at 37°C in a balanced salt solution (BSS; 137 mM NaCl, 5.3 mM KCl, 0.168 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 10 mM HEPES, 33.3 mM glucose, 43.8 mM sucrose) containing 10 U/ml papain (Worthington Biomedical Co., NJ), and 50 mM DL-2-amino-5-phosphonovaleric acid (APV; RBI, MA) followed by washing twice in BSS containing 50 μM APV, 1% bovine serum albumin (BSA), 1% trypsin inhibitor then three times with BSS containing 50 μM APV, 0.1% BSA, 0.1% trypsin inhibitor. Finally cells were washed twice in a defined medium (DM), consisting of HAM's F12/DME-high glucose (Irvine Scientific, CA) supplemented with 50 μg/ml insulin, 100 ug/ml transferrin, 100 μM putrescine, 0.5 μM hydrocortisone, 20 ng/ml progesterone and 30 μM selenium, dissociated by trituration through a glass micropipette and plated onto 25 mm-diameter glass coverslips (Bellco Glass Inc., NJ) pre-coated with either poly-D-lysine (Collaborative Biomedical Products, MA) or mouse laminin (Collaborative Biomedical Products, MA). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in DM+supplements for two days at which time coverslips were transferred to dishes containing confluent cortical cell feeder layers and placed neuron side uppermost to prevent contact between neurons and feeder layer cells. Coverslips were transferred to new feeder layer cultures every three days thereafter. Feeder layer cultures were prepared from cortices obtained from P0–P5 mice, plated in 35 mm poly-D-lysine-coated culture dishes and maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS-MEM). Feeder cultures were fed every four days with FBS-MEM. Prior to use as feeder layers, FBS-MEM was replaced with DM and cells allowed to condition the medium for 24 h before addition of neuronal coverslip cultures.

Electrophysiology

Electrophysiological recordings were obtained using the whole-cell configuration of the patch-clamp technique.[29] Recording pipettes were not polished and had open pipette resistances of 2–5 MΩ. The pipettes were filled with an internal solution composed of (in mM): $CaCl_2$ (0.1), $MgCl_2$ (2), EGTA (1.1), HEPES (10), K-gluconate (120), NaCl (20), pH 7.2. The external solution contained (in mM): NaCl (140), $MgCl_2$ (4), HEPES (5), $CaCl_2$ (1) and KCl (3), pH 7.2. Spontaneous postsynaptic potentials (PSPs) were recorded from individual neurons. An initial set of records (72 s) was obtained in normal external solution. In some

cases 100 μM 4-aminopyridine (4-AP), was added to the external solution to increase the frequency of spontaneous synaptic events. To determine the pharmacological sensitivity of these events, PSPs were monitored for an additional 72 s following bath application of 5 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 μM APV and/or 2 μM bicuculline methchloride (BMC). Data were collected and analysed using a List EPC-7 patch-clamp amplifier, a Dell 386/486 computer, and pCLAMP software (Axon Instruments, v.5.5.1). All recordings were performed at room temperature.

Polymerase chain reaction primers used in the study

F7	TTAGCGACTGGCAGAGGG
B7	CCAAGAACACCATCTCCA
F111	TGGGTCAGGGTATTCCTGGAA
B112	AGGTTGAGCATGGTGTGCGG
F13	TTTGCAGACCACCCTTGTACCC
B6	TGGTGTTGACCTTCACAGTGGAG
F24	AGTCAGTGGGGGACCTAGAAACAC
B2	AAGCTCAGTTCAAAGTGGTTGCTC
F72	ATCACCAGAATGGGACACCGTC
B73	CATCAGGTGGATGTGAGGGTTC
F65	ATGCCAGGAGGAAACTGAAGGGAG
B66	TCATTCCAGGGTCGCACATC
F96	CAACCTTTGGCTGCATGTGG
B97	ACTGCTTGTCTGGCTGGAAGAG
F98	CCAATTCAGTCACCTGGAACCC
B99	CCTGCACACATCTGGTTGCATC

RESULTS

Agrin expression in somatosensory cortex

During the first postnatal week, afferent fibres originating from neurons in mouse ventrobasal thalamus enter the somatosensory cortex and form excitatory synaptic contacts with neurons in layers IV, and V/VI.[2,3,4] Excitatory and inhibitory intracortical synaptic contacts are also established during this same period.[1,33] To examine the temporal correlation between agrin gene expression and formation and maturation of neuronal synapses, we used competitive PCR to measure the level of agrin mRNA present in total RNA isolated from somatosensory cortices of P0–P21 and adult mice (Fig. 1). Levels of agrin mRNA were low in RNA isolated from P0 somatosensory cortices but rose rapidly during the first few postnatal days of development increasing three-fold by P1–P3, a level that was maintained to the end of the first postnatal week. In contrast, agrin mRNA levels declined steadily during the second and third postnatal weeks reaching adult levels by the end of this period that were similar to the low level of expression observed at P0.

Previous studies have shown that alternative splicing at the y site in agrin pre-mRNA is cell specific: agrin transcripts containing a four amino acid insert (y4) predominate in neurons, whereas those lacking this insert (y0) derive from non-neuronal cells.[30,47,55] To determine the relative contribution made by neurons and non-neuronal cells to the total pool of agrin mRNA in somatosensory sensory cortex, we measured the relative abundance of y4 and y0 transcripts amplified from total RNA by
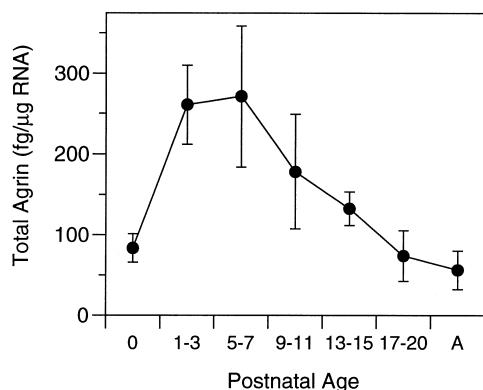
Fig. 1. Developmental changes in the levels of agrin mRNA expressed in somatosensory cortex. The concentration of agrin mRNA/µg total RNA isolated from somatosensory cortices of mice during the first three weeks of postnatal development or from adult animals was determined by competitive PCR. Agrin mRNA levels, shown in terms of the equivalent mass of competing template, changed markedly over the period of time examined with peak levels of expression coincident with the period of synapse formation within somatosensory cortex. Postnatal age is days (numbers) or adult (A). Each data point represents the mean value obtained from four independent RNA isolates. Bars indicate S.E.M.

RT-PCR using primers flanking the y site. An example of such an experiment is shown in Fig. 2A, where it is apparent that $agrin_{y4}$ mRNAs are far more abundant than $agrin_{y0}$, regardless of postnatal age. This impression was confirmed by phosphorimager analysis which showed that $agrin_{y4}$ mRNAs represent 96% of all agrin transcripts in postnatal somatosensory cortex suggesting that much if not all of this mRNA is of neuronal origin.

Alternative splicing of eight and 11 amino acid exons at the z site gives rise to four agrin proteins designated z0, z8, z11, and z19, with distinct physiological properties: isoforms containing either or both exons ($agrin_{z8}$, $_{-z11}$, and $_{-z19}$) have high AChR aggregating activity, whereas the isoform lacking an insert at the z region ($agrin_{z0}$) has little or no AChR aggregating activity.[17,18] To examine the pattern of alternative splicing of the z region in somatosensory cortex, RNA isolated from cortices of different aged mice was analysed by RT-PCR using primers flanking the z region. PCR products representing all four z region variants could be amplified from somatosensory cortical RNA. However, their relative abundance changed markedly during development (Fig. 2B, C). At P0, $agrin_{z0}$ mRNA was the most abundant of all agrin mRNAs but declined almost two-fold during the first postnatal week. During the third postnatal week z0 mRNA levels rose again, peaking at P15–P17, after which they continued to decline with the lowest level of expression observed in RNA isolated from adult somatosensory cortex. $Agrin_{z11}$ mRNAs represented only a minor fraction of all agrin mRNAs in developing somatosensory cortex. Maximal levels ($7.6 \pm 1.8\%$), were observed at P0 that
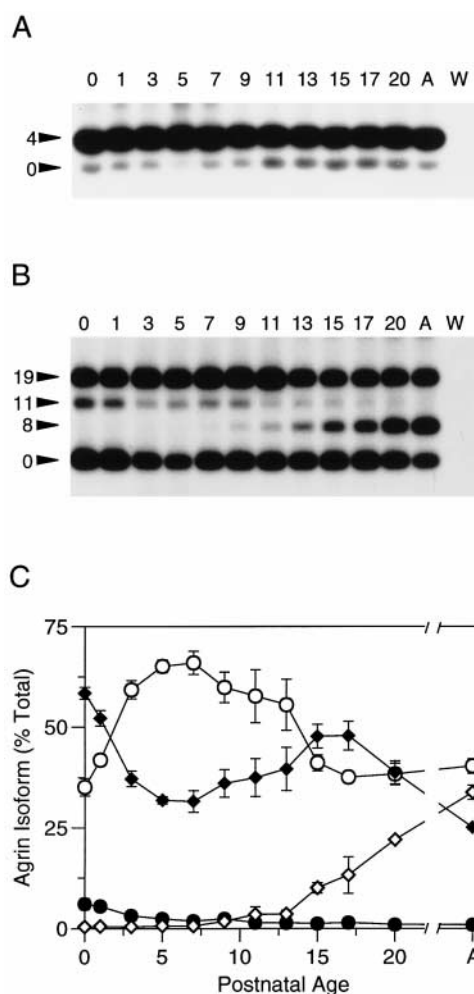

Fig. 2. Expression of alternatively-spliced agrin mRNAs in developing somatosensory cortex. Autoradiograms from typical experiments in which the relative abundance of agrin mRNAs resulting from alternative splicing of exon 28 (A) or exons 32 and 33 (B) were examined in RNA isolated from P0–P20 (numbers) or adult (A) cortices by RT-PCR. No PCR products are apparent in control lanes (W) in which water was substituted for RNA during the first strand cDNA synthesis. Greater than 95% of all agrin transcripts present in somatosensory cortex contain the four amino acid insert at the y site. Quantitative analysis of this and gels from three other RNA preparations showed no significant differences in the relative abundance of the y4 and y0 transcripts during development. In contrast, marked changes during postnatal development were apparent in the relative abundance of agrin mRNAs alternatively spliced at the z site. (C) Phosphorimager analysis of data obtained from four independent RNA preparations shows that, when measured as a fraction of the total agrin mRNA, the highest levels of $agrin_{z0}$ (filled diamonds) and $_{-z11}$ mRNA (filled circles) in somatosensory cortex occur at the earliest developmental stages examined whereas $agrin_{z19}$ mRNA levels (open circles) peak at the end of the first postnatal week and $agrin_{z8}$ mRNA (open diamonds) which first began to increase during the third postnatal week was highest in adult. Postnatal age is days (numbers) or adult (A). Bars indicate S.E.M.

declined steadily to barely detectable levels by the end of the first postnatal week. More robust changes,

however, were evident in the developmental profiles obtained for $agrin_{z8}$ and $_{z19}$ mRNAs. At birth, approximately one-third of all agrin transcripts encode the z19 isoform, a value that increased by two-fold at P5 but declined steadily thereafter. In contrast, $agrin_{z8}$ mRNAs were barely detectable during the first postnatal week but increased during subsequent development with maximal relative abundance ($33.3 \pm 1.4\%$) observed in RNA isolated from adult somatosensory cortices.

To examine the changes in the pattern of alternative splicing during development in more detail, the absolute amount of each transcript/mg total RNA was calculated based on the level of agrin mRNA expression determined by competitive PCR and the relative abundance of each mRNA isoform (Fig. 3). Beginning at P0, levels of expression for both $agrin_{z0}$ and $_{z11}$ mRNAs increased by about three-fold during the early postnatal period, peaking at P1–P3 and declining steadily thereafter. $Agrin_{z19}$ mRNA expression levels also increased during the early phase of postnatal development but the increase was more marked (six-fold) and peaked later (P5–P7) than that for z0 and z11 transcripts. In contrast, the concentration of $agrin_{z8}$ mRNA increased throughout postnatal development with levels in the adult greater than 10 times higher than at birth, the greatest increase occurring after the first week.

Agrin expression in somatosensory cortical cultures

The patterns of agrin gene expression observed in somatosensory cortex are complex and likely to be influenced by a wide range of cell–cell interactions. To provide an additional avenue along which mechanisms regulating agrin gene expression could be explored, we characterized agrin expression in a culture system amenable to experimental manipulation and analysis at the cellular level. Cells acutely dissociated from P0 somatosensory cortices were grown in DM, a simple defined medium, conditioned by a feeder layer of non-neuronal cells. At the time of plating, the majority of cells had one or two short processes extending from phase bright cells bodies (Fig. 4A). However, over the next two weeks the cells elaborated numerous processes that extended between individual cells or groups of cells (Fig. 4B, C). Based on morphology, the majority of the cells developing in these cultures are neuronal.

Using RT-PCR we examined the pattern of alternative splicing of agrin mRNA at the y site (Fig. 5A). Similar to the findings *in vivo*, at all ages in culture greater than 99% of all agrin mRNAs included the four amino acid exon 28 at the y site previously shown to be a neuron specific variant.[30,47,55] The small decrease in the relative abundance of $agrin_{y4}$ mRNA observed at two weeks *in vitro* to about 75% of all agrin transcripts, may reflect an increase in the population of non-neuronal cells able to survive in the DM or adhering to the coverslip from the feeder
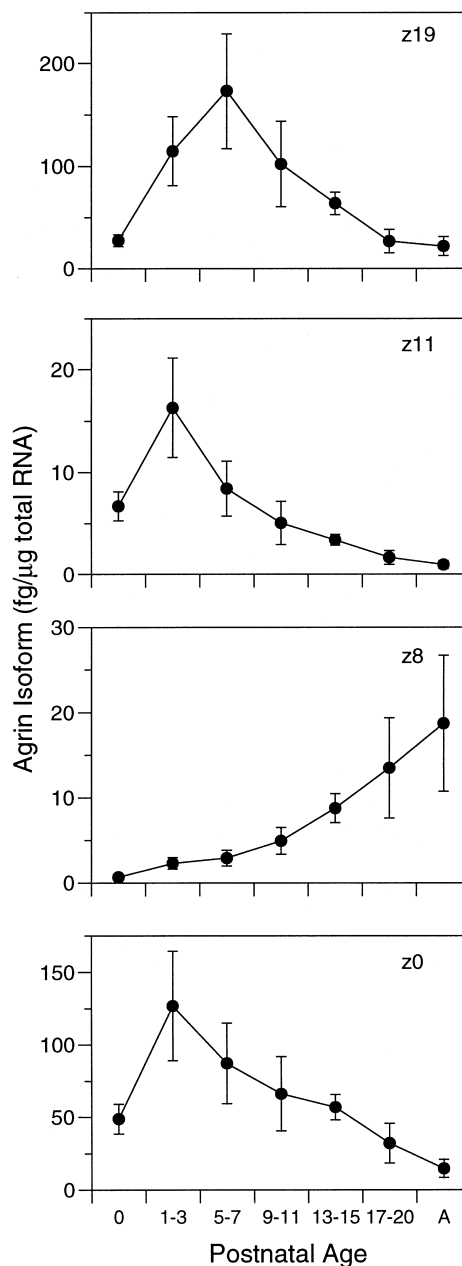

Fig. 3. Levels of alternatively-spliced z region agrin mRNAs expressed in somatosensory cortex change during development. Absolute levels of each z region transcript were calculated based on the total amount of agrin mRNA/µg total RNA (Fig. 1) and the fraction of total agrin mRNA represented by each transcript (Fig. 2) at each postnatal age. As illustrated in the graph, each agrin isoform exhibits a distinct pattern of expression in the cortex during postnatal development. Postnatal age is days (numbers) or adult (A). Each data point represents the mean of a minimum of five determinations, each from independent RNA isolates. Bars indicate S.E.M.

cell layer. An even more striking similarity between the pattern of agrin gene expression during development of somatosensory cortical neurons *in vivo* and *in vitro* became apparent when we examined alternative splicing at the z site (Fig. 5B, C). Reminiscent of

changes observed *in vivo*, the relative abundance of agrin$_{z0}$ mRNA at two days *in vitro* was high, then declined during the first week, followed by an increase in the second week. As noted *in vivo*, much of the change in the relative abundance of agrin$_{z0}$
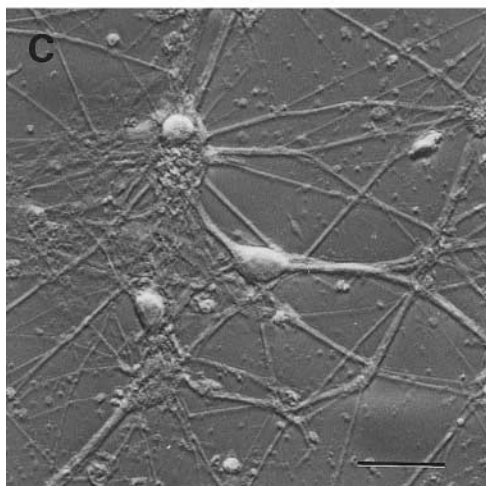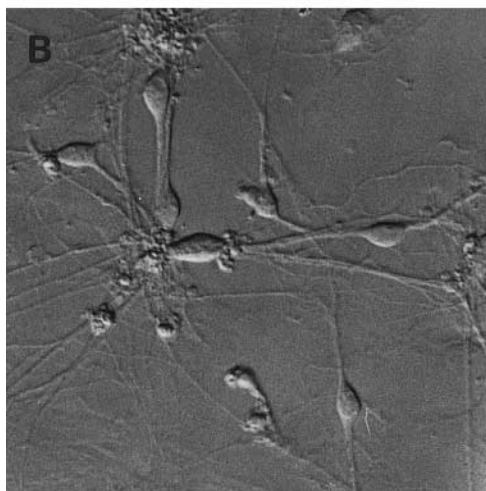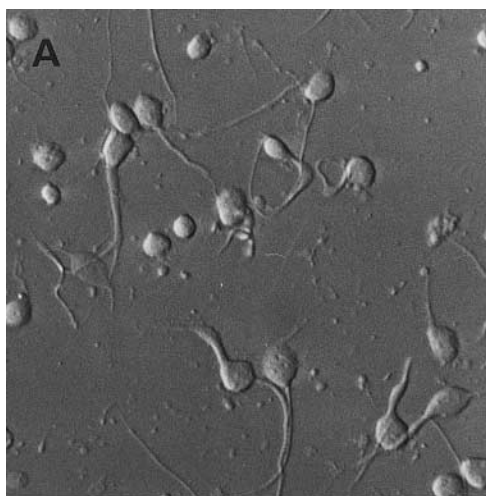
appears to be accounted for by corresponding changes in agrin$_{z19}$ transcripts that begin at a low level, peak during the first week and decline during subsequent development. In contrast, the fraction of agrin transcripts represented by z11 mRNAs decreased with culture age, whereas z8 mRNAs were only clearly detected after the first week.

Previous studies suggest that all neurons in cortex express agrin. The overall similarity in patterns of agrin gene expression in somatosensory cortex and somatosensory cortical neurons grown in cell culture suggests that the classes of neurons present in cell culture are similar to those in somatosensory cortex. To test this hypothesis we used a multiplex RT-PCR approach to examine expression of agrin, CAM IIK, a marker for glutamatergic neurons,[6] and the high molecular weight form GAD$_{67}$ as a marker for cortical GABAergic neurons,[16] in RNA isolated from a single plating of somatosensory cortical neurons. As shown in Fig. 6, PCR products representing agrin, CAM IIK and GAD$_{67}$ mRNAs could be co-amplified from a single RNA preparation consistent with the presence of both excitatory and inhibitory neurons in these cultures.

To examine whether functional synaptic connections were formed between cultured neurons, we used whole-cell patch-clamp electrophysiology to monitor the membrane potential of individual neurons over random 12 s time-intervals to determine if spontaneous PSPs could be detected. The results of a typical experiment are illustrated in Fig. 7 which shows spontaneous PSPs, with two distinct profiles, recorded from neurons during the first week in culture. Whereas the high frequency barrage of PSPs (filled arrowheads) seen in control solutions was unaffected by bath application of the GABA$_A$ receptor antagonist BMC, it was completely blocked by 5 mM CNQX+50 μM APV, which block non-*N*-methyl-D-aspartate (NMDA) and NMDA type glutamate receptors. In contrast, a series of discrete PSPs (open arrowheads), recorded from the same cell were completely blocked by BMC but unaffected by the mixture of gluatamate receptor blockers. Other cells exhibited patterns of PSPs whose pharmacological profile was predominantly GABAergic or glutamatergic (data not shown). Thus, during the first week *in vitro*, coincident with changes in the pattern of agrin gene expression, synaptic connections mediated by

Fig. 4. Cultured somatosensory neurons survive and differentiate in a defined medium conditioned by non-neuronal cells. Neurons were plated onto poly-D-lysine-coated coverslips placed on top of a feeder layer of non-neuronal cells. One hour after plating (A) neurons with phase-bright cell bodies and short processes that remain intact during the dissociation procedure are visible. By six days in culture (B) neurites can be seen connecting individual neurons and small groups of cells. Neurite outgrowth continues in culture such that by 12 days (C) neurons appear interconnected by an extensive neuritic web. Scale bar=30 μm.
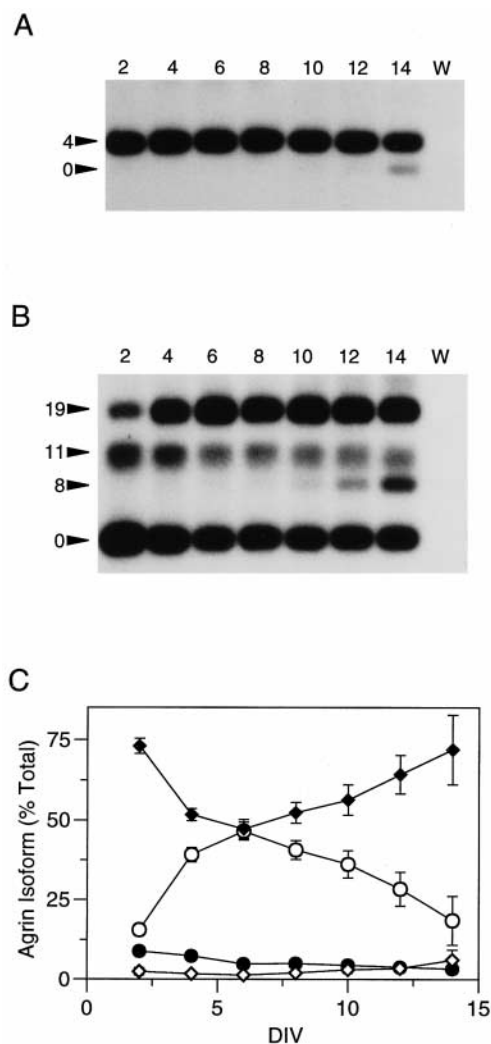
Fig. 5. Expression of alternatively-spliced agrin mRNAs by cultured somatosensory cortical neurons. The relative abundance of alternatively spliced agrin mRNAs in RNA isolated from different age cultures was assayed using RT-PCR with primers flanking the y (A) or z (B) sites. Numbers show days in culture; control lanes in which water was used in place of RNA during the reverse transcription step are indicated by W. Similar to the pattern of expression *in vivo*, virtually all agrin transcripts expressed in culture are y4 mRNAs, y0 mRNAs being at or below the level of detection at all ages examined. In contrast, changes in relative abundance of the z splicing isoforms however were evident in the autoradiograms and when each isoform was expressed as a percent of total agrin mRNA following quantitative analysis of the PCR products using a phosphorimager (C). The transient increase in agrin$_{z19}$, early decline in agrin$_{z11}$ and late appearance of agrin$_{z8}$ are similar in pattern to that seen *in vivo*. Data points represent the mean of values obtained from RNA isolated from four to seven independent platings. Agrin$_{z0}$, filled diamond; $_{z8}$, open diamonds; $_{z11}$, filled circles; $_{z19}$, open circles. Bars indicate S.E.M.

both glutamate and GABA are formed between cultured somatosensory cortical neurons grown in dissociated cell cultures.
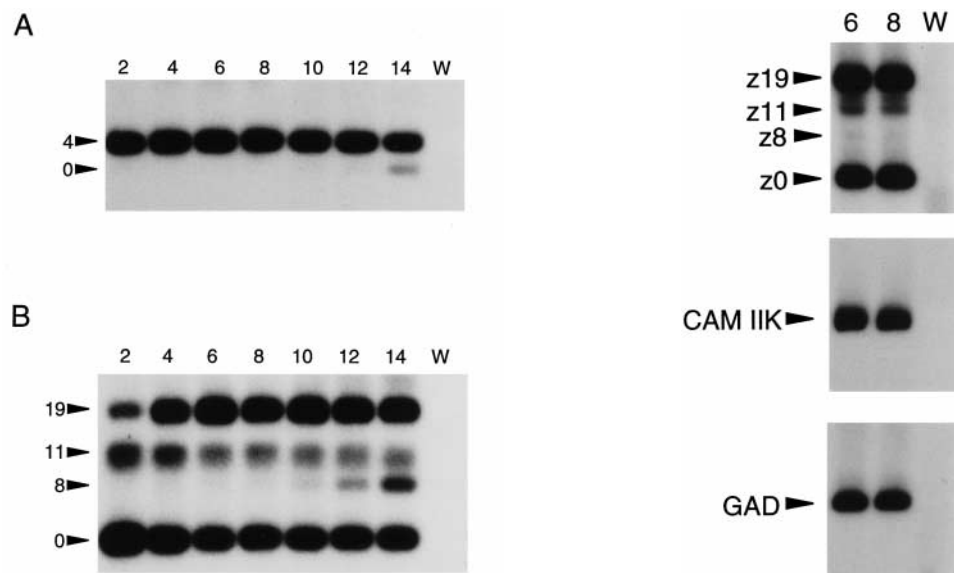
DISCUSSION

In its original formulation, the agrin hypothesis[38] describing agrin's role in the formation and maintenance of the neuromuscular junction, rested on three observations: agrin's known biological activity, its expression by motorneurons, and localization in the neuromuscular junction synaptic basal lamina (reviewed in Ref. 8). Whereas the finding that agrin is expressed throughout the nervous system has raised the possibility that agrin might serve a similar role at synaptic contacts formed between neurons, relatively little progress has been made towards testing the remaining core predictions made by the hypothesis for synapse formation between neurons, in large part due to lack of a suitable CNS system that is amenable to study at the cellular and molecular level. Here we have begun to address this issue by examining agrin gene expression in neurons in the rodent neocortex, both *in vivo* and *in vitro*.

Agrin gene expression in developing somatosensory cortex

Alternative splicing of agrin pre-mRNA is tissue specific and gives rise to agrin isoforms that differ markedly in biological activity. In particular, mRNAs that include exon 28 encoding four amino acids at the y site are found almost exclusively in the nervous tissue and are restricted to neurons or their precursors.[30,35,47,55] Although characteristic of

Control   CNQX+APV   BMC   CNQX+APV   Control
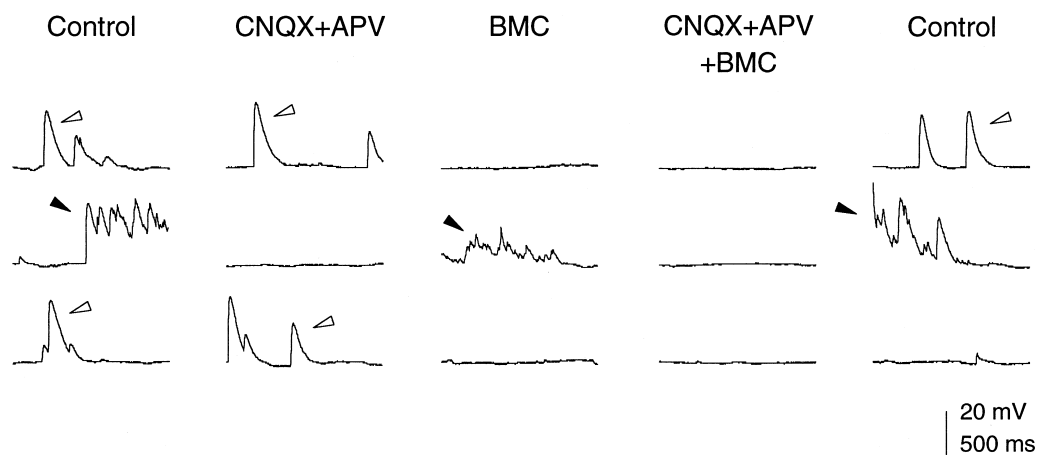   +BMC

20 mV
500 ms

Fig. 7. Functional excitatory and inhibitory synaptic contacts form between cultured somatosensory cortical neurons. Electrophysiological record taken from a single neuron at seven days in culture. In control external medium, PSPs with two distinct profiles can be seen. Discrete PSPs are shown in the upper and lower trace (open arrowheads) while the middle trace contains an example of a high frequency barrage (filled arrowheads). Blockade of the high frequency barrage by 5 µM CNQX+50 µM APV suggests that it was mediated by glutamate whereas application of 2 µM BMC blocked the discrete PSPs indicating that these events are mediated by GABA. Only when CNQX, APV, and BMC were applied simultaneously was all spontaneous activity blocked. Recovery of both glutamatergic and GABAergic events was observed when the cell was returned to control external medium. Solutions were bath applied and contained 100 µM 4AP, to increase the frequency of spontaneous events, in addition to the drugs listed. The cell was held at −75 mV and a minimum of six 12 s recording episodes were obtained in each solution.

somatic tissues such as skeletal muscle, liver, and kidney,[30,35,47] *in situ* hybridization studies have reported significant levels of agrin$_{y0}$ mRNA in blood vessel endothelia in brains of embryonic and early postnatal rats.[55] Based on the relative abundance of agrin$_{y0}$ and $_{-y4}$ variants, our data suggest that non-neuronal cells contribute less than 5% of all agrin mRNAs in somatosensory cortex and that almost all postnatal agrin expression in somatosensory cortex therefore has a neuronal origin. In as much as we were unable to detect any significant change in relative abundance of the two y site variants over time, our data are consistent with those of an earlier study[30] using similar techniques to look at agrin expression in RNA isolated from whole rat brain. Agrin$_{y0}$ expression in mouse somatosensory cortex however, was considerably lower than in rat,[30] a difference that might be species-specific or reflect regional differences in agrin expression. These data support our earlier findings using *in situ* hybridization demonstrating that in adult rat brain agrin gene expression is predominantly neuronal.[41]

Alternative splicing at the y and z sites is regulated in a cell specific manner[30,47,53] and has been shown to influence agrin's biological activity.[17,18,47,60] As shown here and elsewhere[30,36,57] alternative splicing of agrin in nervous tissues is also developmentally regulated such that y4z11, y4z19, and y4z8 mRNAs that encode active agrin, characteristically peak at early, middle, and late stages of development, respectively. This tightly regulated stereotypic pattern of expression suggests that each isoform serves a function that is adapted for each developmental stage. Little is known however about specific roles of the different agrin isoforms or the mechanisms that regulate their expression.

Whereas cortical neurogenesis occurs primarily during embryonic development the formation of cortical laminae and segregation of neurons among them is primarily a postnatal event; only layers I, V and VI are present at birth, the remaining cortical layers differentiating during the first postnatal week.[46] During this time thalamic afferent fibres enter the cortex[3,4,51] and establish synaptic contacts with neurons primarily located in layers V/VI and IV.[1,2] Intracortical synapses are also formed at this time.[1,33] This active period of development coincides with marked changes in agrin gene expression. At birth, total agrin mRNA levels in somatosensory cortex are low but within a few days increase several fold to peak levels that are maintained throughout the first postnatal week. Underlying these changes in total agrin mRNA are variation in y4z0, y4z11 and y4z19 transcript levels. These isoforms therefore, are more likely to play a role in early developmental events whereas increases in agrin$_{y4z8}$ mRNA that occur in the second postnatal week are more likely to be important in cortical maturation.

Agrin gene expression in cultured cortical neurons

Agrin mRNA is also present in cortical neuron cultures where it shows a pattern of expression similar to that observed during development of somatosensory cortex *in vivo*. Based on morphology, the majority of the cells in the cultures are neurons

and, consistent with this observation, virtually all of the agrin mRNA extracted from cultured cells is the neuron-specific y4 isoform. Interestingly, all four agrin mRNAs resulting from alternative splicing at the z site are also expressed in culture and, over time, exhibit changes in relative abundance that mimic those *in vivo*. These results suggest that many of the cellular interactions necessary for normal patterns of agrin gene expression observed during development *in vivo* also occur in cell culture.

We cannot rule out the possibility that developmental changes in alternative splicing are cell autonomous. Previous studies however, have suggested that maintenance of the normal pattern of alternative splice of agrin mRNAs is target-dependent[56] and that agrin gene expression can be influenced by neuronal activity.[42] Soluble growth factors may also be important as demonstrated by the observation that nerve growth factor treatment specifically up-regulates levels of agrin$_{y4z8}$ mRNA in PC12 cells.[52] Our data demonstrate that cultured somatosensory cortical neurons form functional synaptic contacts with one another that result in a measurable level of spontaneous neuronal activity that may also be sufficient to fulfill any requirements or dependence upon target tissue. Non-neuronal cells used as feeder layers are a likely source of growth factors. It will be interesting to learn whether pharmacologic blockade of either glutamatergic and/or GABAergic transmission within the cultures or growth of neurons over feeder layers derived from cortices of mice lacking functional genes for specific growth factors has any effect on agrin gene expression in these cultured neurons.

CONCLUSION

In summary, the pattern and developmental regulation of agrin gene expression in mouse somatosensory cortex *in vivo* and in cell culture is consistent with the hypothesis that agrin plays a role in the formation of neuron–neuron synapse in the mammalian CNS. One possibility, based on agrin's function at the neuromuscular junction, is that agrin plays a direct role in organizing postsynaptic components at neuronal synapses. Consistent with this proposal, MusK, a muscle specific tyrosine kinase, that acts as a functional agrin receptor at the neuromuscular junction,[14,26] and alpha-dystroglycan, an agrin binding protein,[7,11,22] are both expressed in brain.[14,21,61] An alternate or additional function for agrin, however, is suggested by results showing that agrin is a NCAM-binding protein[59] and can act as an adhesive substrate influencing neurite outgrowth and differentiation,[10] raising the possibility that agrin might be important in guiding axonal growth and dendritic arborization in cortex. Recent generation of agrin-deficient mice now makes it possible to test these hypotheses directly. As expected, mice lacking a fully functional agrin gene exhibit significant defects in neuromuscular synaptogenesis,[20] Although these animals die shortly before birth, neurons from cortices of embryonic agrin deficient mice can be grown in cell culture and examined for alterations in synapse formation or neurite outgrowth.

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