Agrin isoforms and their role in synaptogenesis

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Agrin is thought to mediate the motor neuron-induced aggregation of synaptic proteins on the surface of muscle fibers at neuromuscular junctions. Recent experiments provide direct evidence in support of this hypothesis, reveal the nature of agrin immunoreactivity at sites other than neuromuscular junctions, and have resulted in findings that are consistent with the possibility that agrin plays a role in synaptogenesis throughout the nervous system.

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Introduction

The basal lamina, plasma membrane and cytoplasm of muscle fibers at neuromuscular junctions are characterized by aggregates of proteins. Some of these aggregates play a direct role in synaptic transmission and are thus crucial for neuromuscular function. For example, the plasma membrane of the muscle fiber contains a high concentration of acetylcholine receptors (AChRs), and the myofiber basal lamina, which occupies the 50 nm wide cleft between the axon terminal and muscle fiber, contains a high concentration of acetylcholinesterase (AChE). The axon terminal induces the muscle fiber to form the protein aggregates during synaptogenesis in the embryo and its presence is required for their maintenance in the adult.

Several lines of evidence have led to the hypothesis that the basal lamina protein, agrin, mediates axon-terminal induction and maintenance of the protein aggregates [1–5]. For example, it has been amply shown that agrin mimics the effect of motor neurons in aggregating AChRs on myotubes, that the cell bodies of motor neurons synthesize agrin and transport it to the axon terminals at the neuromuscular junction, and that active agrin is bound to basal lamina in the synaptic cleft.

During the past year experiments have provided direct evidence for the first time that agrin is released by motor neurons and that motor neuron-released agrin induces the formation of the postsynaptic protein aggregates on muscle fibers. In addition, it is now evident that, through alternative splicing, the agrin gene codes for a number of agrin isoforms that differ in function and distribution among many different tissues throughout the body. Several new findings further indicate that agrin is contained by neurons other than motor neurons and that it may mediate the formation of the postsynaptic apparatus at neuron–neuron synapses throughout the nervous system. This review summarizes some of the key experiments in this field of research.

Agrin released by axon terminals directs muscle fibers to form postsynaptic apparatus

Motor neurons cultured with myotubes induce the myotubes to form aggregates of AChRs at neuromuscular junctions, much like those formed *in vivo* [6,7]. Reist *et al.* [8••] raised an antiserum against ray agrin that blocked its AChR-aggregating activity on cultured chick myotubes. When they cultured chick motor neurons with chick myotubes in the presence of the antibodies they found that it blocked the formation of the motor neuron-induced AChR aggregates on the myotubes. This demonstrated that the antibodies block the activity of chick agrin, as they block that of ray agrin, and provides the first direct evidence that agrin plays a role in the formation of the postsynaptic apparatus at developing neuromuscular junctions.

Muscle fibers, as well as motor neurons, synthesize agrin isoforms and express them on their surface [9]. Accordingly, the above experiment did not reveal whether the agrin directly involved in mediating the motor neuron-induced AChR aggregation was provided by motor neurons or muscle fibers. When Reist *et al.* [8••] cultured rat motor neurons with chick myotubes in the presence of the antibodies to ray agrin, they found that the motor neuron-induced formation of AChR aggregates on the myotubes was not inhibited. Thus, these antibodies did not block the AChR-aggregating activity of agrin released by rat motor neurons, nor did they prevent chick myotubes from responding to it. This finding [8••], coupled with the antibody blocking of AChR aggregation in chick motor neuron–chick myotube co-cultures, provides

Abbreviations

AChE—acetylcholinesterase; **AChR**—acetylcholine receptor; **ARP**—agrin-related proteins; **PCR**—polymerase chain reaction.

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strong evidence that agrin released by the motor neuron induces the formation of AChR aggregates at the neuromuscular junction.

Cohen and Godfrey [10••] examined the problem in a different way, but obtained results leading to the same conclusion. They co-cultured frog motor neurons with muscle fibers and used histochemistry to study the timing of appearance of agrin and AChR aggregates at the neuromuscular junction; antibodies that recognize agrin of *Rana pipiens* but not of *Xenopus laevis* were used as agrin markers. They found that when AChRs began to aggregate at the neuromuscular junctions formed by *R. pipiens* motor neurons and *X. laevis* muscle cells, anti-*R. pipiens* agrin antibody staining was present at the junction. On the other hand, when *X. laevis* motor neurons were grown with *R. pipiens* myotubes, the antibody staining was found at various spots on the muscle cell surface, but not at neuromuscular junctions. These findings demonstrate that detectable levels of agrin isoforms synthesized by motor neurons in the motor neuron–muscle cell cultures were present at the appropriate time and place to induce the formation of AChR aggregates on muscle cells, while agrin isoforms synthesized by muscle cells were not.

The agrin gene codes for several protein isoforms that differ in function

Agrin cDNAs have been isolated from ray, chick and rat gene libraries. Because the only agrin that has been purified is from ray, ray agrin cDNA was the first to be cloned [3,11••]. Northern blot analysis and sequence data have revealed that the ray cDNA codes for the carboxyl-terminal half of the protein. Ray cDNA has since been used to isolate homologous cDNAs from chick and rat libraries [12••–14••]. These cDNAs also only code for the carboxyl-terminal half of their proteins, but full-length sequences have been obtained for chick and rat by overlapping cDNAs from primer extension libraries.

The proteins encoded by the full-length cDNAs for chick and rat are approximately 1900 amino acids long, with predicted molecular weights of approximately 200 kD. The mRNA size for both species is about 8 kb. Two messages, of approximately 7.5 and 9 kb, are detected in northern blots of ray tissue; the two sizes may be due to variation in the size of the 5′ and/or 3′ untranslated regions. As illustrated in Fig. 1, agrin has a number of domains that show similarities with regions of other extracellular proteins. The nature, number and polarized distribution of the different domains is consistent with the idea of agrin being multifunctional, i.e. interacting with more than one protein.

Studies on ray agrin and its cDNA revealed that the carboxyl-terminal half of the full-length protein is sufficient for aggregating AChRs, AChE and other postsynaptic proteins on the surface of cultured myotubes [11••]. Tsim *et al.* [13••] and Ruegg *et al.* [14••] transfected constructs of three chick cDNAs, each of which coded for the carboxyl-terminal half of their proteins, into COS cells. Each of the constructs generated protein that could be immunoprecipitated by anti-agrin antibodies from the medium bathing the COS cells. One of the constructs, cDNA CBA-1, generated protein that induced myotubes to form AChR and AChE aggregates similar to those induced by purified ray agrin, and was thus designated agrin. The proteins generated by the other two cDNA constructs were inactive and were designated agrin-related proteins, ARP-1 and ARP-2. Their amino acid sequences were identical to that of agrin except that near the carboxyl-terminus they both lacked an 11 amino acid stretch at a position designated B and in addition ARP-2 lacked a 4 amino acid stretch at a position designated A (Fig. 1). The use of chimeric constructs revealed that both of the missing amino acid stretches were required for AChR-aggregating activity of agrin. Southern blot and exon/intron analysis provided evidence that all three proteins are derived from alternative splicing of precursor mRNA from a single gene.

The ray and rat cDNAs code for proteins corresponding to ARP-1 in chick, i.e. they have the 4 amino acid insert at position A(A_4) but no insert at position B(B_0). As expected, when a construct of the partial ray agrin cDNA was transfected into COS cells, these cells generated protein that was inactive in AChR aggregation on cultured chick myotubes [14••]. On the other hand, when Scheller and his group [15] transfected full-length rat cDNA lacking the B_{11} insert into COS or Chinese hamster ovary cells, and co-cultured the cells with rat myotubes, above background levels of AChR aggregates were formed at some of the transfected cell–myotube contacts. The minor activity of rat ARP-1 observed on rat myotubes might be due to differences in species, assay technique or to the use of rat full-length protein. (It is not yet known whether motor neurons or any other cells that express agrin *in vivo* secrete the full-length protein.) Indeed, when Scheller and colleagues [16••] later examined the effect of full-length rat ARP-1 on mouse and chick muscle cells they observed that this full-length protein failed to aggregate AChRs; similarly we have found that while the carboxyl-terminal half of chick agrin is active on rat myotubes, the carboxyl-terminal half of chick ARP-1 is not (S Kröger, UJ McMahan, unpublished data).

In the light of the findings described above, Scheller and colleagues [16••] began searching for mRNA in rat that coded for isoforms with the 11 amino acids by polymerase chain reaction (PCR) on the oligonucleotides flanking position B. They found not only isoforms with an 11 amino acid insert at position B, but also two other isoforms with inserts of 8 and 19 amino acids at the same position. The 19 amino acid insert was a combination of the 8 and 11 amino acid stretches. When full-length ARP-1 cDNA constructs containing inserts coding for the 8, 11 or 19 amino acids at position B were transfected into COS or Chinese hamster ovary cells, the cells generated protein that was significantly more active than ARP-1 on rat myotubes and, unlike ARP-1, active on chick and mouse myotubes. We have also recently detected mRNA encoding the B_{19} isoform in chick, and the B_{19}, B_{11} and B_8 isoforms in ray ([14••]; M Ruegg *et al.*, unpublished

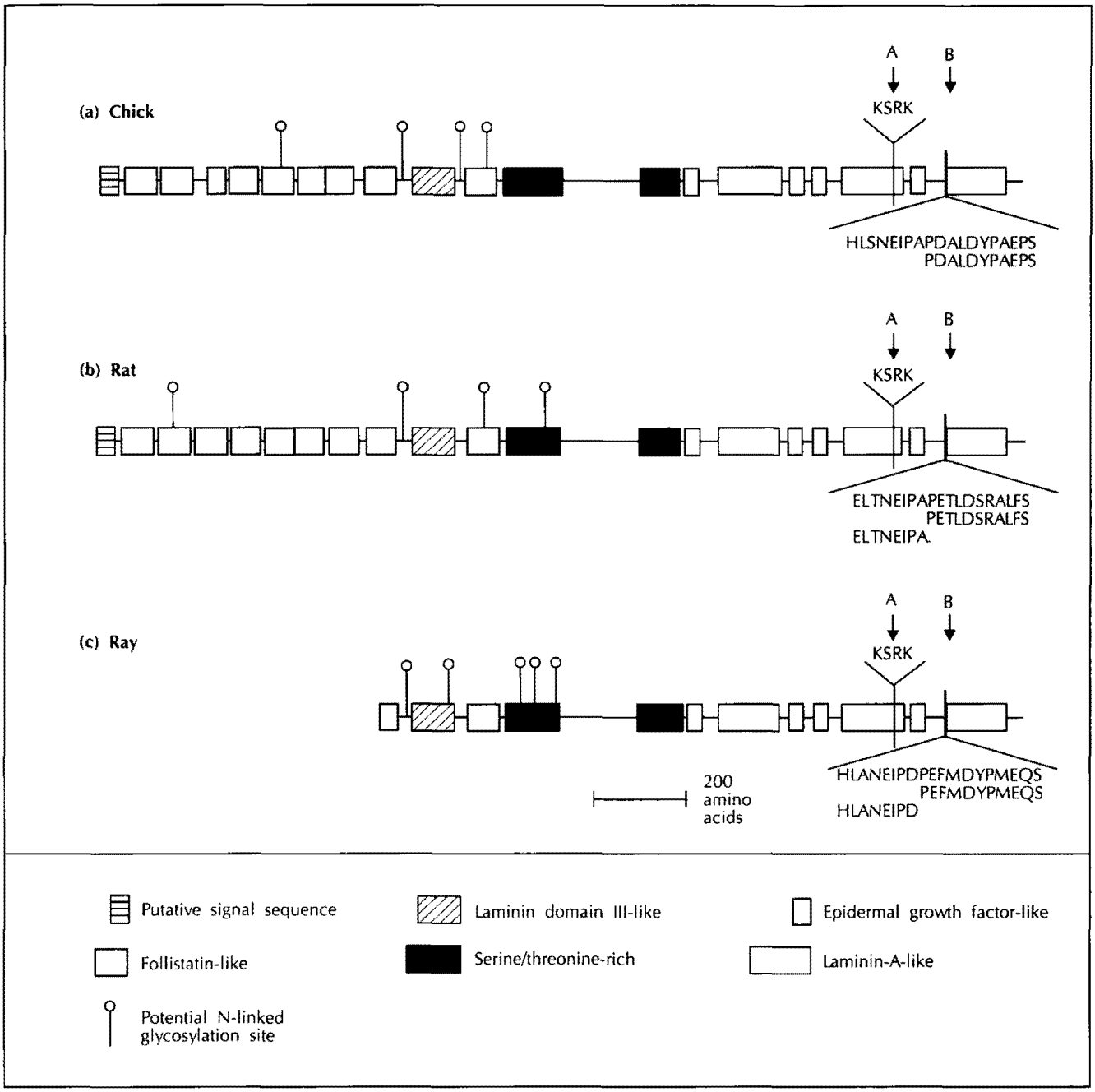


Fig. 1. Schematic representation of the domain structure of agrin isoforms predicted from chick, rat and ray cDNAs. The positions of A and B inserts are indicated. Alternative B inserts are shown in each case. The amino acid sequences of the inserts are in the one-letter code. Adapted from Tsim *et al.* [13••], Ferns and Hall [22] and Smith *et al.* [11••].

data). Extensive PCR analysis of chick central nervous system tissues revealed that all detectable mRNAs containing B inserts also have the A_4 insert [14••]. If it is correct to assume that the native B_8 and B_{19} isoforms contain an insert at position A and that the native B_{19} and B_8 isoforms do not have another insert that blocks the active domain, these results suggest that the agrin gene codes for at least three isoforms of agrin that are active in AChR aggregation on myotubes across species and at least two isoforms that are not. The following experiments indicate that the three native B protein isoforms are indeed active.

Motor neurons express agrin isoforms with B inserts

As a step toward determining which isoforms of agrin neurons contain, Tsim *et al.* [13••] dissociated cells of the embryonic day 6 chick spinal cord and separated them on a metrizamide gradient to obtain two fractions: a motor neuron-enriched fraction and a fraction enriched in other cells including non-motor neurons. Using PCR methods they found that the amount of B_{11} mRNA was

greater than B_0 mRNA in motor neuron-enriched preparations, and the amount of B_0 was greater than B_{11} in non-motor neuron enriched preparations, which led to the suggestion that motor neurons synthesize agrin B_{11}. More recently, using improved PCR methods we examined the relative abundance of mRNAs encoding agrin B_0, B_{11} and B_{19} from total chick spinal cord and spinal cord fractions enriched for motor neurons in several different ways (M Ruegg *et al.*, unpublished data). In every case of motor neuron enrichment B_{11} and B_{19} were enriched, but the greater enrichment by far was in B_{19}.

Enrichment of rat spinal motor neurons revealed an enrichment of mRNAs encoding B_8, B_{11} and B_{19}. Again the greatest enrichment by far was in mRNA encoding B_{19} isoforms. On the other hand, the electric lobe of ray brain, which contains the large, closely packed motor neurons that innervate the electric organ from which agrin has been purified, was vastly enriched in mRNA encoding B_8 isoforms over the rest of the brain. Thus, the agrin synthesized by many if not all motor neurons in the spinal cord of chick and rat appears to be the B_{19} isoform, while agrin synthesized by motor neurons of the ray's electric lobe appears to be the B_8 isoform. The modest enrichment of B_8 and B_{11} in the rat motor-neuron enriched preparations and B_{11} in chick motor-neuron enriched preparations may be due to non-motor neuron contaminants, different populations of motor neurons or the co-expression of different isoforms by the same motor neurons.

Agrin isoforms in the basal lamina of non-neural tissue

Agrin immunoreactivity is present in basal lamina in a variety of non-neural tissues [17]. Ruegg *et al.* [14••] examined by PCR chick mRNA extracted from skeletal muscles taken at different times during embryogenesis and after hatching and from myotubes grown in tissue culture. Only B_0 isoforms were detected. We have now examined a variety of other non-neural tissues in the chick embryo including heart, lung, kidney and liver by PCR. In these tissues the only agrin isoforms detected were B_0 (Table 1). Godfrey [18] has reported that anti-agrin immunopurified extracts from some non-neural tissues have a low level of AChR-aggregating activity. As experiments described above indicate that B inserts are required for AChR-aggregating activity [13••], the B_0 isoforms of agrin present in non-neural tissue seem unlikely sources of the activity.

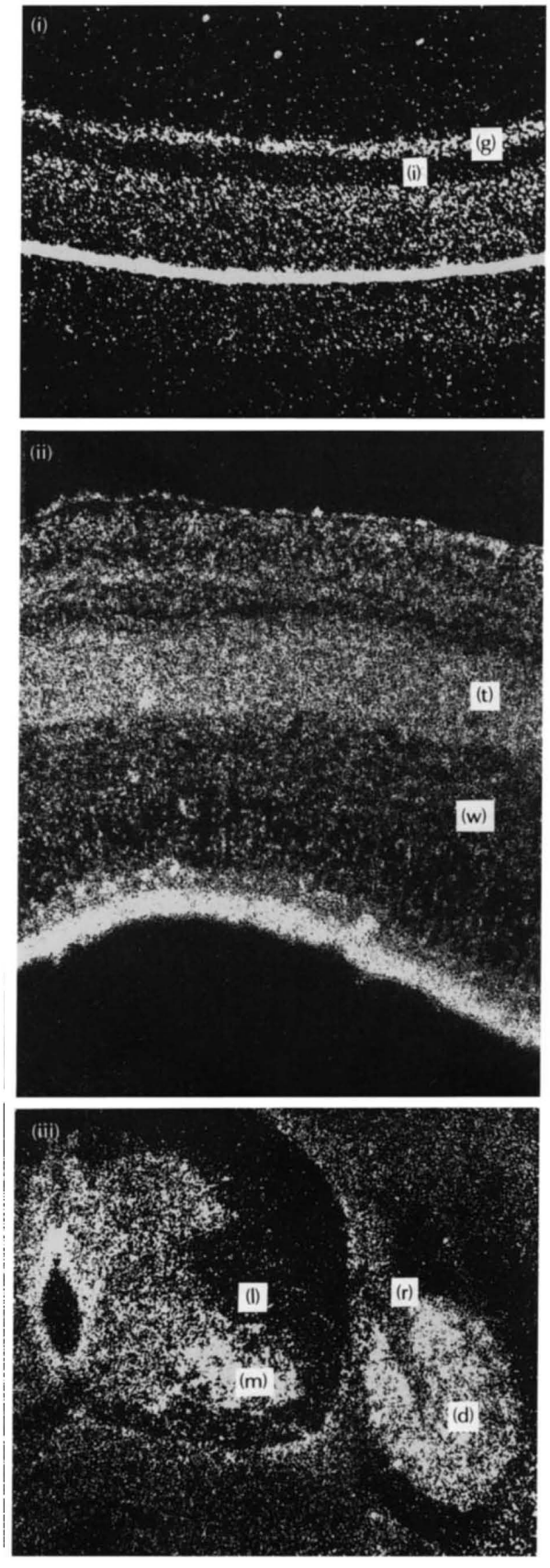
Distribution of agrin isoforms in nervous tissue

In embryo and adult animals of several species, antibodies to agrin stain the basal lamina of Schwann cells in nerves and ganglia of the peripheral nervous system and the basal lamina that occupies the space between capillary endothelium and astrocytes in the central nervous system [19]. The antibodies also intensely stain the cell bodies of motor neurons [20]. Recent *in situ* hybridization studies by Smith *et al.* [11••] have indicated that in the brain of the adult ray certain groups of neurons other than motor neurons express mRNA that encodes agrin isoforms. Low but above background levels of labelling were also observed for white matter, which contains mRNA only of non-neural cells. These cells include endothelial cells and astrocytes, which border on the capillary basal lamina.

Table 1. Distribution of agrin B_0, B_{11} and B_{19} isoforms in chick embryos.

	B_0	B_{11}	B_{19}
Central nervous system			
Forebrain	+	+	+
Optic tectum	+	+	+
Cerebellum	+	+	+
Spinal cord	+	+	+
Retina	+	+	–
Optic nerve	+	–	–
Neural tube	+	–	–
Peripheral nervous system			
Ciliary ganglion	+	–	+
Dorsal root ganglion	+	+	+
Sciatic nerve	+	–	–
Non-nervous system tissue			
Skeletal muscle	+	–	–
Liver	+	–	–
Kidney	+	–	–
Heart	+	–	–

We have now extended these studies by making a detailed examination of the central and peripheral nervous systems by *in situ* hybridization and PCR. As the expression of agrin mRNA in the cell bodies of motor neurons is greatly increased during the period of embryogenesis when the neurons are forming neuromuscular junctions (G Escher *et al.*, abstract, Society of Neuroscience, 1991; 17:179) we concentrated on the 10–13 day embryonic chick central nervous system, when synaptogenesis overall is high. The results are summarized in Table 1 and Fig. 2 (S Horton *et al.*, unpublished data). The *in situ* hybridizations revealed heavy labelling in many regions of the brain and spinal cord that were rich in the cell bodies of neurons, where neuronal proteins are synthesized. There was much less labelling in regions in which neuron cell bodies were sparse or absent. PCR using mRNA from regions of the central and peripheral nervous systems containing neuronal cell bodies revealed mRNA that coded for isoforms with B inserts. On the other hand, the optic nerve, a central nervous system white matter tract that contains mRNA of glial and endothelial cells but not neurons, showed only B_0 isoforms. Similarly, peripheral nerve that contained the mRNA of Schwann and other non-neuronal cells but not neurons, also contained agrin mRNA that coded only for isoforms with no B inserts. Together these findings suggest that neurons other than motor neurons synthesize isoforms of agrin that have B inserts, and that in most if not all cases where non-neuronal cells in the nervous system synthesize agrin isoforms, the isoforms lack the B inserts.



We also used PCR to examine chick neural tube mRNA at stages before the birth of neurons and glial cells to determine the isoforms of agrin synthesized by the progenitor cells. The only isoforms detected were B_0 (Table 1). As isoforms containing B inserts are detected in chick embryo motor neurons at day 5–6 (during the time they differentiate from progenitor cells), it seems that the differentiation of motor neurons and other neuronal types that contain B inserts is accompanied by a switch in alternative splicing of agrin isoforms.

Conclusions

Two different experiments [8••,10••] involving the use of antibodies to agrin in nerve–muscle co-cultures provide evidence that motor neurons release agrin at neuromuscular junctions and that this agrin mediates the motor neuron-induced aggregation of AChR, AChE and other postsynaptic protein aggregates on myotubes. They also provide strong evidence against the often raised possibility that agrin isoforms produced by muscle fibers trigger the formation of AChR aggregates at neuromuscular junctions (e.g. [21]). The problem remains, however, as to whether the findings of the *in vitro* experiments can also be applied to neuromuscular junction formation *in vivo*. The findings [14••] that indicate that motor neurons *in vivo* synthesize agrin isoforms containing amino acid inserts required for AChR aggregating activity, while muscle fibers do not, strongly suggest that the situation is similar *in vivo* and *in vitro*.

The *in situ* hybridization and PCR studies on the distribution of agrin isoforms in the central nervous system indicate that many neuron types contain agrin isoforms with B inserts and in chick, at least, all isoforms with B inserts also have A inserts, both of which are required for AChR-aggregating activity. These findings, together with the previous observation that extracts of regions of the central nervous system containing negligible numbers of motor neurons can be as rich in active agrin as the spinal cord [22], support the hypothesis [3] that agrin directs the formation of postsynaptic protein aggregates at neuron–neuron synapses throughout the nervous system, as at the neuromuscular junction. The *in situ* hybridization and PCR studies further indicate that non-neural cells also synthesize agrin isoforms but they lack the B inserts required for AChR-aggregating activity on muscle fibers. Accordingly, these studies, coupled with PCR experiments on several non-neural tissues revealing only agrin isoforms lacking the B inserts, raise the possi-

Fig. 2. *In situ* hybridization using agrin anti-sense riboprobe showing (i) retina, (ii) optic tectum and (iii) spinal cord in 10 day chick embryo. Grains are most intense over neuronal cell body layers such as the ganglion cell layer of the retina (g) and tectal plate (t), and over spinal motor neurons (m) and dorsal root ganglion (d). Hybridization signal is very sparse over white matter fiber layers such as the retinal inner plexiform layer (i), tectal white stratum (w), lateral spinal cord (l) or dorsal root (r).

bility that isoforms containing B inserts are specific to neurons. As neuronal precursor cells in the embryo lack the B inserts it would seem that the nerve cell, by alternative splicing involving inserts comprising less than 1 % of the amino acid sequence of the full length protein, has adapted a rather widespread extracellular matrix protein for specific use in the formation of cell–cell contacts of fundamental importance for neural communication.

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