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Axonal targeting of agrin in cultured rat dorsal horn neurons

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SUMMARY

Agrin, a synaptic basal lamina protein synthesized by motoneurons is involved in the aggregation of nicotinic acetylcholine receptors (nAchRs) at the neuromuscular junction. Agrin transcripts are broadly expressed in the central nervous system (CNS) including non-cholinergic regions. This wide distribution of agrin mRNAs raises the question of its function in these areas. To approach this question, we analysed the expression and cellular distribution of agrin in primary cultures of rat embryonic dorsal horn neurons. Polymerase chain reaction analysis demonstrated that the four agrin isoform (B_0, B_8, B_{11}, B_{19}) mRNAs are expressed as early as 4 days in vitro, before the formation of functional synaptic contacts. Western blots also showed that agrin-like proteins are secreted in conditioned medium from 7 days cultures. We analysed the subcellular distribution of agrin by double immunolabeling and fluorescence microscopy. We found that agrin is synthesized by almost all neurons and was present in the somata and in the axons but not in dendrites within the sensitivity of the detection. This intra-axonal localisation of agrin could only be seen after permeabilization. Furthermore, agrin immunoreactive axons were found adjacent to gephyrin, the postsynaptic glycine receptor-associated protein. Altogether, our results suggest that, as established at the neuromuscular junction, agrin may be involved in pre- to postsynaptic interactions in the central nervous system.

Key words: Agrin, Central nervous system, Axon, Gephyrin, Spinal cord

INTRODUCTION

In the central nervous system, several ionotropic receptors for neurotransmitters are concentrated at postsynaptic differentiations. For example, glycine receptors (GlyRs) (Triller et al., 1985, 1987) and some subunits of GABA and of glutamate receptors form clusters under the presynaptic zone (Baude et al., 1992; Petralia and Wenthold, 1992). Mechanisms underlying the postsynaptic receptor clusterization and, more precisely, the involvement of presynaptic innervation are largely unresolved in the central nervous system. In comparison, much more information is available concerning the formation of neuromuscular junction (NMJ). One of the first events during the development of NMJ is the accumulation of nicotinic acetylcholine receptors (nAchRs) under the nerve terminal (Hall and Sanes, 1993). This early interaction between the afferent nerve and the muscle fiber is thought to be mediated by agrin, a synaptic basal lamina protein synthesized by motoneurons, and released by the motor axon at the nerve terminal (Magill-Solc and McMahan, 1988, 1990; McMahan, 1990; Ruegg, 1996).

Interest in this molecule has led to the isolation of three agrin cDNAs in rat (Rupp et al., 1991) chick (Tsim et al., 1992) and marine ray (Smith et al., 1992). Analysis of the predicted amino acid sequence revealed that agrin is a large multidomain protein of about 200 kDa (Rupp et al., 1991; Tsim et al., 1992). Expression studies showed that the carboxy-terminal half of the protein is sufficient for inducing nAchR aggregation on cultured myotubes (Ferns et al., 1993; Gesemann et al., 1995; Nitkin et al., 1987; Ruegg et al., 1992; Tsim et al., 1992). The tuning of this clustering activity is regulated by alternative splicing events. More precisely, while several splice sites have been found in both chick (Ruegg et al., 1992; Tsen et al., 1995b; Tsim et al., 1992) and rat (Rupp et al., 1992), one of them, near the C terminus, called B in chick or Z in rat has been associated with the nicotinic acetylcholine receptor (nAChR) aggregating activity. At this site, the lack of inserts results in the expression of a form of agrin with little or no activity. In contrast, insertion of 8, 11 or 8+11 residues yields active forms of agrin, those with 8 and 19 amino acid inserts being the most active (Ferns et al., 1992, 1993; Gesemann et al., 1995). In this paper the variants are named B_0, B_8, B_{11}, B_{19} in accordance with the first observation (Ruegg et al., 1992).

Expression of the alternatively spliced agrin mRNAs appears to be regulated in a cell type-specific as well as in a tissue-specific manner. Thus, PCR experiments performed on motor neuron-enriched fractions as well as in situ hybridizations with isoform specific probes show that transcripts for agrin active forms are concentrated on motoneurons (Ma et al., 1995; Stone and Nikolics, 1995; Tsim et al., 1992). In contrast, as established by RT-PCR studies in the optic and sciatic nerves (McMahan et al., 1992) and more recently in non-neuronal cultures prepared from rat embryonic spinal cord (S. Levi, unpublished observations) the B_0 isoform is the only form present in glial cells. A convincing demonstration of this cell-specific expression has been performed in chick

ciliary ganglia (Smith and O'Dowd, 1994). There, as shown by single cell RT-PCR, the B_0 isoform is the only variant expressed in non neuronal cells while neurons synthesize any combination of one or more agrin mRNAs forms including the B_0 form.

Northern blot, in situ hybridization and RT-PCR analysis have established that agrin mRNAs are largely distributed in the rat and chick CNS (Kröger et al., 1995; Ma et al., 1994; O'Connor et al., 1994; Tsen et al., 1995a). Although the presence of active agrin isoforms is not firmly demonstrated at the cellular level in central neurons other than motoneurons, the wide distribution of agrin mRNA raises the possibility that, as described at the NMJ, agrin might play a role in post-synaptic clusterization.

Progress in characterizing cellular interactions in CNS synapse formation has been hampered by the heterogeneity of cell types and their inaccessibility to specific experimental procedures. To overcome these problems, we have used an in vitro system consisting of primary cultures of rat embryonic dorsal horn neurons. These cultures display several advantages: they do not contain motoneurons and neurons as identified by neurofilament immunoreactivity represent more than 97% of the cell population (Colin et al., 1996). As demonstrated by patch-clamp recording, in comparable cultures, they establish functional inhibitory synapses from the 5-9th day in vitro (DIV) (Jackson et al., 1982). Furthermore, recent ultrastructural studies have confirmed the presence of morphologically mature synapses at this stage (Colin et al., 1996). This in vitro preparation is convenient to elucidate the role of agrin in the CNS since, as in vivo, GlyR form postsynaptic clusters at the neuronal surface (Nicola et al., 1992). The formation of these microdomains in vitro are concomitant with the establishment of synaptic contacts (Nicola et al., 1992). A protein named gephyrin is associated with the cytoplasmic side of the GlyR (Nicola et al., 1992; Triller et al., 1985, 1987), and plays a key role in the formation and/or the maintenance of the GlyR clusters (Kirsch et al., 1993).

In this study, we have analysed the expression and the cellular distribution of agrin in dorsal horn neurons in vitro. We show that active agrin isoforms are expressed prior to synaptogenesis and that agrin is accumulated in axons, some of which are facing gephyrin aggregates.

MATERIALS AND METHODS

Primary cultures

In order to avoid the presence of motoneurons, primary cultures were prepared from the dorsal horn of rat (Sprague-Dawley) spinal cords at day 14 of gestation as previously described (Nicola et al., 1992). The protocol was modified by the addition of DNAse I (200 U/ml, Boehringer Mannheim) and bovine serum albumin (0.4%, w/v; Sigma Chemical Co., St Louis, MO) during cell dissociation. Transcripts for NT-3, a neurotrophic factor specifically expressed by motoneurons (Henderson et al., 1993) could not be detected by RT-PCR in our cultures. These non-motoneuronal cells were plated at a density of 10^5 cells/cm^2 on sterilized glass coverslips (12 mm in diameter) in 4-well plates (Nunc) for immunofluorescence studies and on plastic in 35 mm dishes (Corning) for RT-PCR. Coverslips and Petri dishes were previously coated with poly-DL-ornithine (Sigma Chemical Co., St Louis, MO) (10 μg/ml).

Amplification of transcripts by RT-PCR

Total RNA was prepared from spinal cord neurons cultured for 1, 4, 7 and 10 days using the RNAnow extraction procedure (Biogentex, Seabrook, TX) and was treated with DNAse I (Pharmacia, Uppsala, Sweden). Then, single stranded cDNA was synthesized using Avian myeloblastosis virus reverse transcriptase (RT) (Promega, Madison, WI) and random hexanucleotide primers (Pharmacia, Uppsala Sweden). Aliquots of cDNA equivalent to 100 ng of total RNA were amplified in a polymerase chain reaction (PCR) which was carried out for 35 cycles under the following conditions: denaturation, 94°C for 30 seconds; annealing, 59°C for 30 seconds and extension, 72°C for 1 minute. Sequences of oligonucleotides were similar to those used by O'Connor et al. (1994): 5,323-5,344 (sense) and 5,712-5,734 (antisense) of the rat agrin cDNA (Rupp et al., 1991). After separation on 3% Nu-Sieve agarose (FMC, Roakland, Maine), amplification products were photographed. Samples containing PCR primers and RT-mix without reverse-transcriptase were negative in the PCR reaction (data not shown).

Antibodies

Three monoclonal antibodies (mAb) were employed in this study: mAb7A(1/100) which recognizes gephyrin (Pfeiffer et al., 1984), AP14, which detects MAP2a (Binder et al., 1984,) and Tau-1 raised against tau (Binder et al., 1985). As secondary antibody, we used Cy3-coupled goat anti-mouse IgG (1/200) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Four polyclonal antibodies against recombinant chick agrin protein were used. Antibody 3228 (anti-c95$_{A4B11}$ in Gesemann et al., 1995) was raised against the COOH-terminal region of agrin. Antibodies 706 and 707 (Denzer et al., 1995) and antibody 022 (anti-cfull$_{07A0B0}$ in Denzer et al., 1995) were raised against the full length chick agrin cDNA. The four antibodies cross-reacted with mouse and rat basal lamina and yielded the same results for immunoblots (1/1,000) and immunohistochemistry (1/250). As secondary antibody, we used

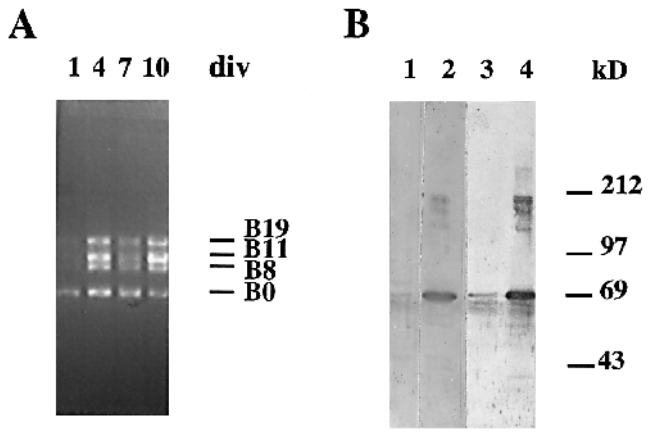

Fig. 1. Detection of mRNA and secreted proteins. (A) RT-PCR analysis of agrin isoform mRNAs during development. Total RNA was isolated from spinal neuron cultures at 1, 4, 7, 10 DIV and PCR was performed on corresponding cDNAs (see Materials and Methods). PCR products were separated on 3% Nu-Sieve agarose gel and visualized by ethidium bromide staining. At 1 DIV, the inactive B_0 form is predominant whereas afterwards, the four isoforms are clearly present. (B) Agrin-like proteins secreted by dorsal horn neurons. Proteins present in the culture medium alone (lane 1 and 3) and in the conditioned medium from 7 DIV cultures (lane 2 and 4) were separated on a 3.6-15% polyacrylamide gel and analysed by immunoblotting with 706 (lanes 1 and 2) and 022 (lanes 3 and 4) antibodies. Note the presence of the 200 kDa agrin-like protein and of agrin immunoreactive proteins of smaller size.

DTAF-coupled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.).

Immunoblots

Conditioned medium from 7 DIV cultures was diluted in an equal volume of Tris buffer (120 mM, pH 6.8) containing 4% SDS, 200 mM DTT and 20% glycerol, boiled for 5 minutes and stored at –20°C. Samples were separated by SDS-PAGE on a 3.6-15% gradient gel. Agrin-like immunoreactivity was detected with peroxidase-linked secondary antibody (Dako, Zug, Switzerland) and TMB (Promega, Madison, WI) as a substrate.

Immunocytochemistry

Cells were fixed with 4% (w/v) paraformaldehyde. The conditions of washing and binding of the primary antibodies were as previously described (Nicola et al., 1992). For the secondary antibody binding, cells were incubated for 45 minutes at room temperature with the secondary fluorescent antibody, the DTAF-coupled goat anti-rabbit IgG alone or mixed with the Cy3-coupled anti-mouse IgG for the double labeling. After extensive washing, the cultures were mounted with Vectashield (Vector laboratories, Inc., Burlingame, CA) and examined with a standard fluorescence microscope (Zeiss) using a rhodamine filter.

Both photographic prints and digitalized pictures (confocal and CCD-camera) are presented. We used normal prints to give the overall and untouched impression of the staining pattern and digitalized pictures to make important points on cellular localization and co-distribution of agrin and gephyrin. Confocal microscopy was used to discriminate intracellular from surface labeling. In this case, excitation was obtained with an Argon ion laser set at 514 nm for Cy3 excitation, and the emitted light was filtered through an appropriate long-pass filter (530 nm). Pixel size and focus steps were 0.15 mm (objective ×63, NA 1.4) with images of 512×512 pixels. For each field, a digitalized series of optical sections at different planes of focus were collected in a host computer (Silicon Graphics) and processed using ImageSpace (Molecular Dynamics). The background noise was reduced, and the contrast enhanced by applying a median (3×3×3) Gaussian filter to the original set of data. Control experiments in the absence of the primary antibody, or with preimmune serum of the same rabbit used for antibody production did not yield any staining above background.

RESULTS

Detection of agrin transcripts and secretion of agrin like proteins

To analyze the expression of agrin variants in the B region during the maturation of the cultures, we used the sensitive RT-PCR approach with oligonucleotide primers flanking the B site. RNAs were extracted from three independent cultures at 1, 4, 7, 10 DIV and subjected to RT-PCR. At 1 DIV, the B_0 mRNA was predominant while the B_8, B_{11} and B_{19} transcripts were barely detectable (Fig. 1A). Afterwards, i.e. at 4 DIV, 7 DIV

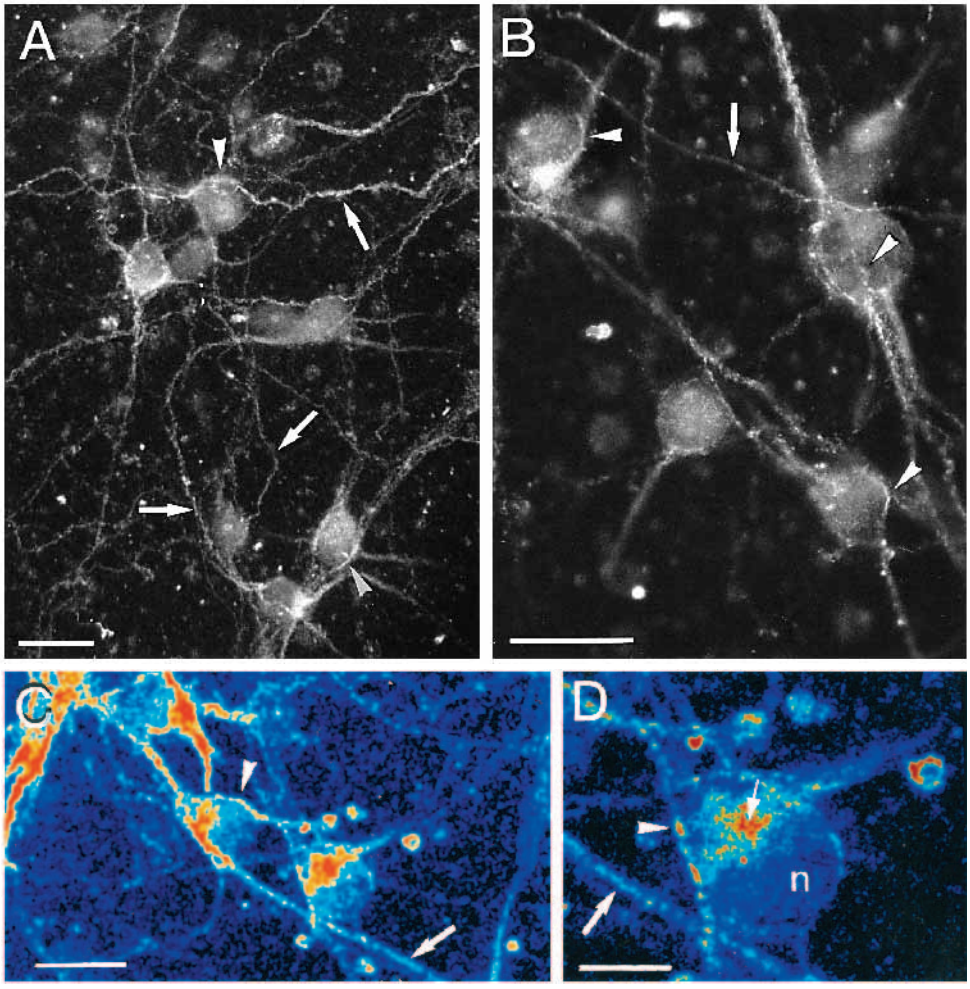

Fig. 2. Detection of agrin-IR in 7 DIV cultured neurons. (A,B) Low- and high-power micrographs showing that the immunofluorescence predominates in neurites (arrows) which may contact somata (arrowheads). Note also the presence of a fuzzy immunoreactivity of the somata. (C,D) Confocal sections (low and high power, respectively) showing the intracellular localisation (small arrow) of the agrin immunoreactivity in the vicinity of the nucleus and at contact points with neurites (other symbols: same as in A,B). Bars: A, B, C, 20 μm; D, 10 μm.

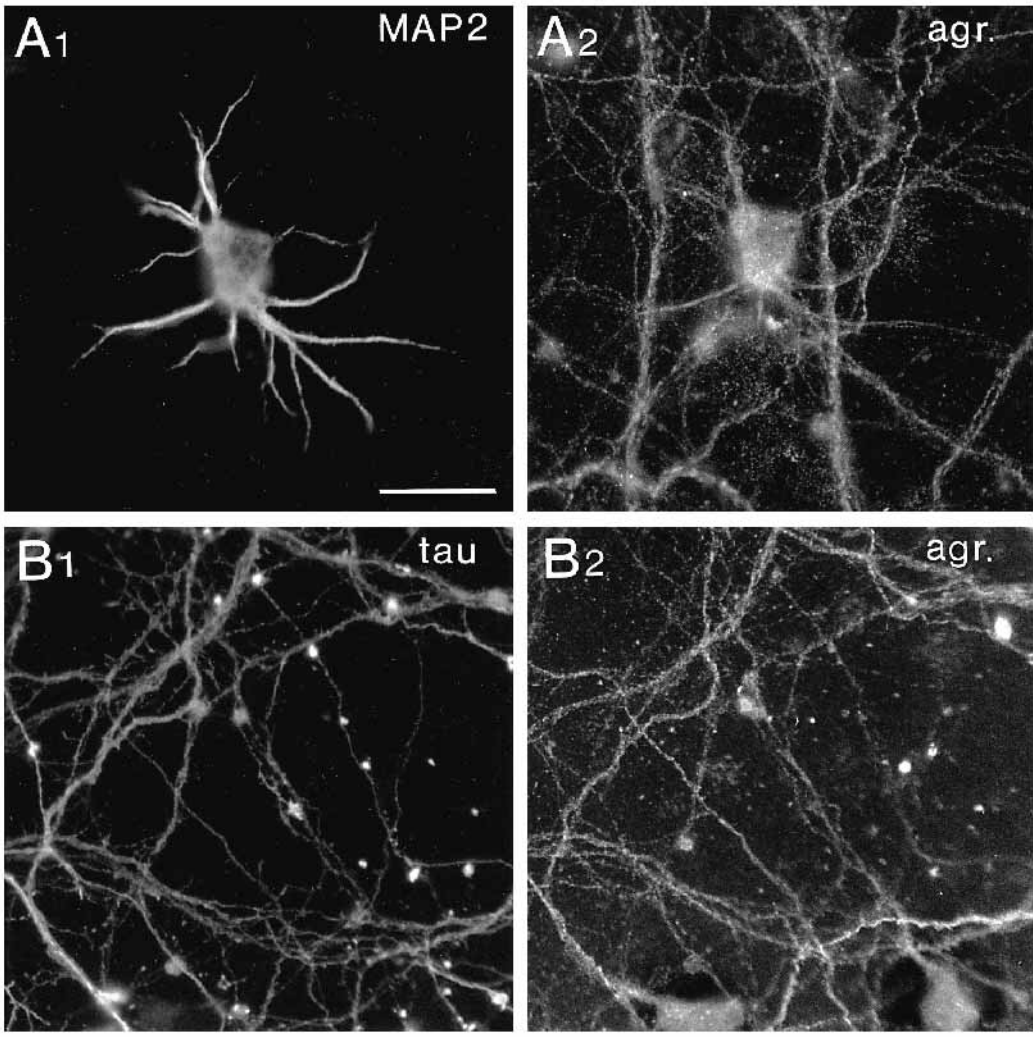

Fig. 3. Evidence that agrin-IR is present in axons. Double immunolabeling showing that agrin immunoreactivity predominates in somata and in MAP-2 negative neurites (A1,A2) which are otherwise tau positive (B1,B2). Detection of anti-agrin with a secondary DTAF coupled antibody, and anti-MAP and anti-tau with a secondary CY3 coupled antibody. Bar, 20 μm.

and 10 DIV, all agrin isoform mRNAs were found to be present in comparable amounts (Fig. 1A). In contrast to what is observed when agrin transcripts in the intact nervous tissue (i.e. neurons and glia) are analysed (Hoch et al., 1993), the B_0 form did not appear to be more abundant than the three other variants, owing probably to the quasi absence of glial cells in our cultures. To confirm their identity, PCR fragments were hybridized with an internal oligonucleotide localized at the position 5,516-5,535 of the rat agrin cDNA (Rupp et al., 1991) which recognized all the agrin isoforms.

Proteins present in conditioned medium of 7 DIV cultures were analysed by immunoblotting. As shown in Fig. 1B, both anti-agrin antibodies 706 and 022 recognized a band of about 200 kDa. Below, within a smear indicating a rapid degradation, bands of about 170 kDa, 150 kDa and 135 kDa were also present. A major band of around 70 kDa, which is also recognized with the other agrin antibodies, was always found. A faint smear, slightly over background could also be detected in high molecular masses around 400 kDa and could correspond to the heparan sulfate proteoglycan (HSPG) forms of agrin recently described in chick tissues (Denzer et al., 1995; Tsen et al., 1995a). The same pattern of bands was also detected with the other anti-agrin antibodies 3228 and 707 (data not shown).

Cellular localization of agrin immunoreactivity

The cellular distribution of agrin-like immunoreactivity (IR) was further characterized in these cultures using the anti-agrin antibodies 3228 and 707 which gave the best immunoreactivities. At 7 DIV, neurons exhibit primary neurites, some of which branch extensively and travel long distances, so that most if not all neurons are contacted by neurites from other cells. At this stage, agrin-like IR was present in almost all cells (Fig. 2), and both soma and some neurites were labeled. Immunoreactive neurites frequently contacted somata, by surrounding (Fig. 3A) or by crossing-over (Fig. 2B). Optical sections obtained with the confocal microscope (Fig. 2C) showed that the somatic staining was genuine, did not extend to the nuclei and did not result from out-of-focus images due to extensive crossing by IR fibers. A small minority of cells was not immunoreactive. The cytoplasmic staining was uneven and often concentrated in the vicinity of the nucleus (Fig. 2C,D). Both in normal and confocal microscopy, the label extending into neurites appeared as punctuate or varicose and could in some instances be traced from the soma of their cells of origin.

The labeled neurites appeared thin and elongated and resembled axons. Double-labeling experiments (Fig. 3), using anti-microtubule-associated protein-2 (MAP-2) and anti-Tau

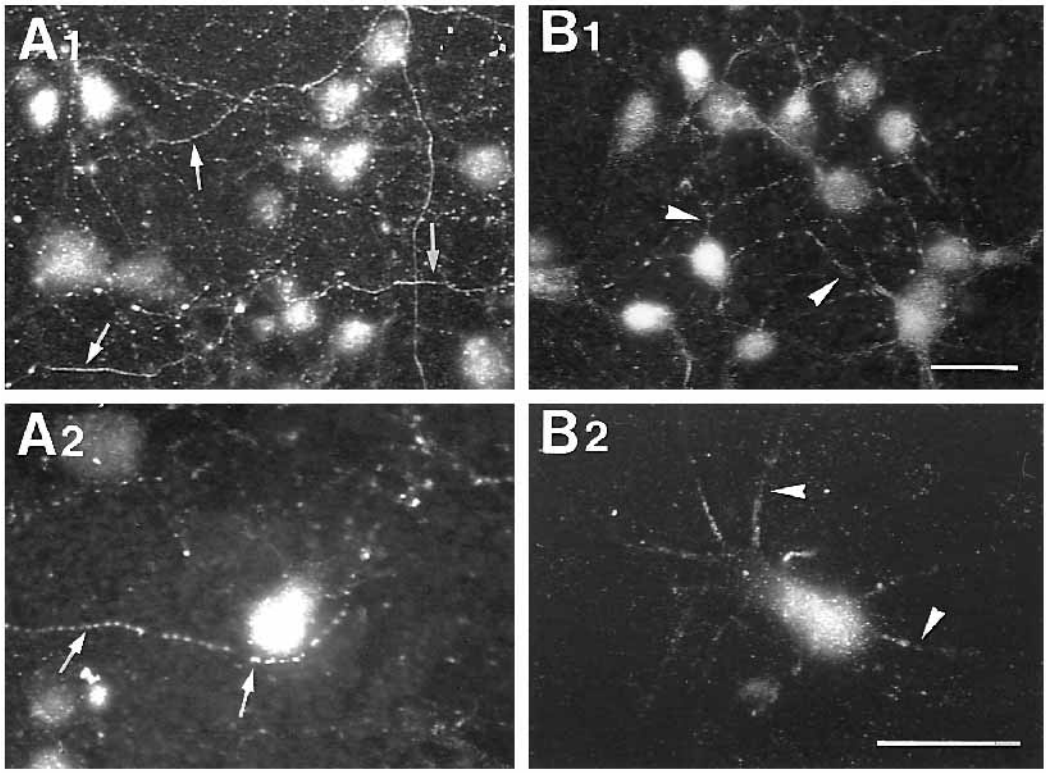

Fig. 4. Effects of Triton on agrin-IR distribution. (A) In the presence of Triton, somata and axons (arrows) are positive. (B) In absence of Triton somata and coarse processes, most probably dendrites (arrowheads) are weakly labeled. (A1,B1) Low power micrographs, and (A2,B2) higher magnification of other fields. Bars, 20 μm.

antibodies to identify the dendritic and the axonal compartments, respectively, and anti-agrin antibodies were also performed. They showed that agrin-IR was not coextensive with MAP2 (Fig. 3A1,A2) but with Tau, indicating that agrin-like IR was enriched in the axonal compartment. Most if not all Tau^+ neurites were also agrin immunoreactive indicating that the axonal enrichment is a property shared by the majority of cells. Sometimes, thick MAP2 positive profiles were paralleled by very thin agrin positive profiles. These images most probably correspond to axons running below, beside or above dendrites on some of their trajectory. Experiments without Triton eliminated the staining of the thin neurites (Fig. 4A1-2) previously identified as axons. Other coarse and short neurites in continuity with the soma were now labeled (Fig. 4B1,B2). They are similar to dendrites identified with MAP2 staining (Fig. 3A1). This IR may correspond to agrin secreted by axons and deposited at the external side of the somato-dendritic plasmalemma.

Given that the majority of axons are positive for agrin, and that axo-somatic contacts are numerous, we further studied whether gephyrin IR was preferentially located at appositions of pre- and postsynaptic specializations. When gephyrin aggregates were detected on a soma (Fig. 5B, C2, arrowheads) they were almost always adjacent to an agrin positive axon (Fig. 5A,C1 arrowhead), with the varicosities being closely associated with the gephyrin aggregates (arrows in Fig. 5A,B). Some gephyrin aggregates were not present on somata and were most probably on dendrites; these spots which were associated with an agrin labeled axon represented most likely axo-dendritic contacts (Fig. 5C2, arrows). Finally, a few gephyrin spots were not adjacent to agrin immunoreactive axons. They most likely correspond to interaction points of somata and dendrites with the bottom of the dish where gephyrin was shown to accumulate transiently (Colin et al., 1996).

DISCUSSION

In this work, we took advantage of an in vitro cell system consisting of non-motoneuronal dorsal horn neurons to analyze the expression of mRNAs coding for agrin isoforms as well as the cellular localization of agrin protein.

In this system, we provide direct evidence that neurons other than motoneurons express active agrin mRNA isoforms. Previous in situ hybridization studies showed that expression of agrin mRNA was not restricted to the cholinergic neuronal system and could be detected throughout the rat and chicken CNS during embryogenesis or in the adult (Kröger et al., 1995; Ma et al., 1994; O'Connor et al., 1994; Stone and Nikolics, 1995). More specifically, agrin mRNA was found in the dorsal horn of the rat spinal cord at E15 or in the adult (O'Connor et al., 1994; Stone and Nikolics, 1995). However, in several studies, the probe used could not distinguish between active or inactive agrin isoforms (O'Connor et al., 1994) and in some cases, it was difficult to attribute the labeling to neurons or glial cells (Stone and Nikolics, 1995). Since pure glial cell cultures express only the B_0 isoform of agrin (S. Levi unpublished observations) and astrocytes (labeled with glial fibrillary acidic protein antibody) represent less than 1% of the cells in our cultures, we attribute the active agrin isoforms to neurons specifically.

At 1 DIV, active agrin isoforms are expressed at barely detectable levels. This is consistent with recent in situ hybridization studies showing that the major agrin isoform expressed at E15 in the rat central nervous system is the B_0 form (Stone and Nikolics, 1995). At 3 DIV (data not shown), both inactive and active form mRNAs are already present. Therefore, expression of active agrin isoform mRNAs occurs before synapse formation which takes place by the end of the first

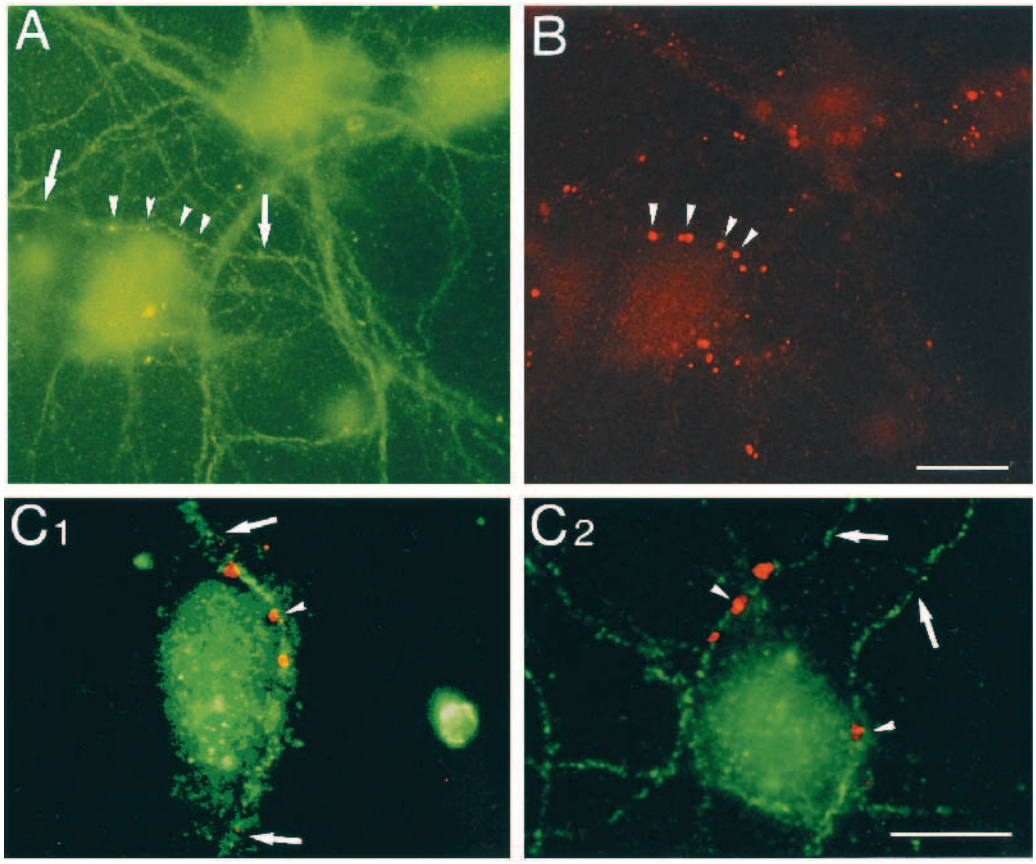

Fig. 5. Accumulation of gephyrin in front of agrin positive axons.
(A,B) Micrographs showing that agrin (A, FITC filters) positive fibers (arrows) are juxtaposed to gephyrin (B, CY3 filters) clusters (arrowheads). (C1,C2) Pseudocolors video images obtained from sequential recording of DTAF (green) and CY3 (red) showing the presence of gephyrin clusters at the contact between immunoreactive axons and the target cell (same symbols as in A,B). Bars, 10 μm.

week of culture. Indeed, in other spinal cord cultures, functional inhibitory synapses start to be detected only from 5 DIV (Jackson et al., 1982). Moreover, around 7 DIV, synaptophysin is found inside neuritic varicosities contacting neurons (Nicola et al., 1992) and, morphologically mature synaptic contacts are observed as shown by electronic microscopy (Colin et al., 1996).

High molecular mass agrin-like immunoreactivity was found in the conditioned medium. Since the agrin antibodies used have all been generated from chick recombinant agrin protein, specificity of the bands is an issue. These antibodies stain rat tissues processed for immunocytochemistry, especially muscle basal laminae and the walls of brain capillaries, which is characteristic of agrin-like IR. The 200 kDa protein has been previously described in rat tissue homogenates (Rupp et al., 1991) and in COS cells transfected by full length rat agrin cDNA (Campanelli et al., 1991). Its molecular mass is consistent with the size predicted from the rat agrin cDNA sequence (Rupp et al., 1991). The 200 kDa protein should therefore represent the core protein of agrin. The other agrin-like proteins ranging from 170 kDa to 135 kDa have been similarly found in rat embryonic (Rupp et al., 1991) and chick (Godfrey, 1991) tissues as well as after agrin extraction from *Torpedo* electric organ (Nitkin et al., 1987). The band of 70 kDa appears to be specific since it is recognized by all agrin antibodies (data not shown) and therefore could represent a proteolytic fragment of agrin. Furthermore, an agrin-IR fragment of a similar molecular mass has been observed in Torpedo electric organ (Nitkin et al., 1987). Agrin in its 400 kDa HSPG form (Denzer et al., 1995; Tsen et al., 1995a) was only weakly detected. Attempts to induce AchR clustering in chick primary cultures using the conditioned medium from 7 DIV cultures have been unsuccessful (experiments kindly performed by Dr M. A. Ruegg, Biozentrum, Basel). This might be due to the fact that agrin activity decays very quickly. Another explanation could be that active agrin is released at very discrete sites by the nerve terminal and therefore is present in too low quantities in the conditioned medium to have a clustering effect. Consistent with this result, experiments using agrin bound to central basal laminae as well as agrin purified from cerebellar granule cells also failed to induce nAchR aggregation (Kröger and Mann, 1996; So et al., 1996).

Strong agrin immunoreactivity was found in almost all neurons at 7 DIV. The agrin immunofluorescence is mainly localized in the somata and, as demonstrated by double labeling with tau antibodies and permeabilization experiments, within the axons. Hence, agrin appears to be preferentially accumulated in the axonal compartment. This distribution is in agreement with the axonal localization of agrin described in the motor neurons at the NMJ after ligation (Magill-Solc and McMahan, 1988) or in chick retina ganglion cells in vivo or in vitro (Kröger et al., 1995). The presence of agrin in axons suggests that agrin could be released at the nerve terminals. The fact that in the absence of permeabilisation a staining could be found at the external side of dendritic and somatic membranes suggests that agrin binding sites are indeed present at this level. This is consistent with the notion that agrin being secreted by axons may act on the somatodendritic pole of the neuron.

Gephyrin aggregates were preferentially apposed to agrin-positive passing axons. At this stage of the culture (7 Div),

gephyrin aggregates are located at the synapse and are associated with GlyR clusters (Béchade et al., 1996). This result supports the possibility that agrin in the CNS could play a role in organizing the post-synaptic clustering of neurotransmitter receptors and in particular, that of GlyR. How could agrin induce the clustering of GlyR? At the NMJ, agrin-induced nAchr clustering occurs through a mechanism that requires tyrosine phosphorylation (Ferns et al., 1996; Meier et al., 1995; Qu and Huganir, 1994) and more precisely, as recently reported, acts via the receptor tyrosine kinase MusK (Glass et al., 1996). However, in preliminary experiments gephyrin clusters were not disrupted with staurosporin (20 nM, 6 hours), a tyrosine kinase inhibitor that blocks agrin-induced AchR cluster formation (Wallace, 1994). This experiment does not rule out the involvement of tyrosine phosphorylation in GlyR clustering.

We have previously reported that gephyrin aggregates are detected at the neuronal surface before the GlyR clusters (Béchade et al., 1996; Kirsch et al., 1993). These findings led us to propose a model for GlyR clustering in which gephyrin is first concentrated under presumptive postsynaptic plasma membrane areas and creates hot spots to which the GlyR binds and becomes concentrated (Béchade et al., 1994). Therefore, a working hypothesis would be that agrin induces primarily the formation of gephyrin aggregates. This possibility is supported by the early expression of active agrin mRNA variants in our cultures which precedes the presence of gephyrin aggregates at the neuronal surface around 6 DIV (Béchade et al., 1996; Kirsch et al., 1993). One may speculate that gephyrin could be an intermediate between agrin secreted by axons and the formation of GlyR clusters.

However, other functions for agrin must be considered as suggested by the multi-domain structure of agrin. The amino-terminal region of agrin exhibits several repeated regions sharing homology with protease inhibitors of the Kazal family (Rupp et al., 1991), later suggested to be domains homologous to follistatin (Patthy and Nikolics, 1993). With respect to this homology, functional analysis of rat recombinant agrin protein established it as a potent inhibitor of several proteases (Biroc et al., 1993), thus suggesting a role of agrin in the regulation of proteolysis in the extracellular matrix and as a consequence its possible involvement in neuritic outgrowth (Lafont et al., 1992). Agrin also contains laminin-like domains which could function in cell adhesion (Rupp et al., 1991). Futhermore, agrin in brain has now been identified as a HSPG (Denzer et al., 1995; Tsen et al., 1995a) which binds to NCAM and could modulate its adhesive function (Burg et al., 1995; Tsen et al., 1995a). Finally, a recent report showed that agrin is selectively adhesive for peripheral motoneurons and inhibits their neuritic outgrowth (Campagna et al., 1995). Agrin knock-out experiments may help to distinguish between these hypotheses (Gautam et al., 1996).

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