ELSEVIER

Molecular Brain Research 91 (2001) 137–147

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

Glycoprotein synthesis at the synapse: fractionation of polypeptides synthesized within isolated dendritic fragments by concanavalin A affinity chromatography

Sergio Villanueva[a], Oswald Steward[b,*]

[a]*Facultad de Medicina, Instituto de Ciencias Biomedicas, Universidad de Chile, Casilla 70005-7, Santiago 6530499, Chile*
[b]*Reeve–Irvine Research Center and Departments of Anatomy & Neurobiology and Neurobiology & Behavior, University of California at Irvine, College of Medicine, 1105 Gillespie Neuroscience Research Facility, Irvine, CA 92697-4292, USA*

Accepted 24 April 2001

Abstract

The synthesis of glycosylated proteins at postsynaptic sites was evaluated by combining metabolic labeling of isolated pinched-off dendritic fragments (synaptodendrosomes) with glycoprotein isolation by Con A affinity chromatography. Three major labeled proteins were detected (apparent molecular weights of 128, 42 and 19 kDa) along with seven minor polypeptides. Treatment of the glycoprotein fraction with *N*-glycosidase F led to shift in the apparent molecular weight of the bands. Also, label incorporation into glycoprotein species was blocked by tunicamycin. Thus, the three prominent polypeptides and most of the minor components of this fraction corresponded to bona fide *N*-glycoproteins. Incubation of synaptodendrosomes with cycloheximide also inhibited label incorporation into the isolated glycoproteins, indicating that the labeling resulted from local de novo synthesis. Subcellular fractionation revealed that the labeled glycoproteins were present in soluble and particulate fractions, mainly microsomes and synaptic membranes, and one of the species (42 kDa) appeared in the incubation medium, indicating secretion. In addition, these glycoproteins were dissimilarly distributed in several brain regions, and were expressed differentially during development, reaching their highest level of synthesis during the period of synaptogenesis. These results provide evidence for local dendritic synthesis of particular glycoprotein components of the synapse.

Theme: Development and regeneration

Topic: Formation and specificity of synapses

Keywords: Dendrite; Glycoprotein; Glycosylation; mRNA; Protein synthesis; Synapse

1. Introduction

Local protein synthesis at postsynaptic sites has gained considerable attention in recent years due to its possible involvement in synapse development [30,34], synapse replacement following denervation [25,33] and plastic changes associated with learning and memory [12,16]. Although the mechanisms that regulate translation at the synapse have begun to be revealed [44,24], several questions about basic features of this process remain unanswered (see Ref. [36] for a recent review). One important question pertains to the characteristics and identity of the locally synthesized proteins.

Studies using in situ hybridization techniques (reviewed in Refs. [8,37]) have identified some mRNAs that are present in abundance in dendrites, which is presumptive evidence that these proteins may be synthesized at synaptic sites. However, a recent study utilizing RT-PCR amplification of mRNA that binds postsynaptic densities suggests that the number of mRNAs present at postsynaptic sites could be substantially higher than estimated from in situ hybridization analyses [38]. In this regard, studies that used reverse transcriptase-based polymerase chain reaction (RT-PCR) amplification of the mRNAs present in dendritic growth cones of cultured neurons [5,21] revealed a large number of mRNAs in these young neurites. Thus, it is still

*Corresponding author. Tel.: +1-949-824-8908; fax: +1-949-824-2625.

E-mail address: osteward@uci.edu (O. Steward).

0169-328X/01/$ – see front matter
PII: S0169-328X(01)00132-2

an open question whether protein synthesis in dendrites involves many different transcripts, or just a few.

Studies of protein synthesis in subcellular fractions enriched in dendrites (synaptoneurosomes [43] and synaptodendrosomes [28,29]) have provided a different type of information about the nature of protein synthesis at the synapse. These studies have defined the effects of depolarization [18] and neurotransmitters [42] on translational activity in dendritic fragments, and also revealed changes in local synthesis over development [41]. Biochemical studies have also revealed that several of the proteins synthesized at the synapse are localized in membranes and synaptic junctions [28,41], and at least one of the mRNAs that is present in abundance in dendrites in mature animals encodes an integral membrane protein [11].

The idea that some synaptic membrane proteins can be locally synthesized is also supported by the observation that the mRNA for a glycine receptor subunit is present in dendrites [27], and there is at least some evidence that the mRNAs for ionotropic glutamate receptor subunits are present in dendrites at low levels during periods of synapse replacement [10]. The mRNA for the 1,4,5-inositol triphosphate receptor type I is also present in dendrites of some neurons [7], although this receptor is not a synaptic protein; instead, it is part of the endomembrane system. All these integral membrane proteins are glycosylated in their native form, raising the question of how they are glycosylated when synthesized within dendrites.

In terms of the possibility of local glycosylation, there is evidence for structures compositionally homologous to endoplasmic reticulum (ER) and Golgi apparatus (GA) in dendrites. Immunocytochemical studies have revealed the presence of several protein markers of ER and GA in the dendrites of spinal cord neurons in vivo [9] and hippocampal neurons in culture [39,40] or in vivo [26]. Moreover, amputated dendrites that are immunopositive for ER and GA markers, are capable of incorporating ^{3}H-labeled sugars into proteins in a tunicamycin-sensitive way [40]. Although these results support the idea that glycosylation can occur in dendrites, the identity and basic features of the locally synthesized glycoproteins are still unknown. Indeed, it is not even known whether this post-translational processing affects most of the synaptically synthesized proteins or only a limited subset of them.

The present study is an initial characterization of the glycoproteins synthesized within pinched-off dendritic fragments (synaptodendrosomes) isolated from different brain regions in developing rats. Synaptosomes were pulse-labeled with [^{35}S]methionine, and the newly synthesized proteins were isolated by Con-A chromatography. Three prominent labeled bands were present in the Con A fraction, along with several other minor labeled bands. All the prominent bands were found in microsomes and differentially partitioned between soluble and synaptic membrane fractions. The degree of labeling of all of these glycoproteins varied across brain regions and as a function of developmental age. These results provide evidence for local dendritic synthesis of glycoprotein components of the synapse.

2. Materials and methods

2.1. Materials

N-Glycosidase F (N-gF) was from Boehringer Mannheim GmbH (Mannheim, Germany). Con A-Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). Disposable centrifugal concentrators (Centriplus 10) were from Amicon (Beverly, MA). SDS–PAGE reagents and MW markers were purchased from Bio-Rad (Richmond, CA). Tunicamycin was obtained from Calbiochem (La Jolla, CA). [^{35}S]Methionine (Tran^{35}S-label) was from ICN (Costa Mesa, CA). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). All other chemicals were obtained from Sigma (St Louis, MO).

2.2. Preparation of synaptodendrosomes

The Institutional Animal Care and Use Committee approved all procedures. Unless otherwise noted, whole brains from 17- or 18-day-old rat pups were used (Harlan; Indianapolis, IN). Synaptodendrosomes were isolated as described by Rao and Steward [28]. Synaptodendrosomal pellet was washed once in 0.25 M sucrose, 25 mM Na–Hepes pH 7.4 and centrifuged at 23 000×*g* for 4 min. The final pellet was resuspended in incubation medium and protein content was determined by the Bradford assay [2].

2.3. Metabolic labeling

Synaptodendrosomes (1 mg protein/ml) were incubated at 37°C in an artificial cerebrospinal fluid containing physiological levels of glucose and non-neuroactive amino acids (except L-methionine) [22]. The medium also contained chloramphenicol to inhibit mitochondrial protein synthesis [18,28]. Medium composition was: 124 mM NaCl, 26 mM $NaHCO_3$, 5 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.1 mg/ml chloramphenicol, 10 mM D-glucose, 500 μM L-glutamine, 50 μM of each one of the following amino acids: L-serine, L-alanine, L-lysine, L-threonine, L-arginine, L-tryptophan, L-asparagine, L-proline and 5 μM of each one of the following amino acids: L-histidine, L-tyrosine, L-leucine, L-phenylalanine, L-valine, L-isoleucine. The medium was continually bubbled with a mixture of 95% O_2/5% CO_2 at a flow rate of 5 ml/min. After a preincubation period of 15 min, metabolic labeling was initiated by the addition of 0.2 mCi of [^{35}S]methionine per ml of incubation medium. Unless otherwise noted, labeling proceeded for 135 min and was halted by mixing incubation medium with 10 vol.

ice-cold 125 mM NaCl, 25 mM Na–Hepes, 10 mM L-methionine, 1 mM EDTA pH 7.4 and centrifuging at 23 000×*g* for 4 min. The labeled synaptodendrosomal pellet was washed once in ice-cold 0.25 M sucrose, 25 mM Na–Hepes pH 7.4. Synaptodendrosomes were then lysed with 10 mM Tris–HCl pH 8.1 (10 ml per mg protein) and proteins were precipitated with trichloroacetic acid (TCA), washed with pure methanol and solubilized in 2% SDS, 10 mM Tris base. After protein determination [31], protein concentration was adjusted to 2 mg protein/ml and samples were stored at −80°C until Con A-Sepharose fractionation.

2.4. Subcellular fractionation of labeled synaptodendrosomes

Labeled synaptodendrosomes were subfractionated using the method of Cotman and Taylor [3] as a guideline, except that the iodonitrotetrazolium violet incubation step was omitted to avoid protein oxidation. In brief, a synaptodendrosomal lysate was obtained as described above and this suspension was centrifuged at 23 000×*g* for 15 min to obtain a lysate supernatant and a lysate pellet. The lysate supernatant was centrifuged for 1 h at 100 000×*g* to obtain a cytosolic fraction in the new supernatant and a crude microsomal fraction in the sediment, whereas the lysate pellet was resuspended in 0.8 ml of 0.32 M sucrose, 50 μM $CaCl_2$ per mg of synaptodendrosomal protein. This suspension was layered on to a discontinuous sucrose gradient (0.8, 1.0 and 1.2 M sucrose prepared in 50 μM $CaCl_2$) and centrifuged at 75 000×*g* for 1.5 h. Myelin, synaptic membrane (SM) and mitochondria fractions were collected from the 0.32/0.8 M sucrose interface, the 1.0/1.2 M sucrose interface and the pellet, respectively.

To obtain the synaptic junction (SJ) fraction, the SM fraction was diluted with three volumes of 10 mM Tris–HCl pH 8.1 and pelleted. The pellet was resuspended with 0.1 ml of 10 mM Tris–HCl pH 8.1 per mg of starting synaptodendrosomal protein and protein concentration was determined. The suspension was diluted to 1 mg/ml with 10 mM Tris–HCl pH 8.1 and then one volume of 1% Triton X-100, 2 mM EGTA pH 7.2 was added. The mixture was gently stirred for 10 min at 4°C and then centrifuged for 1 h at 68 000×*g* through 1.0 M sucrose, 50 μM $CaCl_2$ to pellet the SJs.

Proteins from synaptodendrosomal subfractions were TCA-precipitated and solubilized as outlined above for whole synaptodendrosomes.

2.5. Con A affinity chromatography

Samples were thawed and centrifuged at 100 000×*g* for 1 h at 4°C to eliminate any insoluble material. After this point all the procedures were done at room temperature, unless otherwise noted. The supernatant was diluted 1:10 with a solution of 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgSO_4$, 20 mM Tris–Cl pH 7.4 (binding buffer) containing 3 mM NaN_3 and concentrated to the initial volume using disposable centrifugal concentrators (MW cut-off: 10 kDa). This procedure was repeated one additional time, and the final concentrate was diluted 1:10 with a Con A-Sepharose suspension (0.2 ml of gel per mg of sample protein) previously washed and equilibrated with binding buffer (BB) containing 3 mM NaN_3. The mixture was incubated for 2 h in a rotary mixer and then it was poured into a disposable plastic column. After flow-through collection, the column was sequentially washed with BB, eluted with BB containing 0.3 M α-D-methylmannopyranoside (α-MMP) and washed again with 0.5 M NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 M sodium borate pH 6.5 (SETB buffer). Fifteen gel bed volumes of each solution were used in the corresponding step, collecting six consecutive fractions in each case. The first four fractions of each eluate were pooled and concentrated to approximately 0.5 ml using disposable centrifugal concentrators. Protein from concentrates was precipitated overnight at 0°C by addition of TCA to a final concentration of 5%. Precipitates were washed twice with pure methanol, air-dried and dissolved in 0.1% SDS, 5 mM Tris base for radioactivity counting, protein determination and SDS–PAGE.

2.6. N-gF treatment

Protein samples were incubated for 24 h at 37°C in a medium containing 10 U/ml N-gF, 1% β-mercaptoethanol, 0.5% Nonidet P-40, 0.1% SDS, 3% glycerol, 0.3 mM NaN_3, 10 mM EDTA, 5 mM Tris base, 30 mM sodium phosphate pH 7.2 in a final volume of 50 μl. Control samples were incubated in the absence of enzyme under the same conditions. Incubation was stopped by the addition of concentrated SDS–PAGE loading buffer and boiling for 3 min. A reference tube consisting of 5 μg of ovalbumin instead of sample was included to evaluate the completion of the reaction.

2.7. Gel electrophoresis and band quantification

SDS–PAGE of the samples was carried out on 5–15% gradient gels (1.5 mm thick and 16 cm in length) [17]. Gels were stained with Coomassie Blue (CB), destained and dried on to cellophane. Dried gels were scanned using a Personal Laser Densitometer (Molecular Dynamics; Sunnyvale, CA) and then they were exposed to phosphor screens for 5 days. Serial 1:10 dilutions of [^{35}S]methionine standards (100 000 to 100 dpm at the day of labeling experiment) that were blotted (2 μl each) on to a nitrocellulose strip were exposed along with the gels. Phosphor screens were scanned using a PhosphorImager (Molecular Dynamics) and label incorporation into individual bands (after background subtraction) was calculated using the software provided by the company.

2.8. Statistical analyses

Significance of the differences between samples was evaluated statistically by paired *t*-tests. All results are expressed as the mean±S.E.M.

3. Results

3.1. Glycoprotein synthesis and purification

The degree of labeling of bands isolated by Con A increased over time in a manner that was similar to the increase in labeling of total protein (Fig. 1A). Incorporation of [^{35}S]methionine was linear during the first hour and then the rates of incorporation decreased over the next 2–3 h reaching a plateau before 4 h. At longer incubation periods, however, some differences were evident: while total protein labeling was constant up to 7 h, label incorporation to the glycoprotein fraction decreased almost linearly during the same period. Thus, we carried out all the subsequent experiments using an incubation period of 135 min to facilitate label detection in the glycoprotein fraction.

The column elution profile is illustrated in Fig. 1B. More than 80% of loaded dpm were not bound by Con A-Sepharose and appeared in the flow-through and the initial wash with BB. Elution with BB containing 0.3 M α-MMP resulted in the release of 3.2±0.6% (n=6) of loaded dpm. This fraction also contains some non-specifically-retained labeled polypeptides that are slowly released from the resin (see below); thus, we estimate that specifically retained glycoproteins account for approximately 1–2% of the total radioactivity that is released. Approximately 15% of loaded dpm were tightly bound to the gel, and could be released only by equilibrating the column with a high elution strength solution (SETB buffer).

3.2. SDS–PAGE characterization of labeled glycoproteins

SDS–PAGE/fluorographic analysis of column fractions revealed the presence of three prominent labeled bands in the α-MMP eluate (lane E in Fig. 2B). These bands were enriched in the eluate in comparison with the flow-through or the initial wash with BB (lanes FT and IW, respectively, in Fig. 2B). The apparent MW of the prominently labeled bands is 128, 42 and 19 kDa (solid arrowheads, Fig. 2B); these did not coincide with major bands in the CB-stained gel (Fig. 2A). A fourth prominent labeled band was present at 32 kDa in the glycoprotein fraction (open arrowhead, Fig. 2B). However, it is also present in all fractions and was enriched in the final wash with SETB buffer (lane FW in Fig. 2B).

Some other lightly-labeled bands were sometimes detected in variable amounts in the glycoprotein fraction (see below). Many of these were also present in higher amounts in the initial wash (especially bands at 17 and 14 kDa, see Fig. 2B), suggesting that they do not correspond to proteins specifically bound to Con A. Although the remaining faintly-labeled bands may correspond to minor glycoproteins, their amounts were near the limit of detection. It should be noted that the electrophoretic pattern of the glycoprotein fraction was fairly constant at labeling times ranging from 30 min to 4 h (not shown), making it unlikely that some of the low MW labeled polypeptides

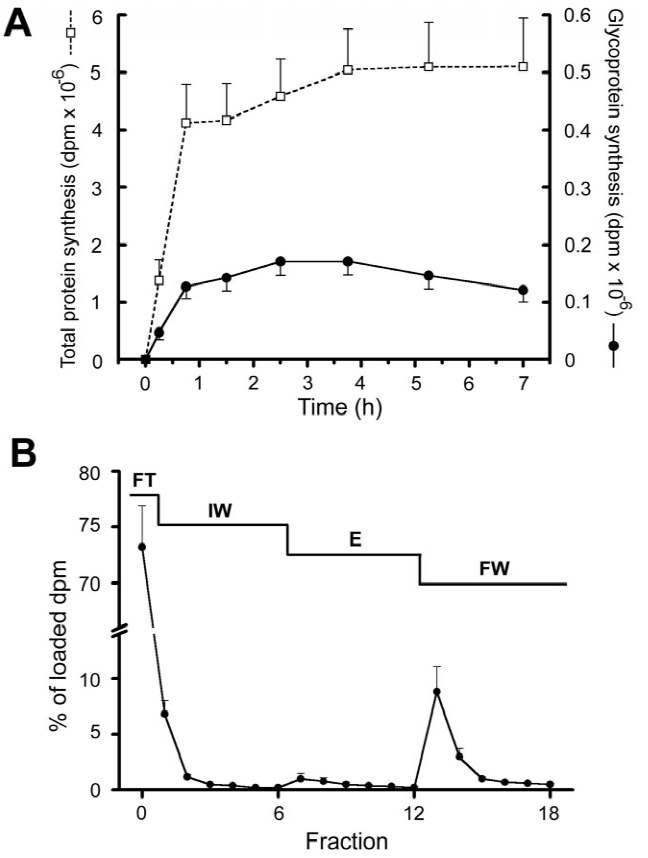

Fig. 1. Glycoprotein labeling and purification. Synaptodendrosomes prepared from whole brains of 18-day-old rats were incubated with [^{35}S]methionine as described in Material and methods. (A) Time course of protein synthesis. Synaptodendrosomes (2 mg of protein) were labeled for the times indicated in the figure and one-half of the protein amount was further processed to obtain a glycoprotein fraction as described in Material and methods. Total dpm were determined in the unfractionated synaptodendrosomal proteins (open squares, left axis) and glycoprotein fraction (solid circles, right axis). Data correspond to the mean±S.E.M. of three experiments. (B) Con A-Sepharose fractionation of synaptodendrosomal proteins. Labeled synaptodendrosomal proteins were fractionated using Con A affinity chromatography as described in Material and methods. Radioactivity was determined in an aliquot of each fraction and expressed as a percent of the loaded dpm. Fraction 0 corresponds to the flow-through (FT) of the column, fractions 1–6 to the initial wash (IW) with binding buffer, fractions 7–12 to the eluate (E) with binding buffer containing 0.3 M α-MMP (glycoprotein fraction) and fractions 13–18 to the final wash (FW) with SETB buffer. Data are the mean±S.E.M. of six preparations.

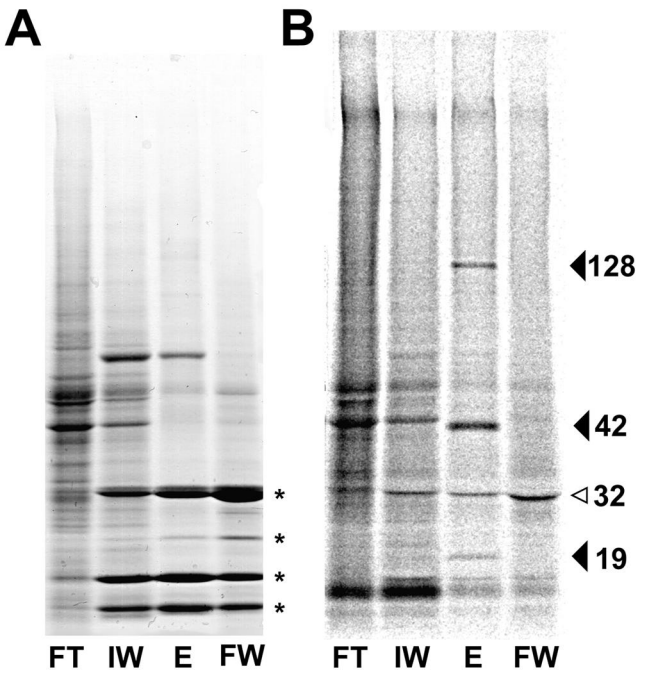

Fig. 2. Protein pattern of Con A-fractionated synaptodendrosomal proteins. Synaptodendrosomal proteins from whole brains of 17-day-old rats were metabolically labeled with [^{35}S]methionine, chromatographed by Con A-Sepharose and 50 μg of each fraction were resolved using SDS–PAGE as described in Material and methods. Lane assignments are: flow-through (FT), initial wash (IW), α-MMP eluate (E), and final wash (FW). (A) Laser densitometry of the CB-stained gel. Asterisk denotes non-sample polypeptides, which are contributed by Con A-Sepharose. (B) Phosphorimage of the gel shown in (A). MWs (in kDa) of bands enriched in α-MMP eluate (solid arrowheads) or final wash (open arrowhead) are indicated on the right. Results are representative of six preparations.

were derived from the proteolytic cleavage of high MW labeled proteins.

Under the present experimental conditions, slightly more than a half of the total unlabeled protein recovered in the α-MMP eluate was present in four polypeptides released from the gel matrix (asterisks, Fig. 2A). This proportion was even higher in the final wash with SETB buffer and somewhat lower in the initial wash with BB. These bands correspond to Con A and unidentified Con A derivatives and/or contaminants, because they are present in commercial preparations of Con A (not shown). Their appearance in our eluates is probably due to the high ratio of gel/protein employed in our studies combined with the presence of residual SDS in our sample because incubation of the resin alone with a solution of 0.002% SDS for 2 h at room temperature produced a similar effect. Attempts to lower SDS concentration in our samples were unsuccessful due to protein insolubility under these conditions. Also, when the ratio of resin/synaptodendrosomal protein was reduced to one-fourth of the usual values, the contamination with exogenous polypeptides was proportionally lowered, but the retention of major labeled glycoproteins on the Con A column was inefficient. Thus, the conditions employed represented a compromise between protein solubilization and binding to Con A. The partial coincidence in the electrophoretic migration of the larger polypeptide released from gel matrix and the labeled 32-kDa band produced a marked quenching in the detection of the latter (see lanes FW in Fig. 2A,B). In spite of its quenching, the band at 32 kDa is by far the most prominent labeled constituent of the final wash with SETB buffer, suggesting that most of the radioactivity associated with this fraction may be attributable to that band.

3.3. Effects of N-gF, cycloheximide and tunicamycin on labeled glycoproteins

Treatment of the proteins from the α-MMP eluate with N-gF caused a clear shift in the migration of 128, 42 and 19-kDa bands (Fig. 3A), indicating that all of them are bona fide *N*-glycoproteins. However, the band at 32 kDa

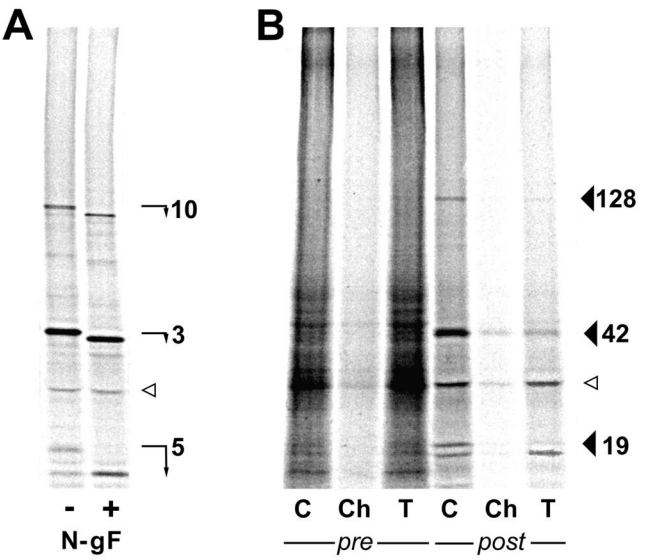

Fig. 3. Effects of N-gF, cycloheximide and tunicamycin on labeled synaptodendrosomal glycoproteins. (A) Phosphorimage of synaptodendrosomal glycoprotein samples treated with N-gF. Synaptodendrosomes prepared from whole brains of 17-day-old rats were metabolically labeled with [^{35}S]methionine and their proteins were fractionated using a Con A-Sepharose column as described in Material and methods. Glycoprotein fraction constituents were incubated in the absence (−) or presence (+) of 10 U/ml of N-gF as described in Material and methods and separated using SDS–PAGE (25 μg protein per lane). MW shifts (in kDa) for the three main glycoproteins are indicated on the right. Open arrowhead denotes the migration of the 32-kDa protein, which is not affected by N-gF treatment. Results are representative of three experiments. (B) Phosphorimage of samples from cycloheximide- or tunicamycin-treated synaptodendrosomes. Synaptodendrosomes prepared from whole brains of 18-day-old rats were labeled in the absence (control, C) or presence of 500 μg/ml of cycloheximide (Ch) or 50 μg/ml of tunicamycin (T) as described in Material and methods, and their protein components (50 μg per lane) were analyzed by SDS–PAGE before (*pre*) or after (*post*) Con A-Sepharose affinity chromatography. MWs (in kDa) of the three prominent components of the glycoprotein fraction (solid arrowheads) are indicated on the right. Open arrowhead shows the position of the 32-kDa band. Results are representative of three experiments.

(open arrowhead in Fig. 3A) was not shifted by N-gF treatment, indicating that this band was not significantly *N*-glycosylated. Several faintly labeled bands at 115, 83, 60, 48 and 39 kDa were more focused (and consequently became more prominent) after treatment with N-gF, showing the existence of additional labeled *N*-glycoproteins in our preparation. Other minor bands at 17 and 14 kDa, in contrast, were unaffected by N-gF treatment (Fig. 3A).

The effects of cycloheximide and tunicamycin on glycoprotein synthesis are shown in Fig. 3B. Cycloheximide, an inhibitor of eukaryotic protein synthesis, significantly decreased ($P<0.05$; $n=3$) the [^{35}S]methionine incorporation into unfractionated synaptodendrosomal protein by 76.8±13.2 and by 81.9±18.1% into the glycoprotein fraction in comparison to control values (see lanes Ch and C in *pre* and *post* panels of Fig. 3B, respectively). Tunicamycin, an inhibitor of *N*-glycosylation, did not affect the labeling of total synaptodendrosomal protein (compare lanes T and C in panel *pre*, Fig. 3B), but did decrease the labeling of bands at 128, 42 and 19 kDa in the α-MMP eluate (see bands indicated by solid arrowheads in lanes T and C of panel *post*, Fig. 3B). In contrast, the labeling of the 32-kDa polypeptide (open arrowhead in Fig. 3B) was not affected by tunicamycin, again implying that this band does not correspond to an *N*-glycoprotein. The labeling of a 17-kDa polypeptide, which was sometimes prominent in our preparations (especially in the initial wash fraction), was also unaffected by tunicamycin (Fig. 3B).

3.4. Subcellular localization of newly-synthesized glycoproteins

Subcellular fractionation of labeled synaptodendrosomes and further Con-A chromatographic isolation of glycoproteins from subfractions, revealed that the three major glycoprotein bands were concentrated in microsomes, cytosol and synaptic membrane fractions (Fig. 4). The band at 19 kDa was enriched in synaptic membrane fraction (compare lanes SM and Sd in Fig. 4), where it was present at 21.6±4.7 times the amount found in the original synaptodendrosomal fraction ($n=3$). This was the only labeled band detected in synaptic junctions (lane SJ, Fig. 4), although its amount in that fraction was notably lower than in synaptic membrane or cytosolic fractions. In considering these data, it is important to recall that the preparation of SJs involves treatment with non-ionic detergent to solubilize membrane components. Such treatment removes most integral membrane proteins leaving behind only the ones that are tethered to the synaptic cytoskeleton.

Bands at 128 and 42 kDa were mainly concentrated in the crude microsomal fraction (compare lanes Ms and Sd in Fig. 4), although they were also present in cytosolic and synaptic membrane fractions (lanes Cs and SM, Fig. 4). In addition, the 42-kDa protein could also be recovered from

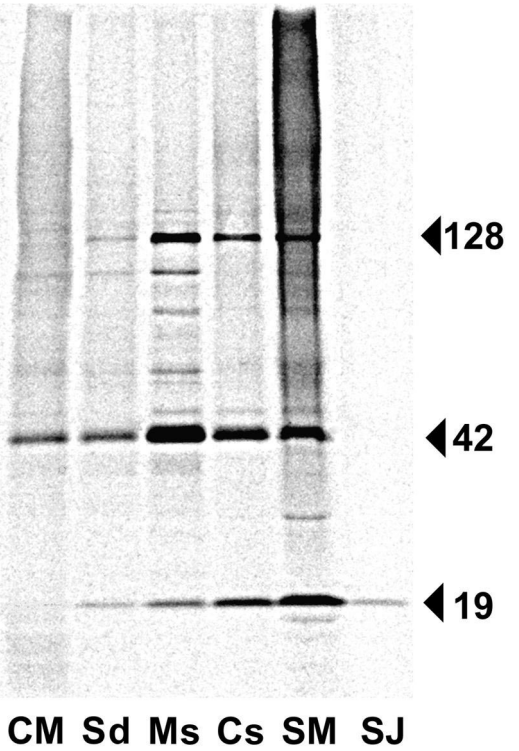

Fig. 4. Subcellular distribution of glycoproteins synthesized in synaptodendrosomes. Synaptodendrosomes prepared from whole brains of 17-day-old rats were metabolically labeled with [^{35}S]methionine, subfractionated and the glycoprotein constituents of each subcellular fraction and culture medium were resolved by SDS–PAGE as described in Material and methods. Lane assignments are: culture medium (CM), whole synaptodendrosome (Sd), microsomes (Ms), cytosol fraction (Cs), synaptic plasma membrane (SM), and synaptic junction (SJ). Migration of the three major glycoproteins is indicated at the right of the phosphorimage. Results are representative of three preparations.

the culture medium (lane CM, Fig. 4). The selective presence of this protein in the culture medium may reflect its constitutive secretion (see Discussion section).

It should be noted that four labeled bands at 104, 83, 60 and 48 kDa, which are barely detected in glycoprotein fractions isolated from whole synaptodendrosomes, are clearly present in glycoprotein fractions obtained from synaptodendrosomal microsomes (lane Ms, Fig. 4). At the same time, the tunicamycin- and N-gF-insensitive bands at 32, 17 and 14 kDa were associated with the synaptic membrane fraction. This fraction presented a high specific activity of labeling (8.9±3.0 times the dpm per μg of protein found in glycoproteins from unfractionated synaptodendrosomes; $n=3$), but substantial amounts of radioactivity were associated with poorly resolved bands that were difficult to distinguish from the background (lane SM, Fig. 4).

In addition, Con A purification of glycoproteins from light-membrane fraction (which is mainly composed of non-synaptic membranes of glial origin) and mitochondrial fraction yielded negligible amounts of radioactivity, precluding the detection of any labeled polypeptide in these

fractions. This observation supports the notion that glycoprotein labeling in our preparation is due to the activity of non-mitochondrial polyribosomes of neuronal origin.

3.5. Brain regional distribution of labeled glycoproteins

The brain regional distribution at 17 days of age was very similar for the 42- and 19-kDa proteins (Fig. 5). Both bands were present in all the regions studied, but were more abundant in diencephalon and basal ganglia (lanes D and BG in Fig. 5, respectively), and clearly impoverished in the brain stem (lane BS, Fig. 5). In contrast, at this age the band at 128 kDa was absent in synaptodendrosomes isolated from brain stem, hippocampus and cerebral cortex (lanes BS, H and Cx, Fig. 5) but was enriched in cerebellum and diencephalon (lanes Cb and D, respectively, in Fig. 5). In addition, a previously undetected doublet at an apparent MW of 147 kDa was detectable in glycoprotein fractions purified from cerebellar synaptodendrosomes (asterisk, lane Cb in Fig. 5).

3.6. Developmental regulation of glycoprotein synthesis

Finally, we studied the developmental changes in the labeling of the key synaptodendrosomal glycoproteins. As is shown in Fig. 6, maximal levels of [^{35}S]methionine incorporation were measured at 17 days of age while minimal levels were found at adult age (90 days) for all three bands. Despite these similarities, some differences were found in the developmental pattern of synthesis of the 128-kDa protein in comparison with the other two major glycoproteins (Fig. 6). First, bands at 42 and 19 kDa were labeled to a high extent at the earliest age studied (7 days), whereas labeling of the 128-kDa protein was near the limit of detection at this age. Second, labeling of the band at 128 kDa at 27 days of age was markedly lower than its peak value (at 17 days), whereas this difference was less in the case of the proteins at 42 and 19 kDa. Finally, there was

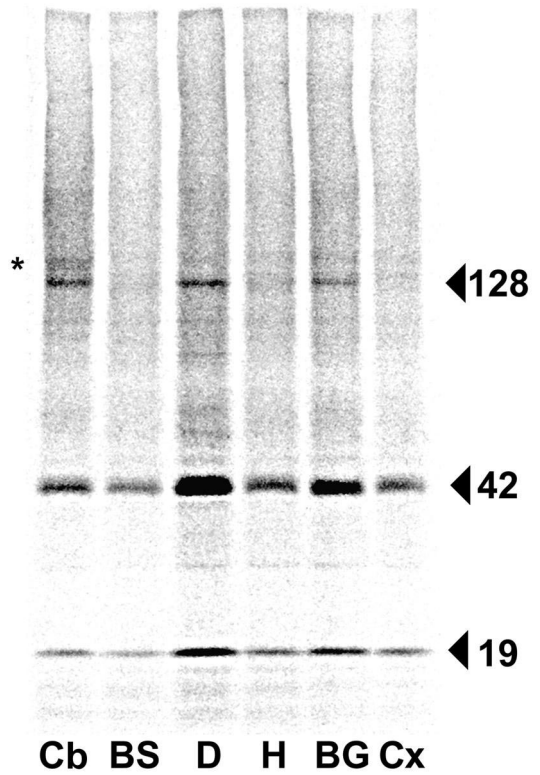

Fig. 5. Brain regional distribution of labeled glycoproteins. Synaptodendrosomal fractions were prepared from cerebellum (Cb), brain stem (BS), diencephalon (D), hippocampus (H), basal ganglia (BG), and cerebral cortex (Cx) of 17-day-old rats and incubated with [^{35}S]methionine as described in Material and methods. Synaptodendrosomal proteins were fractionated by Con A-Sepharose chromatography and the glycoprotein fraction components were resolved using SDS–PAGE (25 μg per lane) as described in Material and methods. MWs (in kDa) of the main glycoproteins are shown at the right of the gel phosphorimage. The asterisk on the left of the gel indicates a labeled doublet at 147 kDa that is especially notorious in glycoprotein fractions isolated from cerebellar synaptodendrosomes. Results are representative of three preparations.

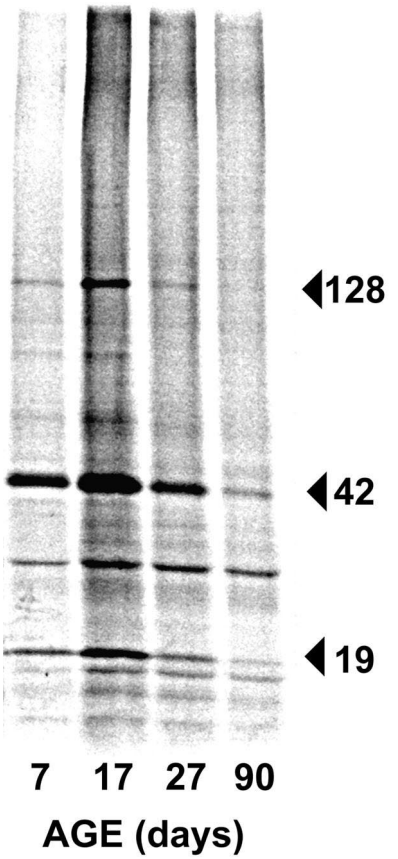

Fig. 6. Developmental changes in synaptodendrosomal glycoprotein synthesis. Synaptodendrosomes were prepared from whole brains of rats at 7, 17, 27 and 90 days of age and incubated with [^{35}S]methionine as described in Material and methods. Proteins were fractionated using Con A-Sepharose chromatography and 25 μg of proteins from the glycoprotein fraction were separated by SDS–PAGE as described in Material and methods. Migration of major bands is indicated at the right of the gel phosphorimage. Results are representative of three preparations.

no detectable labeling of the 128-kDa band in synaptodendrosomes from adult animals, while labeling of 42- and 19-kDa proteins was still clearly detectable. It is worth noting that the two labeled bands at 32 and 17 kDa that usually co-purified with the three main glycoproteins, showed a developmental pattern of synthesis that was entirely different, since their labeling diminished very little from 17 through 90 days of age (Fig. 6).

4. Discussion

Our results reveal that about 10 glycoproteins are synthesized within isolated dendritic fragments. Three are heavily labeled constituents of the Con A-binding fraction of locally-synthesized synaptodendrosomal proteins (MWs of 128, 42 and 19 kDa), whereas the remaining ones are lightly labeled (5–7 polypeptides in the MW range of 147–39 kDa). Since the minor components were present at levels only slightly above of limits of detection, our analyses and further discussion focus on the three major bands.

4.1. Glycosylation of proteins at synapses

A number of studies performed in vivo [9,26] as well in vitro [39,40] have revealed the existence of a dendritic endomembrane network with features of ER and GA. At the ultrastructural level, some of these membranous cisternae extend into dendritic spines and sometimes connect with a spine apparatus [32]. The possibility that these dendritic inner membranes are involved in post-translational processing is directly supported by immunocytochemical and biochemical studies revealing the presence of glycosyltransferase activities in dendrites of cultured hippocampal neurons [40]. This machinery represents a potential site for the glycosylation of proteins synthesized by synapse-associated polyribosome complexes [37].

Previous studies have demonstrated the synthesis of approximately 30 prominent bands after metabolic labeling of isolated dendritic fragments (synaptodendrosomes) [41]. The present results show that none of the prominent bands are major components of the Con A-purified fraction. This finding, together with the observation of only three major bands in the glycoprotein fraction (accounting for around 1% of the overall radioactivity incorporated to proteins), indicates that only a small portion of the proteins synthesized in dendrites are glycoproteins.

Other factors must also be considered in terms of protein glycosylation in synaptodendrosomes. First, Con A affinity chromatography retains only mannose-enriched *N*-glycoproteins, which may comprise a significant portion, but perhaps not all locally-synthesized synaptic glycoproteins. Second, the protein solubility constraints in our samples forced us to maintain some residual SDS in the solutions, which may had interfered with the retention of glycoproteins that bind Con A in a weak way. Third, heterogeneity in the glycosylation of some proteins hindered their detection, as evidenced by the fact that N-gF pretreatment revealed at least five components in the glycoprotein fraction that were not resolved in the untreated samples (Fig. 3A). Finally, it is important to recall that the results presented here were obtained in unstimulated synaptodendrosomes (i.e. they represent the constitutive synthesis of glycoproteins), because it has been demonstrated that synaptic activity markedly influences the translation of some dendritic mRNA at synapses [23,35]. All of these factors may have partially hindered the detection of some glycoproteins whose mRNAs have been reported to be in dendrites [7,21,27]. In addition, it is necessary to bear in mind that our experimental approach permits only the evaluation of an average glycosylation activity of many synapses, leaving open the possibility that some individual synaptic sites may perform a quantitatively more significant protein glycosylation. In fact, previous studies have shown significant differences in sugar incorporation to proteins between distinct dendritic synapses [40].

4.2. Main features of the major locally-synthesized glycoproteins

Incorporation into the three major labeled glycoprotein bands was blocked by tunicamycin, indicating that they are newly *N*-glycosylated polypeptides. This conclusion is further supported by the observation that the three bands exhibited a mobility shift following N-gF treatment, as is expected to occur with a bona fide *N*-glycoprotein. Moreover, the retention of the complete amount of radioactivity in each labeled polypeptide after N-gF treatment (Fig. 3A) reveals that the radioactive isotope was incorporated into the peptide moiety, eliminating the possibility that labeling was due to post-translational incorporation of ^{35}S into the sugar moiety of the glycoprotein. Furthermore, the fact that cycloheximide inhibited incorporation into the three major constituents of the Con A-binding fraction demonstrates that their polypeptide backbones were newly synthesized, confirming that they correspond to locally-synthesized and locally-glycosylated proteins.

Subcellular fractionation studies revealed several interesting features of the three principal glycoproteins. First, the three major-labeled glycoproteins were enriched in microsomes, supporting the idea that endomembranes (the main components of this subcellular fraction) were involved in their synthesis. Second, all the major-labeled polypeptides were present in significant amounts in synaptic membranes and cytosolic fractions. Since the cytosolic fraction is obtained by osmotic lysis of synaptodendrosomes, it also contains the soluble proteins that are stored in membranous intracellular compartments, which are broken as a consequence of the osmotic shock. Thus, the presence of labeled glycoproteins in this fraction may also

indicate their trafficking or residence within the lumen of the endomembrane system (ER, GA, secretory vesicles, etc.). In fact, the detection of the 42-kDa-labeled glycoprotein in the culture medium raises the possibility that this polypeptide is packaged in secretory vesicles, which have been described at synaptic domains of dendrites [20,32]. In this case, the partition of the proteins between soluble and synaptic membrane fractions may reflect their reversible association with some components of the latter (see next section).

The present results also reveal differences in dendritic glycoprotein synthesis in different brain regions. Although some of these differences may be attributable to the extent of synaptogenic activity at the age of our studies (see below), the marked variations in the level of labeling for particular polypeptides in different brain regions suggests that there are authentic regional differences in glycoprotein synthesis. The more evident example is the 128-kDa protein, which was synthesized at high comparative levels in cerebellum, whereas the other two main bands were more lightly labeled. In addition, the 128-kDa band was almost undetectable in cortex, hippocampus and brain stem, whereas labeling of the other major glycoproteins in these regions was evident. In any case, the relative levels of glycoprotein labeling in different brain areas showed a good correlation with the overall capacity for protein synthesis previously described [41].

The developmental variations in glycoprotein labeling revealed a peak in the synthesis of the three major polypeptides in the middle of the third week of postnatal development, an age that coincides with the maximal rate of dendritogenesis and synapse consolidation in rat brain [1,4]. Thus, in contrast to general protein synthesis in dendrites, where several patterns of developmental changes are seen [41], synthesis of the major glycoproteins appears to occur in a more limited time frame, coincident with synaptogenic activity. However, the much more transient expression of the 128-kDa polypeptide in comparison to the other two prominent proteins indicates that not all the locally synthesized glycoproteins are necessarily synthesized during the same stages of development.

4.3. Possible functional implications of synaptic glycosylation

Local glycosylation of proteins within dendrites provides synapses with a mechanism, complementary to protein synthesis, which makes possible a complete and autonomous production of post-translationally-modified mature proteins at individual synaptic sites on neurons. In general, glycoproteins play an important role in several cellular activities, including cell adhesion, protein sorting, the development of polarity, soluble ligand recognition and cell–cell recognition (see Ref. [19] for review). In the case of the glycoproteins characterized in the present work, the assignment of a function must await their unequivocal identification. However, the characteristics of the glycoproteins described in the present work invite some speculations regarding their possible role in dendritic function. For example, the presence of the glycoproteins in the soluble fraction and the appearance of at least one (42 kDa) in detectable amounts in the culture medium suggest that some of the locally synthesized glycoproteins may be released from dendrites. Recent studies have revealed the existence of a secretory machinery in dendrites [20,26,32] and the presence of lectin proteins in brain [6,14], which may function as receptors for secreted glycoproteins. Interestingly, one of these endogenous lectins exhibits binding capabilities specific for a group of soluble glycoproteins, which includes a pair of polypeptides displaying electrophoretic migration similar to two of the major glycoproteins described in this work (42 and 128 kDa) [13]. The fact that this lectin is enriched in the synaptic region [15], invites the speculation that these glycoproteins may act as intersynaptic messengers, influencing the activity of contiguous synapses, or as retrograde messengers, modifying the function of the presynaptic terminal. The higher levels of synthesis of these polypeptides during synaptogenesis suggest that their potential role in intercellular signaling may be especially relevant for synapse maturation. Further work evaluating the effects of synaptic activity on the local synthesis and secretion of glycoproteins must be done in order to test the precedent hypothesis and clarify the relation between synaptogenesis and the synthesis of the glycoproteins described in this study.

5. Conclusion

To our knowledge these data are the first demonstration of a local synthesis of particular glycoproteins within dendrites. Further characterization of these proteins will be required to advance our understanding of their possible function at the synapse.

Acknowledgements

This work was supported by N.I.H. grant NS12333 to O.S.; S.V. was the recipient of a research fellowship award from the Fogarty International Center of the N.I.H.

References

[1] G.K. Aghajanian, F.E. Bloom, The formation of synaptic junctions in developing rat brain: a quantitative electron microscopic study, Brain Res. 6 (1967) 716–727.

[2] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[3] C.W. Cotman, D. Taylor, Isolation and structural studies on synaptic complexes from rat brain, J. Cell Biol. 55 (1972) 696–711.
[4] C.W. Cotman, D. Taylor, G. Lynch, Ultrastructural changes in synapses in the dentate gyrus of the rat during development, Brain Res. 63 (1973) 205–213.
[5] P. Crino, J. Eberwine, Molecular characterization of the dendritic growth cone: regulated mRNA transport and local protein synthesis, Neuron 17 (1996) 1173–1187.
[6] K. Drickamer, Two distinct classes of carbohydrate-recognition domains in animal lectins, J. Biol. Chem. 263 (1988) 9557–9560.
[7] T. Furuichi, D. Simon-Chazottes, I. Fujino, N. Yamada, M. Hasewaga, A. Miyawaki, S. Yoshikawa, J.L. Guenet, K. Mikoshiba, Widespread expression of inositol 1,4,5-triphosphate receptor type I gene (Insp3r1) in the mouse central nervous system, Receptors Channels 1 (1993) 11–24.
[8] F.B. Gao, Messenger RNAs in dendrites: localization, stability, and implications for neuronal function, Bioessays 20 (1998) 70–78.
[9] A. Gardiol, C. Racca, A. Triller, Dendritic and postsynaptic protein synthetic machinery, J. Neurosci. 19 (1999) 168–179.
[10] A.H. Gazzaley, D.L. Benson, G.W. Huntley, J.H. Morrison, Differential subcellular regulation of NMDAR1 protein and mRNA in dendrites of dentate gyrus granule cells after perforant path transection, J. Neurosci. 17 (1997) 2006–2017.
[11] A. Herb, W. Wisden, D.M.V. Catania, D. Marechal, A. Dresse, P.H. Seeberg, Prominent dendritic localization in forebrain neurons of a novel mRNA and its product, dendrin, Mol. Cell. Neurosci. 8 (1997) 367–374.
[12] K.M. Huber, M.S. Kayser, M.F. Bear, Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression, Science 288 (2000) 1254–1256.
[13] P.I. Jaison, P.S. Appukuttan, Identification of endogenous soluble glycoprotein receptors of bovine gray matter 14 kDa β-galactoside-binding lectin, Indian J. Biochem. Biophys. 31 (1994) 91–96.
[14] T.M. Jessell, M.A. Hynes, J. Dodd, Carbohydrates and carbohydrate-binding proteins in the nervous system, Annu. Rev. Neurosci. 13 (1990) 227–255.
[15] R. Joubert, S. Kuchler, J.P. Zanetta, D. Bladier, V. Avellana-Adalid, M. Caron, C. Doinel, G. Vincendon, Immunohistochemical localization of a β-galactoside binding lectin in rat central nervous system. I. Light- and electron-microscopical studies on developing cerebral cortex and corpus callosum, Dev. Neurosci. 11 (1989) 397–413.
[16] H. Kang, E.M. Schuman, A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity, Science 273 (1996) 1402–1406.
[17] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 277 (1970) 680–685.
[18] M.L. Leski, O. Steward, Protein synthesis within dendrites: ionic and neurotransmitter modulation of synthesis of particular polypeptides characterized by gel electrophoresis, Neurochem. Res. 21 (1996) 681–690.
[19] H. Lis, N. Sharon, Protein glycosylation. Structural and functional aspects, Eur. J. Biochem. 218 (1993) 1–27.
[20] M. Maletic-Savatic, R. Malinow, Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part I: trans-Golgi network-derived organelles undergo regulated exocytosis, J. Neurosci. 18 (1998) 6803–6813.
[21] K. Miyashiro, M. Dichter, J. Eberwine, On the nature and differential distribution of mRNAs in hippocampal neurites: implications for neuronal functioning, Proc. Natl. Acad. Sci. USA 91 (1994) 10800–10804.
[22] F. Nishimura, M. Nishihara, M. Mori, K. Torii, M. Takahashi, Excitability of neurons in the ventromedial nucleus in rat hypothalamic slices: modulation by amino acids at cerebrospinal fluid levels, Brain Res. 691 (1995) 217–222.
[23] Y. Ouyang, D. Kantor, K.M. Harris, E.M. Schuman, M.B. Kennedy, Visualization of the distribution of autophosphorylated calcium/calmodulin-dependent protein kinase II after tetanic stimulation in the CA1 area of the hippocampus, J. Neurosci. 17 (1997) 5416–5427.
[24] Y. Ouyang, A. Rosenstein, G. Kreiman, E.M. Schuman, M.B. Kennedy, Tetanic stimulation leads to increased accumulation of Ca^{2+}/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons, J. Neurosci. 19 (1999) 7823–7833.
[25] L.L. Phillips, S.J. Nostrandt, D.M. Chikaraishi, O. Steward, Increases in ribosomal RNA within the denervated neuropil of the dentate gyrus during reinnervation: evaluation by *in situ* hybridization using DNA probes complementary to ribosomal RNA, Mol. Brain Res. 2 (1987) 251–261.
[26] J.P. Pierce, K. van Leyen, J.B. McCarthy, Translocation machinery for synthesis of integral membrane and secretory proteins in dendritic spines, Nat. Neurosci. 3 (2000) 311–313.
[27] C. Racca, A. Gardiol, A. Triller, Dendritic and postsynaptic localization of glycine receptor α subunit mRNAs, J. Neurosci. 17 (1997) 1691–1700.
[28] A. Rao, O. Steward, Evidence that protein constituents of postsynaptic membrane specializations are locally synthesized: analysis of proteins synthesized within synaptosomes, J. Neurosci. 11 (1991) 2881–2895.
[29] A. Rao, O. Steward, Evaluation of RNAs present in synaptodendrosomes: dendritic, glial, and neuronal cell body contribution, J. Neurochem. 61 (1993) 835–844.
[30] A.J. Scheetz, A.C. Nairn, M. Costantine-Paton, NMDA receptor-mediated control of protein synthesis at developing synapses, Nat. Neurosci. 3 (2000) 211–216.
[31] P.K. Smith, R.I. Krohn, A.K. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1995) 76–85.
[32] J. Spacek, K.M. Harris, Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat, J. Neurosci. 17 (1997) 190–203.
[33] O. Steward, Alterations in polyribosomes associated with dendritic spines during the reinnervation of the dentate gyrus of the adult rat, J. Neurosci. 3 (1983) 177–188.
[34] O. Steward, P. Falk, Selective localization of polyribosomes beneath developing synapses: a quantitative analysis of the relationships between polyribosomes and developing synapses in the hippocampus and dentate gyrus, J. Comp. Neurol. 314 (1991) 545–557.
[35] O. Steward, S. Halpain, Lamina-specific synaptic activation causes domain-specific alterations in dendritic immunostaining for MAP2 and CAMKII, J. Neurosci. 19 (1999) 7834–7845.
[36] O. Steward, E.M. Schuman, Protein synthesis at synaptic sites on dendrites, Annu. Rev. Neurosci. 24 (2001) 299–325.
[37] O. Steward, P. Falk, E.R. Torre, Ultrastructural basis for gene expression at the synapse: synapse-associated polyribosome complexes, J. Neurocytol. 25 (1996) 717–734.
[38] Q.B. Tian, K. Nakayama, A. Okano, T. Suzuki, Identification of mRNAs localizing in the postsynaptic region, Mol. Brain Res. 72 (1999) 147–157.
[39] H. Tiegde, J. Brosius, Translational machinery in dendrites of hippocampal neurons in culture, J. Neurosci. 16 (1996) 7171–7181.
[40] E.R. Torre, O. Steward, Protein synthesis within dendrites: glycosylation of newly synthesized proteins in dendrites of hippocampal neurons in culture, J. Neurosci. 16 (1996) 5967–5978.
[41] S. Villanueva, O. Steward, Protein synthesis at the synapse: developmental changes, subcellular localization and regional distribution of polypeptides synthesized in isolated dendritic fragments, Mol. Brain Res. 91 (2001) 148–153 (this issue).
[42] I.J. Weiler, W.T. Greenough, Metabotropic glutamate receptors trigger postsynaptic protein synthesis, Proc. Natl. Acad. Sci. USA 90 (1993) 7168–7171.
[43] I.J. Weiler, S.A. Irwin, A.Y. Klintsova, C.M. Spencer, A.D. Brazel-

ton, K. Miyashiro, T.A. Comery, B. Patel, J. Eberwine, W.T. Greenough, Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation, Proc. Natl. Acad. Sci. USA 94 (1997) 5395–5400.

[44] L. Wu, D. Wells, J. Tay, D. Mendis, M.A. Abbott, A. Barnitt, E. Quinlan, A. Heynen, J.R. Fallon, J.D. Richter, CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of α-CaMKII mRNA at synapses, Neuron 21 (1998) 1129–1139.