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Maturation of the axonal plasma membrane requires upregulation of sphingomyelin synthesis and formation of protein–lipid complexes

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Neuronal maturation is a gradual process; first axons and dendrites are established as distinct morphological entities; next the different intracellular organization of these processes occurs; and finally the specialized plasma membrane domains of these two compartments are formed. Only when this has been accomplished does proper neuronal function take place. In this work we present evidence that the correct distribution of a class of axonal membrane proteins requires a mechanism which involves formation of protein–lipid (sphingomyelin/cholesterol) detergent-insoluble complexes (DIGs). Using biochemistry and immunofluorescence microscopy we now show that in developing neurons the randomly distributed Thy-1 does not interact with lipids into DIGs (in fully developed neurons the formation of such complexes is essential for the correct axonal targeting of this protein). Using lipid mass spectrometry and thin layer chromatography we show that the DIG lipid missing in the developing neurons is sphingomyelin, but not cholesterol or glucosylceramide. Finally, by increasing the intracellular levels of sphingomyelin in the young neurons the formation of Thy-1/DIGs was induced and, consistent with a role in sorting, proper axonal distribution was facilitated. These results emphasize the role of sphingomyelin in axonal, and therefore, neuronal maturation.

Keywords: axon/DIGs/GM1/hippocampal neurons/sphingomyelin

Introduction

The different morphological and molecular changes accompanying neuronal differentiation, from the time when these cells are round and in the process of migration to that of complex axonal and dendritic trees, have been characterized in detail in cultured hippocampal neurons (Craig and Banker, 1994). Shortly after plating neurons have a round cell body without any processes (stage 1). In the course of the next 4–24 h several processes emerge, all with similar morphological characteristics and length (stage 2). Later, one of the short processes elongates whereas the others remain stationary. This is known as stage 3 of neuronal development and it constitutes the first sign of neuronal polarization. Stage 4 of development occurs after ~4 days in culture and involves both axonal and dendritic elongation. At this stage cytoskeletal proteins, like the microtubule associated protein 2 (MAP2), begin to segregate to the dendritic territory (Caceres *et al.*, 1984). Stage 5 neurons (from 7 days in culture) have long and highly branched axons and dendrites with numerous intercellular contacts. At this time in culture cytoskeletal proteins such as MAP2 and tau, and certain dendritic membrane proteins present a polarized distribution (Caceres *et al.*, 1984; Craig *et al.*, 1994; Rao *et al.*, 1998). However, fine molecular segregation, like the restriction of certain surface proteins and the clustering of neurotransmitter receptors and channels required for efficient functional polarization, takes place at later times (Craig *et al.*, 1994). From this it follows that neuronal functional membrane asymmetry arises by the developmental maturation of two mechanisms: first the sorting pathway and then anchoring and clustering. In this work we focus on the possible mechanisms underlying the maturation of the sorting pathway.

Analysis of the distribution of membrane proteins in cultured neurons at different developmental stages revealed that those which present a clear polarized distribution in fully mature stage 5 neurons are randomly distributed in young stage 3 neurons (Dotti and Simons, 1990; Dotti *et al.*, 1991; Bradke and Dotti, 1997; this work). This makes the use of stage 3 and stage 5 neurons a good experimental system to study the mechanisms involved in neuronal membrane sorting: a mechanism effective in stage 5 cells should be absent or ineffective in stage 3 cells and its induction in the latter should facilitate the appearance of sorting. In this work we tested the hypothesis that one of such mechanisms is the formation of protein–sphingomyelin (SM)/cholesterol detergent-insoluble complexes (DIGs) (Parton and Simons, 1995). These complexes are formed during protein processing in the Golgi apparatus, and remain as such on the surface of living cells (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Although their physiological role is unclear (see Simons and Ikonen, 1997 for hypothesis), their importance in membrane trafficking has been extensively documented. In fact, inhibition of protein–glycosphingolipid DIG lipids (SM and cholesterol) or deletions of the protein domain interacting with lipids randomize the distribution of apical and axonal proteins (Mays *et al.*, 1995; Keller and Simons, 1998; Ledesma *et al.*, 1998).

In a recent work we showed that two membrane proteins, the glycosyl-phosphatidyl inositol (GPI)-anchored Thy-1 and the viral hemagglutinin (HA) require interaction early in the secretory pathway with sphingolipids [SM and glycosphingolipids (GSL)] and cholesterol into DIGs for proper delivery to the axonal surface of mature neurons (Ledesma *et al.*, 1998). This requirement is identical to

that reported for the apical delivery of the same proteins in epithelial cells (reviewed in Simons and Ikonen, 1997). The interaction with lipids confers on the proteins in DIGs resistance to extraction with detergents such as 3-[(3-cholamidopropyl)-dimethylammonio]-propansulfate (CHAPS), Triton X-100 or Triton X-114 at 4°C (Skibbens *et al.*, 1989; Brown and Rose, 1992; Fiedler *et al.*, 1993). We show in this work that in developing stage 3 neurons the missorting of Thy-1 and of the glycolipid GM1 correlates with the intrinsic poor capacity of these cells to form DIGs and that this is due to paucity in SM, but not cholesterol or glucosylceramide, levels. In agreement, we show that increasing the cellular levels of SM facilitates the appearance of axonal but not dendritic membrane polarization.

Results

Components of DIGs are not polarized in stage 3 neurons

We and others have shown that membrane proteins with a polarized axonal or dendritic distribution in fully mature neurons are present in both axon and dendrites of younger (stage 3) neurons (Dotti and Simons, 1990; Dotti *et al.*, 1991; Killisch *et al.*, 1991; Craig *et al.*, 1994). The viral HA glycoprotein and endogenous Thy-1, which are axonal and require interaction into DIGs for proper localization in mature neurons (Ledesma *et al.*, 1998), are no exception to this rule and are found in all the neurites of young neurons (not shown, but see Dotti and Simons, 1990; Dotti *et al.*, 1991 for confirmation).

Given the correlation between the axonal distribution of HA and Thy-1 and DIG formation in fully mature stage 5 neurons (Ledesma *et al.*, 1998), and the lack of polarization of these proteins in the developing stage 3 neurons, we decided to test whether the ganglioside GM1, which has been found in DIGs in different cell types, including neurons (Henke *et al.*, 1996), is also present on the axonal surface of fully mature stage 5 neurons and randomly distributed in stage 3 neurons. Cells were incubated before fixation with cholera toxin FITC. This toxin binds specifically to the GM1 on the cell surface (Fishman *et al.*, 1993). In stage 5 neurons GM1 is preferentially axonal as shown by double immunofluorescences comparing with either the dendritic marker MAP2 (Figure 1B) or the axonal marker Tau (Figure 1C). The quantification of GM1-MAP2 images reveals that in fully mature stage 5 neurons, 89% of GM1 is present in the axons whereas 11% is in the dendrites. In stage 3 neurons, however, the distribution of GM1 is equivalent in both axons and dendrites (Figure 1A). Similar results were obtained in cells fixed with methanol or paraformaldehyde.

Stage 3 neurons do not cluster the endogenous Thy-1 into DIGs

We have just provided evidence that proteins and lipids that are present in the axons of mature neurons are not polarized in stage 3 neurons. Then, if DIG formation is an essential mechanism for axonal sorting, these same proteins should not be present in DIGs in the young neurons. This was analyzed biochemically and by light microscopy. We first analyzed the biochemical behavior of the viral glycoprotein HA after infection with Fowl Plague virus (FPV). We observed that newly synthesized and plasma membrane HA partitioned into the detergent-insoluble fraction to a lesser extent in young developing neurons than in the fully mature cells (57 and 87%, respectively), suggesting poor capacity of the former to make DIGs. Moreover, in a flotation experiment using sucrose gradient centrifugation (Brown and Rose, 1992) only 25–30% of the HA from stage 3 cells appeared in the lightest fractions (which correspond to the buoyant density of lipids), in contrast to the 100% of the HA derived from stage 5 neurons (not shown). This further strengthened the view that stage 3 neurons have a deficient interaction between HA and DIG lipids. However, due to the fact that viral infection induced protein aggregation and that some of the HA under analysis was derived from infected glial cells (which in ~7% contaminate the young but not old cultures), we focused our studies on the behavior of an endogenous protein exclusively found in neurons: the GPI-anchored protein Thy-1. Stage 3 cells were treated with Triton X-114 at 4°C and the behavior of Thy-1 analyzed both biochemically and by immunofluorescence (Figure 2). In stage 3 cell extracts Thy-1 was only detected in the soluble fraction (Figure 2A). We have previously reported that in stage 5 cells a significant amount of the protein is insoluble (Ledesma *et al.*, 1998). The fact that essentially all of the Thy-1, intracellular and membrane, is soluble in the young cells indicates that the mechanism of DIG formation (in the Golgi apparatus) and DIG maintenance (at the level of the plasma membrane) has not yet matured. In agreement with this view only 5% of the total protein content of stage 3 cell extracts, as determined by silver staining of SDS–PAGE, is detergent insoluble. It is important to note that the fraction of insoluble proteins gradually increases during neuronal development: the percentage of insolubility was 5, 22, 38 and 48% of total protein content in extracts taken at 3, 4–5, 6 and 7 days in culture, respectively.

GPI anchor has been shown to act as a sorting determinant (Brown *et al.*, 1989) and as a signal for inclusion of proteins in DIGs (Rodgers *et al.*, 1994). Two pieces of data support the hypothesis that the lack of Thy-1 into DIGs in stage 3 cells is due to a true intrinsic developmental insufficiency to make DIGs and not to incomplete processing of the protein at this stage (lack of GPI-anchor). First, Thy-1 is on the surface of stage 3 neurons (Dotti *et al.*, 1991) indicative of normal GPI anchor (cleavage without glypiation results in secretion and lack of cleavage results in intracellular retention (Caras *et al.*, 1989; Moran and Caras, 1992). Secondly, Thy-1 present in the soluble fraction of stage 3 extracts partitions into the detergent phase upon temperature-induced phase separation, typical of normal GPI-anchored proteins (Lisanti *et al.*, 1988), and 65% of it moves into the aqueous phase after the anchor is broken by treatment with phosphatidylinositol-phospholipase C (PI-PLC) (Figure 2A).

Furthermore, lack of Thy-1 into DIGs in the young cells was confirmed at the single cell level (Figure 2B). In stage 3 cultures exposed to 1% Triton X-114 at 4°C for 4 min Thy-1 immunoreactivity was lost completely from the processes of 76% of the neurons and faint labeling could be seen only in the cell bodies. In contrast, in stage 5 cells Triton X-114 extraction did not affect Thy-1 distribution and immunoreactivity was still found

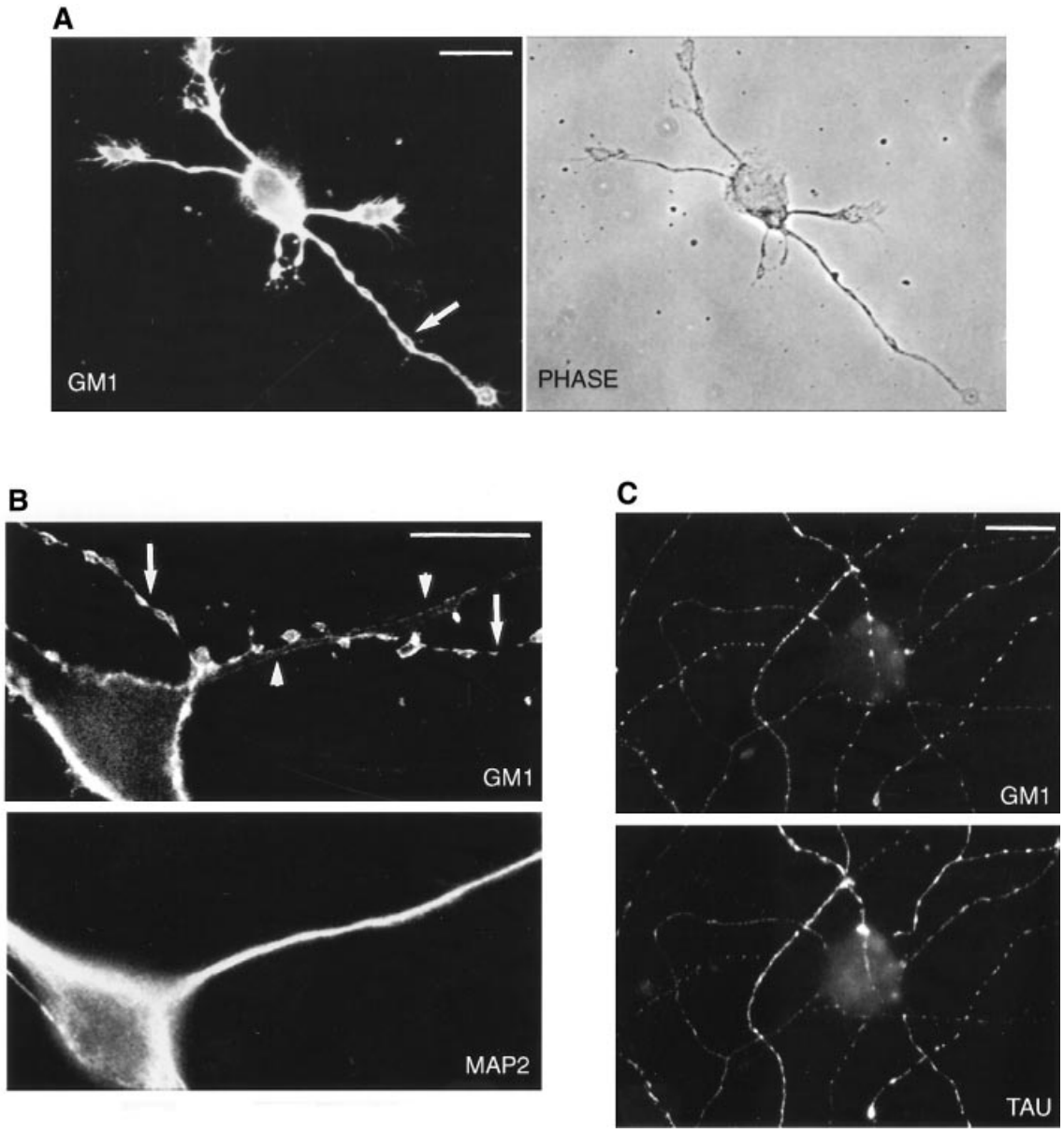

Fig. 1. GM1 distribution in stage 3 and stage 5 neurons. (**A**) The labeling corresponding to the FITC cholera toxin bound to GM1 in the surface of a stage 3 neuron is shown on the left panel (GM1). The right panel (PHASE) shows the phase contrast image of the same cell. GM1 is present on the surface of both axon (arrow) and dendrites of stage 3 neurons. (**B**) Double immunofluorescence image of a stage 5 neuron using FITC cholera toxin (GM1) and the cell body dendritic marker MAP2. Note that the dendrite (arrowheads) is devoid of GM1 staining. Axons (unlabeled with MAP2) are GM1 positive (arrows). The labeling on the periphery of the cell body may represent axons running in close apposition. This is an event that can sometimes be observed in old cultures in which axons form intricate networks. (**C**) Double immunofluorescence of a stage 5 neuron using FITC cholera toxin and the axonal marker Tau. Both GM1 and Tau colocalize to all processes. Cell body and dendrites are not visible. Scale bar represents 10 μm in all cases.

in the axon of 95% of these cells. Similar results were obtained using Triton X-100. Cold treatment alone did not have any effect on the levels of expression or localization (not shown).

In summary, although stage 3 neurons process the sorting signal of Thy-1 to become incorporated into DIGs normally, the mechanism responsible for making the interaction with lipids has not yet matured. Therefore, we next wished to determine whether this is due to a developmental paucity in the synthesis of DIG lipids: sphingolipids, cholesterol or both.

SM levels are low and cholesterol levels are high in stage 3 neurons

In epithelial cells DIGs are enriched in GSL, cholesterol and SM (Fiedler *et al.*, 1993; Melkonian *et al.*, 1995). SM is essential for conferring insolubility on GPI-anchored proteins and SM incorporated into pure liposomes has detergent-insoluble characteristics similar to that of DIGs in intact cells (Schroeder *et al.*, 1994). On the other hand cholesterol is also important in conferring insolubility in both epithelial and neuronal cells (Keller and Simons, 1998; Ledesma *et al.*, 1998). Thus, the lack of insolubility

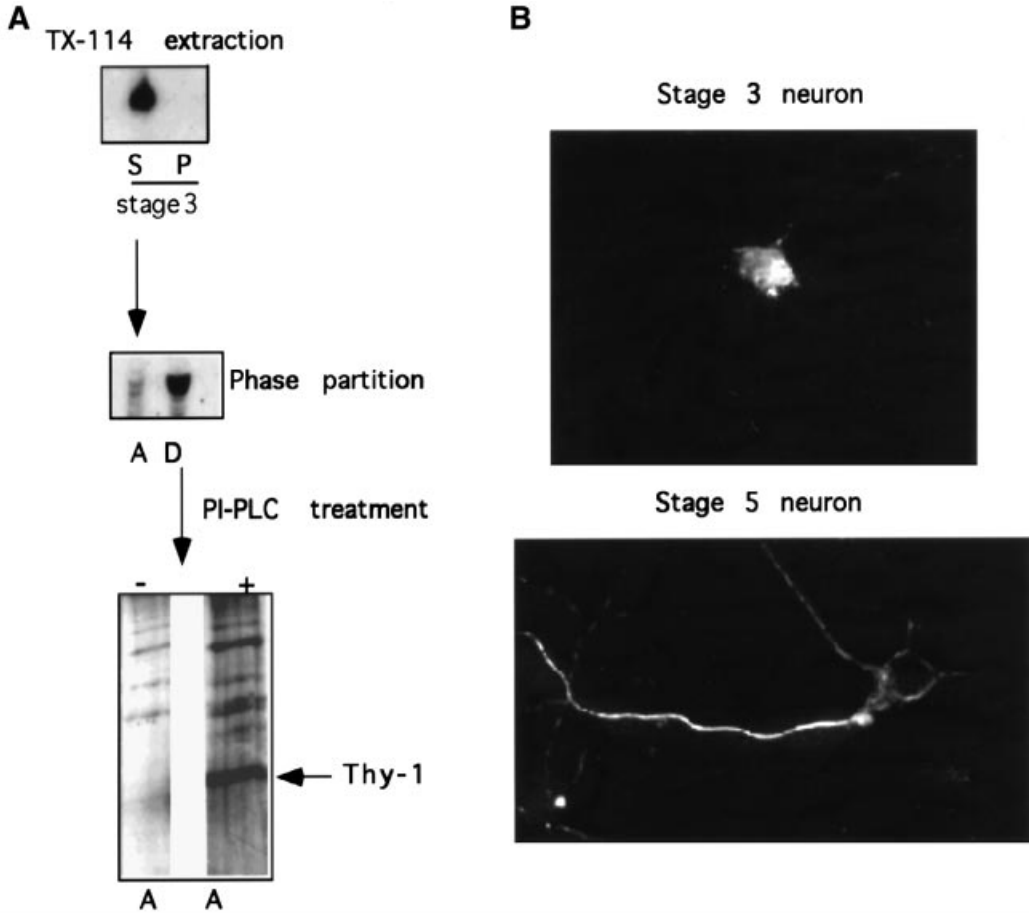

Fig. 2. Triton X-114 insolubility of Thy-1. (**A**) Western blot analysis of Triton X-114 insolubility of Thy-1 in stage 3 hippocampal neurons. The soluble (S) and insoluble (P) fractions after the detergent extraction were analyzed using a monoclonal antibody anti-Thy-1. In these cells essentially all Thy-1 is soluble. Detergent extraction of this fraction with 1% Triton X-114 at 37°C results in Thy-1 partitioning in the detergent phase (D), consistent with the presence of the GPI-anchor. This was also confirmed by treatment with PI-PLC, which removes the lipidic anchor, therefore shifting the protein (shown by silver staining) into the aqueous phase (A) in the treated samples (+). Thy-1 is not present in the aqueous phase (A) of controls incubated in the same conditions without PI-PLC(–). (**B**) Thy-1 solubility in stage 3 cells and insolubility in stage 5 neurons can be revealed by immunofluorescence microscopy. Coverslips with stage 3 or stage 5 cells were incubated for 4 min on ice with 1% Triton X-114 in microtubule stabilizing buffer. The cells were then fixed and processed for immunofluorescence using the monoclonal anti-Thy-1. In agreement with the result obtained in the Western blot, the detergent was able to extract Thy-1 from the majority of the developing neurons (stage 3 neuron) whereas insoluble Thy-1 remained in the axons of adult cells (stage 5 neuron).

and inclusion into DIGs of axonal proteins in the polarized stage 3 hippocampal neurons could be due to the paucity of SM and/or cholesterol in these cells. To test this hypothesis, we analyzed and compared the levels of SM in stage 3 and stage 5 cultures. Mass spectrometry of total cell lysates revealed an increase of all SM species in stage 5 neurons (Figure 3A). Referring the data obtained to the amount of protein, 0.63 pmol of total SM/μg of protein were found in stage 3 cultures, whereas there were 2.38 pmol of total SM/μg of protein in stage 5 cultures. This represents SM levels lower by a factor of 3.7 in stage 3 than in stage 5 neurons.

To further investigate whether these differences are due to increased biosynthesis or to prolonged half life, the rate of synthesis of SM was determined using truncated ceramide. This ceramide analog has been shown to be metabolized as its natural counterpart (Karrenbauer *et al.*, 1990) and it permits analysis of enzymatic activities in the absence of detergents, therefore under the most physiological conditions. Stage 3 and stage 5 cells were incubated with [^{3}H]truncated ceramide ([^{3}H]t-Cer) and the amounts of newly synthesized truncated derivatives, glucosylceramide (t-GlcCer) and SM (t-SM), were quantified by thin-layer chromatography (TLC) (Figure 3B). In both stage 3 and stage 5 cells the rate of synthesis of t-GlcCer was similar. However, t-SM synthesis was 4-fold lower in stage 3 than in stage 5 cells, confirming that stage 3 neurons have a poor capacity to produce SM.

Next, we tested whether endogenous cholesterol levels were also low in the developing stage 3 neurons. Cholesterol concentrations were normalized with respect to total phospholipid and determined by the method described by Gamble *et al.* (1978). The ratio phospholipid/cholesterol was 1.8 ± 0.5 in stage 3 neurons and 2.7 ± 0.7 in stage 5 neurons. This represents cholesterol levels higher in stage 3 than in stage 5 of development by a factor of 1.5.

Altogether these data suggest that the lack of DIG formation for HA and Thy-1 during stage 3 is due to a deficit in SM but not cholesterol or glucosylceramide.

Addition of ceramide results in higher intracellular insoluble SM and increases the resistance of Thy-1 to detergent extraction

Up to this point we have shown that in young neurons there is a striking correlation between lack of proper

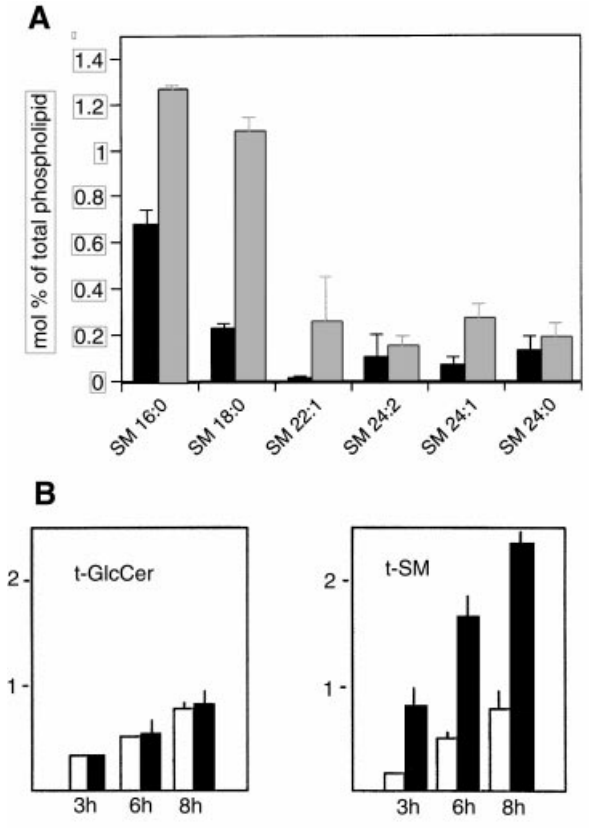

Fig. 3. (**A**) Quantitation of SM in total cell lysates of stage 3 and stage 5 neurons by ESI-MS/MS analysis. Lipid extraction of stage 3 and stage 5 neurons was performed in the presence of internal lipid standards. Extracts were dissolved in $CHCl_3$/MeOH (1:2). Ammonium acetate was added to a final concentration of 10 mM. Precursor Ion Scanning *m*/*z* 184 was used for detecting PC and SM species only. Mass and isotope correction of the spectra and quantitation were performed as indicated in Materials and methods. Phosphate determination was performed according to Rouser *et al.* (1970). The amounts of SM species detected in stage 3 (black bars) and stage 5 (gray bars) neurons are expressed in mol% of total phospholipid. The data represent the mean values ± SD from four independent experiments using different cultures. In all the SM species analyzed there are significantly lower levels in stage 3 than in stage 5 neurons. (**B**) TLC analysis of [^{3}H]t-Cer derivatives in the neuronal medium. Same amount (2×10^5 cells) of stage 3 (white bars) and stage 5 (black bars) neurons were incubated with [^{3}H]t-Cer and equivalent aliquots of the medium were collected at 3, 6 and 8 h. The t-Cer (t-Cer) and its derivatives (t-GlcCer and t-SM) were analyzed by TLC and quantified with a 2D-TLC-scanner. The mean values in arbitrary units ± SD of three different experiments of the amounts of t-GlcCer or t-SM obtained at the different times are plotted in histograms.

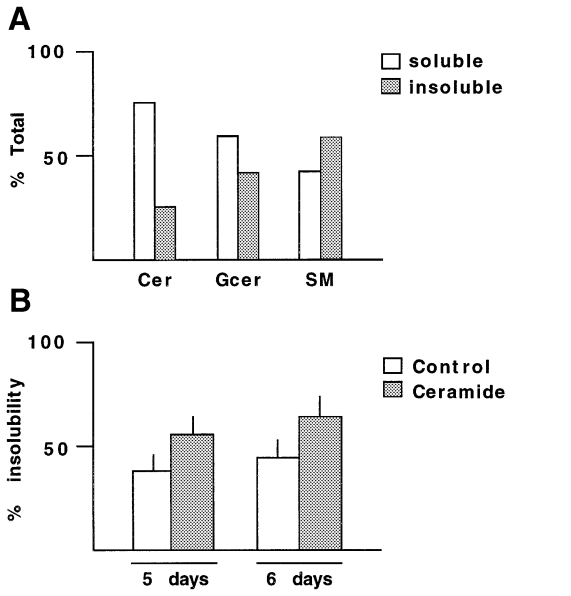

Fig. 4. (**A**) Exogenous NBD-ceramide produces insoluble NBD-SM. Six-day-old hippocampal neurons were treated for 12 h with 2 μM NBD-C6-Cer, then incubated with 1% Triton X-100 on ice for 1 h and centrifuged. The lipids present in the soluble and insoluble fractions were extracted, separated by TLC and quantified. Bars reflect the percentage of NBD-Ceramide (Cer), NBD-Glucosylceramide (Gcer) and NBD-sphingomyelin (SM) in the soluble (white bars) and insoluble (black bars) fractions with respect to the total amount of each lipid. (**B**) Exogenous NBD-ceramide increases Thy-1 insolubility. Five-day-old (5 days) or 6-day-old (6 days) hippocampal neurons were treated (Ceramide) or not (control) with 0.5 μM NBD-C6-Cer for 24 h, extracted on ice with 1% Triton X-100, and the presence of insoluble Thy-1 analyzed by immunofluorescence microscopy. The figure shows the percentage of cells in which Thy-1 could not be extracted (% insolubility). Ceramide treatment increases in a statistically significant 20% the insolubility of Thy-1 in developing stage 4 neurons.

axonal distribution, poor ability of axonal proteins to interact with lipids into DIGs and poor synthesis of a DIG lipid, SM. However, we still lack direct proof that SM is a true functional determinant for the developmental appearance of proper axonal distribution. To address this point, young developing neurons were incubated with exogenous ceramide, the natural precursor of SM, and to determine whether (i) the treatment induced an increase in the amount of Thy-1 that resisted extraction to Triton X-100, and (ii) the intracellular distribution of the protein changed from nonpolarized to axonal. These results are shown in Figures 4 and 5. To increase endogenous SM we used *N*-{6-[(7-nitro-benzo-2-oxa-1,3-diazol-4-yl)amino]caproyl}-D-erythrosphingosine (NBD-C6-Cer), which has been shown to be converted into NBD-C6-GlcCer and NBD-C6-SM in different cell types (Lipsky and Pagano, 1983; van Meer *et al.*, 1987; Kobayashi and Pagano, 1989), and which despite its modified fatty acid chain is capable of interacting with cholesterol via hydrogen bonds (Bittman *et al.*, 1994).

The addition of NBD-C6-Cer to cells from days 1–3 in culture (stages 2 and 3 of development) resulted in cell death within a few hours even at low concentrations of the compound. On the contrary, addition of 0.5 μM NBD-C6-Cer to 4- or 6-day-old neurons (developmental stage 4) for 24 h did not affect cell viability or morphology (this is evident in the photographs shown in Figures 5Ba and 6Ba). The lack of toxicity of the compound at this concentration was also tested at longer times of incubation (up to 3 days) and with older neurons (fully mature stage 5). The number of cells in untreated and treated cultures was equivalent (3–6 cells/field) and there was no significant change either in the average length of the primary neurites (30 ± 20 μm for 5-day-old neurons) or in their morphology. Hence, this time-window (4–6 days *in vitro*) was used for the studies on solubility and distribution of Thy-1.

We analyzed first whether the addition of NBD-C6-Cer resulted in the presence of detergent-insoluble NBD metabolites (glucosylceramide and SM), as occurs with endogenous ceramide. Stage 4 cells treated with NBD-C6-Cer were extracted in Triton X-100 on ice and the

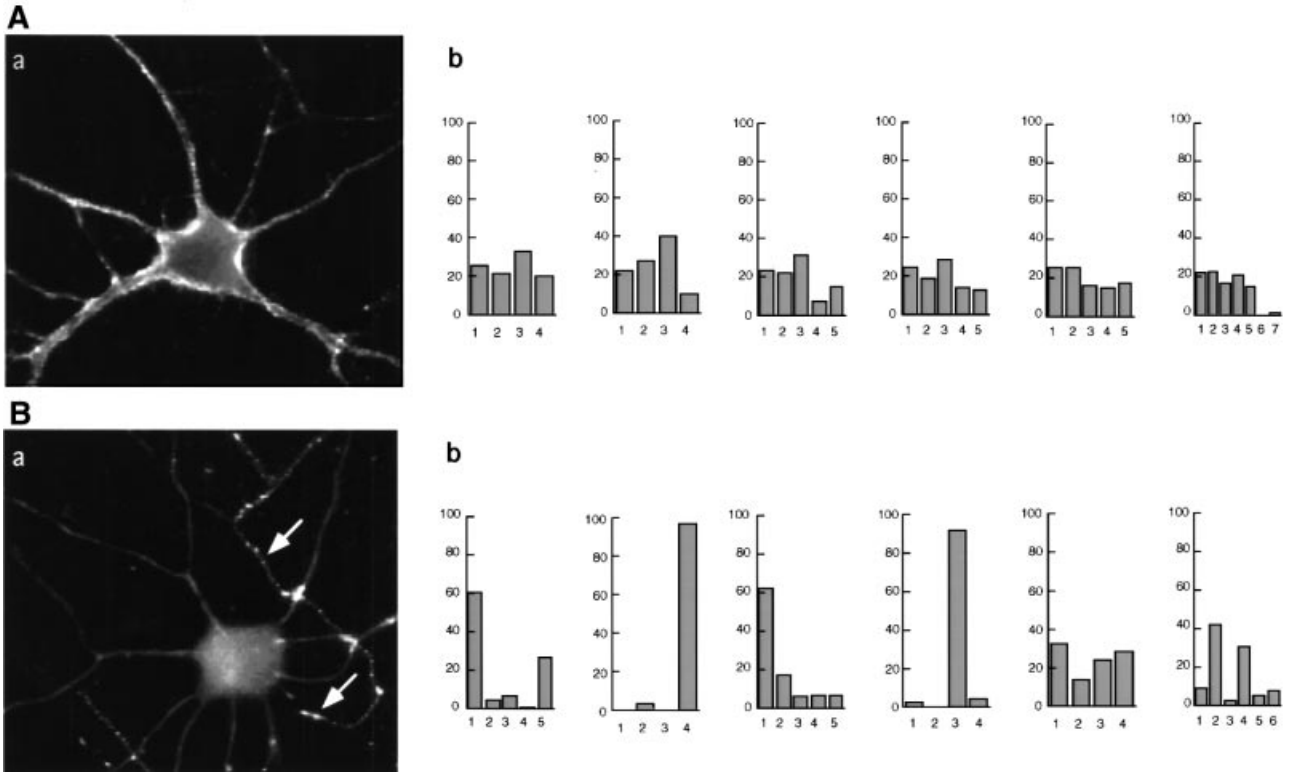

Fig. 5. Thy-1 distribution after NBD-C6-Cer treatment in stage 4 neurons. (**A**) (a) Fluorescence image of surface Thy-1 staining in a control stage 4 neuron (6 days in culture). (b) Quantification of Thy-1 distribution in each of the processes of the cell corresponding to the primary trunks. Secondary and tertiary branches were not considered. The processes are randomly indicated by numbers on the horizontal axis. Six different control stage 4 neurons are shown as representative examples. The values are represented as percentage of the total Thy-1 intensity within all the selected areas in the neurites of each cell (see Materials and methods). In all the cases Thy-1 is roughly equally distributed to all the neurites. (**B**) (a) Fluorescent image of surface Thy-1 labeling of a stage 4 neuron treated with NBD-C6-Cer for 24 h. Thy-1 is restricted to one process (shown by arrows) whereas the others remained unlabeled. The quantification of Thy-1 distribution in each of the primary neurites (randomly indicated by numbers on the horizontal axis) of six different stage 4 ceramide-treated neurons is shown in (b) as percentage of the total Thy-1 in each cell. Of the ceramide-treated neurons analyzed 67% presented a polarization of Thy-1 distribution.

lipids present in the soluble and insoluble fractions were quantified by TLC (Figure 4A). Whereas NBD-ceramide was mostly soluble (75%) a significant proportion of its metabolites: NBD-glucosylceramide and NBD-SM (41 and 58% respectively) were present in the insoluble fraction. Comparison of the intracellular values of SM in control cells, as determined by mass spectrometry, with those of NBD-SM, as determined by TLC, revealed that NBD-ceramide addition produced a 25–30% increase in the total intracellular levels of SM. This result shows that addition of NBD-C6-Cer is a valuable tool to increase intracellular detergent-resistant SM.

We next asked, does NBD-C6-Cer addition increase the resistance of Thy-1 to detergent extraction? Cells grown for 5 days in culture were treated or not with ceramide for 24 h, extracted with 1% Triton X-100 for 4 or 8 min on ice, washed, fixed and the resistance of Thy-1 to detergent extraction determined by immunofluorescence. Any cell in which labeling is higher than the staining observed for the secondary antibody alone is considered detergent resistant no matter what the level of fluorescence intensity. A similar experimental procedure was utilized in cells grown for 6 days in culture. Four different cultures were used, analyzing 60 control and 60 ceramide-treated 5-day-old neurons and 60 control and 60 ceramide-treated 6-day-old neurons from each culture. The result of such analysis is shown in Figure 4B. Thy-1 resisted detergent extraction in 39.8 $\pm$ 7.4% of control untreated neurons fixed after 6 days in culture; the addition of ceramide on day 5 in culture followed by extraction and fixation on day 6 increased the number of Thy-1 positive cells after detergent extraction to 57.6 $\pm$ 9.4%. A similar trend was observed in cells treated with ceramide on day 6 and extracted and fixed on day 7. In control neurons fixed after 7 days in culture Thy-1 resisted extraction in 45.9 $\pm$ 9.6%; this percentage rose to 63.1 $\pm$ 10.8% after ceramide treatment. There was no significant variation depending on the length of detergent extraction. The differences found are statistically significant as revealed by the *t*-test ($p = 0.0046$ for 5-day-old neurons and $p = 0.0158$ for 6-day-old neurons). Besides the increase in Thy-1 insolubility, ceramide treatment also induced a 15% increase in total protein insolubility with respect to the control cells, as evidenced by SDS–PAGE.

The above results show on one hand that there is an endogenous maturation of the DIG machinery (Thy-1 was insoluble in a higher number of 7- than 6-day-old control neurons) and on the other hand the favorable effect of ceramide addition (increases in a significant 20% the number of cells in which Thy-1 remains insoluble in both 6- and 7-day-old neurons). A possible reason for not reaching 100% efficient insolubility in these not yet mature neurons is that not enough NBD-ceramide is converted into insoluble SM. The reason for this could be a developmental

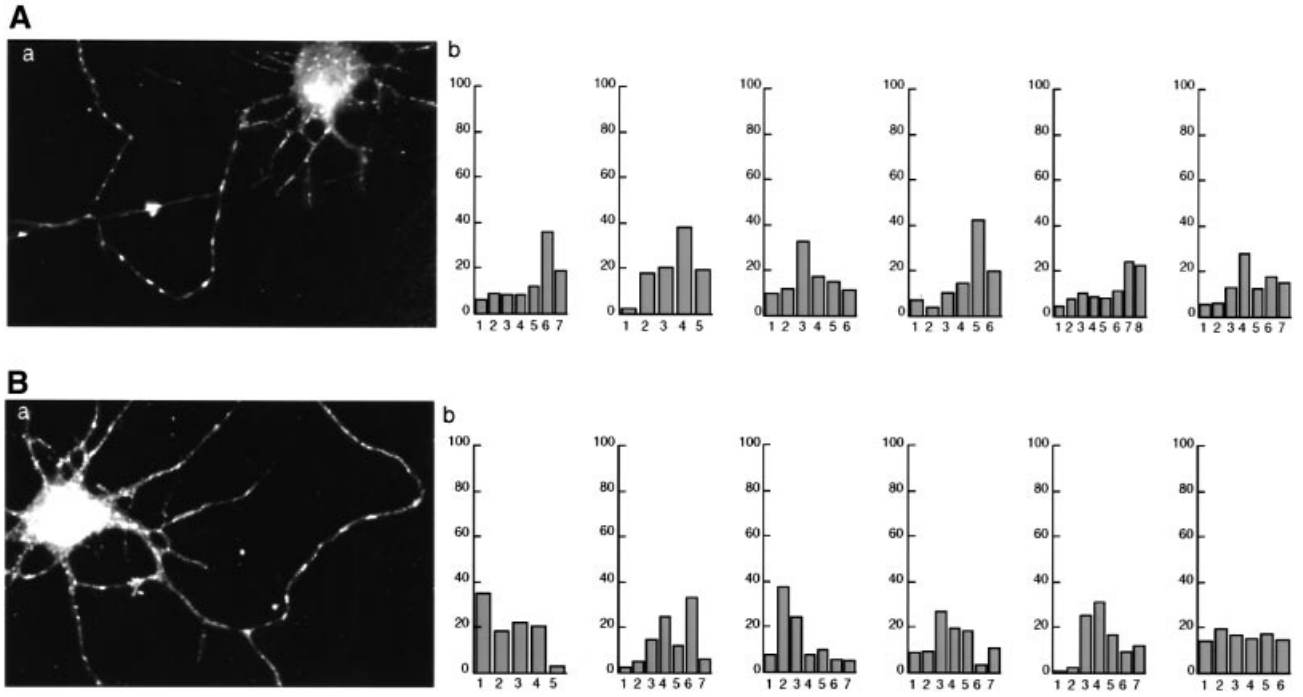

Fig. 6. Transferrin receptor distribution after NBD-C6-Cer treatment in stage 4 neurons. (**A**) (a) Fluorescence image of TfR staining in a control stage 4 neuron in culture. The long and thin axon as well as the shorter dendrites all contain TfR. The quantification of TfR distribution in each of the primary processes (randomly indicated by numbers on the horizontal axis) of 6 different control neurons is shown in (b). The values are represented as percentage of the total TfR staining in each cell. (**B**) (a) Fluorescent image of TfR labeling of a stage 4 neuron treated with NBD-C6-Cer for 24 h. It is clear that the axon (the longest process) still contains receptor. The quantification of the protein distribution in each of the primary processes (randomly indicated by numbers on the horizontal axis) of six different ceramide-treated neurons is shown in (b) as percentage of the total TfR intensity within all the selected areas in the neurites of each cell. No preferential delivery of the protein to one process was detected either in the control or in the ceramide-treated neurons. In some cases one or two neurites contain larger amounts of TfR but never more than 2-fold the amount in the rest of the processes.

deficiency of SM synthase levels or activity (indeed, Figure 4A reveals a large pool of NBD-ceramide that remains unmetabolized).

Addition of ceramide advances Thy-1 polarization

The last series of experiments shows that increasing the intracellular levels of insoluble SM is important for the appearance of protein–lipid complexes. Yet, the important physiological question is: does the increase in insoluble SM facilitate the appearance of polarized distribution? Four- and 5-day-old neurons from three independent cultures were treated or not with 0.5 μm NBD-C6-Cer for 24 h. Then the cells were washed and the distribution of Thy-1 was analyzed by immunofluorescence. In untreated stage 4 neurons, fixed on day 5 or 6 in culture, Thy-1 was found roughly equally distributed to all processes, similarly to the distribution found in stage 3 neurons. Thus, polarization of Thy-1 was 0% under control conditions. The quantification of this experiment is shown in Figure 5Ab and a typical example of Thy-1 staining in a control stage 4 neuron is presented in Figure 5Aa. On the contrary, in cultures treated with 0.5 μM NBD-C6-Cer for 24 h 67% of the neurons presented Thy-1 labeling restricted to one (and sometimes two) process (considering 3-fold more protein than in the others). An example of ceramide-induced Thy-1 polarization is shown in Figure 5Ba. Figure 5Bb shows the quantification of Thy-1 distribution in six different neurons treated with NBD-C6-Cer. Consistent with the favorable effect of higher levels of SM in the polarized distribution of an axonal protein, we observed that the chronic depletion of this lipid, using the inhibitor Fumonisin B, had a deleterious effect on the formation of axon-like processes (not shown).

To test if the ceramide addition also accelerated the polarization of dendritic membrane proteins, which normally do not require interaction with lipids in DIGs for proper sorting (Ledesma *et al.*, 1998), we repeated the experiment and analyzed, in an equivalent number of cells (30 control and 30 ceramide-treated from three independent cultures) the distribution of the transferrin receptor (TfR), which is dendritic in fully mature stage 5 neurons (Parton *et al.*, 1992). Under our culture conditions untreated 6-day-old neurons still have the TfR randomly distributed to all the processes (0% of the control cells had TfR polarized, Figure 6A). The addition of ceramide to sister cultures did not change this uniform distribution (0% of ceramide-treated neurons had TfR polarized, Figure 6B). The amount of TfR found in each of the processes of six different treated cells is shown in Figure 6Bb as a percentage of the total staining analyzed in each cell. Although in some cells TfR was more abundant in one or two neurites, this also happened in control neurons (Figure 6Ab) ruling out the possibility of an increased staining due to stimulation of growth by the experimental treatment. Moreover, the processes showing a higher labeling had in many cases the morphological appearance of axons, inconsistent with dendritic sorting and consistent with preferential transport to the still growing axon (Bradke and Dotti, 1997). Finally, in no case was this increment in labeling >2-fold than in the other neurites and our

criteria for a polarized distribution is a 3-fold increment in the staining. Such increase was only observed for Thy-1 in 67% of the ceramide-treated neurons.

To rule out that the addition of ceramide was accelerating Thy-1 sorting by increasing protein synthesis, we measured the protein concentration in treated and untreated dishes. The value obtained was 16.6 ± 1.2 μg/dish for the untreated neurons and 16.9 ± 0.7 μg/dish for the ceramide-treated. This was also analyzed by silver staining of SDS–PAGE of control and ceramide-treated samples

In all, manipulation of the intracellular level of SM produced a very consistent picture: it improved the formation of DIGs and facilitated axonal but not dendritic sorting.

Discussion

The data presented in this work imply that one important requirement for the acquisition of a mature axonal membrane is the ability to generate DIGs. Thus, we show that young but morphologically polarized neurons (stage 3 neurons) have a poor ability to form DIGs, correlating with the unpolarized distribution of certain proteins and lipids which in fully mature cells (stage 5 neurons) are enriched on the axonal surface. Moreover, we show that both unpolarized distribution and poor ability to form DIGs can be explained by the low levels of an essential DIG lipid, SM. In agreement with this, when we increased intracellular SM in the young neurons insolubility and polarization of an axonal protein improved. Hence, it is reasonable to think that the developmental increase in SM levels plays an essential role in neuronal maturation.

The establishment of membrane polarity

Young stage 3 neurons are clearly morphologically polarized and still have a very poor capacity to form DIGs. Fibroblasts, in contrast, do not have evident morphological polarity and are still capable of forming DIGs (Skibbens *et al.*, 1989; Keller and Simons, 1998). Are fibroblasts more polarized than stage 3 neurons? This may be the case. Indeed it has been shown that fibroblasts, despite their 'unpolarized' morphology, have developed the capacity to generate membrane subdomains equivalent to those found in the apical and basolateral surface of polarized epithelia (Yoshimori *et al.*, 1996; Keller and Simons, 1998). The lack of molecular polarity in developing neurons is, however, transitory and reflects a differentiation event; upon reaching maturity protein–lipid DIGs are present and play an important role in the axonal delivery of certain membrane proteins, both heterologous and endogenous (Ledesma *et al.*, 1998). Similar developmental changes in detergent solubility and sorting have been described in oligodendrocytes (Kramer *et al.*, 1997). Then, the random delivery of HA and Thy-1 to the axonal and dendritic surface in the young stage 3 neurons may occur following a 'default' pathway, as proposed earlier to explain the generation of the fibroblast plasma membrane (Pfeffer and Rothman, 1987). In support of this model, we recently described the polarized bulk-flow transport of membrane and cytosolic components of mature dendrites towards the axon of stage 3 cells (Bradke and Dotti, 1997). The establishment of a spatially different plasma membrane occurs at later developmental stages. Our data imply that one mechanism important for conferring axonal plasma membrane identity is the establishment of the molecular machinery responsible for the formation of protein–lipid DIGs. DIG formation is, however, only one of the mechanisms that must operate to guarantee the correct composition of the axonal membrane proteins. We observed that the axonal protein synaptophysin does not bind the lipids present in the insoluble complexes and remains in the heaviest fractions after a sucrose gradient centrifugation (M.D.Ledesma, unpublished observation). The mechanisms by which this and other axonal proteins are specifically targeted to the axon remain to be determined. It also remains to be addressed whether axonal-mediated sorting by DIGs is a direct or indirect (e.g. exclusion of dendritic proteins) mechanism.

Consistent with the lack of sorting of DIG proteins, a DIG lipid, the ganglioside GM1, is also found in both axons and dendrites in young cells. In fully mature neurons it appears preferentially enriched on the axonal surface. Thus, DIGs could be a sorting mechanism not only for certain proteins but also for lipids. This is the first evidence for the preferential delivery of a GSL to axons (for a review see Schwarz and Futerman, 1996). Previous studies described an unpolarized distribution of GM1 in hippocampal neurons in culture (Sofer and Futerman, 1995). This discrepancy may have a simple explanation: different experimental procedures. First, we used cells with more than 10 days in culture whereas Sofer and Futerman used 6-day-old cells. At this time neurons have not yet fully matured the membrane sorting pathway (see Figures 5 and 6). Secondly, we incubated cells with cholera toxin for 1–2 min on ice whereas in the work mentioned it was done for 30 min at 13–16°C. The results obtained here argue in favor of the sorting of GM1 as a late event during development; at a time when the neurons establish synapsis. Whether or not the axonal segregation of GM1 reported here has any role in the appearance or maintenance of the synapse, structurally or functionally, remains to be determined. However, early work on the role of gangliosides in neurotransmitter release would support this view (Wu and Ledeen, 1991).

Role of different components in DIGs

Cholesterol and sphingolipids (SM and GSL) are the essential lipid components of DIGs; reduction in their levels in mature hippocampal neurons affect both insolubility and sorting of axonal proteins (Ledesma *et al.*, 1998). In this work we have analyzed the role of these lipids and DIG formation in the maturation of the axonal sorting pathway. We have found that the amount of cholesterol is high in stage 3 neurons with respect to that of stage 5. In addition, the synthesis of the precursor of GSL, glucosylceramide, is similar in stage 3 and stage 5 of development and a strong labeling of the ganglioside GM1 could be seen on the surface of stage 3 neurons. All these data would argue against the possibility that the lack of formation of DIGs at this stage is due to a deficiency in cholesterol or GSL. One can envision that at early stages of neuronal differentiation these lipids play a role in cell survival and neurite outgrowth. In agreement with this is the observation that ongoing synthesis of glucosylceramide is required for axon growth during stage 3 (Schwarz *et al.*, 1995), and that young neurons are much more sensitive

to cholesterol depletion than adult neurons (M.Simons, unpublished results).

In contrast to cholesterol and GSL we have found very low levels of SM in the young stage 3 neurons. One obvious conclusion would be that it is the deficit of this lipid that is responsible for the poor ability of these cells to make DIGs, and therefore for the lack of axonal maturity. Supporting this we show that the addition of ceramide increases the levels of insoluble SM and Thy-1 insolubility, and more importantly accelerates its polarization: numerous cells presented an almost exclusive delivery of the protein to one neurite. Although we can not completely rule out that the addition of ceramide also acted by other means (i.e. signaling pathway), the fact that NBD-ceramide did not change the levels of protein synthesis or transport, and failed to accelerate the correct sorting of the 'dendritic' TfR argues in favor of a specific effect of ceramide-derived SM on DIG formation.

Putting together the results from adult (Ledesma *et al.*, 1998) and young neurons (this work) we could then conclude that only when the correct ratio between cholesterol, GSL and SM is established would axonal sorting of certain membrane proteins occur and that SM may be the limiting step for the developmental establishment of such an important event. Indeed, the 'polarizing' effects of ceramide addition occurred in neurons with more than 4 days in culture (stage 4 of development) resulting in cell death at earlier times. This suggests that stage 2 and stage 3 neurons are not yet prepared to metabolize the added ceramide to SM [the former would then accumulate, having an apoptotic effect (Schwarz and Futerman, 1996)]. One possible explanation for that could be a deficiency in the necessary enzymes at these early stages (we show that not only the levels but also the rate of SM synthesis is very low in comparison with that of stage 5 neurons). This is also in agreement with the observation that stage 3 neurons metabolize NBD-C6-Cer mainly to NBD-C6-GlcCer and not NBD-C6-SM (Schwarz and Futerman, 1997).

From the results discussed above we could postulate that DIG lipids play different roles in axonal sorting with cholesterol, GSL and SM essential for creating the complexes and conferring detergent insolubility and SM for, somehow, making the axonal sorting signals operative.

An interesting example that supports a physiological role for DIGs in axonal maturation is the Niemann–Pick disease (Type A). This is a disorder that results from the deficient activity of acid sphingomyelinase which hydrolyzes SM to ceramide. SM accumulates in lysosomes and it is not degraded into ceramide, therefore new SM can not be produced. As a consequence patients present a rapid and progressive neurodegenerative course that leads to death at the age of 2 or 3 years (for a review see Brady, 1987). Recently, an acid sphingomyelinase-deficient mouse line has been generated that is phenotypically comparable to Niemann–Pick disease Type A (Horinouchi *et al.*, 1995). Interestingly, among other alterations, an axonal dystrophy in both central and peripheral nervous systems was detected without signs of dysmyelination or demyelination (Kuemmel *et al.*, 1997). These data support our view on the essential role of ceramide metabolites and DIGs in neuronal axonal maturation.

Materials and methods

Cell culture

Cultures of hippocampal cells were prepared from the brains of 18-day-old rat embryos as described in Goslin and Banker (1991). These neurons survive for several weeks and undergo full polarization when cultured in serum-free medium (N_2) in the presence of a supporting layer of astrocytes. For our experiments cells were kept in culture for 48–72 h (stage 3 neurons), 4–6 days (stage 4 neurons) or 8–15 days (stage 5 neurons).

Virus infection

Stocks of FPV were obtained as described (Matlin *et al.*, 1981; Fuller *et al.*, 1985). Cultured hippocampal cells were infected with FPV: 1–10 p.f.u./cell in N_2 medium. After 1 h infection at 37°C the virus-containing medium was replaced by fresh N_2 and the cells were further incubated for different times depending on the experiments.

Immunofluorescence of surface membrane proteins and GM1 distribution

For surface labeling of proteins FPV-infected or non-infected cells were incubated with either a polyclonal antibody against HA (Matlin *et al.*, 1981) or the mouse anti-Thy 1.1 (clone number MRC OX-7 from Serotec, Camon), respectively, diluted in culture medium for 20 min at 37°C. The cells were fixed with 4% PFA and finally incubated with the species-specific secondary antibodies: rhodamine conjugated anti-rabbit IgG from Dianova or fluoresceine conjugated anti-mouse from Amersham.

For the labeling of TfR, protein cells were fixed in 4% PFA for 15 min and permeabilized with 0.1% Triton X-100 for 5 min. The cells were incubated with a monoclonal antibody against the rat TfR (PharMingen). A rhodamine-conjugated anti-mouse antibody (Cappel) was used as a secondary antibody.

For the GM1 staining stage 3 and stage 5 neurons in coverslips were washed twice with phosphate-buffered saline (PBS) and incubated with 8 μg/ml cholera toxin B subunit FITC (Sigma) diluted in 1% BSA in PBS for 1–2 min on ice. Then the cells were washed with PBS and fixed with methanol at –20°C for 5 min. Cells were then incubated with blocking solution (2% bovine serum albumin, 0.2% fish-skin gelatin and 2% fetal calf serum in water) for 30 min at room temperature (RT). The polyclonal antibody 514 against MAP2 (a gift from Dr C.Sanchez, CBM, Madrid, Spain) or the monoclonal antibody against Tau protein (Tau-1, Boehringer Mannheim) were used for 1 h at RT and after washings with PBS the cells were treated with a rhodamine conjugated anti-rabbit or anti-mouse antibodies respectively. Microscopy was performed with an Axiophot microscope (Zeiss).

For the quantification of protein expression and distribution by immunofluorescence, images were captured at non-saturating integration levels with a charge-coupled device camera (Cohu 4913, Cohu, San Diego, CA) connected to a Macintosh computer (Power Macintosh 7300/166) equipped with an image recorder (Openlab). Images were stored by using NIH IMAGE. For the axonal versus dendritic quantification of GM1, labeled cells were density sliced and segments in each of the neurites were randomly chosen and delineated, the areas were binarized and the signal within the area was quantified (total surface/pixel intensity). Areas were normalized to the same size. The value obtained for the process identified as the axon (MAP2 negative) was compared with the value obtained for the dendrites (MAP 2 positive) in each cell. The data presented are the average percentage of GM1 found in the axon with respect to the total GM1 present in all the dendrites of a cell. Twenty fields from two independent experiments performed with different cell cultures were analyzed.

Detergent extractions

Triton X-114 insolubility of Thy-1 was analyzed following a protocol adapted from Lisanti *et al.* (1988). Cells were extracted for 1 h at 4°C in TBS (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA and a mixture of antiproteases) containing 1% Triton X-114. Insoluble material was pelleted by a 10 min centrifugation at 15 000 *g* at 4°C. Proteins in the soluble and insoluble fractions were analyzed by SDS–PAGE (15%). Thy-1 immunoreactivity was assessed by Western blot using a monoclonal antibody described above. Detection of the presence of the GPI-anchor in Thy-1 protein, phase separation and PI-PLC treatment of stage 3 supernatants after detergent extraction were performed according to Dotti *et al.* (1991).

For the immunofluorescence assays cells on coverslips were incubated

in cold MSB (2 mM $MgCl_2$, 10 mM EDTA, 60 mM PIPES pH 7.00) for 1 min and extracted with either 1% Triton X-114 or X-100 in MSB for 4 or 8 min on ice. Then the cells were washed twice in cold MSB, fixed with 4% PFA and processed for immunofluorescence using anti-Thy-1 antibody.

Analysis of [^{3}H]t-Cer derivatives in the neuronal medium

Stage 3 or stage 5 neurons plated in 3 cm dishes were incubated with 1 ml N_2 medium containing 1 mCi [^{3}H]t-Cer (Karrenbauer *et al.*, 1990) and 2.5 nmol t-Cer at 37°C/5% CO_2. Aliquots were taken from the medium at 3, 6 and 8 h. After concentration on RP-18 columns short-chain lipids appearing in the medium were separated by TLC. For quantification of labeled products chromatograms were analyzed on a 2D-TLC-scanner (Berthold digital autoradiograph).

Phospolipid analysis

Lipids were extracted according to Bligh and Dyer (1959) in the presence of internal standards. Lipid standards (di-lauroyl-PC, di-myristoyl-PC, di-arachidoyl-PC and di-behenoyl-PC) were obtained from Avanti Polar Lipids (Alabaster, AL) except for *N*-oleoyl-SM (Sigma). *N*-myristoyl-SM and *N*-pentacosanoic-SM were kindly provided by Roger Sandhoff (Biochemie-Zentrum Heidelberg). Dried lipid extracts were resuspended in chloroform/methanol (1:2). Prior to mass spectrometric analysis 10 mM ammonium acetate in methanol was added and the sample was centrifuged in a bench top centrifuge for 5 min at 14 000.

Mass spectrometric analysis of hippocampal neurons

Mass spectrometric analyses were performed on a Quattro II tandem quadrupole mass spectrometer (Micromass UK Ltd, Altrincham, UK) equipped with a nano-ES source operating in positive ionization mode with a flow rate of 20–50 nl/min. Nano-electrospray capillary tips (type D) were purchased from Micromass. Tandem mass spectrometric analyses were performed using argon as collision gas at a collision cell pressure of 2×10^{-3} mbar. Precursor ion scanning for *m*/*z* 184 fragment ions specific for PC and SM were performed with a collision energy of 30 eV. For each spectrum 50–100 scans of 4 s duration were averaged. Lipid quantification was performed according to Brugger *et al.* (1997).

Cholesterol determination

Cholesterol was measured following the method described by Gamble *et al.* (1978). Standards curves were made with cholesterol (Sigma) from 25 to 500 ng. Four independent experiments were performed with different pools of stage 3 and stage 5 neurons. The fluorescence was measured in an Aminco-Bowman spectrophotometer (excitation, 325 nm; emission, 415 nm).

TLC analysis of NBD lipids insolubility

Stage 4 neurons (6 days in culture) were incubated in the neuronal medium for 12 h with 2 μM NBD-C6-Cer. Then the cells were washed, scraped and the cell membrane pellet was obtained after centrifugation at 100 000 *g* at 4°C for 1 h. The pellet was extracted in 1% Triton X-100 for 1 h at 4°C and soluble and insoluble fractions were obtained after centrifugation as previously indicated. The lipids in the fractions were extracted according to Bligh and Dyer (1959). The supernatant and the pellet after detergent extraction and a sample containing 1 μg of NBD-C6-Cer as a control were separated by TLC. The bands corresponding to the NBD-ceramide and its metabolites: NBD-glucosylceramide and NBD-SM were visualized under UV light, the image was scanned and the bands quantified using the NIH IMAGE program.

Analysis of the polarizing effect after NBD-C6-Cer treatment

Four- and 5-day-old neurons (stage 4 of development) were treated or not with 0.5 μm NBD-C6-Cer (Molecular Probes) for 24 h. The ceramide was added to the cultures either in ethanol (reaching a final concentration of 1% ethanol) or as complexes with defatted bovine serum albumin (BSA; Pagano and Martin, 1994). Then the cells were washed and processed for immunofluorescence using an antibody against Thy-1 or TfR. Thy-1 and TfR distribution was analyzed in 30 ceramide-treated and 30 control neurons for each protein (10 control and 10 ceramide-treated cells from three independent cultures). For these quantitations images were captured at non-saturating integration levels with a charge-coupled device camera (Cohu 4913, Cohu, San Diego, CA) connected to a Macintosh computer (Power Macintosh 7300/166) equipped with an image recorder (Openlab). Images were stored by using NIH IMAGE. Thy-1 or TfR labeled cells were collected and segments of ~10 μm (many processes in these developing cells are only this length) were delineated in each neurite at 5–10 μm from the cell body. The areas were quantitated (total surface/pixel intensity) and were normalized to exactly the same size. The value obtained for each neurite was represented as the percentage of the total Thy-1 or TfR intensity within all the selected areas in each cell. We then measured ratio on each neurite versus total and ratio neurite versus neurite. We considered a polarized distribution when a process had >3-fold the amount of protein present in the other neurites.

The amount of protein in control and NBD-ceramide-treated samples from three different experiments using three different cultures was monitored by the Bio-Rad protein assay based on the method of Bradford. Silver staining of the gels after SDS–PAGE of control and ceramide-treated samples was also performed.

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