Fluorescent analogues of plasma membrane sphingolipids are sorted to different intracellular compartments in astrocytes
Harmful effects of chronic ethanol exposure on sphingolipid trafficking and metabolism

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Abstract Sphingolipids are basic constituents of cellular membranes and are essential for numerous functions such as intracellular signalling. They are transported along the exocytic and endocytic pathways in eukaryotic cells. After endocytosis, fluorescent-labelled sphingolipids are sorted to distinct intracellular organelles prior to recycling (via early/recycling endosomes) or degradation (late endosomes/lysosomes). Here we examine, in primary cultures of rat astrocytes, the internalisation routes followed by C_6-NBD-glucosylceramide (NBD-GlcCer) and C_6-NBD-sphingomyelin (NBD-SM) and the effects of ethanol on their endocytic trafficking. Endocytosed plasma membrane NBD-GlcCer and NBD-SM are diverted to the Golgi apparatus and lysosomes, respectively. These different internalisation pathways are maintained regardless of the differentiation stage of astrocytes. Chronic ethanol exposure did not alter this endocytic sorting, but delayed the internalisation of both NBD-sphingolipids. Moreover, ethanol also stimulated the in situ metabolism of NBD-ceramide to NBD-GlcCer and NBD-SM. We conclude that in rat astrocytes internalised plasma membrane NBD-sphingolipids are sorted to different subcellular compartments. The exposure to chronic ethanol perturbed the lipid endocytic process and stimulated the de novo synthesis of NBD-sphingolipids, shifting the balance of sphingolipid metabolism in favour of the sphingomyelin pathway.
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Key words: NBD-lipid; Glial cell; Alcohol; Endocytosis; Golgi apparatus; Endosome

1. Introduction

Sphingolipids (SLs) are components of the plasma membrane of vertebrate cells. They are particularly abundant in the central nervous system, where they are enriched in discrete flask-shaped, non-coated plasma membrane invaginations named caveolae, which act as centres for signalling activity within cells (see [1] for a review). It has been claimed that glycolipid-enriched domains are targets for a variety of diseases of the nervous system including Alzheimer and prion diseases, and neurolipidoses [2]. For this reason the study of the composition, biogenesis and recycling of these glycolipid-enriched domains is particularly interesting in neurones and astrocytes [2].

Like proteins, lipids are also transported along the biosynthetic and endocytic pathways. This has been extensively investigated in epithelial cells by examining the trafficking of fluorescent, short acyl chain lipid analogues (especially the NBD and BODIPY groups, C_6-NBD/BODIPY-lipids). Newly synthesised fluorescent analogues of (glyco-)SLs (glucosylceramide, sphingomyelin, or lactosylceramide) are sorted to different plasma membrane (apical/basolateral) domains. The results of studies on endocytosis and sorting depend on the cell type and the differentiation stage of the cell [3,4]. Trafficking of NBD/BODIPY-SLs has been examined in neurones and oligodendrocytes. Unlike other mammalian cells in culture, neurones internalise plasma membrane C_6-NBD-glucosylceramide (NBD-GlcCer) and C_6-NBD-sphingomyelin (NBD-SM) to the Golgi apparatus regardless of the stage of differentiation [5]. Conversely, oligodendrocytes internalise BODIPY-LacCer and BODIPY-sulphatide to endosomes in quantities that depend on the differentiation stage. However, no information is available on trafficking of SL analogues in astroglial cells. Such information is important, given the involvement of these cells in the formation and maintenance of the blood–brain barrier. It has been reported that astrocytes are particularly vulnerable to the toxic effects of ethanol and that exposure to ethanol during foetal development perturbs astrogliogenesis and alters the cell viability of astrocytes in culture [6]. In particular, astrocytes chronically exposed to ethanol (25–30 mM) show lower secretion of neural cell adhesion molecules, growth factors (nerve growth factor and neurotrophins) and their receptors, and a decrease in protein trafficking [6]. These alterations are concomitant with the foetal alcohol syndrome, which is caused by heavy drinking during pregnancy and is the leading cause of mental retardation [7].

Therefore, we examined, firstly, the internalisation and sorting of the SL analogues NBD-GlcCer and NBD-SM in growing and differentiated rat astrocytes, and secondly, the effect of chronic ethanol exposure on their trafficking and metabolism. Internalised NBD-GlcCer and NBD-SM were sorted to the Golgi complex and lysosomes, respectively. This sorting

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was not affected by ethanol, which nevertheless delayed endocytosis and accelerated the de novo synthesis of NBD-GlcCer and NBD-SM from C_6-NBD-ceramide (NBD-Cer).

2. Materials and methods

2.1. Materials

Brefeldin A (BFA) and nocodazole (NZ) were purchased from Sigma (St. Louis, MO, USA). Wheat germ agglutinin (WGA) was from Molecular Probes (Eugene, OR, USA). Sodium dithionite was from Merck (Darmstadt, Germany). NBD-GlcCer, NBD-SM, NBD-Cer and the coumarin fluorescent derivative of ceramide (7-diethylaminocoumarin-3-carboxylic acid, DECA-ceramide (DECA-Cer), were synthesised as described [8,9].

2.2. Primary cultures of rat astrocytes and ethanol treatment

Primary cultures of rat astrocytes from 21-day-old rat foetuses were prepared from brain hemispheres as previously described [10,11]. Foetuses were obtained from rats in sterile conditions, and the cerebral hemispheres were dissected free of meninges and mechanically dissociated by pipetting 10 times with a 10-ml pipette in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA). Cell suspension was vortexed at maximum speed for 1 min and filtered through a nylon mesh (pore diameter 80 μm). Cells were maintained in the same culture medium containing 20% foetal bovine serum and antibiotics. Cells were then grown in a humidified atmosphere of 5% CO_2 and 95% air, at 37°C. In these conditions, cells grow quickly for 4–7 days as a consequence of their high proliferative activity [12,13]. After 1 week of culture, cells stop growing and begin to differentiate [10]. During this period, the serum content of the culture medium was reduced to 10%. Other cell cultures were left to grow for 21 days. Ethanol was added to the medium containing proliferative and differentiated astrocyte primary cultures on the first day of culture (day 0). Medium with ethanol was replaced every 2 days. The ethanol concentration in the medium was checked daily and adjusted to a final concentration of 25 mM (ethanol evaporation after 24 h was 10–20%), which is equivalent to the ethanol concentration in the blood (a) of pregnant chronic drinkers or (b) when three to five alcoholic drinks are consumed within 1 h by women weighing about 60 kg [14]. The purity of astrocyte cultures was assessed by immunofluorescence using a mouse anti-glial fibrillary acidic protein monoclonal antibody (Sigma). Contamination by neurones was also assessed by immunofluorescence using an anti-MAP-2 antibody (Boehringer Mannheim, Indianapolis, IN, USA).

All experiments were approved by the appropriate institutional review committee and performed in strict compliance with the European Community Guide for the Care and Use of Laboratory Animals.

2.3. Plasma membrane insertion of fluorescent lipids

NBD-SLs and DECA-Cer were stored in chloroform/methanol (2:1 v/v), dried under a nitrogen atmosphere, and solubilised in absolute ethanol (0.5% final concentration).

2.4. Back-exchange of membrane-inserted fluorescent lipids

To remove any fluorescent lipid remaining at the plasma membrane before observation under the fluorescence microscope, cells were subjected to the back-exchange procedure. This was carried out by incubating the cells at 4°C for 20 min with 3% bovine serum albumin (BSA) (w/v) in Hanks' solution, followed by extensive rinses in this solution. Thereafter, cells were incubated at 20°C for 2 min with Hanks' solution containing sodium dithionite (80 mM final concentration).

2.5. Cytochemical single and double labelling experiments

Primary cultures of rat astrocytes grown on glass coverslips were washed in ice-cold Hanks' medium, transferred to a cold room and then incubated with NBD-SLs (2–4 μM) for 30 min to label plasma membrane. The cells were subsequently rinsed in cold Hanks' solution and warmed to 37°C for various times to induce endocytosis. Excess of fluorescent lipid in the plasma membrane was then removed by the back-exchange procedure described above.

Double labelling experiments with DECA-Cer (to stain the Golgi apparatus) and NBD-GlcCer were performed by incubating the astrocytes with NBD-GlcCer (2–4 μM) and DECA-Cer (1 μM) for 10 min at room temperature. Cells were then rinsed in Hanks' solution, warmed to 37°C for 90 min, and back-exchanged at 4°C. Colabelling experiments with TRITC-WGA (to stain late endosome/lysosomes) were performed by incubating astrocytes first with TRITC-WGA (15 μg/ml) for 30 min at 37°C. Cells were then washed in Hanks' solution and then incubated at 37°C (without WGA) for 90 min. Thereafter, cells were incubated with NBD-SM (2–4 μM) for 10 min at 4°C for insertion in the plasma membrane and subsequently warmed to 37°C for internalisation. Finally, a back-exchange procedure was performed.

Indirect immunofluorescence was carried out as previously described [15]. Anti-giantin antibody dilution was used at 1:500.

Fluorescence microscopy was performed with an Olympus BX60 fluorescence microscope equipped with suitable filter packs that allowed the specimens to be excited at specific wavelengths. A personal computer-connected and cooled CCD camera (Olympus DP50) was attached to the microscope for image acquisition. All the images were captured at non-saturating integration levels.

2.6. Lipid extraction and thin-layer chromatography analysis

After the NBD-SL internalisation experiments, astrocytes were back-exchanged at 4°C with 3% BSA in Hanks' solution to separate NBD-SLs present in the plasma membrane from those in the intracellular membranes. Extracted NBD-SLs were subsequently analysed by thin-layer chromatography (TLC) on silica gel 60 HPTLC plates using chloroform/methanol/NH_3/H_2O (35:15:2:0.5) as the running solvent system. Fluorescent GlcCer and SM were identified by comparison with NBD-labelled standards. For quantitative analysis, individual spots were scraped from the HPTLC plates and eluted from the silica with 1% (v/v) Triton X-100 solution by vigorous shaking and incubated at 37°C for 60 min. Silica particles were centrifuged, and the fluorescence in the supernatant was measured in a fluorimeter (Kontron Instruments Fluorimeter, SFM 25, Kontron Instruments, Southampton, UK) at excitation and emission wavelengths of 465 nm and 530 nm, respectively.

2.7. In situ metabolism using NBD-Cer

Control and ethanol-treated proliferative astrocytes were washed in Hanks' culture medium and then incubated for various times at 37°C with 2 μM of NBD-Cer/BSA. Cells were washed with ice-cold Hanks' solution and scraped from the culture dish, and the lipids were extracted and processed by TLC as described above.

2.8. BFA and NZ treatments

Cultured astrocytes were first labelled with NBD-GlcCer as described above and then incubated with BFA (5 μg/ml) and NZ (20 μg/ml) for 30 and 60 min, respectively, at 37°C.

3. Results and discussion

3.1. In rat astrocytes, internalised plasma membrane NBD-GlcCer and NBD-SM are transported to the Golgi complex and lysosomes, respectively

We first examined the internalisation of NBD-labelled SLs from the plasma membrane in astrocytes at proliferative (7 zdays in culture) and differentiated (21 days in culture) stages. Cells were incubated with NBD-GlcCer or NBD-SM at 4°C, washed, and warmed to 37°C for 30 min. At this time, NBD-GlcCer stained a juxtanuclear structure in both proliferative (Fig. 1A) and differentiated astrocytes (not shown). According to its morphology and subcellular positioning, this structure resembled the Golgi complex. This assumption was confirmed by double labelling experiments with DECA-Cer (Fig. 1B), whose spectral properties differ significantly from the NBD fluorescent group. Fluorescent-tagged ceramide is a well-established in vivo marker of the Golgi complex (see [9] for a review). The identification of the Golgi complex was further substantiated when we examined the effects of two agents that alter the organisation of the Golgi complex: BFA and NZ, which promote respectively the fusion of Golgi mem-

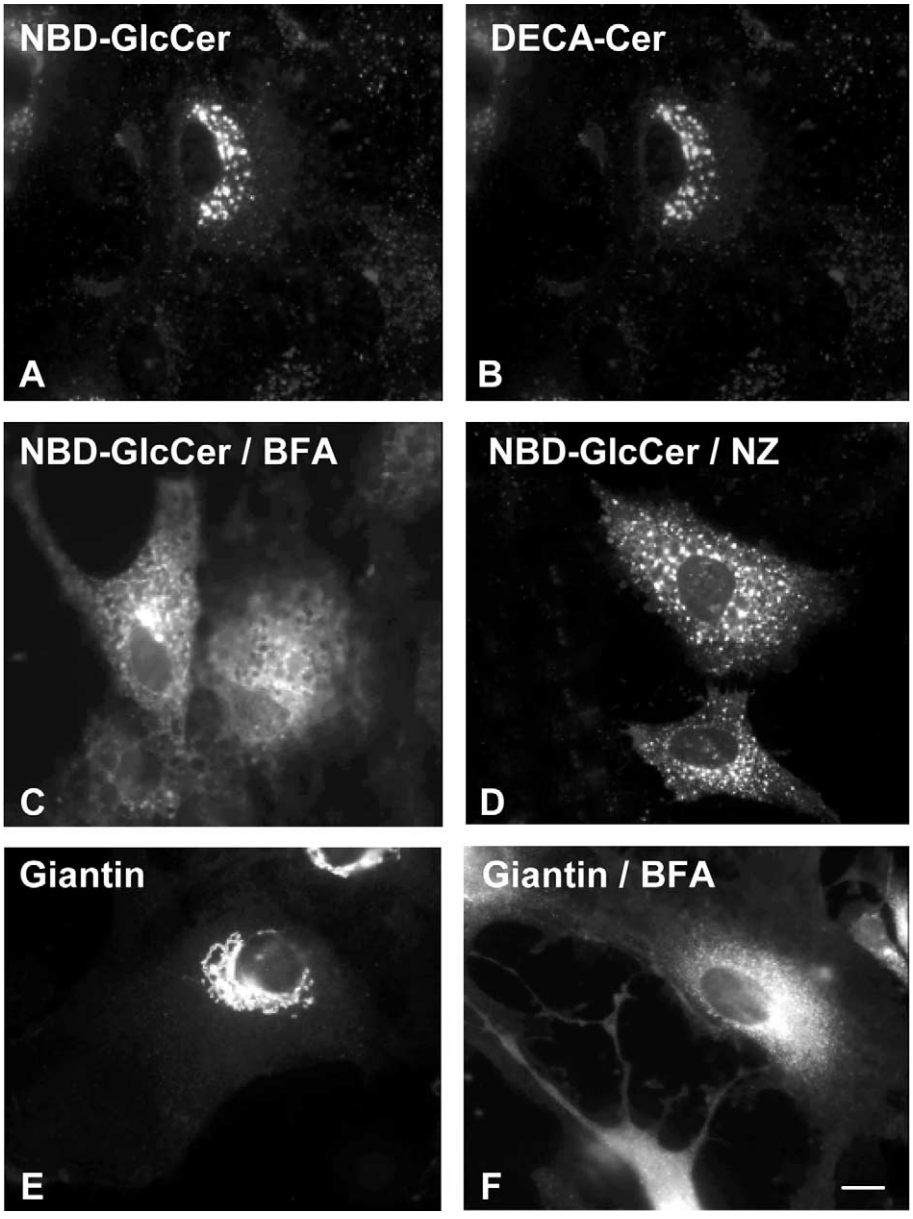


Fig. 1. Plasma membrane NBD-GlcCer is internalised and transported to the Golgi complex in rat astrocytes. Primary cultures of rat astrocytes were cultured to proliferative stage (7 days in culture). Cells were co-incubated with NBD-GlcCer (A) and DECA-Cer (B) for 30 min at 4°C, washed, warmed for 30 min at 37°C and back-exchanged with BSA (see Section 2). Internalised NBD-GlcCer co-localised with DECA-Cer in the Golgi complex. For BFA and NZ treatments, cells were incubated first with NBD-GlcCer as described above, and then treated with BFA (C) or NZ (D) as indicated in Section 2. As expected, treatments with BFA and NZ showed the respective ER-like staining pattern and Golgi fragmentation and cytoplasmic dispersion of NBD-GlcCer. Untreated (E) and BFA-treated (F) astrocytes were also immunostained to the Golgi-resident protein giantin. Bar, 10 μm.

branes into the endoplasmic reticulum (ER) and the fragmentation of Golgi membranes as a consequence of microtubule disruption. In BFA-treated astrocytes, most of the NBD-GlcCer staining showed the characteristic reticular ER-like staining pattern (Fig. 1C) although in some cells Golgi-like and cytoplasmic punctate labelling was also observed, which most likely corresponded to the *trans*-Golgi network and endocytic vesicles, respectively. After NZ treatment, NBD-GlcCer staining appeared in numerous fluorescent cytoplasmic fragments dispersed throughout the cytoplasm that correspond to Golgi ministacks (Fig. 1D). Taken together, the results strongly indicate that in most rat astrocytes, internalised plasma membrane NBD-GlcCer is transported to the Golgi complex. To rule out the possibility that the staining of the Golgi complex by endocytosed NBD-GlcCer was attributable to its hydrolysis to NBD-Cer, we assessed NBD-GlcCer metabolism along the internalisation pathway, as examined in rat hippocampal neurones [5]. NBD-GlcCer in the plasma membrane pool remained unaltered (data not shown) whereas in the intracellular pool less than 10% of internalised NBD-GlcCer was converted to NBD-Cer at the time in which NBD-GlcCer reached the Golgi complex (30 and 60 min in control and ethanol-treated cells, respectively) (Fig. 2). This percentage of NBD-Cer is too low to explain the strong Golgi complex staining observed after NBD-GlcCer internalisation. At the beginning of endocytosis (15 min), NBD-GlcCer co-

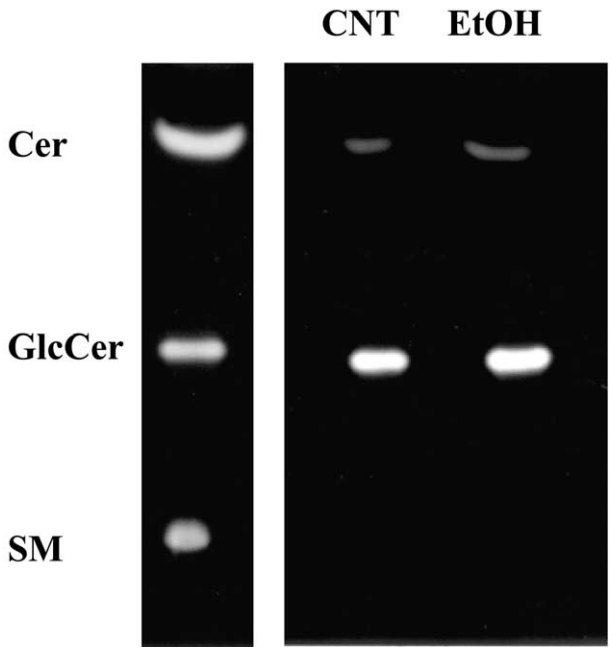


Fig. 2. The staining of the Golgi apparatus by internalised NBD-GlcCer is not caused by its total conversion to NBD-Cer. The NBD-lipid content of the intracellular pool in untreated (CNT) and ethanol-treated (EtOH) astrocytes was monitored by TLC analysis after 60 min of internalisation of NBD-GlcCer.

localised with TRITC-transferrin in endocytic vesicles (data not shown), which suggests that NBD-GlcCer is transported to the Golgi complex via early/recycling endosomes.

We also examined the internalisation route followed by NBD-SM. As described for NBD-GlcCer, cells were incubated with NBD-SM at 4°C, washed, and warmed for 60 min at 37°C. Unlike NBD-GlcCer, NBD-SM was visualised in numerous cytoplasmic punctate structures regardless of the astrocyte stage (Fig. 3A,C). Double labelling experiments in which astrocytes were incubated with NBD-SM and TRITC-WGA, an in vivo marker of late endosomes and lysosomes [16], revealed extensive co-localisation of fluorescent lipid and WGA in vesicular structures after 60 min of endocytosis (Fig. 3B,D). Therefore, unlike NBD-GlcCer, plasma membrane NBD-SM is internalised to degradative compartments. This endocytic transport is usual in non-polarised cultured mammalian cells. Most of the plasma membrane fluorescent lipid analogues are transported to degradative compartments with the exception of NBD-GlcCer in undifferentiated HT29 cells [17] and both NBD-GlcCer and NBD-SM in human skin fibroblasts [18,19] and rat hippocampal neurones [5]. On the other hand, alterations in the sorting of internalised SLs in SL storage diseases have also been reported [20]. Our data suggest that SLs are sorted following endocytosis: NBD-GlcCer to the Golgi complex and NBD-SM to lysosomes. This sorting could be generated at the plasma membrane. Rafts, which are low-density detergent-insolu-

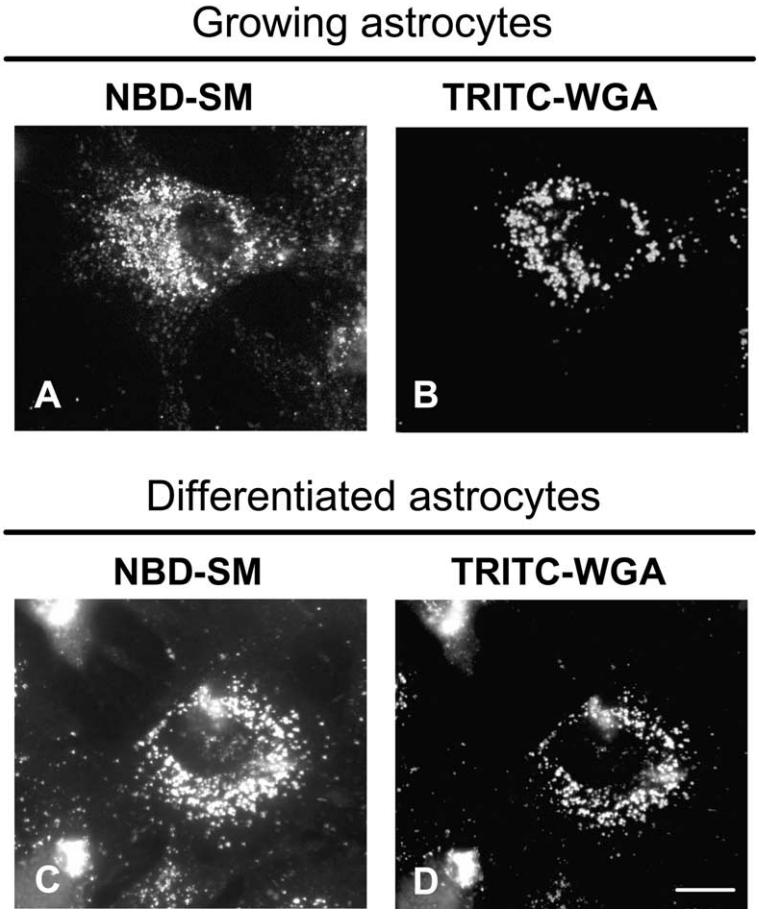


Fig. 3. Plasma membrane NBD-SM is internalised to lysosomes regardless of the astrocyte differentiation stage. Proliferative (7 days in culture; A,B) or differentiated (21 days in culture; C,D) astrocytes were first pulse-labelled with TRITC-WGA (B,D), washed and thereafter co-incubated with NBD-SM (A,C) for 30 min. Bar, 10 μm.

ble microdomains (DIMs), are enriched in SLs (mainly sphingomyelin) and cholesterol (see [21,22] for reviews). Interestingly, when an inhibitor of glucosylceramide synthase inhibited the synthesis of the major neutral glyco-SLs GlcCer and LacCer, DIMs remained unaltered in both their lipid (cholesterol and sphingomyelin) and protein profiles [23]. Therefore, GlcCer and SM may be sorted at the cell surface as a consequence of their partitioning into separate SL domains determined by their cholesterol content (see below). Cholesterol plays a significant role in intracellular trafficking of BODIPY-LacCer and globoside. These lipid analogues are endocytosed to the Golgi complex but are re-targeted to endosomes and lysosomes in fibroblasts derived from SL storage diseases [31], where cholesterol accumulates in degradative compartments. When cholesterol is depleted from these fibroblasts, both BODIPY-SLs are transported to the Golgi. In contrast, loading excess cholesterol into normal fibroblasts mistargeted BODIPY-LacCer to late endosomes and lysosomes. Therefore, in rat astrocytes, NBD-GlcCer could be partitioned into a plasma membrane domain that is low in cholesterol and subsequently endocytosed to the Golgi complex. In contrast NBD-SM is partitioned into a cholesterol-enriched raft domain, which is subsequently internalised to degradative compartments. Therefore, the different endocytic sorting of NBD-GlcCer and NBD-SM in rat astrocytes could begin at the plasma membrane, where the amount of cholesterol would

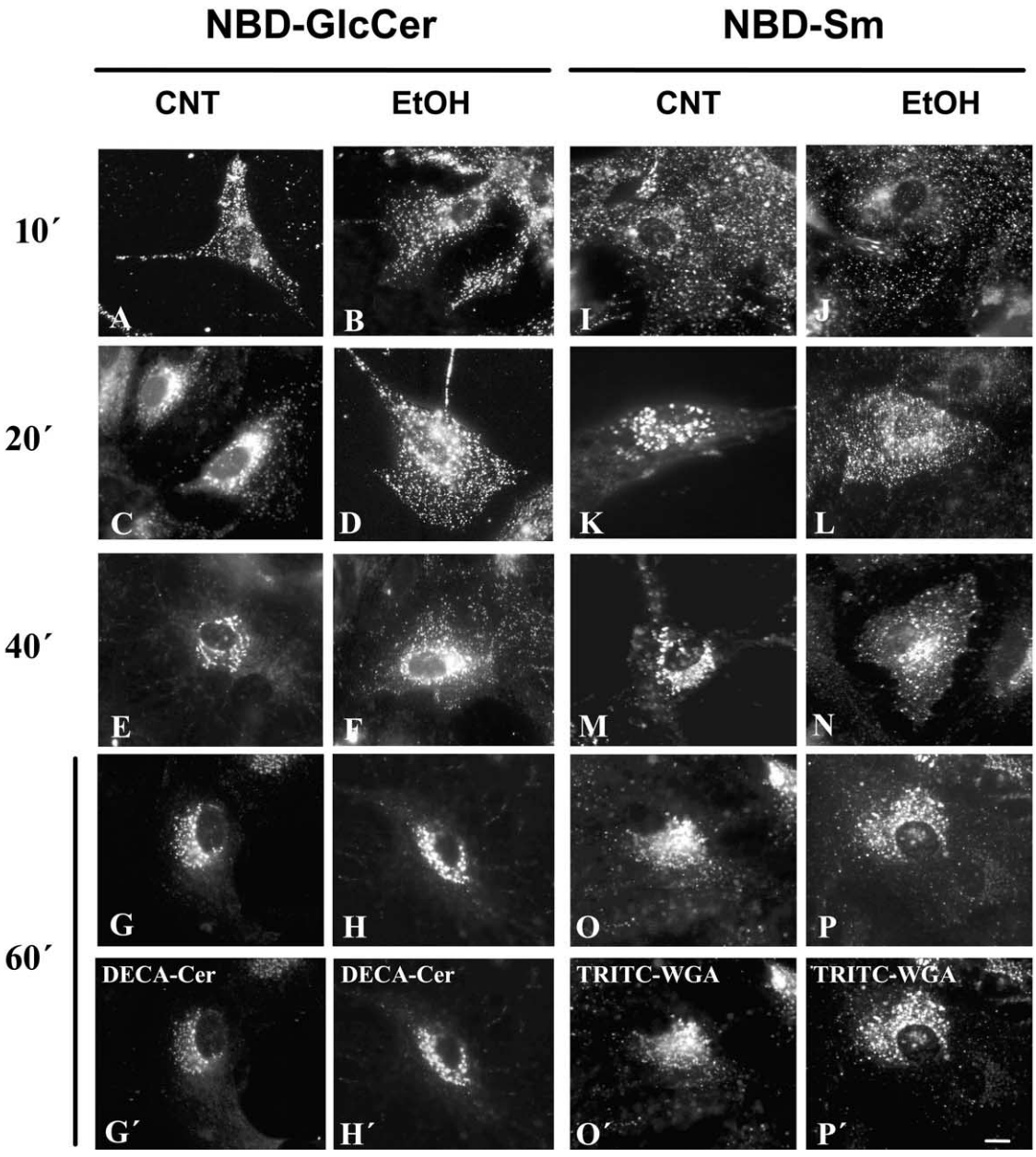


Fig. 4. Ethanol delays the arrival of endocytosed NBD-GlcCer and NBD-SM to the Golgi complex and lysosomes, respectively. Control and ethanol-treated (30 mM) proliferative astrocytes (7 days in culture) were incubated for 30 min at 4° C with NBD-GlcCer (A–H) or NBD-SM (I–P), washed, warmed for 10 min (A,B,I,J), 20 min (C,D,K,L), 40 min (E,F,M,N) or 60 min (G,H,O,P) at 37°C and back-exchanged. In panels G/G′–P/P′, astrocytes were first pulse-labelled with DECA-Cer (G′ and H′) or TRITC-WGA (O′ and P′), washed and thereafter NBD-GlcCer (G,H) or NBD-SM (O,P) was internalised for 60 min. Bar, 10 μm.

determine their destination. This could also influence the mode of internalisation of both SLs (clathrin-dependent versus clathrin-independent mechanisms), which in turn has been postulated to be a major factor in determining the subsequent targeting of fluorescent-labelled SLs to the Golgi complex versus late endosomes/lysosomes [19,24]. Alternatively, the presence of separate SL domains could also explain that this differential SL endocytic sorting could take place in endocytic compartments rather than the plasma membrane. In this respect, in polarised HepG2 cells, fluorescent analogues of GlcCer and SM are transported along the transcytotic pathway but are subsequently targeted, in a subapical compartment, to different plasma membrane domains [25]. These authors provide evidence that these SLs are first separated in luminal domains of this compartment, which in turn are regulated by cytoplasmic proteins. Therefore, once internalised, NBD-GlcCer and NBD-SM could be separated into different domains in early endocytic compartments, and then sorted to the Golgi and late endosomes/lysosomes, respectively.

The physiological relevance of this sorting is unknown but it may supply the high demands of membrane transport and signalling events. For example, GlcCer could be re-utilised by continuous recycling to plasma membrane via *trans*/*trans*-Golgi network. Conversely, SM is directly transported to degradative components, where it is hydrolysed to ceramide and sphingosine-1-phosphate, which are either re-utilised for the de novo synthesis of SLs or may function as second messengers or regulators of signal transduction (see [26] for a review).

3.2. Ethanol impairs the kinetics of internalisation of NBD-GlcCer and NBD-SM but not their target compartments

We next examined the effects of chronic doses of ethanol (30 mM) on endocytic transport of these SL analogues (Fig. 4). Ethanol did not affect sorting of NBD-GlcCer and NBD-SM (to the Golgi complex and lysosomes, respectively) (Figs. 4H,P). However, the internalisation of NBD-GlcCer (Fig. 4A–H) or NBD-SM (Fig. 4I–P) was delayed (Fig. 4D,F in comparison with C,E for NBD-GlcCer; and L,N in comparison with K,M for NBD-SM). Although the same amount of NBD-SLs was added to control and ethanol-treated cells, this alteration could be the result of an ethanol-induced decrease in the amount of fluorescent SL analogue incorporated into plasma membrane. To examine this possibility, control and ethanol-treated astrocytes were cultured at the same cell density and incubated with NBD-GlcCer or NBD-SM (4 μM each, final concentration) in Hanks' culture medium for 30 min at 4°C. This medium was then replaced and cells were further incubated for 60 min at 4°C to inhibit endocytosis. Cells were then fixed and processed for TLC. Ethanol increased the amount of NBD-GlcCer or NBD-SM incorporated into the plasma membrane (Fig. 5A,B). The results indicate that the impairment in the NBD-SL endocytosis caused by ethanol is not the mere consequence of the decrease in the amount of NBD-lipid that is incorporated into the plasma membrane. Conversely, ethanol increased the incorporation of NBD-lipid into the plasma membrane. More likely, ethanol alters the composition of the plasma membrane, which in turn interferes with the formation of endocytic vesicles. This impairment could be attributable to the alterations caused by ethanol on actin and microtubule cytoskeletons of rat astrocytes [15], since both cytoskeletons provide a structural scaffold for the formation and/or locomotion of endocytic vesicles generated by clathrin-dependent and -independent mechanisms (see [27] for a review).

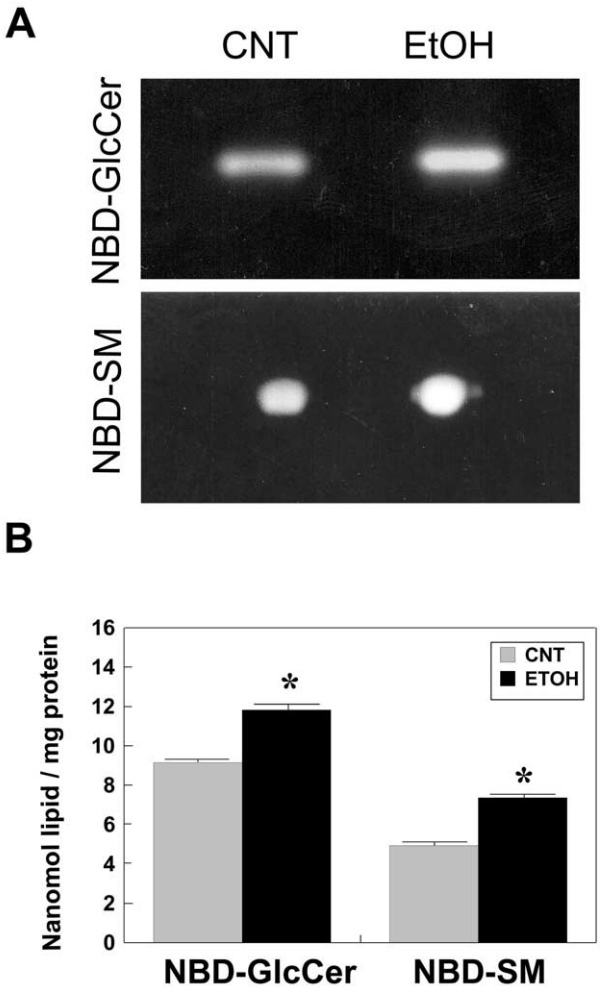


Fig. 5. The incorporation of NBD-GlcCer and NBD-SM into the plasma membrane was increased by ethanol. Control and ethanol-treated (30 mM) proliferative astrocytes were incubated for 30 min at 4°C with NBD-GlcCer or NBD-SM (2 μM). Cells were washed and lipids were then extracted, separated by TLC (A) and quantified (B) as indicated in Section 2. The data are the mean ± S.D. of four independent experiments. Asterisks indicate statistically significant differences (Student's *t*-test, $P \leq 0.05$).

3.3. Ethanol stimulates the de novo synthesis of fluorescent GlcCer and SM

To assess the effect of ethanol on NBD-SL biosynthesis, control and ethanol-treated astrocytes at the proliferative stage were examined for the de novo synthesis of fluorescent GlcCer and SM from the fluorescent precursor NBD-Cer. The amount of NBD-GlcCer and, in particular, NBD-SM increased significantly in ethanol-treated cells 2 h after the addition of NBD-Cer (Fig. 6), and remained high for at least 12 h. SM metabolism is a signal transduction pathway that affects apoptosis in many cell types (see [28–30] for recent reviews).

3.4. Conclusions

We used plasma membrane fluorescent SL analogues to study endocytosis in rat astrocytes at proliferative and differ-

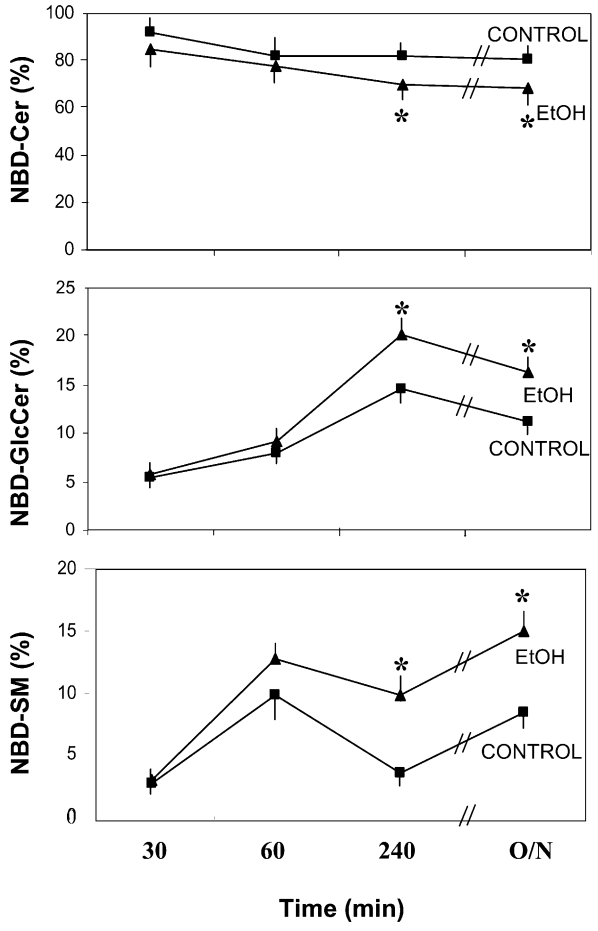


Fig. 6. Ethanol stimulated de novo synthesis of NBD-GlcCer and NBD-SM in rat astrocytes. Control and ethanol-treated astrocytes were incubated for 60 min at 37°C with 2 μM NBD-Cer, washed and back-exchanged. Thereafter, lipids were extracted after 30 min, 1 h or 2 h, separated by TLC, and quantified. Data are the means ± S.D. of three independent experiments. Asterisks indicate statistically significant differences (Student's *t*-test, $P \leq 0.05$).

entiated stages. We show that internalised NBD-GlcCer is transported to the Golgi complex whereas NBD-SM goes to degradative compartments. These different NBD-SL internalisation pathways are maintained irrespective of the stage of differentiation. These results provide the first evidence for SL sorting along the endocytic pathway in rat astrocytes. The physiological relevance of this sorting is unknown, but it may supply the demands of membrane transport of these cells and signalling events. Finally, chronic ethanol exposure does not affect the sorting, although it delays the arrival of both NBD-SLs in their respective compartments. Moreover, it shifts the balance of SL metabolism in favour of the SM pathway, which in turn could increase the susceptibility of astrocytes to apoptosis.

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