The internalization of a short acyl chain analogue of ganglioside GM₁ in polarized neurons

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SUMMARY

In order to study the endocytosis of membrane lipids during the development of neuronal polarity, we examined the internalization of a short acyl chain fluorescent derivative of ganglioside GM₁, N-(6-(4-nitrobenz-2-oxa-1,3-diazole-7-yl)-aminohexanoyl)-GM₁ (C₆-NBD-GM₁), in hippocampal neurons cultured at low density. C₆-NBD-GM₁ was internalized by temperature- and energy-dependent mechanisms, and after short times of incubation, accumulated in endosomes in the axon, cell body and dendrites of neurons maintained for up to 4-5 days in culture. C₆-NBD-GM₁ was subsequently transported in a retrograde direction to a pool of recycling endosomes in the cell body, with little transport to lysosomes, as indicated by the lack of degradation of C₆-NBD-GM₁ even after long times, and the re-appearance of intact C₆-NBD-GM₁ at the cell surface after recycling; similarly, little degradation of C₆-NBD-GM₁ was detected in N18TG-2 neuroblastoma cells. In hippocampal neurons maintained for longer than 6 days in culture, there was little internalization of C₆-NBD-GM₁ along the length of axons, but the amount of endocytosis from dendrites was similar to that observed in younger neurons. These results demonstrate that gangliosides turnover rapidly in dendritic membranes at all stages of neuronal development, whereas ganglioside turnover in axons is much less rapid, at least in mature, polarized neurons.

Key words: Neuron, Ganglioside, Endocytosis

INTRODUCTION

Gangliosides are major components of neuronal membranes where they have been postulated to play a variety of important roles (Ledeen and Yu, 1992). A large amount of biochemical information is available about ganglioside synthesis and degradation (Schwarzmann and Sandhoff, 1990; van Echten and Sandhoff, 1993), but much less is known about the intracellular transport of gangliosides in living cells. The internalization of gangliosides has been studied using various probes. Popular among these is cholera toxin, which binds with high affinity to ganglioside GM₁ (Fishman et al., 1993), and has recently been used to localize GM₁ to caveolae at the cell surface of A431 cells (Parton, 1994). However, cholera toxin is internalized to the Golgi apparatus of non-neuronal (Lencer et al., 1995) and neuronal cells (Sofer and Futerman, 1995), whereas GM₁ is degraded in lysosomes (Schwarzmann and Sandhoff, 1990). This suggests that the internalization of cholera toxin to the Golgi apparatus is a reflection of the intracellular transport pathway of the toxin rather than of the ganglioside.

In order to directly analyze the endocytosis of gangliosides in living cells, we have synthesized a short acyl chain fluorescent analogue of ganglioside GM₁, N-(6-(4-nitrobenz-2-oxa-1,3-diazole-7-yl)-aminohexanoyl)-GM₁ (C₆-NBD-GM₁; Fig. 1). Fluorescent, short acyl chain lipid analogs rapidly and spontaneously transfer from exogenous sources into biological membranes (Pagano and Sleight, 1985), and have been used to study lipid endocytosis in a variety of cultured cells (reviewed by Hoekstra and Kok, 1992). In the current study, we examine C₆-NBD-GM₁ endocytosis in hippocampal neurons cultured in such a way that axons and dendrites develop by a known sequence of events and can be distinguished both morphologically and biochemically, leading to the generation of fully-polarized neurons (Dotti et al., 1988; Goslin and Banker, 1991). Development of these neurons has been divided into five stages (Dotti et al., 1988). Initially (0-2 days in culture) neurons appear unpolarized extending several short neurites identical in morphology and growth rate (stage 1 and 2). Polarity is first expressed when one of these processes begins to grow rapidly, becoming significantly longer than the others (stage 3); this process is destined to become the axon. In stage 4 (days 4-5 in culture), the minor processes elongate and acquire the typical characteristics of dendrites. The formation of synaptic contacts characterizes the final stage of maturation (stage 5).

Axonal and dendritic endocytic compartments have been characterized in hippocampal neurons (Mundigl et al., 1993; Parton, 1992). In stage 3 cells, exo-endocytic recycling of synaptic vesicles takes place along all the axon surface (Fletcher et al., 1991; Matteoli et al., 1992). Thus, synaptic vesicle-associated proteins recycle locally through axonal early endosomes (Matteoli et al., 1992) but proteins internalized by bulk membrane flow are transported to late endosomes and lysosomes localized in the cell body (Augenbraun et al., 1993; Parton et al., 1991, 1992). In stage 5 cells, axonal early
endosomes are confined to presynaptic terminals and varicosities. In contrast, early endosomes are distributed throughout the dendrites and cell body at all stages of development (Parton et al., 1992). No information is currently available about the internalization of lipids in these or other polarized neurons.

We now report that C6-NBD-GM1 is internalized by vesicular transport mechanisms to discrete puncta identified as recycling endosomes, and that the rate of C6-NBD-GM1 endocytosis decreases from axons but not from dendrites during neuronal development.

**MATERIALS AND METHODS**

**Cell culture**

Hippocampal neurons were cultured at low density as described (Goslin and Banker, 1991; Harel and Futerman, 1993). Briefly, the dissected hippocampi of embryonic day 18 rats (Wistar), obtained from the Weizmann Institute Breeding Center, were dissociated by trypsinization (0.25%, w/v, for 15 minutes at 37°C). The tissue was washed in Mg/Ca-free Hank’s balanced salt solution (Gibco) and dissociated by repeated passage through a constricted Pasteur pipette. Cells were plated in minimal essential medium (MEM) with 10% horse serum, at a density of 12,500-25,000 cells per 13 mm glass coverslip (Assistent, Germany) that had been precoated with poly-L-lysine (1 mg/ml). After 3-4 hours, coverslips were transferred into 24-well Multidishes (Nunc) containing a monolayer of astroglia. Coverslips were placed with the neurons facing downwards and were separated from the glia by paraffin ‘feet’. Cultures were maintained in serum-free medium (MEM) which included N2 supplements (Goslin and Banker, 1991), ovalbumin (0.1%, w/v) and pyruvate (0.1 mM). Neurons cultured at high density (230,000 cells per 24 mm glass coverslip in 100 mm Petri dishes; Nunc) were used for biochemical analysis (Hirschberg et al., 1996; Schwarz et al., 1995).

Axons were distinguished from dendrites by light microscopic criteria. Most neurons have one axon which is a long, thin process of uniform diameter, whereas mature neurons have 4-5 dendrites which are much shorter and taper from the proximal to the distal end (Dotti et al., 1988). The designation of processes as either axons or dendrites was confirmed using antibodies specific for axonal- (i.e. GAP 43) or dendritic- (i.e. MAP 2) proteins (Goslin and Banker, 1991).

N18TG-2 cells (a mouse neuroblastoma cell line) were provided by Professor Uriel Littauer (Department of Neurobiology, Weizmann Institute of Science). N18TG-2 cells were grown in flasks or Petri dishes (Hamprecht et al., 1985) in 10% fetal calf serum in MEM (Dulbecco’s minimal essential medium) in a water-saturated atmosphere of 5% CO2 at 36°C. For microscopy, cells were grown for 2-3 days on 13 mm acid-washed glass coverslips precoated with poly-L-lysine (20 μg/ml).

**Labeling of neurons with C6-NBD-GM1**

C6-NBD-GM1 was prepared by N-acylation of lyso-GM1 using succinimidyl 6-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl) aminohexanoate (Antes et al., 1992; Neuenhofer et al., 1985; Schwarzmann and Sandhoff, 1987). Complexes of C6-NBD-GM1 with defatted-bovine serum albumin (BSA) were prepared according to the method of Pagano and Martin, 1994.

Coverslips were removed from the Multiwell dishes and incubated in MEM containing 50 mM Heps (pH 7.3), 4 mM NaHCO3, 11 mg/ml pyruvic acid, 1 mM glutamine and 0.6% (w/v) glucose (Hepes-buffered medium) for 15 minutes at 37°C. Cells were then cooled (13-16°C) for 5 minutes prior to addition of C6-NBD-GM1/BSA (5 μM) for a further 30-40 minutes to label the plasma membrane (PM) (Koval and Pagano, 1989). Cells were subsequently warmed to 37°C for various times followed by elimination of NBD-fluorescence at the external leaflet of the PM by incubation with sodium dithionite (80 mM, 3 minutes, 25°C) (McIntyre and Sleight, 1991) immediately before microscopic examination. Occasionally, neurons were incubated with C6-NBD-GM1/BSA directly at 37°C for up to 1 hour, and subsequently returned to MEM/N2 medium in Multiwell dishes containing a glial monolayer in the CO2 incubator. Similar protocols were used for labeling of N18TG-2 cells.

In some experiments, neurons were labeled at 16°C with C6-NBD-GM1 for 30 minutes, and after washing, transferred to either Krebs-Ringer-Heps (KRH) buffer (128 mM NaCl, 3 mM KCl, 1 mM Na2HPO4, 1.2 mM MgSO4, 2.7 mM CaCl2, 20 mM Heps, 11 mM glucose), KRH/55 mM K+ buffer (76 mM NaCl, 55 mM KCl, 1 mM Na2HPO4, 1.2 mM MgSO4, 2.7 mM CaCl2, 20 mM Heps, 11 mM glucose), or KRH/55 mM K+/Ca2+ free buffer (76 mM NaCl, 55 mM KCl, 1 mM Na2HPO4, 1.2 mM MgSO4, 1 mM EGTA, 20 mM Heps, 11 mM glucose), and warmed to 37°C for 35 minutes, followed by treatment with dithionite, as above.

**Analysis of C6-NBD-GM1 metabolism**

Hippocampal neurons grown at high density were incubated with C6-NBD-GM1/BSA as described above. Cells were removed from the coverslips by scraping with a rubber policeman into 9 ml of water. The cell suspension was centrifuged at 4,500 g, and the resulting cell pellet resuspended using a Potter-Elvehjem homogenizer in 1 ml of 10 mM MES buffer, pH 5.5. In order to permeabilize lysosomes, homogenates were dialyzed for 18 hours against MES buffer, pH 5.5, at 4°C. Lysates were then incubated with 10 μM C6-NBD-GM1 at 37°C with continuous stirring. After 4.5 hours,
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Lysates were frozen and lyophilized prior to lipid extraction, purification, and separation by TLC.

**Analysis of C₆-NBD-GM₁ recycling**

To determine whether C₆-NBD-GM₁ recycles back to the PM from intracellular compartments, one dish containing 4 coverslips of neurons cultured at high-density was incubated with C₆-NBD-GM₁/BSA (5 µM) in Hepes-buffered medium for 1 hour at 37°C. Neurons were subsequently washed, incubated with 2·1% (w/v) defatted-BSA (DF-BSA) to 'back-exchange' C₆-NBD-GM₁ located at the external leaflet of the PM (Koval and Pagano, 1989), and then incubated with dithionite to remove any residual cell surface fluorescence. Neurons were then washed and returned to dishes containing MEM/N2 medium and 1% DF-BSA in the CO₂-incubator, but without a glial monolayer. After various times, the medium was removed from the dishes, cells washed 3 times, and cells removed from the coverslips by scraping with a rubber policeman. The medium and washes were combined, centrifuged (4,500 g av, 20 minutes, 4°C) to remove cell debris, and then passed over a Sep-Pak C₁₈ column. Lipids were eluted from the column by methanol. Lipids were extracted from cells with chloroform/methanol/1% pyridine (5:5:1, by volume) for 48 hours at 40°C, dried under a stream of nitrogen, dissolved in methanol/water/chloroform (94:96:6, by volume), passed over a C₁₈ column, and eluted as above. Fluorescence was quantified in the medium and in the cell extract using a SLM-8000 spectrofluorimeter (SLM instruments, Urbana, IL). The molar concentration of recovered NBD-fluorescence was determined by reference to a stock standard solution (λ ex = 468 nm, λ em = 530 nm).

**Other procedures**

C₆-NBD-glucosylceramide (GlcCer), C₆-NBD-galactosylceramide (GalCer), and N-[5-(5,7-dimethyl BODIPY™)-1-pentanoyl]-GlcCer (C₅-DMB-GlcCer) were synthesized by N-acylation as described (Pagano et al., 1991; Schwarzmann and Sandhoff, 1987).

Energy depletion was performed by pre-incubating neurons for 15 minutes at 37°C with 5 mM sodium azide and 50 mM 2-deoxyglucose in Hepes-buffered medium minus glucose. Neurons were subsequently cooled to 16°C for 5 minutes, incubated with C₆-NBD-GM₁ for 30 minutes, and then warmed to 37°C for 30 minutes. Prior to observation, neurons were treated with dithionite. The metabolic inhibitors were present throughout the incubations.

Rhodamine-conjugated wheat germ agglutinin (Rh-WGA) (Vector Laboratories, Inc., Burlingame, CA) was used as a vital stain of endocytic compartments (Matteoli et al., 1992).

Fluorescence microscopy was performed using Plan Apochromat ×63/1.4 NA and Plan Neofluar ×40/1.3 NA oil objectives of a Zeiss Axiovert 35 microscope equipped with appropriate filters for NBD-, DMB-, and rhodamine-fluorescence. Cells were photographed using a Contax 167MT camera and Kodak Tmax p3200 film.

**RESULTS**

The internalization of C₆-NBD-glycosphingolipids in hippocampal neurons

Previous studies (reviewed by Hoekstra and Kok, 1992) have demonstrated that fluorescent, short acyl chain sphingolipid analogs with polar or ‘bulky’ head groups (i.e. analogs of sphingomyelin, GlcCer, GalCer and lactosylceramide), rapidly transfer from either donor liposomes or BSA into the external...
leaflet of the PM. In most cases, the analogs are unable to undergo spontaneous transbilayer movement and are internalized by endocytic mechanisms. However, a recent study has shown that some sphingolipid analogs do undergo transbilayer movement at either the PM or in endosomes (Martin and Pagano, 1994). We therefore examined the energy- and temperature-dependence of internalization of various short acyl chain glycosphingolipid analogs in cultured hippocampal neurons. Three of the lipid analogs tested, C6-NBD-GlcCer, C6-NBD-GalCer, and C5-DMB-GlcCer, were internalized by a combination of energy- and temperature-dependent mechanisms (presumably vesicle-mediated endocytic transport), and of transbilayer movement followed by monomer transport through the cytosol. This latter process results in non-specific labeling of intracellular membranes, limiting the suitability of these analogs to study lipid endocytosis in cultured neurons.

In contrast, C6-NBD-GM1 was internalized exclusively by a temperature- and energy-dependent mechanism. Six-day-old hippocampal neurons (stage 4) were incubated with C6-NBD-GM1 at 16°C. C6-NBD-GM1 rapidly transferred from a BSA complex and labeled the PM of axons, dendrites and the cell body (Fig. 2A,D). If neurons were maintained at 16°C, no intracellular fluorescence could be detected after elimination of cell surface labeling using either dithionite (McIntyre and Sleight, 1991) (Fig. 2B,E) or BSA (‘back-exchange’; see Koval and Pagano, 1989) (not shown), neither of which gain access to the cytosol or to the inner leaflet of the PM. When cells were warmed to 37°C for various times prior to elimination of cell surface fluorescence by dithionite, discrete intracellular fluorescent puncta were observed (see Figs 3 and 7 below). However, incubation with the metabolic inhibitors, sodium azide (5 mM) and 2-deoxyglucose (50 mM), completely blocked the internalization of C6-NBD-GM1 when cells were warmed to 37°C (Fig. 2C,F). The temperature- and

**Fig. 3.** C6-NBD-GM1 is transported in a retrograde direction and accumulates in endocytic structures in the cell body. Six-day-old neurons were incubated with 25 μg/ml Rh-WGA in Hepes-buffered medium for 1 hour at 37°C, washed, and then transferred to Multiwell dishes containing glial co-cultures in the CO2 incubator. After 17-18 hours, neurons were removed from the incubator, washed, incubated with 5 μM C6-NBD-GM1 in Hepes-buffered medium for 1 hour at 37°C, washed again, and then treated with dithionite. Cells were photographed using appropriate optics for either (B) NBD or (C) rhodamine fluorescence; (A) a phase contrast micrograph of the same cell. Note that NBD-fluorescence is present in the dendrites and cell body, but absent in the axon (arrowhead); the axon continues well beyond the photographic field. In D-F, 6-day-old neurons were incubated with Rh-WGA in Hepes-buffered medium for 1 hour at 37°C, washed, incubated with C6-NBD-GM1 for 1 hour, washed, and then returned to the CO2 incubator for an additional 14 hours. Prior to observation, cells were treated with dithionite. (D) Phase contrast, (E) NBD-fluorescence, (F) rhodamine fluorescence. After 14 hours incubation, the two fluorescent probes are colocalized in the same puncta (arrow) in the perikaryal region. Bar, 10 μm.
energy-dependence of internalization suggests that C6-NBD-GM1 is internalized by an endocytic, vesicle-mediated process, and can be used as a tool to study the transport of GM1 along the endocytic pathway in cultured neurons.

The site of accumulation of C6-NBD-GM1 in hippocampal neurons

We next determined the sites of intracellular accumulation of C6-NBD-GM1. Six-day-old neurons were pre-incubated for 1 hour at 37°C with Rh-WGA, washed to remove excess Rh-WGA, and then incubated for a further 17-18 hours in the CO2 incubator. Rh-WGA is internalized by absorptive endocytosis from axons and dendrites (Matteoli et al., 1992) and transported to the cell body where it accumulates in a late endocytic, non-degradative compartment (Raub et al., 1990) (Fig. 3C,F). Subsequent incubation for either 5 minutes (not shown) or 1 hour (Fig. 3B) with C6-NBD-GM1 directly at 37°C resulted in the appearance of discrete puncta of internalized NBD-fluorescence in the cell body and dendrites. Identical results were obtained if cells were incubated with C6-NBD-GM1 at 16°C and subsequently warmed to 37°C (see Fig. 7). In 6- to 7-day-old neurons, no puncta were observed in axons with the exception of the proximal 30-40 μm (Fig. 3A,B). Considerable overlap of C6-NBD-GM1 and Rh-WGA fluorescence was observed in the cell body after a 1 hour incubation with C6-NBD-GM1 (Fig. 3B,C). After a further 14 hours, there was complete overlap of C6-NBD-GM1 and Rh-WGA fluorescence in the cell body (Fig. 3E,F), and no C6-NBD-GM1 fluorescence remained in any of the processes. These data demonstrate that C6-NBD-GM1 is efficiently internalized from the somatodendritic PM, followed by retrograde transport to the cell body where it accumulates in a late endocytic compartment.

To determine whether the internalization of C6-NBD-GM1 was calcium-dependent and depolarization-sensitive, neurons were incubated with either KRH/55 mM K+ or KRH/55 mM K+/Ca2+-free buffers. No difference in the amount of internalization or in the intracellular site of accumulation of C6-NBD-GM1 was detected in either 3- or 10-day-old neurons compared to control cells incubated under identical conditions in either KRH or Hepes-buffered medium.

To determine the extent of C6-NBD-GM1 metabolism, high-density cultures were incubated under similar conditions to those used for morphological analysis. Small but detectable amounts of C6-NBD-GM2, the immediate product of C6-NBD-GM1 degradation, were observed after 14 hours incubation (Fig. 4A); C6-NBD-GM2 could barely be detected after 1 hour incubation (not shown). C6-NBD-GM1 was also hydrolyzed in vitro to C6-NBD-GM2 and C6-NBD-GM3 at acidic pH (Fig. 4B), demonstrating that C6-NBD-GM1 is a substrate for lysosomal β-galactosidase. However, the small amount of hydrolysis in vivo supports the idea that gangliosides exhibit long half lives (Schwarzmann and Sandhoff, 1990; van Meer, 1989), and demonstrates that little C6-NBD-GM1 is transported from endosomes to lysosomes. The small amount of cellular material did not permit a more quantitative analysis of C6-NBD-GM1 degradation. However, preliminary studies using a radioactive analogue of naturally occurring GM1 (prepared by tritium-labeling of the sphingoid long chain base of GM1 using NaB[3H]4; Hirschberg et al., 1996; Schwarzmann, 1978) indicates that little metabolism of exogenously-added, naturally occurring GM1 occurs in hippocampal neurons.

The recycling of C6-NBD-GM1 to the cell surface

Both the co-localization with Rh-WGA (Fig. 3), and the lack of significant metabolism of C6-NBD-GM1 (Fig. 4), suggest that C6-NBD-GM1 is internalized to a late endocytic, non-degradative compartment. To determine whether C6-NBD-GM1 is able to exit this compartment and recycle back to the cell surface, neurons cultured at high density, in which the late endocytic pool had been pre-labeled with C6-NBD-GM1 (Fig. 3), were incubated for various times in medium containing 1% DF-BSA (continual ‘back exchange’, see for instance van Genderen and van Meer, 1995). The amount of cell-associated NBD-fluorescence decreased during the first 3 hours of incubation, with a concomitant increase in the amount of NBD-fluorescence in the medium, in both 3-day-old and 6-day-old neurons (Fig. 5). No metabolites of C6-NBD-GM1 were detected in the medium by TLC analysis (not shown), demonstrating that C6-NBD-GM1 itself, and not a metabolic product (see Fig. 4), was transported from the late endocytic compartment to the cell surface. During the next three hours incubation, there was little increase in the amount of C6-NBD-GM1 transported to the cell surface, with approximately 25-30% of the total C6-NBD-GM1 remaining inaccessible to back-exchange in both 3- and in 6-day-old neurons. This may indicate that there is a pool of C6-NBD-GM1 that is unable to recycle, or alternatively, that there is a pool of C6-NBD-GM1 that cannot be removed from the PM by ‘back-exchange’.
However, the ability to back-exchange C6-NBD-GM1 from the cell surface during the first three hours incubation (Fig. 5) indicates that C6-NBD-GM1 is transported out of the late endosomal compartment in the cell body (Fig. 3) to the cell surface. Although we did not determine the kinetics of exit from endosomes, the fact that all of the exchangeable C6-NBD-GM1 reached the cell surface during the first three hours incubation indicates that the half-time of recycling cannot be more than 90 minutes, and is probably much less, as is the case in non-neuronal cells (Koval and Pagano, 1989; Mayor et al., 1993; van Genderen and van Meer, 1995).

The internalization of C6-NBD-GM1 in neuroblastoma cells

To determine whether C6-NBD-GM1 is similarly transported to an endosomal compartment in other cell types, we examined its internalization in a neuroblastoma cell line (N18TG-2). C6-NBD-GM1 was internalized to a series of fluorescent puncta distributed throughout the cytoplasm (Fig. 6A,B), with some accumulation in an area corresponding to the microtubule organizing center (Koval and Pagano, 1989). To determine the extent of C6-NBD-GM1 metabolism in N18TG-2 cells, cells were incubated with C6-NBD-GM1 for 4 hours at 37°C. No degradation products of C6-NBD-GM1 were detected after 1 hour incubation (not shown), while after 4 hours, small amounts of C6-NBD-GM2 were detected (Fig. 6C). The lack of significant metabolism of C6-NBD-GM1 in N18TG-2 cells is similar to that observed in hippocampal neurons (Fig. 4A), supporting the idea that C6-NBD-GM1 is internalized mainly to recycling endosomes, and does not reach lysosomes.

The regulation of C6-NBD-GM1 endocytosis during the development of neuronal polarity

We next examined the internalization of C6-NBD-GM1 during the development of neuronal polarity. Neurons of various ages were incubated with C6-NBD-GM1 at 37°C for 40 minutes to label the PM, warmed to 37°C for 1 hour, and then treated with dithionite to eliminate cell surface fluorescence. In 2-day-old neurons (stage 2 and 3), endocytic vesicles were observed in all the processes, including the longest process, which is destined to become the axon (Fig. 7A,B). No difference in the intensity of labeling or in the number of vesicles per unit length was observed between minor and major process. Similarly, after 3 days a significant number of vesicles were labeled in both the axon and minor processes, although there appeared to be a slight reduction in endocytic activity in axons (Fig. 7C,D). After 6- (Fig. 3B) or 9-days (Fig. 7E,F) (stage 4/5), few fluorescent puncta were observed in axons but a large number of intensely-labeled puncta were observed in dendrites and the cell body. A similar distribution of C6-NBD-GM1-labeled vesicles was observed in 14-day-old (stage 5) neurons (Fig. 7G,H).
Fig. 7. Axonal endocytosis of C₆-NBD-GM₁ decreases during the development of neuronal polarity. Hippocampal neurons were incubated with 5 μM C₆-NBD-GM₁ in Hepes-buffered medium for 40 minutes at 16°C, and then warmed to 37°C for 1 hour prior to dithionite treatment. (A,B) Two-day-old neurons; (C,D) 3-day-old neurons; (E,F) 9-day-old neurons; (G,H) 15-day-old neurons. (A,C,E,G) Phase contrast micrographs, (B,D,F,H) immunofluorescence. Note that in older neurons, far fewer C₆-NBD-GM₁-labeled vesicles are observed in axons (arrowheads) than in younger cells. Bar, 20 μm.
To determine if \( C_{6} \)-NBD-GM\(_1\) was internalized exclusively from dendrites, or if some of the fluorescent puncta were actually associated with synaptic vesicles in presynaptic terminals, 10-day-old neurons were incubated with tetramethylrhodamineisothiocyanate-transferrin, which is internalized specifically from the cell body and dendrites in mature neurons (Cameron et al., 1991). Considerable overlap of \( C_{6} \)-NBD-GM\(_1\) and tetramethylrhodamineisothiocyanate-transferrin fluorescence was observed in the somatodendritic area (not shown). These results indicate that during the initial stages of the development of polarity, significant amounts of \( C_{6} \)-NBD-GM\(_1\) are internalized from axons and minor processes, but as neurons mature, endocytic activity decreases from the axon.

**DISCUSSION**

The major finding of the current study is that a short acyl chain derivative of ganglioside GM\(_1\), \( C_{6} \)-NBD-GM\(_1\), is internalized from dendrites throughout neuronal development, but internalization from axons and subsequent retrograde transport to the cell body decreases as neurons mature. This is the first time that the internalization of a membrane lipid has been analyzed in polarized neurons, and suggests that significant differences exist in the rate of turnover of gangliosides, and presumably other membrane lipids, between axons and dendrites.

In axons, the initial endocytic compartment labeled upon internalization of \( C_{6} \)-NBD-GM\(_1\) is probably the same as that labeled by synaptic vesicle markers (Matteoli et al., 1992). Thus, after short incubations of stage 3-4 neurons with \( C_{6} \)-NBD-GM\(_1\), axons contained a large numbers of vesicles along their entire length. However, there was virtually no endocytosis of \( C_{6} \)-NBD-GM\(_1\) from axons in stage 5 neurons, similar to that observed for synaptic vesicle markers (Matteoli et al., 1992). After longer times of incubation in both stage 3-4 and in stage 5 neurons, \( C_{6} \)-NBD-GM\(_1\) was observed in the cell body only, indicating that most \( C_{6} \)-NBD-GM\(_1\) is sorted away from the pool of synaptic vesicles that locally recycle in axons (Matteoli et al., 1992; Parton and Dotti, 1993).

After being transported in a retrograde direction from axons and dendrites, \( C_{6} \)-NBD-GM\(_1\) accumulates in the same compartment as that labeled by Rh-WGA (Raub et al., 1990). Together with the data on recycling of \( C_{6} \)-NBD-GM\(_1\) to the cell surface, this implies that the major site of accumulation of \( C_{6} \)-NBD-GM\(_1\) in the cell body is a non-degradative, recycling compartment of the endosomal system. This is consistent with studies using another short acyl chain sphingolipid analog, \( C_{6} \)-NBD-sphingomyelin (\( C_{6} \)-NBD-SM), which acts as a marker of constitutive recycling vesicles, as demonstrated by kinetic (Mayor et al., 1993) and biochemical analysis (Koval and Pagano, 1989). In Chinese hamster ovary cells, \( C_{6} \)-NBD-SM accumulates in a peri-centriolar recycling compartment, and only small amounts are targeted to lysosomes, indicating that most internalized lipid is recycled back to the PM (Mayor et al., 1993).

The lack of significant hydrolysis of \( C_{6} \)-NBD-GM\(_1\) even after 14 hours in hippocampal neurons also suggests that little \( C_{6} \)-NBD-GM\(_1\) reaches lysosomes. Similarly, in neuroblastoma cells, \( C_{6} \)-NBD-GM\(_1\) accumulated in a series of fluorescent puncta distributed throughout the cytoplasm, and was not metabolized significantly even after 4 hours. However, accumulation of an exogenously-added biotinylated-derivative of GM\(_1\) in endosomes and also lysosomes has been observed by electron microscopy (Schwarzmann et al., 1987), and biochemical analysis indicates that when added from exogenous sources, radioactive analogs of GM\(_1\) with long acyl chains are degraded in neurons, presumably in lysosomes (Riboni et al., 1992, 1993; Riboni and Tettamanti, 1991). The differences in the extent of metabolism of GM\(_1\) between these various reports might be due to differences in the mechanism of uptake and processing of radioactive GM\(_1\) compared to \( C_{6} \)-NBD-GM\(_1\). Alternatively, they may be due to the different cell types used in the various experiments. With respect to the latter, it should be noted that many of the previous studies in neurons were performed using fully differentiated neurons in culture (Riboni and Tettamanti, 1991), whereas hippocampal neurons actively differentiate and develop in culture. This possibility is also consistent with suggestions that the rates of exo- and endocytosis (and presumably synthesis and degradation) of membrane components differ between immature, rapidly growing neurons and mature, non-growing neurons (Futerman and Banker, 1996; Futerman et al., 1995).

Significant amounts of endocytosis of \( C_{6} \)-NBD-GM\(_1\) were observed in dendrites at all stages of development, as indicated by the high density of NBD-puncta along their entire length. The unidirectional nature of endocytic transport in dendrites (in the retrograde direction; see Parton et al., 1992) suggests that vesicles observed in the dendritic arbor are internalized at a site that is at least distal to their observed location, and is not due to anterograde transport from the cell body. The latter is unlikely anyway since after longer times of incubation (i.e. 4 hours), \( C_{6} \)-NBD-GM\(_1\) is found exclusively in the cell body. In cerebellar Purkinje cells, evidence has recently been provided, based on immunolocalization, that a minor ganglioside, GD\(_{1a}\), is specifically localized to proximal dendrites and cell bodies (Furuya et al., 1994), and that ganglioside GM\(_{1b}\) is localized to proximal dendrites in hippocampal neurons (Furuya et al., 1995); both of these studies were based on analysis of tissue slices, and no confirmation has yet been performed in dissociated cell cultures (reviewed by Schwarz and Futerman, 1996). However, if specific gangliosides are indeed localized to specific neuronal membranes, then mechanisms would need to exist to sort and target them back to the correct membrane domain after their internalization. Although GM\(_1\) is found in both axons and dendrites of hippocampal neurons (Sofer and Futerman, 1995), the high levels of internalization of \( C_{6} \)-NBD-GM\(_1\) from dendrites demonstrates that there is rapid turnover of membrane gangliosides in dendritic membranes, whereas the turnover of membrane gangliosides in axons is much less rapid, at least in mature, polarized neurons.

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