We have previously shown that ongoing glucosylceramide (GlcCer) synthesis is required for basic fibroblast growth factor (bFGF) and laminin to stimulate axonal growth in cultured hippocampal neurons (Boldin, S., and Futerman, A. H. (1997) J. Neurochem. 68, 882–885). We now demonstrate that stimulation of axonal growth by bFGF leads to an increase in the rate of GlcCer synthesis. Within minutes of incubation with bFGF, a significant increase in the rate of metabolism of \([^{14}C]\text{hexanoyl ceramide}\) to \([^{14}C]\text{hexanoyl GlcCer}\) is detected, but there are no changes in the rate of \([^{14}C]\text{hexanoyl sphingomyelin}\) synthesis. In vitro analysis of GlcCer synthase activity revealed an approximately 2-fold increase in the rate of \([^{14}C]\text{hexanoyl GlcCer}\) synthesis upon incubation with either bFGF or laminin; other growth factors, which did not effect the rate of axon growth, had no effect on the rate of \([^{14}C]\text{hexanoyl GlcCer}\) synthesis. The increased rate of \([^{14}C]\text{hexanoyl GlcCer}\) synthesis was not affected by preincubation with either cycloheximide or actinomycin, and no elevation of GlcCer synthase mRNA levels was detected, suggesting that GlcCer synthase is up-regulated by a post-translational mechanism. The relevance of these results for understanding the regulation of axonal growth is discussed.

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**EXPERIMENTAL PROCEDURES**

**Materials**—\(n\)-Hexanoic acid \([1-^{14}C]\text{N-hydroxysuccinimide ester}\) (52.2 or 55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). \(\text{D-erythro-Sphingosine}\) and \(\text{L-threo-sphingosine}\) were from Matreya (Pleasant Gap, PA). An mRNA isolation kit and a Titan™ one-tube reverse transcriptase polymerase chain reaction (RT-PCR) system were from Roche Molecular Biochemicals. Silica Gel 60 plates were from Merck KGaA, Darmstadt, Germany. Ultima Gold™ scintillation fluid was from Packard Instrument Co. Nerve growth factor was from Promega (Madison, WI), and epidermal growth factor was from Toyoba Co. Ltd., Osaka, Japan. bFGF, laminin, an anti-bFGF antibody (product number F6162), and other chemicals were from Sigma. Solvents (analytical grade) were from Bio-Lab Laboratories Ltd., Jerusalem, Israel.

**Cell Culture**—Hippocampal neurons plated at high-density (180,000 cells/24-mm polysine-coated glass coverslip) were cultured in serum-free medium as described (14, 16, 17) and grown for 2 days in 100-mm Petri dishes containing a monolayer of astroglia. Coverslips were placed with the neurons facing downwards and separated from the glia by paraffin “feet” (17). For morphological measurements, neurons were plated at low density (12,000 cells/13-mm coverslip) (12, 15).

**In Vivo Analysis of Sphingolipid Synthesis**—Six coverslips containing 2-day-old neurons were removed from dishes containing a glial monolayer and placed with the neurons facing upwards in a 100-mm Petri dish that did not contain a glial monolayer; 4 ml of medium, taken from dishes containing glia, were added to each culture dish. \([^{14}C]\text{Hexanoyl Cer}\) (synthesized by \(N\)-acylation of sphingosine using \(n\)-hexanoic acid \([1-^{14}C]\text{N-hydroxysuccinimide ester}\) (18)) was dissolved in ethanol and added to the culture dishes, which were then returned to the incubator for various times. bFGF (1 ng/ml, dissolved in Hank’s balanced salt solution (HBSS)) or an equivalent volume (10 μl) of HBSS were added to the culture dishes 1 h later. After various times, coverslips were removed from the dishes and washed with HBSS, and neurons were removed by scraping with a rubber policeman into ice-cold water and lyophilized. After resuspension in a small volume of water, lipids were extracted (19) and separated by thin layer chromatography using CHCl₃/CH₃OH/9.8 m M CaCl₂ (60:35:8, v/v/v) as the developing solvent. Thin layer chromatography plates were exposed to a \(^{14}C\)-sensitive imaging plate (BAS-TR2040S, Fuji Photo Film Co., Ltd., Japan), lipids were recovered from the plates by scraping, and radioactiv...
ity was determined by liquid scintillation counting.

In Vitro Analysis of GlcCer Synthesis—Neurons were placed into new culture dishes as above and treated with or without bFGF or other growth factors for various times. Coverslips were subsequently washed three times with HBSS; neurons were removed by scraping with a rubber policeman, and neurons were homogenized in a handheld Potter-Elvehjem homogenizer in 2–3 ml of TK buffer (25 mM KCl and 50 mM Tris, pH 7.4). Homogenates were used fresh. GlcCer synthesis was assayed as described (2). The standard reaction mixture contained 20 μg of protein (determined according to Bradford (20), 5 mM UDP-Glc, 2 μM [14C]hexanoyl Cer, and 10 mM MnCl	extsubscript{2} in a total volume of 2–3 ml of TK buffer. The reaction was terminated after 2 h at 37 °C by addition of CHCl	extsubscript{3}/CH	extsubscript{3}OH (1:2, v/v). Lipids were extracted (19) and separated by thin layer chromatography as described above.

RT-PCR Analysis of GCS—mRNA was isolated from 2-day-old hippocampal neurons using an mRNA isolation kit. Partial GlcCer synthase cDNA (~400 base pairs), based on the sequence of rat GlcCer synthase (5), was obtained by RT-PCR using a Titan	extsuperscript{TM} one-tube RT-PCR system and the following GlcCer synthase primers: 5'-TTGTCGCGTTGCTGTCCTT-3' (forward primer), and 5'-GACTCGTATTCCGGTATCC-3' (reverse primer). RT-PCR products were resolved on 1.5% agarose-gels.

RESULTS

In Vivo Analysis of GlcCer Synthesis—To determine whether bFGF stimulates sphingolipid synthesis, [14C]hexanoyl Cer was added directly to culture dishes containing 2-day-old neurons. After 1 h, bFGF was added, and [14C]hexanoyl GlcCer and [14C]hexanoyl SM synthesis was analyzed for the next 30 min. Immediately prior to addition of bFGF, the rate of [14C]hexanoyl GlcCer synthesis was 35.8 fmol/min, which increased to 274 fmol/min for a 10-min period after addition of bFGF (Fig. 1A and Table I). In contrast, there was no increase in the rate of [14C]hexanoyl SM synthesis (Fig. 1A, Table I). A small increase in the rate of [14C]hexanoyl GlcCer synthesis was also observed in control cultures dishes, to which an equivalent volume of buffer (HBSS) was added. The rate of GlcCer synthesis after addition of buffer was 81.6 fmol/min; this is presumably due to removing the culture dish from the incubator, opening the dish for addition of HBSS, and gentle shaking after addition of the buffer.

The rate of uptake of [14C]hexanoyl Cer by neurons was also significantly increased after addition of bFGF (Fig. 1B and Table I). Two possibilities could explain the increase in the rate of [14C]hexanoyl Cer uptake. (i) bFGF could directly affect the rate of [14C]hexanoyl Cer uptake into neurons, and, as a consequence, the rate of [14C]hexanoyl GlcCer synthesis is elevated simply because of increased availability of intracellular [14C]hexanoyl Cer. (ii) bFGF could directly affect the rate of [14C]hexanoyl GlcCer synthesis, and, as a result, more [14C]hexanoyl Cer is taken up by neurons to provide sufficient substrate for increased [14C]hexanoyl GlcCer synthesis. To distinguish between these two possibilities, which are fundamental to explaining the mechanism of action of bFGF, neurons were incubated with a stereoisomer of Cer, [14C]hexanoyl-erythro-Cer, which is not metabolized to GlcCer (12). Addition of bFGF did not affect the rate of uptake of [14C]hexanoyl-erythro-Cer (Fig. 2B) or the rate of [14C]hexanoyl-erythro-SM synthesis (Fig. 2A), demonstrating that bFGF directly affects the rate of [14C]hexanoyl GlcCer synthesis rather than the rate of [14C]hexanoyl Cer uptake.

Although there was an increase in the rate of [14C]hexanoyl GlcCer synthesis immediately after addition of bFGF (Fig. 1A), reaching a maximum after 20 min, the rate of [14C]hexanoyl GlcCer synthesis decreased after this time (Fig. 1A). No increase in the amount of [14C]hexanoyl GlcCer was detected in the medium 30 min after addition of bFGF, and no significant metabolism of [14C]hexanoyl GlcCer to [14C]hexanoyl lactosylceramide was detected when cells were incubated either with [14C]hexanoyl Cer or directly with [14C]hexanoyl GlcCer (not shown). In contrast, upon direct incubation with [14C]hexanoyl GlcCer, significant metabolism to [14C]hexanoyl Cer was detected, and it is possible that the decrease in the amount of [14C]hexanoyl GlcCer observed 50 min after addition of bFGF is due to its degradation to [14C]hexanoyl Cer.

In Vitro Analysis of GCS—We next examined the specificity of the effect of bFGF by analyzing GCS activity in vitro. In these experiments, neurons were incubated with bFGF or other growth factors for 10 min in culture dishes subsequently removed from the coverslips by scraping, homogenized, and assayed for GCS activity using [14C]hexanoyl Cer as substrate (2, 18). When measured in vitro, GCS activity increased from 2.5 to 4.1 pmol of [14C]hexanoyl GlcCer synthesized per μg of protein/h after treatment with bFGF (Table II). A similar increase in the specific activity of GCS was detected when neu-
Comparison of the rates of uptake and synthesis of \(^{14}C\)hexanoyl sphingolipids in control and bFGF-treated neurons

Data are taken from Fig. 1. Rates were calculated by linear regression analysis. After addition of bFGF, there is a significant increase in the rate of both \(^{14}C\)hexanoyl Cer uptake and of \(^{14}C\)hexanoyl GlcCer synthesis compared with control neurons; the numbers in parenthesis are the ratios of the rate of uptake or synthesis in the 10-min period after addition of bFGF or HBSS versus the 30-min period immediately prior to their addition. The difference in the rates of uptake and synthesis between 0–30 min and 30–60 min is due to the rapid initial rate of uptake of \(^{14}C\)hexanoyl Cer (18, 21) by neurons immediately after its addition to the culture dishes.

### Table I

| Time after addition of \(^{14}C\)hexanoyl Cer | Control neurons | bFGF-treated neurons |
|--------------------------------------------|-----------------|----------------------|
| **Rate of \(^{14}C\)hexanoyl Cer uptake (fmol/min)** | 1292            | 161 (1)              |
| **Rate of \(^{14}C\)hexanoyl GlcCer synthesis (fmol/min)** | 89.5            | 35.8 (1)              |
| **Rate of \(^{14}C\)hexanoyl SM synthesis (fmol/min)** | 64.7            | 12.9 (1)              |

### Table II

Effect of growth factors on GCS activity and on the rate of axon growth

Neurons (plated at a density of 12,000 cells/13-mm coverslip) were incubated with or without growth factors at 48 h in culture, and axon length was measured at 51 h; axon length in control cells at 48 h was 208 ± 6.4 μm. In parallel experiments, neurons (plated at high density) were incubated with growth factors for 10 min at 48 h in culture, removed from the coverslips by scraping, homogenized, and assayed for GCS activity (20 μg of protein, 2 μM \(^{14}C\)hexanoyl Cer, 2-h reaction) in vitro. Values are means ± S.E. of three individual experiments.

| Growth factor \(^a\) | Axon length at 51 h | GCS activity |
|----------------------|---------------------|--------------|
|                      | μm                  | pmol \(^{14}C\)hexanoyl GlcCer synthesized/μg of protein/h |
| Control              | 214 ± 8.4           | 2.55 ± 0.62  |
| bFGF (1 ng/ml)       | 278 ± 8.1<sup>b</sup> | 4.10 ± 0.84<sup>b</sup> |
| Denatured bFGF (1 ng/ml) | 217 ± 9.7           | 1.98 ± 0.28  |
| bFGF (1 ng/ml) + anti-bFGF (250 ng/ml) | 210 ± 9.7          | 2.20 ± 0.32  |
| anti-bFGF (250 ng/ml) |                    |              |
| EGF (50 ng/ml)       | 220 ± 11.4          | 1.93 ± 0.43  |
| NGF (200 ng/ml)      | 221 ± 8.4           | 2.55 ± 0.70  |
| NT3 (400 ng/ml)      | 220 ± 9.6           | 2.45 ± 0.84  |
| Laminin (5 μg/ml)    | 218 ± 12.9          | 2.47 ± 0.61  |

<sup>a</sup> The conditions of incubation for each growth factor were as follows: denatured bFGF, bFGF was heated at 95 °C for 10 min; bFGF and laminin stimulate both, whereas inactivated bFGF and other growth factors do not effect either parameter.<sup>3</sup>

The activity of GCS at various substrate concentrations was examined in neurons treated with or without bFGF; an approximately 2-fold increase in activity was observed in bFGF-treated compared with control neurons under conditions in which the rate of the reaction was not limited by substrate availability (Fig. 3A). Activity was linear with respect to time of the reaction (Fig. 3B) and protein concentration (not shown).

To examine the time course of the effect of bFGF, neurons were incubated for various times with bFGF prior to assay of GCS activity. GCS activity remained higher in bFGF-treated neurons than in their control counterparts for up to 3 h after addition of bFGF (Table III). However, when bFGF was added to the incubation medium for 10 min and then removed (by washing the coverslips and replacing the medium with medium that did not contain bFGF), GCS activity decreased compared with neurons in which bFGF remained present throughout the 3-h incubation (Fig. 4).

Up-regulation of GCS Activity by a Post-translational Mechanism

<sup>3</sup> Note that addition of bFGF directly to the reaction mixture had no effect on GCS activity (not shown).
anism—Because GCS activity was elevated within 5–10 min of addition of bFGF, it appears unlikely that GCS activity is regulated via a transcriptional mechanism as was observed, for instance, upon long-term depletion of substrate levels (8). This was confirmed by semiquantitative RT-PCR analysis, in which no changes in GCS mRNA levels were detected between control neurons and those treated with bFGF for 10 min or 3 h (Fig. 5). Moreover, preincubation with the protein synthesis inhibitor, cycloheximide, or the RNA synthesis inhibitor, actinomycin, had no effect on the bFGF-stimulated increase in GCS activity (Table IV).

**DISCUSSION**

The major finding of the current study is that stimulation of axonal growth by bFGF results in an increase in the rate of GlcCer synthesis due to post-translational modification of GCS. In contrast, the rate of SM synthesis is not affected. A role for GlcCer synthesis in regulating the rate of axonal growth in cultured hippocampal neurons has been established (12, 14, 15), although the precise molecular requirements for which GlcCer synthesis is needed are not known.

It has been reported that the rate of SM synthesis increases compared with GlcCer synthesis during neuronal development (22), perhaps to regulate the supply or the function of glycosylphosphatidylinositol-anchored proteins (23) in growing axons. However, careful comparison of the rates of SM and GlcCer synthesis is required before distinct functions can be ascribed to each lipid at different stages of development, particularly as differences in the ratio of SM and GlcCer synthesis can be detected depending on the ceramide analogues used to measure activity and on ceramide concentration.2 Irrespective of the possible distinct roles for SM and GlcCer synthesis at different stages of neuronal development, the data presented in the current study demonstrate that in young neurons (2 days in culture), GlcCer synthesis is activated by bFGF, whereas the rate of SM synthesis is unaffected. This activation occurs within minutes of bFGF treatment and is presumably a prerequisite for accelerated axonal growth, because incubation with inhibitors of GlcCer synthesis blocks axonal growth (12, 14, 15).

**TABLE III**

| Time after addition of bFGF (min) | Control neurons | bFGF-treated neurons |
|----------------------------------|-----------------|---------------------|
|                                  | Specific activity (pmol [14C]hexanoyl GlcCer synthesized/μg of protein/h) |                      |
|                                  |                 |                     |
| 5                                | 3.52 ± 0.76     | 5.03 ± 0.48         |
| 10                               | 3.16 ± 0.67     | 5.97 ± 1.05         |
| 30                               | 3.21 ± 0.68     | 5.08 ± 1.07         |
| 180                              | 3.08 ± 0.67     | 4.70 ± 1.12         |

**TABLE IV**

| Pretreatment | Control neurons | bFGF-treated neurons |
|--------------|-----------------|---------------------|
|              | Specific activity (pmol [14C]hexanoyl GlcCer synthesized/μg of protein/h) |                      |
| None         | 3.8 ± 0.4       | 5.6 ± 0.5           |
| Actinomycin  | 3.7 ± 0.5       | 6.4 ± 0.1           |
| Cycloheximide| 3.6 ± 0.1       | 6.7 ± 0.6           |
In contrast to some previous studies (8–10) demonstrating transcriptional activation of GCS, our data demonstrate that GCS can also be modulated by a post-translational mechanism. Two earlier studies have suggested a similar mode of activation. In the first (24), a decrease in the activity of GCS was observed upon treatment of Kym-1 rhabdomyosarcoma cells with tumor necrosis factor, presumably to prevent GCS from consuming ceramide generated by the SM cycle (25). In the second study, SM generated by application of endogenous sphingomyelinase to B16 melanoma cells resulted in up-regulation of GCS by both transcriptional and post-translational steps (26). Common to both of these studies is the pathway by which GCS activity is regulated namely as a response to generation of ceramide at the cell surface upon activation of the SM cycle. This appears to be different from the pathway by which GCS is up-regulated in bFGF-treated hippocampal neurons. There is no evidence that the activity of bFGF is mediated by the SM cycle, but rather it appears to be mediated via tyrosine phosphorylation of the cytoplasmic domain of high affinity bFGF receptors (27). In PC12 cells, tyrosine phosphorylation of the bFGF receptor activates mitogen-activated protein kinase (28), and in hippocampal neurons, bFGF causes a rapid increase in tyrosine phosphorylation of a variety of proteins, as yet unidentified (29). Whether activation of one of these pathways is a first step in the rapid up-regulation of GCS minutes after addition of bFGF is not known. However, it should be noted that the active site of GCS is on the cytosolic face of the Golgi apparatus (1–4), rendering the enzyme relatively accessible without intracellular signals and second messengers.

Because GCS has not been crystallized, little secondary and tertiary structural information is available. In a recent study, a histidine residue was detected in the active site of GCS (30). In addition, deletion of the N-terminal domain of GCS (including the transmembrane region) resulted in total loss of activity. As suggested (30), this region may be involved in interaction of GCS with ceramide and may be a potential site for post-translational modification. Unraveling the pathways by which binding of bFGF (and laminin) to their respective cell surface receptors leads to post-translational modification of GCS presents a formidable challenge.

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REFERENCES
1. Coste, H., Martel, M.-B., and Ger, R. (1986) Biochim. Biophys. Acts 858, 6–12
2. Futerman, A. H., and Pagano, R. E. (1991) Biochem. J. 285, 295–302
3. Jeckel, D., Karrenbauer, A., Burger, K. N. J., Van Meer, G., and Wieland, F. (1992) J. Cell Biol. 117, 259–267
4. Marks, D. L., Wu, K., Paul, P., Kamisaka, Y., Watanabe, R., and Pagano, R. E. (1999) J. Biol. Chem. 274, 451–456
5. Ichikawa, S., and Hirabayashi, Y. (1998) Trends Cell Biol. 8, 198–202
6. Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K., and Hirabayashi, Y. (1996) Proc. Natl. Acad. Sci. 93, 4638–4643
7. Paul, P., Kamisaka, Y., Marks, D. L., and Pagano, R. E. (1996) J. Biol. Chem. 271, 2287–2293
8. Meivar-Levy, I., and Futerman, A. H. (1999) J. Biol. Chem. 274, 4607–4612
9. Watanabe, R., Wu, K., Paul, P., Marks, D. L., Kobayashi, T., Pittelkow, M. R., and Pagano, R. E. (1998) J. Biol. Chem. 273, 9651–9655
10. Memon, R. A., Holleran, W. M., Uchida, Y., Moser, A. H., Ichikawa, S., Hirabayashi, Y., Grunfeld, C., and Feingold, R. R. (1999) J. Biol. Chem. 274, 19707–19713
11. Futerman, A. H., and Banker, G. A. (1996) Trends Neurosci. 19, 144–149
12. Boldin, S., and Futerman, A. H. (1997) J. Neurochem. 68, 882–885
13. Harel, R., and Futerman, A. H. (1995) J. Biol. Chem. 268, 14476–14481
14. Schwarz, A., Rapaport, E., Hirschberg, K., and Futerman, A. H. (1995) J. Biol. Chem. 270, 16990–16998
15. Schwarz, A., and Futerman, A. H. (1997) J. Neurosci. 17, 2929–2938
16. Hirschberg, K., Zisling, R., van Echten-Deckert, G., and Futerman, A. H. (1996) J. Biol. Chem. 271, 14876–14882
17. Goslin, K., Asmussen, H., and Banker, G. (1998) in Culturing Nerve Cells (Banker, G., and Goslin, K., eds) pp. 339–370, MIT Press, Cambridge, Massachusetts
18. Futerman, A. H., and Pagano, R. E. (1992) Methods Enzymol. 200, 437–446
19. Eligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
20. Bradford, M. (1976) Anal. Biochem. 72, 248–254
21. Futerman, A. H., Stieger, B., Hubbard, A. L., and Pagano, R. E. (1990) J. Biol. Chem. 265, 8650–8657
22. Ledesma, M. D., Brugger, B., Bunning, C., Wieland, F. T., and Dotti, C. G. (1999) EMBO J. 18, 1761–1771
23. Ledesma, M. D., Simons, K., and Dotti, C. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3966–3971
24. Bourteele, S., Haussler, A., Doppler, H., Hornmuller, J., Ropke, C., Schwarzmann, G., Pfitzenmaier, K., and Muller, G. (1998) J. Biol. Chem. 273, 31245–31251
25. Kolesnick, R. N., and Krome, M. (1998) Annu. Rev. Physiol. 60, 643–665
26. Komori, H., Ichikawa, S., Hirabayashi, Y., and Ito, M. (1999) J. Biol. Chem. 274, 8981–8987
27. Jaye, M., Schlessinger, J., and Dionne, C. A. (1992) Biochim. Biophys. Acta 1135, 185–199
28. Sano, M. (1992) J. Neurochem. 58, 837–844
29. Creuzet, C., Loeb, J., and Barbin, G. (1995) J. Neurochem. 64, 1541–1547
30. Wu, K., Marks, D. L., Watanabe, R., Paul, P., Rajan, N., and Pagano, R. E. (1999) Biochem. J. 341, 395–400
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