Transcriptional Control of Cholesterol Biosynthesis in Schwann Cells by Axonal Neuregulin 1*

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A characteristic feature of many vertebrate axons is their wrapping by a lamellar stack of glially derived membranes known as the myelin sheath. Myelin is a cholesterol-rich membrane that allows for rapid saltatory nerve impulse conduction. Axonal neuregulins instruct glial cells on when and how much myelin they should produce. However, how neuregulin regulates myelin sheath development and thickness is unknown. Here we show that neuregulin receptors are activated by drops in plasma membrane cholesterol, suggesting that they can sense sterol levels. In Schwann cells, neuregulin-1 increases the transcription of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme for cholesterol biosynthesis. Neuregulin activity is mediated by the phosphatidylinositol 3-kinase pathway and a cAMP-response element located on the reductase promoter. We propose that by activating neuregulin receptors, neurons exploit a cholesterol homeostatic mechanism forcing Schwann cells to produce new membranes for the myelin sheath. We also show that a strong phylogenetic correlation exists between myelination and cholesterol biosynthesis, and we propose that the absence of the sterol branch of the mevalonate pathway in invertebrates precluded the myelination of their nervous system.

Myelination heavily influenced the evolution and structure of vertebrate brains, augmenting the reliability and speed of signal propagation in nervous pathways. Glial cells (oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system) extend plasma membrane processes, wrapping axons with specialized membrane named myelin. It is well established that myelin thickness depends largely on axon size (1, 2). How axonal size signals the adequate myelin thickness produced by glial cells has remained largely unknown. However, it has been recently suggested that a product of the Nrg1 (neuregulin 1 gene) signals to myelinating cells about the axon diameter (2) regulating the myelin thickness. In addition, the artificial expression of this protein in unmyelinated axons converts them to a myelinated phenotype, suggesting that threshold levels of expressed neuregulin, and not strictly axon size, determines the myelination status of the neuron (3).

Myelin chemical composition differs greatly from other cellular membranes. Thus, high cellular levels of cholesterol are necessary for myelin membrane growth (4). In contrast to other tissues, the brain is unable to obtain cholesterol from circulating plasma lipoproteins and depends entirely on de novo cholesterol biosynthesis, mostly performed by glial cells (1, 4). The rate-limiting step in vertebrate cellular cholesterol production is the synthesis of mevalonate performed by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (5). Studies in vivo and in vitro have shown that HMGR is highly regulated at the transcriptional level (6). When cholesterol concentration drops in the endoplasmic reticulum (ER), the SREBP-2 transcription factor is released and binds to a sterol-response element (SRE) located on the HMGR promoter. This leads to increased transcription of the HMGR gene, stimulating the cholesterol biosynthesis and safeguarding the adequate cholesterol concentration within the cell (6). Other regulatory elements on this promoter have been described, suggesting that additional transcription factors regulate HMGR expression (7).

Most of the free cellular cholesterol is located within the plasma membrane (1) where levels are tightly regulated. Despite this, no cholesterol sensor has been proposed for this cell compartment. However, it has been shown that the epidermal growth factor receptor (also known as ErbB1), which resides in the plasma membrane, is phosphorylated after acute cholesterol depletion (8). In turn, ErbB1 phosphorylation causes hyperactivation of the PI3K and the mitogen-activated protein kinase (MAPK) pathways, showing that membrane cholesterol depletion can elicit intracellular signaling cascades (9) and suggesting that some ErbB receptors could be part of a mechanism for sensing plasma membrane cholesterol concentration.

* This work was supported in part by Spanish Ministry of Education and Science Grant SAF2004-01011 (to F. V.), “Instituto de Salud Carlos III” Grant PI05/0353, and “Conselleria de Salut de la Generalitat Valenciana” Grant AP-002/06 (to H. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Supported by predoctoral fellowships from the Spanish Ministry of Education and Science.

§ Supported by the postdoctoral program of the “Instituto de Salud Carlos III” from the Spanish Ministry of Health.

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5 The abbreviations used are: HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; CRE, cAMP-response element; CREB, CRE-binding protein; SRE, sterol-response element; PI3K, phosphatidylinositol-3-kinase; MAPK, mitogen-activated protein kinase; SMDF, sensory and motor-neuron derived factor; MJCD, methyl-β-cyclodextrin; GST, glutathione S-transferase; ER, endoplasmic reticulum; qPCR, quantitative PCR; TBP, TATA-binding protein; EGF, epidermal growth factor; SREBP, sterol regulatory element-binding protein.
Here we show that neuregulin receptors (ErbB2, ErbB3, and ErbB4) are transactivated by ErbB1 after acute drops in cholesterol, suggesting that they could also form part of a plasma membrane cholesterol-sensing mechanism. In addition, we show that the activation of the neuregulin 1-ErbB pathway in Schwann cells up-regulates the expression of HMGCR, the rate-limiting enzyme in cholesterol biosynthesis. We propose that neurons, by activating the NRG-ErbB pathway, simulate a drop of cholesterol to which Schwann cells respond augmenting cholesterol biosynthesis. Cholesterol up-regulation will help to increase plasma membrane size, which will wrap around the axon to form the myelin sheath.

**EXPERIMENTAL PROCEDURES**

**Constructs, Promoter Cloning, and Site-directed Mutagenesis**—The HMGCR promoter (nucleotides −309,+77) was amplified from mouse genomic DNA (using primers sense 5′-GGTACCTGAAGTGTCAGTGGTGAGAGAAGTATT and antisense 5′-CGGTGCTGCAGATGAGATCTGAG) and transformed in Escherichia coli DH5α. All constructs were verified by automatic sequencing.

**mRNA Detection and Quantification by Reverse Transcription-PCR and qPCR**—To detect and quantify gene expression, Schwann cell total RNA was isolated and retro-transcribed to cDNA with SuperScript II Reverse transcriptase (Invitrogen). Control reactions were performed by omitting retrotranscription—PCR and qPCR.

Control reactions were performed by omitting retrotranscriptase. First strand cDNA was PCR-amplified with specific primers for SREBP-2 (sense 5′-GGAA and antisense 5′-GCCGCCGCACTTCATCCAGGACC), SRE (sense 5′-GCCGGTCACCAGAAGGTTA), INSIG1 (sense 5′-CTTCTTCATTGGCG and antisense 5′-CGGTGCTTCGAGC), and antisse 5′-GCCGCCGTCAGGACC, and antisense 5′-GCCGCCGCTGCAG.”

**Cholesterol Depletion Assays**—To ensure comparable levels of the receptor per well, pcDNA3-ErbB transiently transfected COS-7 cells were trypsinized, reseeded, and grown to ~80% confluence in 24-well plates. Thereafter, cells were serum-starved for 5 h and incubated with recombinant SMDF for 3 min. Medium was removed, and cells were harvested with 100 μl of β-mercaptoethanol containing SDS sample buffer. Whole cell extracts were heated-denatured, separated by SDS-PAGE, and analyzed by immunoblotting with monoclonal anti-phosphotyrosine antibody from Sigma (1:1,000).

**Reporter Activity Assays**—Schwann cells, MCF-7, Oligo-neu, or COS-7 cells were growth in 60-mm culture dishes and trans-

**Real-time PCR Analysis** was performed using Platinum® Green qPCR Supermix UDG (Invitrogen) with 400 nM of gene-specific primers for rat HMGCR (sense 5′-CTTCATTTAGCAGGGGATGGTACA) and antisense 5′-TCCTCTATAGACCGCATGGTTACA. Reactions were performed in duplicate, and threshold cycle values were normalized to the housekeeping rat TBP mRNA (sense 5′-GAGGCGGAGAACGACTCAGCG and antisense 5′-AGGCTGCTGCCAA ACTTACG). The specificity of the products was determined by melting curve analysis and gel electrophoresis. The ratio of the relative expression of HMGCR to TBP was calculated by using the 2−ΔΔCT formula.

**Cell Culture and Transfection**—Cos-7 and MCF-7 were obtained from the ATCC and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The Oligo-neu cell line was kindly provided by Prof. J. Trotter (University of Heidelberg, Germany) and cultured in SATO medium (10). Schwann cells were cultured from sciatic nerves of neonatal rats as described previously by Brockes et al. (11). All the procedures were performed following European Union and institutional guidelines. Cells cultures were expanded in DMEM supplemented with 3% FBS, 5 μM forskolin, and 50 mM GST-NRG1 and used up to eighth passage except where indicated. Cells were transfected with plasmid DNA using Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s recommendations.

**Purification of Reconstituent Neuregulins and Tyrosine Phosphorylation Assay**—Cloning of pGEX-SMDF was already described elsewhere (12). cDNA encoding EGF-like domain of NRG1 was amplified by PCR and cloned into pGEX-4T-1. Bacterial cells were grown until reaching 0.6–0.8 OD. Thereafter, cells were induced with isopropyl 1-thio-β-d-galactopyranoside at 0.3 μM for 4 h and pelleted. The pellet was resuspended in phosphate-buffered saline, 5 μM dithiothreitol, and sonicated. Triton X-100 was added to reach 1% and centrifuged at 10,000 × g for 10 min. Protein was purified from the supernatant with GSH-agarose beads. After extensive washing, neuregulin was eluted from the beads with 10 mM GSH in 5 mM dithiothreitol, 50 mM Tris-HCl, pH 8.8. The concentration of protein was determined using the method of Bradford (13). Neuregulin-induced tyrosine phosphorylation of ErbB receptors was carried out as described by Ho et al. (14). Briefly, MCF-7 cells were growth in 60-mm culture dishes and trans-

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RESULTS

Neuregulin Receptors Are Activated by Transphosphorylation after Drops in Plasma Membrane Cholesterol—To test whether, similar to ErbB1 (8), other members of the ErB receptor family can also sense the plasma membrane cholesterol level, the cDNA encoding for ErbB2 was transfected into the fibroblast cell line COS-7. Plasma membrane cholesterol was acutely depleted with increasing concentrations of MβCD and receptor phosphorylation status monitored with an anti-phospho-ErbB2 (Tyr-1248)-specific antibody. As shown in Fig. 1a, ErbB2 was strongly phosphorylated in an MβCD dose-dependent manner, suggesting that cholesterol levels modulate the phosphorylation status of ErbB2. Additionally, we found that all the other members of this receptor family, ErbB3 and ErbB4, are also phosphorylated during cholesterol depletion (Fig. 1b).

Because COS-7 cells naturally express ErbB1, which heterodimerizes with other ErB receptors, the possibility exists that cholesterol depletion-induced ErbB1 activation transphosphorylates other ErBb partners. To test this hypothesis, cells were preincubated with 30 nM of the ErbB1-specific inhibitor tyrphostin AG1478 (ErbB1 IC50 = 3 nM) (15). As is shown in Fig. 1c, AG1478 completely inhibited the MβCD-induced phosphorylation of ErbB2, ErbB3, and ErbB2/ErbB3 in transfected COS-7 cells. Thus, our results indicate that ErbB1 can sense drops in plasma membrane cholesterol concentration and transactivate other ErBb receptors.

Activation of the Neuregulin-ErbB Pathway Stimulates HMGR Gene Transcription in Cell Lines of Different Lineages—It has been shown that the epidermal growth factor (a ligand for ErbB1) up-regulates the cholesterol biosynthetic pathway in adenocarcinoma cells (16). To further understand the role of ErbB signaling in cholesterol biosynthesis, we investigated the effects produced by activation of this pathway in different ways on the transcription of the HMGR gene. To this goal, we cloned the mouse HMGR promoter (nucleotides −309 to +77) into a luciferase reporter vector (pGL3-basic). The resulting construct (pHMGR-Luc) and the pcDNA3-ErbB2 and pcDNA-ErbB3 vectors were co-transfected into COS-7 cells, and the resulting luciferase activity was determined. As shown in Fig. 2a, the ErbB2-ErbB3 complex nearly doubled the transcriptional activity of the HMGR promoter (1.87 ± 0.09-fold (n = 6)). This result suggests that neuregulins, by activating the ErbB2-ErbB3 complex, are involved in the control of cholesterol biosynthesis in cultured mammalian cells.

To test this hypothesis further, we took advantage of MCF-7 cells, a human adenocarcinoma cell line that naturally expresses the neuregulin receptors ErbB2 and ErbB3 (17). MCF-7 cells transfected with pHMGR-Luc were incubated with recombinant sensory and motor-neuron derived factor (SMDf), a human type III neuregulin 1 highly expressed in the peripheral nervous system (14). As shown in Fig. 2b, recombinant SMDf (GST-SMDf) promotes the phosphorylation of the ErbB2-ErbB3 complex (inset) and induces the activity of the HMGR promoter in MCF-7 cells (1.9 ± 0.3-fold increase (n = 6)).

Neuregulin-ErbB signaling pathway plays an essential role in glial cell development and myelination (18). To unveil whether it also has a role in cholesterol biosynthesis in glial cells, we turned to Oligo-neu cells, a murine oligodendrocyte cell line immortalized by the expression of a constitutive active form of ErbB2 (10). As is shown in Fig. 2c, two ErBb inhibitors (AG1478 and AG825) decreased significantly the activity of the HMGR promoter in these cells, suggesting that neuregulin-ErbB pathway controls cholesterol biosynthesis also in glial cells. Taken together, our results show that activity of the neuregulin-ErbB pathway controls the HMGR gene expression at the transcri-
Neuregulin 1 Increases the Steady-state Levels for HMGR mRNA in Schwann Cells—It has been shown that the thickness of the myelin sheath in the peripheral nervous system is graded to the amount of neuregulin expressed on the surface of the neuronal axon (2, 3). Because myelin is a cholesterol-enriched specialized plasma membrane, and cholesterol is critical for myelin biogenesis (4), we tested whether the neuregulin-ErbB pathway controls the cholesterol biosynthesis in primary cultures of Schwann cells, the cell type responsible for myelinization in the peripheral nervous system. To this goal, Schwann cells were obtained from excised sciatic nerves of newborn rats and cultured (see “Experimental Procedures”). To normalize gene expression levels, a β-galactosidase reporter was used as control of transfection efficiency and cell viability. No major changes in β-galactosidase activity were found. Data are given as mean ± S.E. The number of experiments (n) is indicated.

Steady-state levels of mRNA depend on the balance between synthesis and degradation rates. To check whether the transcriptional effect of neuregulin is translated into an increase in the steady-state amount of the HMGR mRNA transcript. Thus, our results show that the transcriptional effect of neuregulin decreases in expanded Schwann cell cultures, probably as a consequence of the down-regulation of ErbB receptors.

Steady-state levels of mRNA in mammalian cell lines of different lineage, including glial cells.

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FIGURE 2. The neuregulin-ErbB pathway regulates the transcriptional activity of HMGR gene in different cell lines. a, ErbB pathway activation by overexpression of ErbB2, ErbB3, or a mixture of ErbB2 and ErbB3 receptors in COS-7 cells stimulates the transcriptional activity of the HMGR gene, measured as the relative luciferase activity of a construct containing the HMGR promoter. The activation of the HMGR promoter in cells cultured with LPDS was used as a positive control. b, recombinant SMDF (GST-SMDF) stimulates the HMGR promoter in MCF-7 adenocarcinoma cells. The activity of the recombinant neuregulin was previously evaluated by its capability to induce tyrosine phosphorylation of a 185-kDa band (inset). wb, Western blot. c, blocking ErbB signaling with AG1478 or AG825 decreased the activity of the HMGR promoter in the oligodendrocyte cell line Oligo-neu. Cells were transfected with pHMGR-Luc and the ErbB plasmid indicated or incubated with GST-SMDF increased the HMGR promoter activity by 2.21 ± 0.28-fold (n = 9) in Schwann cells, supporting the tenet that the activation-induced down-regulation of neuregulin receptors masked partially the transcriptional effects of neuregulin. To explore the role of neuregulin on HMGR transcriptional activity in a more physiological model, we repeated the experiments with nonexpanded rat Schwann cell cultures. The presence of fibroblasts in these cultures should not affect the conclusions because they do not express neuregulin receptors and cannot contribute to the transcriptional effects of neuregulin (data not shown).

Steady-state levels of mRNA in Schwann cultures in response to neuregulin 1 application. To avoid any genomic DNA amplification, specific primers for rat HMGR were designed in separated exons that include a large intron interposed between them (see “Experimental Procedures”). To normalize gene expression levels, a housekeeping gene (the rat TATA-binding protein (TBP)) was used. As is shown in Fig. 3c, GST-NRG1 increased up to 181 ± 5% (n = 4) the amount of the HMGR transcript. Thus, our results so far show that the axonal product neuregulin 1 stimulates the transcription of the HMGR gene in cultured Schwann cells and produces an almost 2-fold increase in the total amount of the HMGR mRNA transcript.
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**FIGURE 3.** The neuregulin-ErbB pathway regulates the transcriptional activity of HMGR gene in Schwann cells. **a**, GST-SMDF induces the transcription of HMGR gene in rat Schwann cell primary cultures. Incubation with the recombinant NRG1 EGF-like domain of neuregulin (GST-NRG1) produced a similar effect suggesting that the transcriptional activity of SMDF is mediated by direct ErbB binding and activation. Rat Schwann cell primary cultures were transfected with pHMGR-Luc, serum-starved, and incubated with the indicated amounts of recombinant GST-SMDF, GST-NRG1, or GST. After 48 h, relative luciferase activity was determined. The β-galactosidase activity of a co-transfected pCMV-LacZ reporter was used as control of transfection efficiency and cell viability. No major changes in β-galactosidase activity were found. **b**, nonexpanded primary cultures of rat Schwann cells showed increased responsiveness to GST-NRG1. Nonexpanded rat Schwann cell primary cultures were transfected with the constructs, serum-starved, and incubated with the indicated amounts of recombinant GST-NRG1 or GST. After 48 h, luciferase and β-galactosidase activities were determined. **c**, recombinant GST-NRG1 increases the steady-state levels of the HMGR mRNA in Schwann cells. Total RNA was extracted from nonexpanded cultures of rat Schwann cells and mRNA for HMGR quantified by real time qPCR. The level of mRNA was normalized to the amount of mRNA for a housekeeping gene (TBP). Data are given as mean ± S.E. The number of experiments (n) is indicated.

**Intracellular Pathways Involved in Neuregulin 1 Signaling**—The HMGR promoter contains at least two transcription factor-binding sites, shown schematically in Fig. 4a. The transcriptional control of HMGR in response to cholesterol depletion in hepatocytes and fibroblasts is mainly mediated by a sterol response element (SRE) located in the HMGR promoter (6, 20). Because Schwann cells do express the mRNA for the transcription factor SREBP-2 (Fig. 6b), we decided to explore whether SRE mediates the neuregulin-induced HMGR transcriptional activation in these cells. To this goal, we deleted the SRE of the pHMGR-Luc construct (Fig. 4a). Schwann cells were transfected with the wild type construct or the mutant, and the responsiveness to neuregulin SMDF was compared with the luciferase/β-galactosidase assay. As is shown in Fig. 4b, we were unable to detect any modification in the neuregulin-induced transcriptional activation after SRE disruption (1.94 ± 0.19-fold increase in the mutant versus 2.19 ± 0.19 in wild type), suggesting that the neuregulin effect is mediated by a different transcription factor-binding site in this promoter. It has been shown previously that thyrotrpin-mediated stimulation of the HMGR gene transcription in FRTL-5 rat thyroid cells is mediated by a CRE located downstream of the SRE (21) (see Fig. 4a). To test whether the CRE site mediates the transcriptional effect of neuregulin in Schwann cells, we introduced disruptive mutations in its core by inverse PCR. As is shown in Fig. 4b, mutations in CRE decreased (although not abrogated) the GST-SMDF-induced stimulation of the HMGR promoter (1.33 ± 0.15-fold (n = 6)), suggesting that neuregulin transcriptional control of HMGR is in part mediated by the cAMP-response element. To explore this tenet further, a CRE-luciferase construct (pCRE-Luc) was transfected into cultured rat Schwann cells, and the transcriptional effect of neuregulin was determined by the luciferase/β-galactosidase assay. As is shown in Fig. 4c, GST-SMDF was able to stimulate CRE promoter activity in Schwann cells by 2.37 ± 0.29-fold (n = 6). Even a larger increment (4.52 ± 0.22-fold (n = 3)) was obtained with GST-NRG1. Taken together our results suggest that the transcriptional effect of neuregulin 1 on the HMGR gene in Schwann cells is in part mediated by a CRE/ATF transcription factor. Indeed, when the cAMP-dependent pathway was stimulated with forskolin (Fig. 4d), the transcriptional effect of GST-NRG1 on HMGR gene doubled (6.34 ± 0.69-fold (n = 6)) versus 3.24 ± 0.44-fold (n = 6) in nonuntreated cultures.

Neuregulin activates two major signal transduction cascades in mammalian cells, the PI3K and the MAPK pathways (19, 21). To check which pathway mediates the transcriptional effects of neuregulin on the HMGR promoter, we took advantage of highly specific inhibitors. Interestingly, blocking the PI3K pathway with LY294002 fully abrogated the neuregulin transcriptional effect on the HMGR promoter. Moreover, PI3K inhibition down-regulates the basal activity of the promoter as well (Fig. 5a). In contrast, the selective inhibition of the MAPK pathway with PD98059 had no effect on the GST-NRG1 transcriptional effect. Furthermore, it produced a substantial increase in the basal activity of the promoter (3.6 ± 1.3-fold increase, n = 3). In summary, our results indicate that, in cultured Schwann cells, neuregulin favors the binding of a member of the CREB/ATF family of transcription factors to the HMGR promoter by activating the PI3K pathway. In support of this tenet, the inhibition of this pathway with PD98059 also abrogated the transcriptional stimulation of neuregulin on a pCRE-Luc construct transfected into Schwann cells (Fig. 5b).

Because our data so far suggested that neuregulin 1 increases the transcription of HMGR by stimulating the PI3K pathway in Schwann cells, we decided to check whether the activation of this pathway by additional mechanisms produces similar effects to that elicited by neuregulin 1. To this purpose, we transfected Schwann cells with a vector encoding myr-Akt, a constitutively active form of one of the major PI3K downstream effectors. As is shown in Fig. 5c, myr-Akt expression produced a notable increase in the transcriptional activity of the HMGR promoter in Schwann cells (14.36 ± 3.2-fold (n = 5)). Thus, our results support the view that activation of the PI3K pathway by extracellular signaling molecules stimulates cholesterol biosynthesis in Schwann cells. Interestingly, myr-Akt expression also produced a notable increase in the transcription of the CRE-luciferase construct (8.31 ± 0.8-fold (n = 7)), supporting the tenet that, in Schwann cells as in other cell types, CREB is a
downstream target of Akt (22–27). However, we also found that the deletion of the CRE in the HMGR reductase promoter did not reduce the myr-Akt-dependent transcriptional activity (Fig. 5d), suggesting that the sustained activation of Akt is able to induce the HMGR promoter at saturation level by a CRE-independent mechanism (28).

Cholesterol Depletion Does Not Activate the Transcription of the HMGR Gene in Schwann Cells—When animal cells are deprived of cholesterol, the SREBP-2 protein is transported from the ER to the Golgi by binding to the protein Sec24. Once in the Golgi, SREBP-2 is proteolytically processed, shedding its N-terminal domain, a transcription factor that binds to SREs and up-regulate genes for cholesterol synthesis. This feedback mechanism allows mammalian cells to survive when cholesterol supply is sparse (5, 6).

Cholesterol metabolism in the central nervous system differs to the rest of the body. Although most of the peripheral tissues can obtain cholesterol from plasma lipoproteins, brains synthesize all its cholesterol, most of the synthesis being performed by glial cells (1). To explore whether, akin to other tissues, cholesterol starving regulates the transcription of HMGR in glial cells, cultured Schwann cells were transfected with the pHMGR-Luc and pCMV-LacZ constructs. Thereafter cultures were incubated in a medium with abundant cholesterol (DMEM plus 10% of fetal bovine serum (FBS)) or without cholesterol (DMEM supplemented with 10% of lipoprotein-deficient serum (LPDS)). 48 h later cells were lysed, and luciferase and β-galactosidase activities were determined. No major changes in β-galactosidase activity were found. Data are given as mean ± S.E. The number of experiments (n) is indicated. *, p = 0.004. Student’s t test was used.

FIGURE 4. A CRE site in the HMGR promoter mediates the neuregulin transcriptional effects. a, diagrammatic view of the constructs used. The HMGR promoter was cloned in the pG3L basic vector (Promega) upstream of the luciferase gene. Two transcription factor-binding sites are highlighted as follows: the canonical binding site for SREBP, SRE (−163, −153); and CRE (−103, −95). SRE or CRE were mutated by inverse PCR (pHMGR-ΔSRE-Luc and pHMGR-ΔCRE-Luc). b, transcriptional activity of GST-SMDF on HMGR promoter decreases when the CRE site, but not when the SRE site, is mutated. c, both GST-SMDF and GST-NRG1 stimulate the transcription of a luciferase gene controlled by a CRE in Schwann cells. d, forskolin (2 μM) increases the transcriptional effect of neuregulin on the HMGR gene. Schwann cells were transfected with the indicated constructs and incubated with the correspondent treatments. 48 h later cells were lysed, and luciferase and β-galactosidase activities were determined. Data are given as mean ± S.E. The number of experiments (n) is indicated. *, p = 0.004. Student’s t test was used.
SREBP-2, SCAP, INSIG1 and -2, and site 1 and 2 proteases were designed, and mRNAs for the proteins were amplified by reverse transcription-PCR. As shown in Fig. 6b, all these mRNAs are expressed in Schwann cells. Therefore our results show that, although Schwann cells probably express SREBP-2 and the machinery involved in its processing, they do not adjust transcriptionally cholesterol biosynthesis to external availability. As a consequence its cholesterol homeostasis remains dependent on cues obtained from neurons and/or other cell types.

**Is There a Correlation between Cholesterol Biosynthesis Capability and Myelination?**—Myelination is a characteristic of most vertebrate nervous systems. The most ancient myelinated brains belong to cartilaginous fishes (sharks and rays). However, not all vertebrates myelinate; agnathes like hagfishes and lampreys lack myelinated axons. Although some invertebrates show axons covered by multilayered glial cell membranes, resembling myelin membranes, a “true” myelin sheet is not found in invertebrates (29).

To unveil evolutionary links between cholesterol biosynthesis capabilities and nervous system myelination, we searched for the genes coding for pivotal enzymes in sterol biosynthesis in the completely sequenced genomes of a diverse group of metazoans, including representative vertebrates and invertebrates. Cholesterol is synthesized from farnesyl pyrophosphate by using the sterol branch of the mevalonate pathway (30). This pathway is a ramified metabolic route that, starting with acetyl-CoA, leads to a great diversity of isoprenoid compounds in addition to cholesterol (Fig. 7a).

Thus, we searched in Ensembl data base for the genes codifying for HMGR (the rate-limiting enzyme in the mevalonate pathway) and for the enzymes of the sterol branch of the mevalonate pathway (FDFT1, SQLE, LSS, CYP51, TM7SF2, SC4MOL, EBP, SC5DL, DHCR24, and DHCR7). As is illustrated in Fig. 7b, the genes for the sterol branch of the mevalonate pathway were detected in all the vertebrates. Moreover, all of them (representatives of mammals, birds, and fishes) are able to form myelin in their nervous system. By contrast, the fully sequenced genome of *Ciona intestinalis*, an invertebrate chordate derived from a common ancestor of extant vertebrates, does not show several necessary genes for the cholesterol biosynthesis, including HMGR. Most interestingly, the sea squirt is also unable to form myelin (31). While searching the genomes of other invertebrate nonchordates, for which a complete genomic sequence is available, we found that all of them lacked several fundamental genes of the sterol branch of the mevalonate pathway, supporting the data obtained by other authors (32). The search included arthropods like *Drosophila melanogaster* and *Anopheles gambiae*, and the nematode *Caenorhabditis elegans*. Interestingly it is well known that these species do not form myelin sheaths as well (33–35).

Taken together, our genomic scan, although limited in species number, clearly indicates that there is a strong phylogenetic correlation between the cholesterol biosynthesis capability and the myelination of the nervous system.
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This is achieved by the activation of the PI3K/Akt pathway and is in part mediated by a CRE located within the HMGCR promoter. Although we did not address how the PI3K pathway regulates the CRE occupancy in the HMGCR promoter, it is interesting to point out that a link between PI3K and CRE activation has been reported previously in neural cells (22 and reviewed in Ref. 37). When PI3K is activated, the protein kinase Akt is translocated to the plasma membrane where it becomes active. Here we show that PI3K pathway activation by the expression of myr-Akt (a membrane attached constitutively active form of Akt) activates the HMGCR transcription in Schwann cells (Fig. 5c). It has been previously shown that CREB is a regulatory target for the protein kinase Akt (23–27). In this way we show that myr-Akt induces notably the activity of a CRE-luciferase reporter transfected to Schwann cells (Fig. 5c). Taken together, our data suggest that Akt-mediated CREB phosphorylation is involved in the transcriptional effects of neuregulin 1. In support of this view, it has been reported previously (38) that CREB phosphorylation also stimulates the transcription of the 3-hydroxy-3-methylglutaryl-CoA synthase, another key regulatory enzyme of the mevalonate pathway.

Surprisingly, CRE deletion did not decrease the activity of the HMGCR promoter in myr-Akt-transfected cells (Fig. 5d). These experiments suggest that the CRE is not the only mediator of the neuregulin effect on the HMGCR promoter. In fact, CRE deletion was also unable to abrogate completely the transcriptional activity of neuregulin 1 (Fig. 4b). It is possible that the sustained overstimulation of the PI3K pathway by myr-Akt activates to saturation the HMGCR promoter by a CRE-independent mechanism. In this way, it is interesting to point out that PI3K/Akt pathway activation in Chinese hamster ovary cells promotes as well the transcription of the HMGCR promoter by stimulating SREBP processing and ER-to-Golgi transport of SCAP (28). In summary, our data support the tenet that the activation of the PI3K pathway by extracellular signaling molecules up-regulates cholesterol biosynthesis in myelin-forming cells, both by CRE-dependent and CRE-independent mechanisms.

During our experiments we also noticed that the PI3K pathway controls the basal activity of the HMGCR promoter in Schwann cells (Fig. 5a) as well as in COS-7 cells (data not shown). It has been reported that PI3K activity regulates the activity of the HMGCR promoter by modulating the SREBP-2 biosynthesis in cancer cells (29). Although our deletion studies suggest that SRE does not mediate the transcriptional effects of neuregulins (Fig. 4a), SREBP-2 could be involved in controlling the basal activity of the HMGCR promoter.

In agreement with what has been suggested previously (39), our results clearly show that Schwann cells do not respond transcriptionally to changes in extracellular cholesterol availability. This is consistent with the observation that the nervous system synthesizes de novo all its cholesterol, because it can not obtain it from plasma lipoproteins (1). Furthermore, Schwann cell unresponsiveness to cholesterol depletion seems not to be the consequence of the lack of SREBP-2, INSIG1, INSIG2, site 1 protease or site 2 protease. Because it has been recently shown that the activator-recruited co-factor-mediator co-activator...
complexes are necessary for the transcriptional activity of SREBP proteins (40), we cannot exclude that Schwann cells fail to express some of these proteins, thus precluding SREBP transcriptional effects.

The lack of a negative transcriptional feedback mechanism for cholesterol synthesis in Schwann cells contrasts with other cell types and suggests that cholesterol biosynthesis depends completely on axonal signaling. Interestingly, Schwann cells do express ErbB2 and ErbB3 but not ErbB1 (41), suggesting that the only way to activate the ErbB pathway is by neuregulin-dependent activation of ErbB3/ErbB2 heterodimers. This lack of cell-autonomous cholesterol homeostasis mechanisms can be a recent evolutionary acquisition. It is tempting to speculate that glial cells of primitive nonmyelinating organisms were able to respond transcriptionally to extracellular cholesterol availability. Later, neurons, by producing neuregulins and activating ErbB receptors, exploited cholesterol homeostasis mecha-

**FIGURE 7. Critical enzymes of the sterol branch of the mevalonate pathway are not encoded in the genome of invertebrates.** a, mevalonate pathway produces several biologically relevant isoprenoids from acetyl-CoA, like cholesterol, steroid hormones, ubiquinone, dolichol, and insect juvenile hormones. Cholesterol is synthesized by the sterol branch of the mevalonate pathway (blue box). In red are marked the selected genes for the genomic scan (all the enzymes of the sterol branch of the mevalonate pathway and the HMG-CoA reductase). b, distribution of the genes encoding for cholesterol biosynthetic pathway in the genomes of several metazoans (only species with complete sequenced genomes were considered). The presence of the gene is marked with a +, absent in *C. intestinalis* but present in *Ciona savignyi*, c, present in *C. intestinalis* and absent in *C. savignyi*, d, homology to the fly genes *CG17952*, *CG11162*, and *CG1998* can be detected according to Ref. 22. Only the organisms with a complete cholesterol biosynthetic pathway (all vertebrates) form myelin in their nervous system. HMGCR/HMGCoAr, FDT1/squalene synthase, SQLE/squalene monooxygenase, LSS/lanoster synthase, CYP51/lanosteroidemethylase, TM7SF2/8(14)-sterol reductase, SC4MOL/C-4 methylsterol-oxidase, EBP/3β-hydroxysteroid-Δ(7), 8(7)-isomerase, SC5DL/stereol-5-desaturase, DHCR24/3β-hydroxycholesterol-reductase.
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FIGURE 8. A model for the axonal control of Schwann cell cholesterol biosynthesis and myelination. Membrane-attached axonal neuregulin binds and activates ErbB receptors exposed on the membrane surface of Schwann cells. ErbB activation is interpreted as a drop in cholesterol concentration which induces Schwann cells to up-regulate cholesterol biosynthesis by activating the PI3K pathway and a CREB/ATF transcription factor. Incremented cholesterol biosynthesis produces plasma membrane hyperplasia that wraps around the axon to form the myelin sheath. Question mark indicates a putative CRE-independent pathway involved in HMG-CoA reductase promoter activation by neuregulin.

nisms of glial cells. Now the response of Schwann cells to the NRG-ErbB pathway activation is to induce the expression of HMG, which allows the production of new plasma membrane that wraps the axon and forms the myelin sheath (Fig. 8). It is interesting to point out that the overexpression of HMG protein promotes the proliferation of stacked membranes (named karmellae in yeast and crystalloid ER in mammalian cells) with a striking structural similarity to the myelin sheath (42, 43).

Myelination allowed vertebrates to develop complex brains in relatively small volumes (29). Although glial cells from invertebrates like Drosophila do establish close contacts with neuronal axons, they do not form myelin sheaths (44). It is a well known fact that insects need to be fed cholesterol-containing diets to develop and survive (33). Recent genomic alignments have shown that Drosophila and Mosquito lack several key genes of the sterol branch of the mevalonate pathway (32), elegantly explaining their necessity to obtain cholesterol from the diet. Like insects, nematodes do not form myelin, and they are also unable to synthesize cholesterol (34).

Our search of the genes encoding for the enzymes of the sterol branch of the mevalonate pathway in the completely sequenced genomes of several vertebrates (Homo sapiens, Mus musculus, Gallus gallus, and Takifugu rubripes) and invertebrates (C. intestinalis, D. melanogaster, A. gambiae, and C. elegans) led to a clear-cut correlation between cholesterol biosynthesis and myelination in metazoans. Our data show that, similar to other invertebrates, several enzymes for the cholesterol biosynthesis are absent in the genome of the invertebrate chordate C. intestinalis. Although the genes for two myelin proteins (MAL and PMP22) have been detected in its genome, no myelin is formed in this organism. Probably these genes play a different biological role having been recruited for myelination during the posterior evolution of vertebrates (31).

De novo cholesterol biosynthesis has been experimentally demonstrated in some marine invertebrates (45), but they do not myelinate. Because there is no complete genomic data for these species, it is currently not possible to know whether cholesterol is produced through the sterol branch of the mevalonate pathway or by a different metabolic route.

Whether the sterol branch genes have never been present in the invertebrate genome or have been lost during evolution is unknown. However, given that invertebrates have neuregulin homologs (vein in Drosophila and LIN-3 in C. elegans) (35), it is tempting to speculate that the absence of cholesterol biosynthetic capability mediated by the mevalonate pathway during evolution prevented them from “inventing” myelination as a way to increase nerve conduction velocity and to develop a more complex nervous system.

Acknowledgments—We thank Professor Yossef Yarden for kindly supplying ErbB plasmids, Professor F. Mayor for the myr-Akt plasmid, and Professor J. Trotter for supplying Oligo-neu cells. We also thank Consuelo Martinez-Moratalla and Pedro Morenilla-Ayala for technical assistance.

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