Molecular Biology of the Cell
Vol. 19, 2101–2112, May 2008

Cholesterol Loss Enhances TrkB Signaling in Hippocampal Neurons Aging in Vitro

Mauricio G. Martin,*† Simona Perga,*‡ Laura Trovò,† Andrea Rasola,§ Pontus Holm,‖ Tomi Rantamäki,¶ Tibor Harkany,# Eero Castrén,¶ Federica Chiara,*§ and Carlos G. Dotti†

†VIB and Department of Human Genetics, Catholic University of Leuven, B-3000 Leuven, Belgium; ‡Cavalieri Ottolenghi Scientific Institute, Università degli Studi di Torino, 10043 Orbassano (Torino), Italy; §Department of Biomedical Sciences, Università degli Studi di Padova, 35122 Padova, Italy; #Laboratory of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, and ‖Department of Neuroscience, Karolinska Institute, S-171 77 Stockholm, Sweden; and ¶Sigrid Juselius Laboratory, Neuroscience Center University of Helsinki, FIN-00014 Helsinki, Finland

Submitted September 13, 2007; Revised February 6, 2008; Accepted February 11, 2008
Monitoring Editor: Robert Parton

Binding of the neurotrophin brain-derived neurotrophic factor (BDNF) to the TrkB receptor is a major survival mechanism during embryonic development. In the aged brain, however, BDNF levels are low, suggesting that if TrkB is to play a role in survival at this stage additional mechanisms must have developed. We here show that TrkB activity is most robust in the hippocampus of 21-d-old BDNF-knockout mice as well as in old, wild-type, and BDNF heterozygous animals. Moreover, robust TrkB activity is evident in old but not young hippocampal neurons differentiating in vitro in the absence of any exogenous neurotrophin and also in neurons from BDNF −/− embryos. Age-associated increase in TrkB activity correlated with a mild yet progressive loss of cholesterol. This, in turn, correlated with increased expression of the cholesterol catabolic enzyme cholesterol 24-hydroxylase. Direct cause–effect, cholesterol loss–high TrkB activity was demonstrated by pharmacological means and by manipulating the levels of cholesterol 24-hydroxylase. Because reduced levels of cholesterol and increased expression of choleseterol-24-hydroxylase were also observed in the hippocampus of aged mice, changes in cellular cholesterol content may be used to modulate receptor activity strength in vivo, autonomously or as a way to complement the natural decay of neurotrophin production.

INTRODUCTION

During development, neurotrophins are mandatory for the survival, differentiation, and growth of different neuronal populations (Reichardt, 2006). In the mature nervous system, neurotrophins are important for the modulation of neuronal connectivity and activity-dependent plasticity (Conover and Yancopoulos, 1997; Blum and Konnerth, 2005). Neurotrophins bind and activate receptor tyrosine kinases (RTKs), in turn leading to multiple intracellular signaling pathways, most notoriously those involving mitogen-activated protein kinases and phosphatidylinositol 3-kinase (PI3K) (Kaplan and Miller, 2000; Reichardt, 2006). In the hippocampus, a region of the brain critically involved in certain types of learning and memory, the most prominently expressed neurotrophin receptor is TrkB (Tokuyama *et al.,* 1998), whose cognate ligand is BDNF (brain-derived neurotrophic factor). In agreement with a role in memory-associated processes, loss-of-function studies of both TrkB and BDNF result in changes in affective and cognitive states in mammals and humans (Minichiello *et al.,* 1999; Pozzo-Miller *et al.,* 1999; Xu *et al.,* 2000; Egan *et al.,* 2003; Yeo *et al.,* 2004). Although there is no doubt that BDNF is the main modulator of TrkB activity, a number of evidences indicate that certain roles mediated by the activation of TrkB may occur independently from BDNF. For instance, TrkB conditional knockout mice present clear defects in pre- and postsynaptic morphogenesis in the hippocampus (Luikart *et al.,* 2005), yet this is not the case in BDNF conditional knockout mice (Gorski *et al.,* 2003; Hill *et al.,* 2005). The last observations are in turn consistent with the lack of an overt effect on the development of the hippocampus in BDNF knockout animals (Ernfors *et al.,* 1994; Jones *et al.,* 1994). Furthermore, the absolute need for BDNF for all and every TrkB-mediated activity in the adult hippocampus is challenged by reports showing that the levels and activity of BDNF in the hippocampus start to decay in early adulthood in rats, reaching lowest levels at the end of the first year of life (Gooney *et al.,* 2004; Shetty *et al.,* 2004; Hattiangady *et al.,* 2005; see however Katho-Semba *et al.,* 1998). One simple conclusion from these results is that mechanisms alternative or complementary to BDNF binding have evolved during cellular/neuronal differentiation, to guarantee TrkB activity in the adult brain. The best characterized of such mechanisms is the promiscuity of the lower affinity ligands NT3/NT4 to bind to TrkB (Yan *et al.,* 1993; Davies *et al.,* 1995). Paradigmatically, in vivo studies demonstrated that the lack of BDNF is not always compensated by NT4 and vice versa (Conover *et al.,* 1995;

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–09–0897) on February 20, 2008.

* These authors contributed equally to this work.

Address correspondence to: Federica Chiara (fchiara@bio.unipd.it) or Carlos G. Dotti (carlos.dotti@med.kuleuven.be).

Stenqvist *et al.*, 2005). Robust TrkB signaling in the mature brain can in principle also occur through the increased expression of TrkB subunits, and/or of its p75 coreceptor (Zaccaro *et al.*, 2001; Hartmann *et al.*, 2004; Marshak *et al.*, 2007). Contradicting this possibility, recent studies have shown the down-regulation of full-length TrkB in the hippocampus, starting 2 mo after birth (Silhol *et al.*, 2005). It also appears possible that TrkB signaling in the adult brain could occur via the up-regulation of ligands like adenosine or gangliosides, which have been shown to act as potent inducers of TrkB activity in cells in vitro (Lee and Chao, 2001; Duchemin *et al.*, 2002).

Irrespective of the possibilities of TrkB binding to a combination of classical or noncanonical ligands, one can also envision that cells have evolved non-ligand–mediated mechanisms to guarantee the most efficient transduction of this important signaling receptor. One such mechanism may be the modulation of the content/ratios of different membrane lipids, most notoriously cholesterol. Cholesterol plays a crucial role in the generation of ordered domains in the plasma membrane that laterally segregate certain proteins, thus reducing their rate of lateral diffusion and, by virtue of this, increasing clustering and consequently signaling strength (Edidin, 2003; Hancock and Parton, 2005; and Hancock, 2006). In fact, the number of studies in this regard is steadily increasing, also for RTKs (Paratcha and Ibanez, 2002; Suzuki *et al.*, 2004; Nicolau *et al.*, 2006; Hanzal-Bayer and Hancock, 2007; Jacobson *et al.*, 2007; Pereira and Chao, 2007). These observations prompted us to address the hypothesis that the activity of TrkB in the differentiated hippocampus may be controlled via a physiological modulation of cholesterol levels in the neuronal plasma membrane.

MATERIALS AND METHODS

Cell Culture and Reagents

Primary cultures of embryonic rat hippocampal neurons were prepared as described (Kaech and Banker, 2006). Hippocampal neurons from BDNF knockout mice were obtained from crossing BDNF +/− mice (Ernfors *et al.*, 1994). The two hippocampi from each 14–15-d embryo were put in separate tubes; a piece of cortex from each embryo, for later determination of genotype with BDNF specific primers, was also put in separate tubes. The hippocampi were then trypsinized and dissociated as for the rat hippocampal cultures, followed by plating in individual dishes. Each dish was later classified as BDNF +/+, +/−, or −/−, according to the PCR analysis of the corresponding cortical specimens. For biochemical analysis, 10^5 cells were plated into 3-cm plastic dishes coated with poly-L-lysine (0.1 mg/ml) and containing minimal essential medium with N2 supplements (MEM-N2). Neurons were kept under 5% CO_2 at 37°C. Where indicated, cells were incubated with particular inhibitors for 2 h to assess target inhibition or for 36 h to measure cell death. The monoclonal anti-Flotillin 1 antibody, clone 18, was from Transduction Laboratories (Franklin Lakes, NJ); the polyclonal chicken anti-BACE 1 antibody, raised against Fc-Asp 2-fusion protein, and the monoclonal anti-transferrin receptor antibody were from Zymed Laboratories (San Francisco, CA). Antibodies used to detect pTrk were polyclonal rabbit either from Santa Cruz Biotechnology (Santa Cruz, CA) or from Cell Signaling (Beverly, MA). Antibodies used to detect TrkB were polyclonal rabbit either from Santa Cruz Biotechnology or from BD Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-tubulin were all from Cell Signaling, and the rabbit antiserum anti-p85 was from Upstate Biotechnology (Lake Placid, NY). CYP46A1 antibody was provided by Dr. Russell (University of Texas Southwestern Medical Center, Dallas, TX) and described previously (Lund *et al.*, 1999).

Stimulation by Ligand and Neurotrophin Inhibition

Predifferentiated 10 days in vitro (10 DIV) hippocampal neurons were cultured in a serum-free medium and stimulated in independent experiments with 100 ng/ml BDNF for 30 min, 50 ng/ml nerve growth factor (NGF) for 10 min, or with a conditioned medium derived from 26 DIV neurons for 120 min. Neurotrophin activities were neutralized in 10 DIV cell culture medium by adding agonist antibodies raised against BDNF, NGF, NT3, NT4/5 (anti-neurotrophin antibody sample pack 1; Chemicon, Temecula, CA) at a concentration of 5 mg/ml for 120 h.

Plasma Membrane Purification

Mature rat hippocampal neurons were lysed by osmotic swelling in 25 mM MES Buffer, pH 7.0, 5 mM dithiothreitol, 2 mM EDTA and protease inhibitor cocktail (Roche, Indianapolis, IN), in ice for 15 min. Extracts were homogenized with a syringe (22 gauge) and centrifuged for 5 min at 700 × *g* and at 4°C. Supernatants were brought to a final concentration of 1.6 M sucrose, put on the bottom of an SW50 rotor centrifuge tube (Beckman Instruments, Fullerton, CA), overlaid with a continuous sucrose gradient from 1.6 to 0.4 M, and subsequently centrifuged at 12,0000 × *g* for at least 12 h at 4°C. Eight fractions were collected from the top to the bottom of the tube. The fractions that contain plasma membrane were subjected to centrifugation at 10,0000 × *g* for 18 h at 4°C. The fractions were finally analyzed by Western immunoblot using antibodies against specific markers of membrane compartments.

Separation of DRMs

Detergent-resistant membranes (DRMs) were prepared from hippocampal membranes (see above) by Triton X-100 extraction, and soluble and insoluble (DRMs) fractions were separated as described previously (Tansey *et al.*, 2000). For some experiments, total plasma membrane extracts were brought to 60% sucrose in MES Buffer Saline (MBS), and a sucrose step gradient was overlaid (35 and 5% sucrose). After centrifugation at 100,000 × *g* for 18 h at 4°C, fractions were collected from the top of each tube. Fractions 4 and 5 were identified as the DRM fractions by the presence of the DRM marker Flotillin 1.

Western Blotting, Immunoprecipitations, and Antibodies

Rat or mouse hippocampal tissues were homogenized in PBS containing 9% sucrose, protease inhibitors (CLAP: pepstatin, antipain, and chymostatin, each at a final concentration of 25 mM) and 1 mM sodium orthovanadate using a dounce homogenizer and 10 passages through a 22-gauge syringe. Samples were centrifuged for 10 min at 2500 × *g*, and supernatants were considered as total extracts. A further centrifugation step was performed at 10,000 × *g* for 1 h at 4°C to pellet the membrane fraction.

Total and membrane pellets of hippocampal neurons were extracted with two different detergents depending on the aim of the experiment: Nonidet P-40 (1% Nonidet P-40, 10% glycerol, 100 mM NaCl, 2 mM EDTA, 10 mM Tris/HCl, 500 mM sodium orthovanadate, and protease inhibitors) or Triton X-100 buffer (100 mM MES, pH 7, 150 mM NaCl, 1% Triton X-100) in the presence of 1 mM sodium orthovanadate and protease inhibitors. Extracts were clarified by centrifugation, and the protein concentrations were quantified by the BCA method (Bio-Rad Laboratories, Hercules, CA). Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies for 16 h. Species-specific peroxidase-conjugated secondary antibodies were subsequently used to perform enhanced chemiluminescence detection (Amersham, Little Chalfont, United Kingdom).

For immunoprecipitations, hippocampal neurons were extracted as described above. The soluble and insoluble fractions were diluted with the appropriate buffer to equalize detergent amount (1% Triton and 0.1% SDS). For TrkB and p85 immunoprecipitations, 1 μg of polyclonal antibody/sample was added to protein A–Sepharose beads, and samples were rotated at 4°C overnight. Beads were washed with cold lysis buffer. Immunoprecipitated complexes were separated by 10% PAGE-SDS electrophoresis and subjected to Western blot analysis. Quantification was done by densitometry of the autoradiograms using the NIH Image J software package (http://rsb.info.nih.gov/ij/).

Lipid Extraction and Thin-Layer Chromatography

Lipids were extracted from plasma membranes obtained from rat hippocampal tissues or cultured neurons according to Bligh and Dyer (1959). Extracted lipids (cholesterol, ceramide, and sphingomyelin) were subsequently analyzed by thin-layer chromatography (TLC) on silica gel 60 HPTLC plates using a double-step system (hydrophilic running solvent: chloroform/acetone/acetic acid/methanol/water 50:20:10:10:5) and hydrophobic solvent: hexane/ethyl acetate (5:2) for cholesterol and ceramide. The plate was then dried and developed by spraying with 7% sulfuric acid and heating at 150°C in an oven.

Membrane Cholesterol Quantification, Reduction, and Replenishment

Total cholesterol was measured in membrane extracts from hippocampal neurons in vitro or whole hippocampus as previously described (Abad-Rodriguez *et al.*, 2004). For the induction of membrane cholesterol reduction, 0.4 μM mevilonin and 0.5 mM β-methyl-cyclo-dextrin were added daily to 5-d-old neurons during 4 d (96 h). At the end cholesterol was measured, to ascertain that the treatment did not result in more than 25% reduction. Only validated cases membranes were utilized for further experimentation. Cells were scraped in MES Buffer-CLAP at 4°C, and centrifuged at 1000 × *g*. Postnuclear supernatants were further centrifuged at 10,0000 × *g* for 1 h at 4°C to get the membrane pellet. Protein and cholesterol concentrations were measured after resuspension in MES Buffer-CLAP with 0.1% Triton X-100.

Cholesterol-MCD inclusion complexes were prepared as described (Klein *et al.*, 1995). These complexes, containing 0.3 mM cholesterol, were added to the

medium at a final concentration of 0.3 mM together with 2 mg/ml free cholesterol. The treatment was performed for 15 min, and then cells were washed and scraped with MES Buffer-CLAP 0.1% Triton X-100 at 4°C

Analysis of Gene Expression

Total RNA samples were prepared from 5×10^5 hippocampal neurons at 10, 15, 20, and 25 DIV using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). One hundred nanograms of total RNA were first incubated with poly-T oligonucleotides and subjected to reverse transcription using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. cDNA was used as a template for the real-time PCR analysis based on the SYBR Green PCR Master Mix assay with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Real-time PCR primers were designed using primer-Express software (Applied Biosystems). Expression of genes encoding TrkA, TrkB, and BDNF and of genes involved in cholesterol synthesis and metabolism was analyzed, and transcriptional expression was normalized using the housekeeping gene encoding for the β Actin (*actb*) as reference in order to avoid differences due to possible RNA degradation or different reverse transcription efficacy. Relative expression levels of the targets were calculated with the comparative cycle threshold (C_T) method according to the following formula: sample ΔC_T = (gene C_T − *actb*C_T). To follow expression during ageing, results were normalized to a calibration standard sample of RNA (10 DIV sample). This control was assigned the normalization ratio = 1 and the normalization ratio of each sample was calculated according to the formula: $2^{-\Delta\Delta CT} = 2^{-(\text{sample } \Delta CT \,-\, \text{calibrator } \Delta CT)}$.

Immunofluorescence Microscopy

For the light microscopy detection of Cyp46, neurons on glass coverslips were washed in PBS (0.01M), fixed with 4% paraformaldehyde, and processed for immunofluorescence by using anti-CYP46. For the analysis of phospho-TrkB on the surface of young and old cells, cells were first incubated with the corresponding antibody (see above) at 15°C for 15 min to minimize loss by internalization, and excess antibody washed away and then fixed and processed for immunofluorescence microscopy. The surface distribution of the ganglioside GT1b was detected with anti-GT1b mAb (Seikagaku, Tokyo, Japan). Samples were analyzed on an Olympus IX81 fluorescence microscope (Melville, NY). Quantification was performed using the NIH ImageJ software, and signal intensities were normalized by area.

Plasmid Construction

The *cyp46A1* targeting sequence was designed by using the RNA interference design algorithm at www.dharmacon.com/DesignCenter/DesignCenterPage.aspx, and 46-sense and 46-antisense oligos encoding *cyp46A1*-short hairpin RNA (shRNA) were designed as reported by Rubinson *et al.* (2007). To generate pSi46 plasmid, these oligonucleotides were annealed and cloned into the HpaI and XhoI sites from pLentiLox 3.7 (Rubinson *et al.*, 2007): 46-sense oligo: 5′-TAGATGTACCGTGCGATTCATTCAAGAGATGAATCGCACGGTACATCTTTTTTTC-3′; 46-antisense oligo: 5′-TCGAGAAAAAAAGATGTACCGTGCGATTCATCTCTTGAATGAATCGCACGGTACATCTA-3′.

The full ORF expression clone p46+ including the full *cyp46A1* mouse open reading frame was purchased from RZPD (German Resource Center for Genome Research).

Transfection

Primary dissociated hippocampal cells isolated from rat embryos (on embryonic day 18) were transfected using the Rat Neuron Nucleofector Kit from Amaxa Biosystems (Köln, Germany).

RESULTS

Robust TrkB Activity in the Hippocampus of Old Mice Is Not Reliant on High BDNF Expression

Consistent with previous work showing a decay in BDNF levels in the mature and aged brain (Hattiangady *et al.*, 2005; Silhol *et al.*, 2005), the mRNA levels were lower in 10- and 21-mo-old mouse hippocampi as compared with those in 1-mo-old mice (Supplementary Figure 1A). To test whether decreased BDNF levels during aging were paralleled by a decreased activity of TrkB (the main Trk receptor in the hippocampus; Merlio *et al.*, 1992; Cellerino, 1996), phosphorylated TrkB levels were measured in hippocampal membranes of mice as they grew old. Quite paradoxically, Western blotting revealed weak yet noticeable levels of phosphorylated TrkB in the hippocampi of 21-d-old mice and much higher in 10- and 20-month-old animals (Figure 1A). In support of the levels of receptor phosphorylation leading to activation of downstream survival effectors, a similar curve was observed for the downstream target Akt (Figure 1B). To further test that hippocampal cells can potently activate the TrkB pathway in low-BDNF situations, we performed Western blot analysis of hippocampal membranes from BDNF heterozygous (+/−) and knockout (−/−) mice, which have, respectively, half or no BDNF compared with wild-type (see Ernfors *et al.*, 1994; Agerman *et al.*, 2003). Supporting the above prediction, the membranes of 21-d-old BDNF −/− mice and the membranes from 10-mo-old BDNF +/− and +/+ mice all presented a highly phosphorylated TrkB signal (Figure 1, C and D).

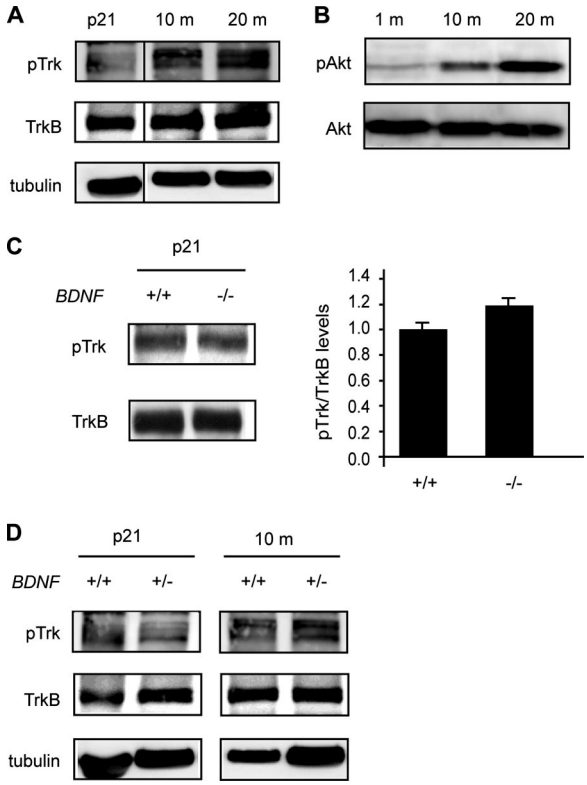

Figure 1. TrkB signaling robustness in vivo is independent of the levels of BDNF. (A) Western blot of hippocampal membranes from postnatal day 21 (P21), postnatal month 10 (10 m) and postnatal month 20 (20 m), with antibodies against active (pTrk) and total (TrkB) Trk receptors. The active form of the receptor increases dramatically with age, reaching highest levels in 20-mo-old mice. (B) A similar curve was observed for the downstream target Akt, suggesting that the levels of receptor phosphorylation lead to activation of survival effectors. (C) Western blot of hippocampal membranes from 21-d-old BDNF knockout (BDNF −/−) and wild-type animals (BDNF +/+) animals, with antibodies against active (pTrk) and total (TrkB) receptor. Note that the absence of ligand (BDNF −/−) does not affect the levels of receptor expression nor activity (compare with BDNF +/+). The relative amount of pTrk/TrkB present in KO mice respect to the wild-type case is shown on the right. (D) Western blot of hippocampal membranes from post natal day 21 (P21) and postnatal month 10 (10 m) wild-type (BDNF +/+) and BDNF heterozygous mice (+/−). Levels of the active receptor (pTrk) increase with age, in both types of animals.

Robust TrkB Activity in Aging Hippocampal Neurons In Vitro in the Absence of Exogenous BDNF

Numerous studies have shown that embryonic hippocampal neurons from wild-type rodents, mouse or rat, acquire in

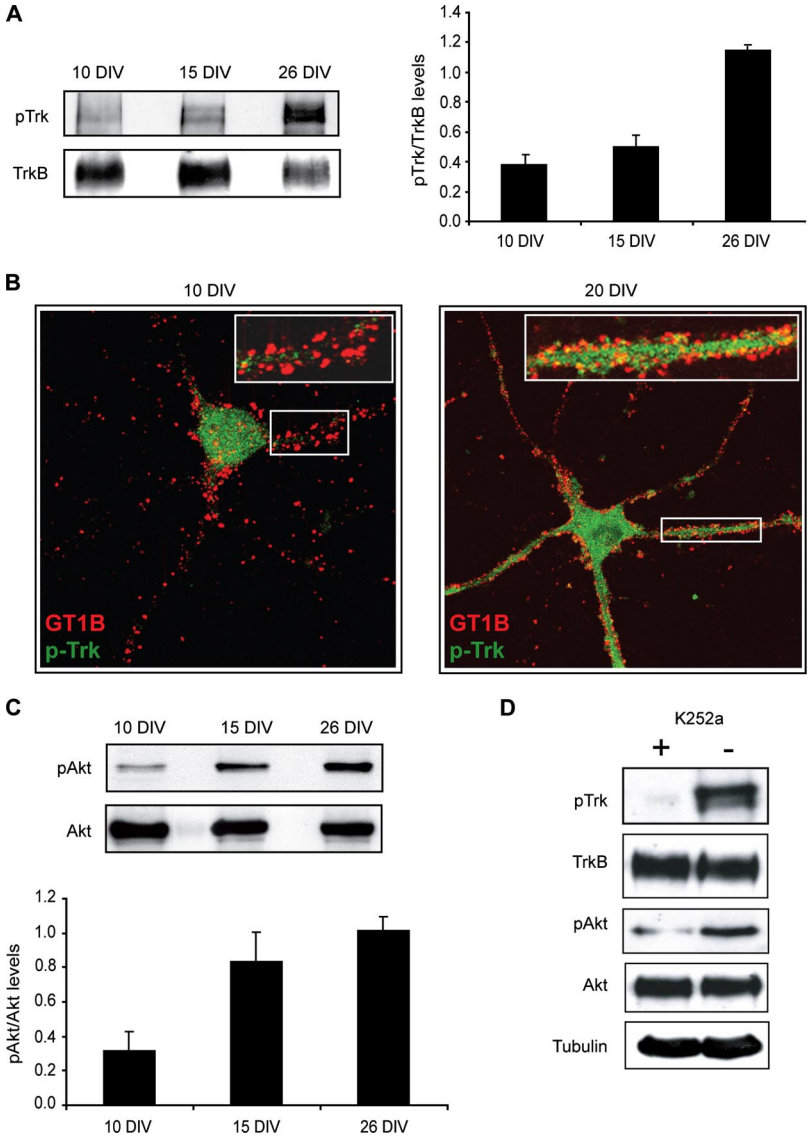

Figure 2. TrkB activity increases during in hippocampal neurons in vitro. (A) Immunoblotting (IB) of membranes from hippocampal neurons maintained for 10, 15, and 26 d in vitro (DIV). Increased receptor activity (pTrk) occurs with days in vitro, despite equal levels of receptor expression. Protein loading was assessed with anti-TrkB (TrkB) antibody. Quantification of blots from different experiments (n = 5) shows the increase of the pTrk/TrkB levels in aged cells. (B) Immunofluorescence microscopy analysis of phospho-TrkB of the surface of young and old cells. Distribution revealed more and more peripherally distributed structures in old neurons as compared with young neurons. This analysis reveals a high degree of colocalization between active Trk (green) and the ganglioside GT1b (red) in the aged neurons (insets). (C) Immunoblot analysis of Akt activity in cellular extracts from 10, 15, and 26 DIV neurons. Akt activity is low in 10 DIV neurons in comparison with 15 and 26 DIV neurons. This age-dependent increase in activity is not due to differences in the amount Akt protein expression. Quantitative analysis is shown on the right. (D) Effect of addition of the Trk inhibitor K252a on Trk phosphorylation and Akt activity. Addition of the inhibitor to 26-d-old cells (+, treated; −, untreated) result in the great reduction of Trk and Akt phosphorylation after 24-h treatment. These results suggest that the high levels of Trk activity found in old cells promote survival.

vitro many of the complex aspects of neuronal architecture and function of their in vivo counterparts. Importantly, these features are acquired without the need of supplementation by BDNF, or any other neurotrophins, to the culture medium (Kaech and Banker, 2006). Furthermore, survival and acquisition of mature molecular and functional phenotype does not seem to require endogenous ligand. In agreement, we observed that the levels of BDNF in the culture medium of these neurons, as determined by ELISA, ranged between 0.07 and 4.38 pM. Because the highest of such values is 30-fold lower than the concentrations required to elicit TrkB phosphorylation by BDNF in these same cells (see Suzuki *et al.*, 2004), we have considered these cells as a most suitable system to study the possible mechanisms that may trigger TrkB activity independently from high levels of BDNF, as just was demonstrated to be the case in situ. Furthermore, this system is also most suitable as TrkB is the main Trk isoform in this culture (Supplementary Figure 1B).

To determine if hippocampal aging in vitro is accompanied by increased TrkB phosphorylation, as in the in vivo situation (see Figure 1A), the levels of phosphorylated TrkB were measured in membranes from hippocampal neurons maintained in culture for 10, 15, and 26 DIV, representative of immediate terminally differentiated, fully mature, and aging neurons, respectively. This study revealed weak levels of TrkB phosphorylation in 10 DIV neurons, 10% higher in 15 DIV neurons, and a further and a most significant increase in aged 26 DIV neurons (Figure 2A). In agreement with the biochemical data, immunofluorescence microscopy analysis of phospho-TrkB distribution revealed more and more peripherally distributed structures in aged (20 DIV) than in 10 DIV neurons (Figure 2B). Because 20 DIV neurons also presented fully active TrkB at the biochemical level, identical to 26 DIV neurons, 20 or 26 DIV were indistinctly taken as representative of the in vitro aged situation.

In support that higher levels of TrkB phosphorylation during aging were transduced downstream, Akt presented an activity curve similar to that of TrkB: low levels at 10 DIV, evident at 15 DIV, and highest by 26 DIV (Figure 2C), and the incubation of 15 DIV neurons with the kinase inhibitor K252a (Lee *et al.*, 2002) blocked the occurrence of Akt phosphorylation (Figure 2D).

To further strengthen the notion that hippocampal neurons in vitro is a good system to study the possible mecha-

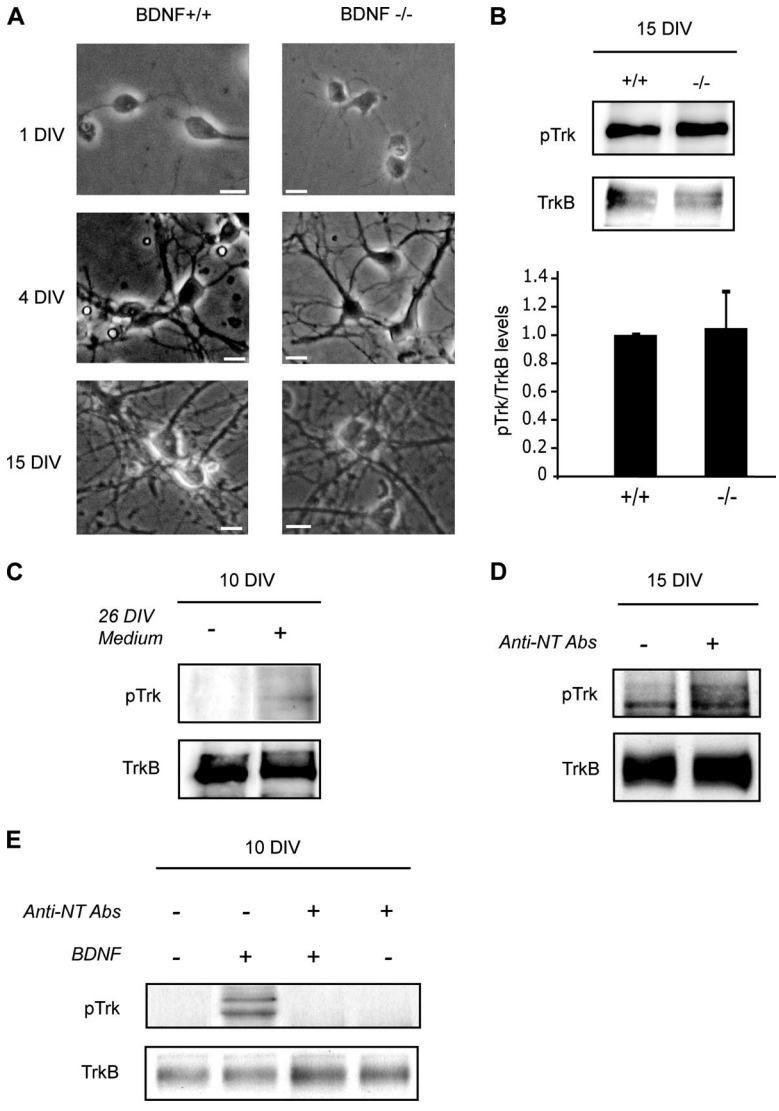

Figure 3. TrkB activity in fully differentiated hippocampal neurons in vitro is ligand-independent. (A) Phase-contrast images of hippocampal neurons from BDNF +/+ and BDNF −/− embryos, maintained in culture for 1, 4, and 15 DIV: cell number and degree of morphological differentiation is similar. (B) Immunoblotting of membranes from hippocampal and cortical neurons, from BDNF −/− and BDNF +/+ embryos, maintained for 15 DIV: the intensity of phosphorylated TrkB signal in the −/− cells is comparable to that of +/+ cells. Protein loading was assessed with anti-TrkB (TrkB) antibody, and the relative amount of pTrk/TrkB present in −/− cells with respect to the wild-type case is shown below. (C) Neurons from wild-type rat embryos maintained in vitro for 10 d (10 DIV) were treated for 30 min with the medium of neurons maintained in vitro for 26 d (26 DIV medium): this treatment does not elicit any TrkB activation in these cells, despite abundant levels of receptor. (D) Western blot analysis of TrkB activation of 15 DIV neurons incubated or not with a cocktail of neurotrophin-blocking antibodies (Anti-NT Abs), starting on day 10 in vitro (see *Materials and Methods*). Note that the antibody mix did not perturb the increase in TrkB signal, whether for total Trk (TrkB) or its phosphorylated form (pTrk). (E) Control of antibody-mix efficacy as neurotrophin-blocking TrkB activation. Neurons kept in vitro for 10 DIV neurons were incubated with 100 mg/ml BDNF; effect on TrkB activity (pTrkB) was measured 30 min later. Treatment triggers intense TrkB activity (pTrkB; BDNF +) in nonneurotrophin antibody-mix–incubated neurons (Anti-NT Abs, −); TrkB activity is absent in cells coincubated with the antibody mix (BDNF +/Anti-NT Abs, +).

nisms behind robust TrkB activity in low neurotrophin conditions, the activity of the receptor was next assessed in hippocampal neurons in vitro from BDNF knockout embryos (obtained by crossing BDNF +/− mice; see *Materials and Methods*), grown in the absence of any exogenous neurotrophin. In these cells, rate of hippocampal neuron survival and the degree of morphological differentiation was, as observed in vivo (Ernfors *et al.*, 1994), undistinguishable from wild-type neurons (Figure 3A). More relevant for the purpose of this work, 15 DIV neurons from BDNF −/− embryos presented a phosphorylated TrkB signal, not different from that of neurons from wild-type littermates at the same age in vitro (Figure 3B). This last result conclusively demonstrates that hippocampal neurons have mechanisms to elicit TrkB activity independently from the presence of the receptor natural ligand. To test whether or not such activity could be attributed to excess production/release of low-affinity ligands, we shifted back to the rat embryo hippocampal neurons from wild-type animals, which present robust TrkB activity in the absence of exogenous neurotrophins (see Figure 2A). For this purpose, the culture medium of 26 DIV neurons, in which low-affinity ligands would be highest if responsible for the presence of high receptor phosphorylation at this stage, was added to 10 DIV cells, that present only weak TrkB activity (see Figure 2 A). This treatment did not induce TrkB activity in 10 DIV cells (Figure 3C), suggesting that activation in the old neurons was not due to increased production of NT3/NT4 or adenosine (see *Introduction*). In further agreement, incubation of 15 DIV neurons with a cocktail of neurotrophin function-blocking antibodies neither prevented nor reduced the spontaneous appearance of TrkB activity in these neurons (Figure 3D). On the other hand, the antibody cocktail was efficient to inhibit the activity induced by supraphysiological amounts of BDNF (Figure 3E), ruling out that their inefficacy to block spontaneous activation in old neurons (Figure 3D) was due to lack of inhibitory activity or the capacity to reach the sites of receptor location on the neuronal surface (synaptic cleft). Surely, these data do not exclude that activation of TrkB during aging may be due to binding to as yet unidentified or nonsoluble ligands, which might have become up-regulated with aging. On the other hand, these data, together with the previous in situ results, clearly demonstrate that differentiated hippocampal neurons posses mechanisms that guarantee TrkB activity even under conditions of minimal availability of canonical neurotrophins.

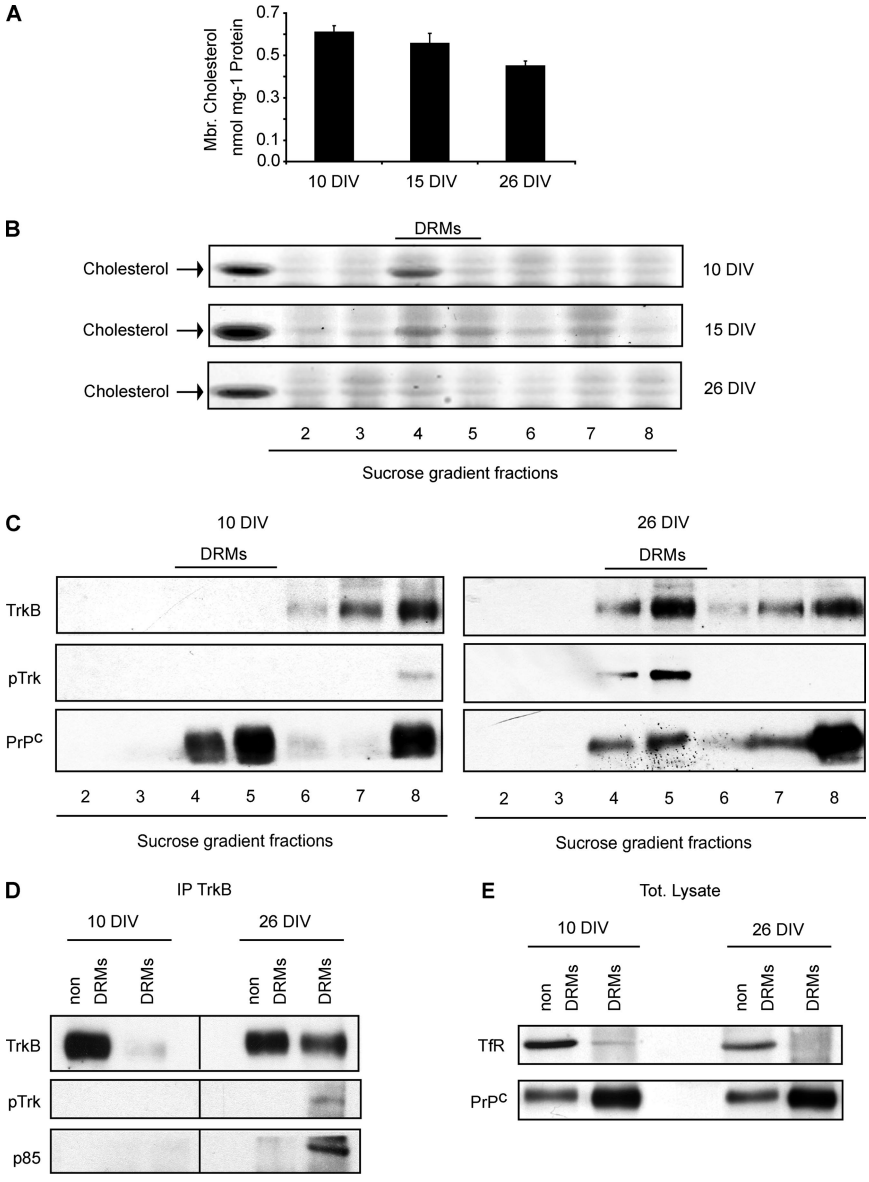

Figure 4. Moderate still continuous loss of cholesterol from the plasma membrane of differentiated hippocampal neurons in vitro determines TrkB DRM partitioning. (A) Colorimetric measurement of plasma membrane cholesterol from hippocampal neurons kept for 10, 15, and 26 DIV. Mean ± SD values (nmol/mg protein, n = 4) were 0.61 ± 0.029, 0.55 ± 0.047, and 0.43 ± 0.023 in 10, 15, and 26 DIV neurons, respectively. (B) TLC analysis of the membrane cholesterol content in DRMs of 10, 15, and 26 DIV neurons; cholesterol is clearly and progressively reduced in DRMs from 15 and 26 DIV neurons. Percentages of cholesterol reduction in DRMs (fractions 4 and 5) and non-DRMs (fractions 6–8) were measured by densitometry and normalized for sphingomyelin values of different sucrose fractions (n = 2). In DRMs, cholesterol was decreased to 43.29% in 15 DIV cells, and to 25.92% in 26 DIV cells. The migration of the cholesterol standard is indicated (cholesterol). (C) Western blotting with anti-total Trk, anti-phospho Trk, and anti-prion protein (PrP^c) antibodies, of 4°C detergent-extracted, sucrose-gradient centrifuged membranes from 10 and 26 DIV hippocampal neurons in vitro. Note that receptor activity is exclusively present in the DRM domains (fractions 4 and 5) of 26 DIV neurons. The PrP^c lane demonstrates that the presence in fractions 4 and 5 truly reflect DRM partitioning. (D) Immunoprecipitations of TrkB from the DRMs and non-DRMs of 10 and 26 DIV neurons, probed with antibodies against phospho Trk and the anti-p85 subunit of PI3K. Note that the downstream survival effector of TrkB is precipitated exclusively from the DRM domains of 26 DIV neurons. (E) Control for correct separation between DRMs and non-DRMs fractions. Lysates utilized in C were hybridized with antibodies against the non-DRM protein Transferrin receptor (IB: TfR) or the DRM protein PrP^c (IB: PrP^c).

Cholesterol Levels Decay throughout Neuronal Differentiation in Vitro

Plasma membrane cholesterol levels play a critical role in receptor signaling strength (see *Introduction*). Hence, we found it most natural to test the possibility that high TrkB activity in aging neurons could be due to the occurrence of changes in the content of this lipid. Therefore, we measured cholesterol levels in the membranes of 10, 15, and 26 DIV hippocampal neurons. Colorimetric measurement revealed that 10 DIV neurons have 10 and 25% more cholesterol than 15 and 26 DIV neurons, respectively (Figure 4A). Together with the data of Figure 2, these last results indicate the existence of an inverse cholesterol level–TrkB activity correlation (see Figure 2).

The biochemical isolation of DRMs is a useful tool to confirm the existence of changes in the organization of cellular membranes (Brown and London, 1997). Therefore we speculated that the decrease in cholesterol content with ageing in vitro would be reflected in the content of this lipid in DRMs prepared from hippocampal neurons cultured for 10, 15, and 26 d (see *Materials and Methods*). TLC of equal protein loads confirmed the clear reduction in cholesterol content in15 and 26 DIV neurons, compared with 10 DIV neurons (Figure 4B). Because changes in cholesterol levels affect the degree of activation of numerous proteins, including Trks, and this is reflected in changes in the way these proteins associate with DRMs (Paratcha and Ibanez, 2002; Hancock, 2006; Jacobson *et al.*, 2007), we next determined the degree of TrkB phosphorylation in DRMs throughout aging. This revealed TrkB activity confined to DRMs at 26 DIV. In contrast, TrkB activity was undetectable in the DRMs of 10 DIV neurons (Figure 4C, p-Trk). To test if the fractions containing active receptors corresponded to DRMs (despite their lower cholesterol levels), the same membranes were blotted with an antibody against the glycosylphosphatidylinositol-anchored PrPc. That this is the case is shown in Figure 4C (PrP^c). In further support that TrkB is present in DRMs of old neurons in vitro and that this activity may be relevant to neuronal survival, the p85 subunit of PI3K, a downstream target of TrkB activity, was precipitated exclusively from the

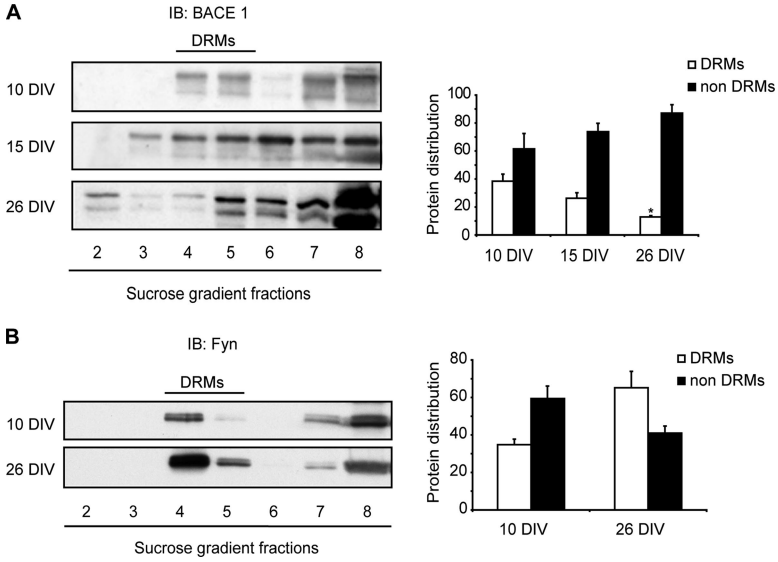

Figure 5. (A) Sucrose gradient membrane fractions from cold detergent-solubilized plasma membranes from 10, 15, and 26 DIV hippocampal neurons followed by immunodetection for the protein BACE 1. Note that BACE1 segregation to DRMs progressively decreases with time. Percentages of BACE1 distribution in DRMs (fractions 4 and 5, □) and nonDRMs (fractions 6–8, ■) were measured by densitometry and reported in the graphics to the right of the Western lot (mean ± SD, n = 4). In exact numbers, BACE 1 was 35.3 ± 5.8% in the DRMs of 10 DIV neurons, decreasing to 25.3 ± 4.1% in 15 DIV neurons and further decreasing to 15.8 ± 6.4% in senescent cells. (B) Sucrose gradient fractions of cold detergent-solubilized plasma membranes from 10 and 26 DIV neurons followed by immunodetection for the protein Fyn. Percentages of Fyn distribution in DRMs (fractions 4 and 5, □) and non-DRMs (fractions 6–8, ■) were measured by densitometry and reported in the graphics on the right (mean ± SD, n = 4). In 10 DIV neurons 34.75 ± 2.8% of Fyn is in DRMs, increasing to 58.9 ± 8.6% in 26 DIV neurons.

DRMs of differentiated neurons only in complex with the active receptor (Figure 4D). On the other hand, the transmembrane protein BACE1, which was shown is associated with DRMs of neurons in a cholesterol-dependent manner (Ledesma *et al.*, 2003; Abad-Rodriguez *et al.*, 2004) became more concentrated in detergent-soluble membranes of the aging neurons (Figure 5A). In good agreement with the presence of TrkB in DRMs of cells with low cholesterol, the TrkB DRM-localizing enzyme Fyn (Pereira and Chao, 2007) was also found considerably enriched in DRMs of aging neurons, compared with younger cells (Figure 5B).

Mild Membrane Cholesterol Reduction Increases TrkB Activity in Young Neurons; Cholesterol Replenishment in Old Neurons Decreases Activity

Increased activity due to cholesterol loss was observed for Fyn (Ko *et al.*, 2005), epidermal growth factor receptor (Oh *et al.*, 2007), and TGF-β (Chen *et al.*, 2007). To test if this is also the case for the increased activity of TrkB in aging neurons, the levels of cholesterol were lowered in 10 DIV neurons, until reaching those of aging neurons (25% reduction, see Figure 4A). This was accomplished by the addition of a cholesterol synthesis inhibitor (mevilonin) and a cholesterol removal drug (β-methyl-cyclodextrin; Ledesma *et al.*, 2003; Abad-Rodriguez *et al.*, 2004; see *Materials and Methods*). Consistent with the existence of a direct causal relationship, treated 10 DIV neurons presented strong TrkB phosphorylation levels, far above those of nontreated neurons of similar age (Figure 6A). This effect occurred in the DRMs of these neurons, similarly to what happens in untreated aging neurons (see Figure 4D). In agreement with the effect on TrkB activity being due to the true reduction of cholesterol levels, the association of Flotilin1 with DRMs was decreased in the cholesterol-deprived 10 DIV neurons (Figure 6B). To further control that the effects on TrkB were due to the loss of cholesterol and not to a β-methyl-cyclodextrin–mediated

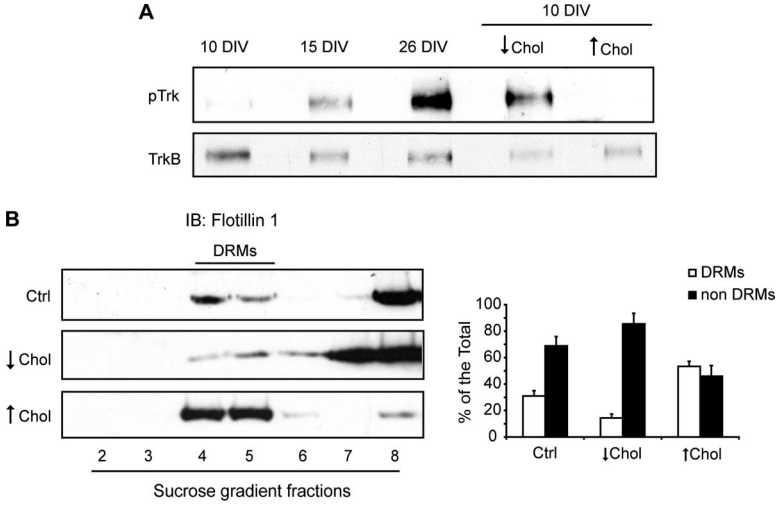

Figure 6. Pharmacological-induced changes in cholesterol levels in young neurons modulate pTrk activity. (A) Western blot of hippocampal membranes from 10, 15, and 26 d in vitro (DIV) control neurons as well as from 10 d in vitro (10 DIV) neurons with cholesterol levels reduced to the values of untreated neurons 26 DIV neurons (see Figure 2A; ↓Chol) and 10 DIV neurons with cholesterol levels replenished after reduction, to reach 10 DIV levels (↑Chol). Note how cholesterol reduction in the 10 DIV cells triggers intense TrkB activity, whereas its replenishment to their natural values makes receptor activity disappear. (B) Sucrose gradient fractions of cold detergent-solubilized plasma membranes from 10 in vitro (DIV) with control (ctrl), pharmacological reduction of membrane cholesterol (↓Chol, 25% less than in control cells) or with cholesterol levels replenished after reduction to control levels (↑Chol). The 25% decrease in membrane cholesterol induces the displacement of Flotillin-1 away from DRMs (↓Chol), yet replenishment restores DRM-preferential partitioning (↑Chol, compare with ↓Chol). Percentages of Flotillin-1 distribution in DRMs (fractions 4 and 5, □) and nonDRMs (fractions 6–8, ■) were measured by densitometry (graphics on the right, mean ± SD, n = 4). Flotillin 1 was 31.3 ± 8% in the DRM domains of 10 DIV neurons. Cholesterol depletion reduced Flotillin-1 in DRMs to 14 ± 3.0%; cholesterol replenishment of the latter cells increased Flotillin 1 in DRMs to 50.4 ± 8.0%.

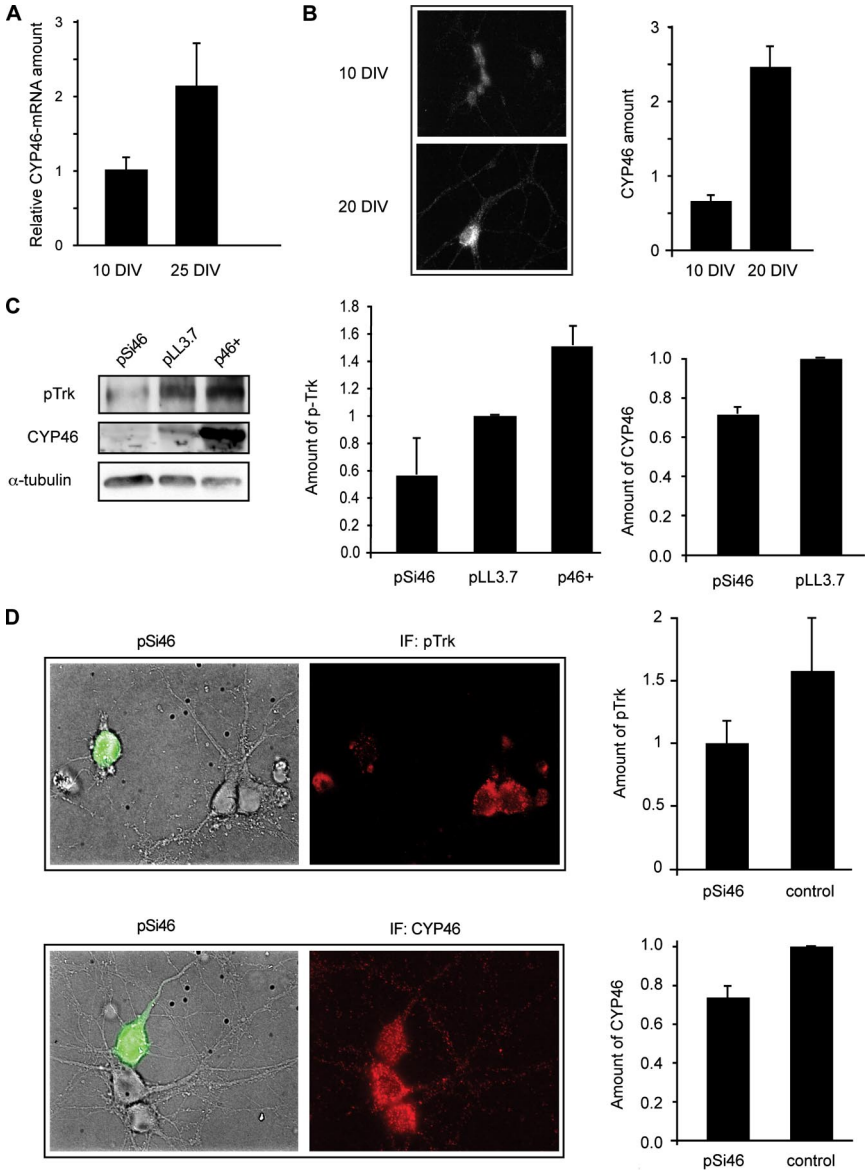

Figure 7. Differentiation-occurring increase in cholesterol-24-hydorxylase is sufficient and necessary for TrkB activation. (A) Compared with 10 DIV neurons, and after normalization for actin mRNA, mRNA expression levels of cholesterol-24-hydroxylase (CYP46) increase at 26 DIV. Variations related to 10 DIV (value of 1) are indicated. (B) Immunofluorescence analysis of the expression levels of cholesterol-24-hydroxylase in hippocampal neurons maintained for 10 and 26 DIV. Antibody concentration and image exposure were identical for all three time points. Higher reactivity occurs in the 26 DIV neurons, consistent with the higher expression of the messenger (see A). (C) Hippocampal neurons in suspension were transfected either with the plasmid expressing *cyp46a1* (p46+) or scrambled vector (pLL3.7) or with the plasmid expressing the *cyp46a1*-siRNA (pSi46). TrkB activity was measured at 7 DIV or 10 DIV. Note how the increased expression of the cholesterol-24-hydroxylase (CYP46) resulted in high levels of active TrKB in these cells, and reduction of the cholesterol 24-hydroxylase by siRNA resulted in lower levels of Trk phosphorylation. The amount of pTrk/tubulin either in pSi46 or p46+ transfected cells respect to the control is shown. Quantification of the Western blottings performed using anti-cholesterol-24-hydroxylase antibody reveals the efficacy of the knockdown strategy. (D) Immunofluorescence microscopy with anti phospho-Trk antibody (p-Trk) reveals that reduction in cholesterol-24-hydroxylase results in reduced levels of active Trk (IF:pTrk). The efficacy of the knockdown strategy was analyzed with anti-cholesterol-24-hydroxylase antibody reveals (see IF: CYP46).

inhibition of the lateral mobility of the receptor, as shown for other proteins (Goodwin *et al.*, 2005), cholesterol was added back to the mevilonin/cyclodextrin-treated 10 DIV neurons. This resulted in the suppression of TrkB signal in these cells (Figure 6A). In further agreement, cholesterol replenishment resulted in the reappearance of Flotilin 1 in the DRMs of these cells (Figure 6B). Treatment of young neurons with mevilonin did not result in receptor activation (Supplementary Figure 1C), consistent with the inefficacy of this treatment alone to reduce the levels of cholesterol in these cells (Simons *et al.*, 1998).

The Cholesterol Catabolic Enzyme Cholesterol 24-Hydroxylase Regulates TrkB Activity in Mature Hippocampal Neurons

To test the involvement of cholesterol loss in TrkB activation by means different from pharmacological reduction/replenishment, which may lead to a number of side effects (Kwik *et al.*, 2003; Hancock, 2006), we searched for the possible molecular event responsible for cholesterol loss in aging cells. To this aim, we performed a real-time PCR-based analysis of the major cholesterol anabolic and catabolic enzymes, utilizing as template mRNA from hippocampal neurons at different stages of development. This study revealed a twofold increase in the expression of the brain-specific catabolic enzyme cholesterol-24-hydroxylase, also known as CYP46 (Lund *et al.*, 1999; Figure 7A), without significant changes in the levels of the metabolic enzymes. In agreement, also the levels of the protein were higher in 20 DIV neurons (Figure 7B). Having established this link, we next modified the neurons' cholesterol levels by altering the expression of cholesterol-24-hydroxylase through canonical gain (cDNA expression) and loss (shRNA knockdown) of function approaches. To test if cholesterol loss can produce increased TrkB activity, *cyp46A1* was overexpressed in 10 DIV neurons, which, as shown earlier, present weak to undetectable TrkB activity. Consistent with the importance of low cholesterol levels, overexpressing cells showed higher levels of TrkB phosphorylation (Figure 7C). In further agreement, knocking *cyp46A1* down in aged cells, led to

reduced levels of TrkB activity detected either by Western blot (Figure 7C) or by immuno-fluorescence (IF) microscopy (Figure 7D).

DISCUSSION

Numerous evidences have accumulated over the years that unequivocally prove that the BDNF-TrkB pathway is a crucial player in the survival of certain neuronal populations during development and in synapse fine-tuning in the mature nervous system (Egan *et al.,* 2003; Blum and Konnerth, 2005; Reichardt 2006). We here showed that BDNF can be dispensable for TrkB activation toward survival in the mature hippocampus, both in vitro and in situ. Because the adult hippocampus and hippocampal cells do, however, present strong TrkB survival activity, a most logical conclusion from the BDNF-independent data are that in the adult brain TrkB activity may be guaranteed by more than one mechanism, acting in parallel or complementarily to BDNF. One such mechanisms is via the modulation of cholesterol content. This conclusion is supported by a temporal correlation, i.e., cholesterol is reduced during ageing in vitro and in situ and also by a direct cause–effect evidence; i.e., 20–30% reduction of cholesterol in young neurons elicited receptor activation. It is difficult to argue that such effect might have derived from inhibition of the lateral diffusion of TrkB (induction of clustering) triggered by the cholesterol-removing drug-utilized, β-methyl-cyclodextrin (Goodwin *et al.,*

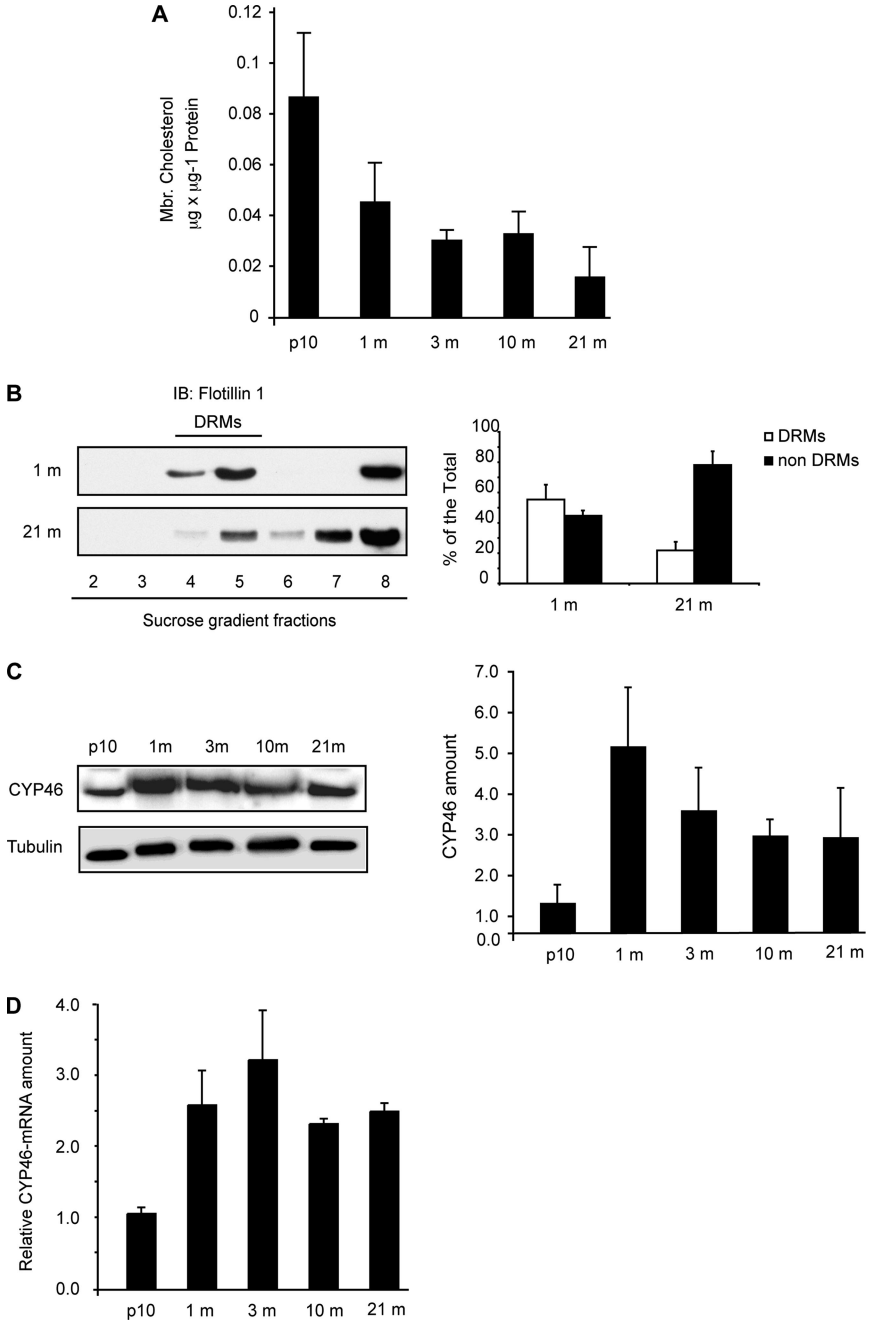

Figure 8. In vivo reduction of membrane cholesterol, changes in membrane DRM partitioning characteristics and increased levels of cholesterol-24-hydroxylase. (A) Colorimetric analysis of membrane cholesterol levels in hippocampal membranes at different postnatal times: 10 d (p10), 1 mo (1 m), 3 mo (3 m), 10 mo (10 m), and 21 mo (21 m). Cholesterol is most abundant in the membranes of early postnatal hippocampus (p10) undergoing significant loss with time, reaching the lowest levels at month 21 (21 m). (B) Western blot analysis with an antibody against the canonical DRM protein Flotillin 1 in detergent extracted, sucrose gradient separated, DRM and non-DRM fractions from 1- and 21-mo-old mice hippocampi. Note the clear DRM partitioning in the membranes from young animals and the more homogeneous distribution in the old animals. (C) Western blotting (left) and quantification (right) of the expression levels of cholesterol-24-hydroxylase in hippocampal extracts from 10 d (p10) and 1-, 3-, 10-, and 21-mo-old mice. CYP46 levels were normalized for tubulin. (D) Real-time PCR analysis of expression levels of cholesterol-24-hydroxylase. Total RNA was obtained from hippocampus from 10 d (p10) and 1-, 3-, 10-, and 21-mo-old mice. As shown in C and D, time-associated increased expression of the enzyme parallels both loss of cholesterol (see above, A) as well as the increased partitioning of TrkB in DRMs in this structure (see Figure 3C).

2005), because TrkB activity in the young cells was reverted to control levels (i.e., undetectable) upon restoring cholesterol to normal (for this age) levels. Whether or not a loss of cholesterol may cause TrkB activity in vivo is a matter that needs further attention. However, in support that this may be the case, reduced cholesterol levels were observed in the hippocampus of aged mice, together with a reduction of Flotilin1 from DRMs and increased expression of cholesterol-24-hydroxylase (Figure 8, A–D), clearly resembling the in vitro data.

The demonstration that cholesterol reduction can lead to increased TrkB activity constitute, at first sight, a most paradigmatic example of cholesterol control of cellular signaling. In fact, for the most cases, cholesterol loss leads to reduced signaling (Simons and Toomre, 2000). On the other hand, recent articles have shown that the opposite effects (i.e., reduction leading to increased signaling) is also possible (Kalvodova *et al.*, 2005; Chen *et al.*, 2007; Oh *et al.*, 2007), indicating that the outcome of cholesterol reduction will depend on the type of receptor, cell type, and stage of differentiation. The increased activity for TrkB under low cholesterol conditions may reflect autoactivation by dimerization due to slower diffusion on the plane of the membrane. Alternatively, cholesterol reduction may have triggered receptor activation in the early secretory pathway or in endosomes, because of altered transport or recycling. In any event, the way that cholesterol modulates TrkB activity in differentiated neurons is certainly different from that in developing neurons, in which receptor activation is reduced by cholesterol-loss treatments (Suzuki *et al.*, 2004). The dissection of the mechanisms of TrkB cholesterol dependence and independence in adult and young neurons is of major biological relevance and will need extensive work before we can understand it. Unfortunately, the overexpression of TrkB variants may not be very useful for this purpose, because it leads to receptor activation independently from ligand and cholesterol concentrations.

Cholesterol loss in differentiated neurons, both in vivo and in vitro, seems to occur via up-regulation of the catabolic enzyme cholesterol-24-hydroxylase. This brain specific enzyme is responsible for the conversion of cholesterol into 24S-hydroxycholesterol, the excreted form of cholesterol (Lund *et al.*, 1999, 2003), an event that progressively increases with age (Lutjohann, 2006). Although this does not rule out that other events contribute to brain cholesterol reduction during ageing, such as a decay in the activity of synthesizing enzymes or a reduction in cholesterol transport from astrocytes, overexpression of cholesterol24-hydroxylase induced TrkB activation in young neurons and its knockdown prevented its occurrence in the old neurons, proving that modulation of this enzyme can act as a sufficient and necessary stimulus for receptor activation. Although the finding that the activity of the enzyme is also high in the differentiated hippocampus in situ makes us feel confident that this pathway might operate in vivo, direct validation is needed. Quite encouraging that this may be the case, the only (up to now) event demonstrated to increase the expression of cholesterol-24-hydroxylase activity is oxidative stress (Ohyama *et al.*, 2006). Unfortunately, the use of cholesterol-24-hydroxylase knockout mice cannot be envisioned for this purpose, as these mice compensate decreased cholesterol excretion by a reduction in de novo synthesis (Lund *et al.*, 2003).

The observation that up-regulation of cholesterol-24-hydroxylase can activate TrkB makes unavoidable to discuss its relevance for Alzheimer's disease (AD). In fact, although still controversial, several groups have found a significant association between intronic mutations in this gene and increased risk of AD (Kolsch *et al.*, 2002; Papassotiropoulos *et al.*, 2003; Borroni *et al.*, 2004; see however, Desai *et al.*, 2002; Wang and Jia, 2007). Although we here report that cholesterol loss, triggered by the up-regulation of cholesterol-24-hydroxylase, leads to TrkB activation, we have previously demonstrated that cholesterol loss of more than 30% can lead to apoptosis (Ledesma *et al.*, 2003; Abad-Rodriguez *et al.*, 2004). In this context, and considering that TrkB activity is important for survival of differentiated cortical neurons under stress (Gates *et al.*, 2000), it is quite conceivable that intronic mutations could predispose to AD through a "gain-of-function" process, i.e., premature and constant loss of cholesterol leading to cell death upon the occurrence of further loss (beyond 30%) triggered when age-associated (i.e., cumulative) stress passes the physiological threshold. But it is also possible that the intronic mutations found in certain cases of AD may predispose to disease by a "loss-of function" mechanism, whereby this enzyme would not respond to physiological stress and therefore prevent the activation of an important antiapoptotic pathway like that of TrkB. To distinguish between these possibilities is another interesting derivation from this work.

ACKNOWLEDGMENTS

We thank Outi Nikkila for preparing the BDNF +/− crosses; Kristel Vennekens (University of Leuven) for the hippocampal cultures; Francesca Gilli and Fabiana Marnetto for help with the real-time PCR experiments; and Laura Doglio, Jose Abad-Rodriguez, and Maria D. Ledesma for discussions and technical advice. The following institutions provided financial support: Swedish Medical Research Council and the Alzheimer's Association (T.H.), Sigrid Juselius Foundation and Academy of Finland (E.C.), and Fund for Scientific Research Flanders (FWO), Federal Office for Scientific Affairs (IUAP P6/43/), and Flemish Government (Methusalem grant; C.G.D.).

REFERENCES

Abad-Rodriguez, J. *et al.* (2004). Neuronal membrane cholesterol loss enhances amyloid peptide generation. J. Cell Biol. *167*, 953–960.

Agerman, K., Hjerling-Leffler, J., Blanchard, M. P., Scarfone, E., Canlon, B., Nosrat, C., and Ernfors, P. (2003). BDNF gene replacement reveals multiple mechanisms for establishing neurotrophin specificity during sensory nervous system development. Development *130*, 1479–1491.

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*, 911–917.

Blum, R., and Konnerth, A. (2005). Neurotrophin-mediated rapid signaling in the central nervous system: mechanisms and functions. Physiology *20*, 70–78.

Borroni, B., Archetti, S., Agosti, C., Akkawi, N., Brambilla, C., Caimi, L., Caltagirone, C., Di Luca, M., and Padovani, A. (2004). Intronic CYP46 polymorphism along with ApoE genotype in sporadic Alzheimer disease: from risk factors to disease modulators. Neurobiol. Aging *25*, 747–751.

Brown, D. A., and London, E. (1997). Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem. Biophys. Res. Commun. *240*, 1–7.

Cellerino, A. (1996). Expression of messenger RNA coding for the nerve growth factor receptor trkA in the hippocampus of the adult rat. Neuroscience *70*, 613–616.

Chen, C. L., Huang, S. S., and Huang, J. S. (2007). Cholesterol modulates cellular TGF-beta responsiveness by altering TGF-beta binding to TGF-beta receptors. J. Cell. Physiol. *Epub ahead of print*.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., and Boland, P. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature *375*, 235–238.

Conover, J. C., and Yancopoulos, G. D. (1997). Neurotrophin regulation of the developing nervous system: analyses of knockout mice. Rev. Neurosci. *8*, 13–27.

Davies, A. M., Minichiello, L., and Klein, R. (1995). Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. EMBO J. *14*, 4482–4489.

Desai, P., DeKosky, S. T., and Kamboh, M. I. (2002). Genetic variation in the cholesterol 24-hydroxylase (CYP46) gene and the risk of Alzheimer's disease. Neurosci. Lett. *328*, 9–12.

Duchemin, A. M., Ren, Q., Mo, L., Neff, N. H., and Hadjiconstantinou, M. (2002). GM1 ganglioside induces phosphorylation and activation of Trk and Erk in brain. J. Neurochem. *81*, 696–707.

Edidin, M. (2003). The state of lipid rafts: from model membranes to cells. Annu. Rev. Biophys. Biomol. Struct. *32*, 257–283.

Egan, M. F. *et al.* (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell *112*, 257–269.

Ernfors, P., Lee, K. F., and Jaenisch, R. (1994). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature *368*, 147–150.

Gates, M. A., Tai, C. C., and Macklis, J. D. (2000). Neocortical neurons lacking the protein-tyrosine kinase B receptor display abnormal differentiation and process elongation *in vitro* and *in vivo.* Neuroscience *98*, 437–447.

Goodwin, J. S., Drake, K. R., Remmert, C. L., and Kenworthy, A. K. (2005). Ras diffusion is sensitive to plasma membrane viscosity. Biophys. J. *89*, 1398–1410.

Gooney, M., Messaoudi, E., Maher, F. O., Bramham, C. R., and Lynch, M. A. (2004). BDNF-induced LTP in dentate gyrus is impaired with age: analysis of changes in cell signaling events. Neurobiol. Aging *25*, 1323–1331.

Gorski, J. A., Zeiler, S. R., Tamowski, S., and Jones, K. R. (2003). Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. J. Neurosci. *23*, 6856–6865.

Hancock, J. F., and Parton, R. G. (2005). Ras plasma membrane signalling platforms. Biochem. J. *389*, 1–11.

Hancock, J. F. (2006). Lipid rafts: contentious only from simplistic standpoints. Nat. Rev. Mol. Cell. Biol. *7*, 456–462.

Hanzal-Bayer, M. F., and Hancock, J. F. (2007). Lipid rafts and membrane traffic. FEBS Lett. *581*, 2098–2104.

Hartmann, M., Brigadski, T., Erdmann, K. S., Holtmann, B., Sendtner, M., Narz, F., and Lessmann, V. (2004). Truncated TrkB receptor-induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor. J. Cell Sci. *117*, 5803–5814.

Hattiangady, B., Rao, M. S., Shetty, G. A., and Shetty, A. K. (2005). Brain-derived neurotrophic factor, phosphorylated cyclic AMP response element binding protein and neuropeptide Y decline as early as middle age in the dentate gyrus and CA1 and CA3 subfields of the hippocampus. Exp. Neurol. *195*, 353–371.

Hill, J. J., Kolluri, N., Hashimoto, T., Wu, Q., Sampson, A. R., Monteggia, L. M., and Lewis, D. A. (2005). Analysis of pyramidal neuron morphology in an inducible knockout of brain-derived neurotrophic factor. Biol. Psychiatry *57*, 932–934.

Jacobson, K., Mouritsen, O. G., and Anderson, R. G. (2007). Lipid rafts: at a crossroad between cell biology and physics. Nat. Cell Biol. *9*, 7–14.

Jones, K. R., Farinas, I., Backus, C., and Reichardt, L. F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell *76*, 989–999.

Kaech, S., and Banker, G. (2006). Culturing hippocampal neurons. Nat. Protocols *1*, 2406–2415.

Kalvodova, L., Kahya, N., Schwille, P., Ehehalt, R., Verkade, P., Drechsel, D., and Simons, K. (2005). Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. J. Biol. Chem. *280*, 36815–36823.

Kaplan, D. R., and Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. *10*, 381–391.

Katho-Semba, R., Semba, R., Takeuchi, I., and Kato, K. (1998). Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescent-accelerated mice: a comparison to those of nerve-growth factor and neurotrophin 3. Neurosci. Res. *31*, 227–234.

Klein, U., Gimpl, G., and Fahrenholz, F. (1995). Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry *34*, 13784–13793.

Ko, M., Zou, K., Minagawa, H., Yu, W., Gong, J. S., Yanagisawa, K., and Michikawa, M. (2005). Cholesterol-mediated neurite outgrowth is differently regulated between cortical and hippocampal neurons. J. Biol. Chem. *280*, 42759–42765.

Kolsch, H., Lütjohann, D., Ludwig, M., Schulte, A., Ptok, U., Jessen, F., von Bergmann, K., Rao, M. L., Maier, W., and Heun, R. (2002). Polymorphism in the cholesterol 24S-hydroxylase gene is associated with Alzheimer's disease. Mol. Psychiatry *7*, 899–902.

Kwik, J., Boyle, S., Fooksman, D., Margolis, L., Sheetz, M. P., and Edidin, M. (2003). Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. Proc. Natl. Acad. Sci. USA *100*, 13964–13969.

Ledesma, M. D., Abad-Rodriguez, J., Galvan, C., Biondi, E., Navarro, P., Delacourte, A., Dingwall, C., and Dotti, C. G. (2003). Raft disorganization leads to reduced plasmin activity in Alzheimer's disease brains. EMBO Rep. *4*, 1190–1196.

Lee, F. S., and Chao, M. V. (2001). Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. USA *98*, 3555–3560.

Lee, F. S., Rajagopal, R., Kim, A. H., Chang, P. C., and Chao, M. V. (2002). Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J. Biol. Chem. *277*, 9096–9102.

Luikart, B. W., Nef, S., Virmani, T., Lush, M. E., Liu, Y., Kavalali, E. T., and Parada, L. F. (2005). TrkB has a cell-autonomous role in the establishment of hippocampal Schaffer collateral synapses. J. Neurosci. *25*, 3774–3786.

Lund, E. G., Guileyardo, J. M., and Russell, D. W. (1999). cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. Proc. Natl. Acad. Sci. USA *96*, 7238–7243.

Lund, E. G., Xie, C., Kotti, T., Turley, S. D., Dietschy, J. M., and Russell, D. W. (2003). Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. J. Biol. Chem. *278*, 22980–22988.

Lutjohann, D. (2006). Cholesterol metabolism in the brain: importance of 24S-hydroxylation. Acta Neurol. Scand. Suppl. *185*, 33–42.

Marshak, S., Nikolakopoulou, A. M., Dirks, R., Martens, G. J., and Cohen-Cory, S. (2007). Cell-autonomous TrkB signaling in presynaptic retinal ganglion cells mediates axon arbor growth and synapse maturation during the establishment of retinotectal synaptic connectivity. J. Neurosci. *27*, 2444–2456.

Merlio, J. P., Ernfors, P., Jaber, M., and Persson, H. (1992). Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. Neuroscience *51*, 513–532.

Minichiello, L., Korte, M., Wolfer, D., Kuhn, R., Unsicker, K., Cestari, V., Rossi-Arnaud, C., Lipp, H. P., Bonhoeffer, T., and Klein, R. (1999). Essential role for TrkB receptors in hippocampus-mediated learning. Neuron *24*, 401–414.

Nicolau, D. V., Jr., Burrage, K., Parton, R. G., and Hancock, J. F. (2006). Identifying optimal lipid raft characteristics required to promote nanoscale protein-protein interactions on the plasma membrane. Mol. Cell. Biol. *26*, 313–323.

Oh, H. Y., Lee, E. J., Yoon, S., Chung, B. H., Cho, K. S., and Hong, S. J. (2007). Cholesterol level of lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated Akt and ERK signal transduction. Prostate *67*, 1061–1069.

Ohyama, Y. *et al.* (2006). Studies on the transcriptional activity of cholesterol 24-hydroxylase (CYP46A1): marked insensitivity toward different regulatory axes. J. Biol. Chem. *281*, 3810–3820.

Papassotiropoulos, A. *et al.* (2003). Increased brain beta-amyloid load, phosphorylated tau, and risk of Alzheimer disease associated with an intronic CYP46 polymorphism. Arch. Neurol. *60*, 29–35.

Paratcha, G., and Ibanez, C. F. (2002). Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme. Curr. Opin. Neurobiol. *12*, 542–549.

Pereira, D. B., and Chao, M. V. (2007). The tyrosine kinase Fyn determines the localization of TrkB receptors in lipid rafts. J. Neurosci. *27*, 4859–4869.

Pozzo-Miller, L. D., Gottschalk, W., Zhang, L., McDermott, K., Du, J., Gopalakrishnan, R., Oho, C., Sheng, Z. H., and Lu, B. (1999). Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. J. Neurosci. *19*, 4972–4983.

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways. Philos. Trans. R. Soc. Lond. B Biol. Sci. *361*, 1545–1564.

Rubinson, D. A. *et al.* (2007). Corrigendum: a lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. *39*, 803.

Shetty, A. K., Rao, M. S., Hattiangady, B., Zaman, V., and Shetty, G. A. (2004). Hippocampal neurotrophin levels after injury: Relationship to the age of the hippocampus at the time of injury. J. Neurosci. Res. *78*, 520–532.

Silhol, M., Bonnichon, V., Rage, F., and Tapia-Arancibia, L. (2005). Age-related changes in brain-derived neurotrophic factor and tyrosine kinase receptor isoforms in the hippocampus and hypothalamus in male rats. Neuroscience *132*, 613–624.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. *1*, 31–39.

Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C. G., and Simons, K. (1998). Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc. Natl. Acad. Sci. USA *95*, 6460–6464.

Stenqvist, A., Agerman, A., Marmigere, F., Minichiello, L., and Ernfors, P. (2005). Genetic evidence for selective neurotrophin 3 signaling through TrkC but not TrkB in vivo. EMBO Rep. *6*, 973–978.

Suzuki, S., Numakawa, T., Shimazu, K., Koshimizu, H., Hara, T., Hatanaka, H., Mei, L., Lu, B., and Kojima, M. (2004). BDNF-induced recruitment of TrkB receptor into neuronal lipid rafts: roles in synaptic modulation. J. Cell Biol. *167*, 1205–1215.

Tansey, M. G., Baloh, R. H., Milbrandt, J., and Johnson, E. M., Jr. (2000). GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron *25*, 611–623.

Tokuyama, W., Hashimoto, T., Li, Y. X., Okuno, H., and Miyashita, Y. (1998). Highest trkB mRNA expression in the entorhinal cortex among hippocampal subregions in the adult rat: contrasting pattern with BDNF mRNA expression. Brain Res. Mol. Brain Res. *62*, 206–215.

Wang, F., and Jia, J. (2007). Polymorphisms of cholesterol metabolism genes CYP46 and ABCA1 and the risk of sporadic Alzheimer's disease in Chinese. Brain Res. *1147*, 34–38.

Xu, B., Gottschalk, W., Chow, A., Wilson, R. I., Schnell, E., Zang, K., Wang, D., Nicoll, R. A., Lu, B., and Reichardt, L. F. (2000). The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J. Neurosci. *20*, 6888–6897.

Yan, Q., Elliott, J. L., Matheson, C., Sun, J., Zhang, L., Mu, X., Rex, K. L., and Snider, W. D. (1993). Influences of neurotrophins on mammalian motoneurons *in vivo.* J. Neurobiol. *24*, 1555–1577.

Yeo, G. S., Connie Hung, C. C., Rochford, J., Keogh, J., Gray, J., Sivaramakrishnan, S., O'Rahilly, S., and Farooqi, I. S. (2004). A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nat. Neurosci. *7*, 1187–1189.

Zaccaro, M. C., Ivanisevic, L., Perez, P., Meakin, S. O., and Saragovi, H. U. (2001). p75 Co-receptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains. J. Biol. Chem. *276*, 31023–31029.