II. MATERIAL AND METHODS

2.1 Cultures of purified CNS neurones

RGCs were isolated by sequential immunopanning as described (Barres et al., 1988). Postnatal day 7 (P7) Wistar rats (animal facility, Faculte de Medicine, Universite Louis Pasteur, Strasbourg) were killed and retinae were dissected out, cleaned and digested [30 min in D-PBS with 33 U/ml Papain (Worthigton Biochemical Corporation, Lakewood, NJ, USA) and 200 U/ml DNAse (Sigma) at 37°C]. Digestion was stopped by incubation in 0.15% Ovomucoid [0.15% (w/w) trypsin inhibitor (Roche Molecular Biochemicals) in D-PBS with 0.15% (w/w) BSA (Sigma A8806)]. Tissue was successively triturated in 0.15% Ovomucoid with 330 U/ml DNAse and 1:75 rabbit-anti rat macrophage serum (Axell, Westbury, NY, USA) using 1 ml pipette tips with 0.2 to 2 mm tip diameter. The cell suspension was centrifuged for 13 min at 128 g and the cell pellet was resuspended in 1% Ovomucoid solution. After another centrifugation step, the cells were resuspended in 0.02% BSA in D-PBS and filtered through a nylon mesh (Nitex, 20 µm, Tetco, Monterey-Park, CA, USA). RGCs were purified by sequential immunopanning, therefore three panning plates were prepared the day before. The fist two plates [(Petri dish Ø 15 cm, Falcon, Becton-Dickinson) incubated for at least 12 h at 4°C with goat anti rabbit IgG (10 µg/ml, Biotrend, Cologne) in Tris/HCl (50 mM, pH 9.5)] were subtraction plates, while the third plate [(Petri dish Ø 10 cm, Falcon, Becton-Dickinson) incubated with goat anti mouse IgM (10 µg/ml, Biotrend, Cologne) in Tris/HCl (50 mM, pH 9.5)] was a selection plate. Plates were washed (PBS) and blocked with 0.2% BSA in D-PBS. Additionally, the selection plate was incubated with mouse anti rat Thy-1 (5 ml culture medium of T11D7e hybridoma cells, American Type Culture Collection, Manassas, VA, USA) for at least 2 h at room temperature. The filtered cell suspension was plated in succession on the two subtraction plates for 36 and 33 min, respectively before filtration and positive selection for Thy-1 on the last plate for 45 min. The plate was washed (D-PBS) until only adherent cells were visible. To take off the selected cells, they were incubated for 10 min in equilibrated Earle's balanced salt solution (EBSS, without Ca^{2+} and Mg^{2+}, Sigma) with 2.5 mg/ml trypsin (Sigma) at 37°C and 5% CO_2. Trypsin digestion was stopped by incubation with 30% heat inactivated fetal calf serum (FCS, Gibco) and cells were removed mechanically by pipetting. Cells were counted using a haemocytometer (Assistant, Sondheim) and plated for dense cultures at 200 cells/mm^2 on poly-D-lysine

(PDL) [MW 30 – 70 kDalton, 10 µg/ml in water (Sigma)] on tissue culture dishes (35 mm Falcon, BD Biosciences). For microcultures cells were plated at 20 cells/mm^2 on tissue culture dishes (35 mm Falcon, BD Biosciences) containing microdots of PDL (70 - 150 kDalton; 100 µg/ml in water; Sigma) that were formed by a custom-built microatomizer. RGCs were cultured in serum-free NB$^+$ medium (see Tab.1, Meyer-Franke et al., 1995). Three times a week, half of the culture medium was replaced by fresh NB+. Five days after plating, Cholesterol (5 µg/ml; Sigma) was added to neuronal cultures from a 1000x ethanolic stock solution as described (Mauch et al., 2001). For some experiments Laminin-1 (ultrapure, mouse; BD Biosciences), merosin (human, Gibco) or laminin fragments from the α1 (aa 2091-2108; CSRARKQAASIKVAVSADR; Sephel et al., 1989) and the γ1 chain (aa 1575-1564; RNIAEIIKDI; Liesi et al., 1989) (Bachem) were added to the culture medium at 0.25 µg/ml.

Table 1: NB$^+$ medium for cultivation of RGCs (modified from Meyer-Franke et al.,1995)

Components	**Concentration**
Neurobasal medium (Invitrogen)	
B-27 supplement (Invitrogen)	2% (v/v)
human brain-derived neurotrophic factor (BDNF) (PeproTech/TEBU)	25 ng/ml
rat ciliary neurotrophic factor (CNTF) (PeproTech/TEBU)	10 ng/ml
forskolin (Sigma)	10 µM
glutamine (Invitrogen)	2 mM
insulin (Sigma)	5 µg/ml
N-acetylcysteine (Sigma)	60 µg/ml
penicillin (Invitrogen)	100 units/ml
progesterone (Sigma)	62 ng/ml
putrescine (Sigma)	16 µg/ml
sodium selenite (Sigma)	40 ng/ml
bovine serum albumin (BSA); crystalline grade #A4161, Sigma)	100 µg/ml
sodium pyruvate (Invitrogen)	1 mM
streptomycin (Invitrogen)	100 µg/ml
triiodothyronine (Sigma)	40 ng/ml
holo-transferrin (Sigma)	100 µg/ml

2.2 Preparation of GCM

GCM was obtained from cortical glial cells that were prepared similarly as described (Pfrieger & Barres, 1997; McCarthy and de Vellis, 1980). P7 rats were killed by decapitation and cortices were dissected out and cleaned. The preparation of cell suspension followed the same steps as described for RGCs (2.1). After resuspension in BSA, cells were centrifuged and the cell pellet was resuspended in DMEM/FCS culture medium (see Tab.2). Cells were cultured in PDL-coated [PDL, MW 30 – 70 kDalton, 10 µg/ml in water (Sigma)] tissue culture flasks (25 cm^2, TPP) in a medium that does not support survival of neurones (see Tab.2). After one week, culture flasks were washed with PBS and glial cells were then cultured in NB+ (see Tab.1) except that BDNF, CNTF and B27 were omitted. Three times a week, half of the GCM was harvested and replaced by fresh NB+. GCM was centrifuged for 5 min at 3000 g to remove cellular debris and added to 5 d old RGC cultures by replacing 5 out of 7 parts of culture medium with 3 parts of GCM and 2 parts of fresh NB+.

Table 2: Cortex culture medium DMEM/FCS

Components (all Invitrogen)	**Concentration**
DMEM without glutamine and phenol red, with 1000 mg/ml glucose	
FCS, heat inactivated, sterile filtered	10%
glutamine	2 mM
penicillin	100 units/ml
sodium pyruvat	1 mM
streptomycin	100 µg/ml

2.3 Electrophysiological recordings

For recordings, I selected neurons that grew singly or in small groups (< 12 cells) in microcultures. Whole-cell currents were recorded at room temperature (20 - 24°C) on an inverted microscope (Axiovert 135TV, Zeiss) with patch pipettes made of borosilicate glass [2 – 5 MOhm, (World Precision Instruments) prepared using a pipette puller (P97, Sutter Instruments)] using an Axopatch 200B amplifier (Axon Instruments), a data acquisition board (PCI-MIO-16E1, National Instruments) and custom-written Labview programs (National Instruments). The intracellular recording solution contained 100 mM

potassium gluconate, 10 mM KCl, 10 mM EGTA, 10 mM HEPES adjusted to pH 7.4 with KOH (all Sigma). The extracellular solution contained 120 mM NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES adjusted to pH 7.4 with NaOH (all Sigma). The membrane potential was clamped at -70 mV. Currents were low-pass filtered at 5 kHz and digitized at 20 kHz. For each cell, spontaneously occurring postsynaptic currents were recorded during 30 seconds. Then, evoked synaptic responses were elicited by extracellular field stimulation [constant current pulses, 30-70 mA/1 ms (Isolator-11, Axon Instruments) delivered via two platinum electrodes (distance ~4 mm)] at 1 Hz with three trains of 20 pulses each (Nagler et al., 2001). Whole-cell currents were recorded continuously during stimulation to monitor asynchronous transmitter release (Goda & Stevens, 1994) occurring at least 20 ms after the stimuli (Nagler et al., 2001). For simplicity, I will refer to synaptic currents that are synchronously evoked and occur within 20 ms after a stimulus as evoked currents and to those occurring in residual interstimulus interval of 980 ms as asynchronous currents. To determine the size of miniature excitatory postsynaptic currents (EPSCs) (quantal size), spontaneous (autaptic) EPSCs from singly growing RGCs were analyzed, as in these cells action-potential evoked transmitter release is suppressed by the voltage-clamp (Nagler et al., 2001).

Synaptic currents were analysed offline with custom-written Labview routines in a blinded fashion. Spontaneous and asynchronous synaptic currents were detected automatically based on size and timing criteria. The frequency of asynchronous events was corrected for the rate of spontaneous synaptic currents. The charge transfer amplitudes were calculated automatically. For charge transfer amplitudes the baseline-corrected membrane current was integrated over 8 ms starting at the onset of a synaptic event. For evoked currents, the onset was determined visually. Since evoked synaptic currents often overlapped with the large sodium current that was activated by extracellular stimulation, charge transfer of transmission failures was subtracted from evoked EPSCs.

2.4 Filipin staining

To determine the neuronal cholesterol content in neurites, cultured neurons were fixed (4 % paraformaldehyde for 30 min) and incubated for 2 hours with filipin (10 µg/ml, stock-solution: 1 mg/ml in 95% ethanol, Sigma), an antibiotic that selectively binds to sterols with a hydroxy group at the 3rd C-atom (Norman et al., 1972). Filipin fluorescence was then excited by monochromatic light (356 nm, provided by a xenon-lamp and a

monochromator, Polychrome Junior, TILL Photonics) and fed into the epi-illumination port of an upright microscope (Axioskop II FS, Zeiss). Fluorescence was viewed through an appropriate emission filter (Filter Set 2, Zeiss) and a 40x objetive (water-immersion, N.A. 0.8, Zeiss). Images were acquired by an air-cooled monochrome CCD camera (1280 x 1024 pixels, 8-bit digitization width, PCO Computer Optics, Kelheim, Germany). The measured fluorescence intensity is correlated to the concentration of cholesterol in the cell (Muller et al., 1984). For analysis, filipin fluorescence intensity was determined manually in background area devoid of cells. To determine the intensity in neurites in an unbiased manner, four neuritic areas crossing the vertical and horizontal middle axis of each image were marked manually, the mean intensity over 3 x 3 pixels in each of these areas was calculated and then the average intensity of all areas was calculated. Extensive tests showed that it was impossible to compare intensities of filipin fluorescence between independent staining experiments probably due to the instability of the compound. To avoid possible artefacts and allow for comparison of data, the intensities of filipin fluorescence in neurites of RGCs after different periods of GCM- or cholesterol-treatment were normalized to the average of respective intensities from untreated control cultures that were run in parallel.

2.5 Immunocytochemistry

Microcultures were processed for immunocytochemistry as described (Meyer-Franke et al., 1995). Cells growing on tissue culture plates were washed (PBS), fixed (7 min in methanol at -30 °C), blocked [30 min at room temperature; 50% goat serum in antibody buffer containing 150 mM NaCl, 50 mM Tris, 1% BSA (Sigma A2153), 100 mM l-lysine, 0.04% sodium azide, pH 7.4], and incubated overnight with primary antibodies (diluted in antibody buffer). Presynaptic terminals and postsynaptic receptor clusters were stained with a mouse monoclonal anti-synapsin I antibody (Cl 46.1, 1:500, Synaptic Systems) and a rabbit polyclonal antibody against glutamate receptor 2/3 (GluR2/3) (1:200, Upstate Biotech; 0.2% Triton X added during primary incubation) and visualized with Alexa 488 (Molecular Probes) and Cy3-conjugated secondary antibodies (0.1% Triton X added; 1 hour room temperature; Jackson ImmunoResearch Labs), respectively. Dendrites and microtubule were visualized by mouse monoclonal antibodies against MAP2 (HM-2; 1:400; Sigma) and against α-tubulin (3A2, 1:1000, Synaptic Systems) respectively. Matrix Gla protein (MGP) was stained with a rabbit polyclonal antibody derived against the

C-terminal peptide ERYAMVYGYNAAYNRYFRQRRGAKY (a kind gift from Gerard Karsenty, Baylor College of Medicine, Houston). Heme oxygenase 1 (HO1) was detected using a monoclonal mouse antibody (HO-1-2, 1: 500, Stressgen). Immunofluorescence was viewed using an Hg lamp as light source (HBO100, Zeiss), appropriate filter sets (Cy3: XF33, Cy2: XF100; Omega Optical/Photomed) and a 40 x objective (Zeiss). Images were acquired by an air-cooled monochrome CCD camera (1280 x 1024 pixels, 8-bit digitization width, PCO Computer Optics, Kelheim, Germany). Control experiments showed absence of background staining by secondary antibodies (data not shown). The densities of synapsin-, GluR2/3 and doubled-stained puncta on neurites was determined by a semi-automatic Labview routine. First it was differentiated between fluorescent puncta on neurites and on somata for each microisland. To accomplish this, somata were outlined manually in phase-contrast images and fluorescent puncta were detected using a somata-outlining mask. To detect fluorescent puncta, images were first processed with a Laplacian filter to enhance puncta-like features. This involved discrete convolution of the image with the following matrix:

$$\begin{bmatrix} -1 & -1 & -1 & -1 & -1 \\ -1 & -1 & -1 & -1 & -1 \\ -1 & -1 & 25 & -1 & -1 \\ -1 & -1 & -1 & -1 & -1 \\ -1 & -1 & -1 & -1 & -1 \end{bmatrix}$$

The filtered image was then sectioned with a fixed threshold [median intensity of the filtered image plus antibody-specific offsets; synapsin: 150 analog-to-digital units (adu); GluR2/3: 130 adu]. Puncta were selected based on pixel area (synapsin: 4-250; GluR2/3: 2-250 pixels) and their mean fluorescence intensity (from unprocessed image; threshold intensity at 0.95 cumulative relative frequency of all intensities plus antibody-specific offsets; synapsin: 40 adu; GluR2/3: 30 adu). Double-stained puncta had to meet intensity criteria of both stains and show at least two pixels overlap. To determine the neuritic area in each image, phase contrast images were processed by a Prewitt filter, segmented (threshold intensity at 0.95 cumulative relative frequency of all intensities in the filtered image) and eroded to remove single-pixel particles. All white pixels in the soma-excluded area were then counted. Puncta densities are stated per 100 x 100 pixels. MAP2 immunostainings were analysed manually by counting the number of MAP2 positive neurites per soma whose length exceeded the soma diameter.

2.6 RNA preparation

For gene expression analysis, RGCs were purified by sequential immunopanning from P6 wistar rats and cultured at 177-218 cells/mm^2 on PDL coated tissue culture dishes in NB$^+$ as described under 2.1. After 6 days in culture the cells were treated for 30 hours as given in table 3.

Table 3: Treatment of RGCs for gene expression analysis (added to 2 ml of NB$^+$).

Condition	Treatment
Control to GCM treatment	2.5 ml DMEM$^+$, 2 µl BDNF (50µg/ml, final conc. 44 ng/ml)
GCM	2.5 ml GCM, 2 µl BDNF (50µg/ml, final conc. 44 ng/ml)
Control to cholesterol treatment	2 µl EtOH (100%)
Cholesterol	2 µl Cholesterol in EtOH (5 mg/ml, final conc. 5 µg/ml)

RNA was prepared using the RNeasy Mini Kit (Qiagen). Cells were scrapped off with a rubber policeman in 350 µl RNeasy lysis buffer containing 1% β-mercaptoethanol, followed by addition of 350 µl 70% ethanol. Samples were mixed gently, applied to an RNeasy mini-column and processed following the manufacturer's protocol. Purified total RNA was eluted in 60 µl H_2O and stored at -80°C. Prior to use, RNA was quantified by absorbance at 260 and 280 nm. Quality was checked by capillary electrophoresis using the RNA 6000 Nano Assay in combination with the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). For this 1 µl sample RNA together with 1 µl ladder was loaded onto an RNA 6000 Nano chip and processed following the manufacturer's protocol.

2.7 Gene expression analysis

Gene expression analysis was performed using the Affymetrix GeneChip system. This system is based on oligonucleotide probes, which are synthesized on arrays. Biotin-labeled RNA fragments were hybridized to probe arrays and detected with a combination of streptavidin phycoerythrin conjugate and an antibody against streptavidin coupled to fluorescence. Hybridized gene chips were scanned at the excitation wavelength of 488 nm. The amount of light emitted at 570 nm is proportional to the bound target at each location

on the probe array. The sample amplification, labeling, hybridization and scanning were performed as described in the Affymetrix protocol. Briefly, mRNA was reverse transcribed using T7-oligo(dT) primer and complemented by second strand cDNA synthesis. After a cleanup of double-stranded cDNA, biotin-labeled antisense RNA was generated by T7 RNA polymerase and biotinylated ribonucleotides. Before chip hybridization, the biotin-labeled antisense RNA was purified, quantified and fragmented. Metal-induced hydrolysis was used for statistical fragmentation. Samples were hybridized on the Affymetrix GeneChip Rat Expression Set 230, which consists of two arrays A and B. The GCM or cholesterol treated samples were hybridized in parallel with controls, and processed during the same time. Equivalent amounts of biotinylated antisense RNA were hybridized for compared samples. Each experiment was repeated three times with independent cell preparations.

All primary expression data were analyzed using the Affymetrix 4.0 Microarray Suite (MAS) software. This software determines a detection call for each probe set. The detection algorithm uses probe pair intensities to generate a detection p-value and assigns a present, marginal, or absent call. Each probe pair in a probe set is considered as having a potential vote in determining whether the measured transcript is detected (present) or not detected (absent). The vote is described by a value called the discrimination score. The score is calculated for each probe pair and is compared to a user-definable threshold Tau. Probe pairs with scores higher than Tau vote for the “presence” of the transcript. Probe pairs with scores lower than Tau vote for the “absence” of the transcript. Additionally, a signal value is calculated which assigns a relative measure of abundance to the transcript. The signal is calculated using the One-Step Tukey’s Biweight Estimate which yields a robust weighted mean that is relatively insensitive to outliers. Furthermore this software allows calculating a comparison analysis. In a comparison analysis, two samples, hybridized to two gene chip probe arrays of the same type, are compared against each other in order to detect and quantify changes in gene expression. Two sets of algorithms are used to generate change significance and change quantity metrics for every probe set. As for the single array analysis, the Wilcoxon’s Signed Rank test is used in comparison analysis to calculate a change p-value and an associated change related to user-defined cut-offs. A second algorithm produces a quantitative estimate of the change in gene expression in the form of signal log ratio. As for the signal, this number is calculated using a one-step Tukey’s Biweight method by taking a mean of the log ratios of probe pair intensities across the two arrays. A base 2 log scale is used. Finally, for each gene the values for signal,

detection, detection p-value, signal log ratio, change and change p-value were calculated. These data were copied into an MS Access database and matched with the annotation files. The following formulas were used to calculate the fold change from signal log ratio values:

Fold change = $2^{\text{Signal Log Ratio}}$ for Signal Log Ratio > 0 and
Fold change = $(-1) * 2^{-(\text{Signal Log Ratio})}$ for Signal Log Ratio < 0.

In addition, the coefficient of variance (CV) of each gene was calculated over three independent experiments, to select for relevant changes. Results were selected by relevance criteria given under results point 3.2.1. All positive selected genes were checked for their identity in a discontinuous BLAST search, using the sequence derived form (Zhang & Madden, 1997). Identified ESTs were included in the results and indicated. All selected genes were grouped by their function or localization as indicated by the literature.

2.8 SDS-polyacrylamid-gel electrophoresis (SDS-PAGE)

SDS-PAGE was done as described by Laemmli (1970), using the Miniprotean II system (Biorad). Gels were prepared with different concentrations of Acrylamid (Tab. 4), depending on the size of investigated proteins.

Table 4: Composition of SDS-polyacrylamid minigels

Components (all Sigma)	**separation gel 5%**	**separation gel 10%**	**collection gel 4%**
H_2O	2.85 ml	2.03 ml	1.5 ml
30% Acrylamid/Bisacrylamid (29:1)	0.83 ml	1.65 ml	0.33 ml
1.5 M Tris/HCl pH 8.8	1.25 ml	1.25 ml	-
0.5 M Tris/HCl pH 6.8	-	-	0.63 ml
10% SDS	50 µl	50 µl	25 µl
10% Ammoniumperoxodisulfat	25 µl	25 µl	12.5 µl
Temed	2.5 µl	2.5 µl	2.5 µl

RGCs growing in dense or microcultures were washed and harvested in sample buffer [0.125 M Tris/HCl pH6.8, 2.5% SDS, 0.025% bromphenol blue, 10% (v/v) glycerol] on ice using an rubber policemen. For reducing conditions, 1% (v/v) β-mercaptoethanol was added and samples were incubated at 99°C for 5 min. Determination of molecular weight

was performed by a molecular weight marker mix (Precision Plus Protein Standard, BioRad) in a separated slot onto the same gel together with samples. Electrophoresis was performed in running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) by 80 V during the first 15 min and continued by 100 V.

2.9 Immunobloting

Separated proteins were transferred from SDS-Page gels to nitrocellulose membranes (Amersham Biosciences) using the Mini Trans-Blot Transfer Cell (BioRad). Transfer was performed in transfer buffer (25 mM Tris, 19.2 mM glycine, 20% methanol) at 100 V for one to two hours, depending on the size of transferred proteins. Subsequently, membranes were incubated in blocking buffer [5% low fat milk powder (Regilait) or 5% BSA (Sigma A2153, for MAP2) in TBS-T (20 mM Tris/HCl pH 7.6, 137 mM NaCl, 0.1% tween 20)] for one hour at room temperature. Membranes were reacted with specific antibodies including polyclonal anti phospho-MAP2 [Thr1620/1623, (1:10.000) or Ser136, (1:1000), Cell Signaling Technology], monoclonal anti MAP2 (1:1000, clone HM-2, Sigma), polyclonal anti GluR2/3 (1:1000, Upstate), monoclonal anti tubulin (1:10.000, clone 3A2, Synaptic Systems), monoclonal anti HO1 (1:5000, clone HO-1-2, Stressgen) and polyclonal anti Histon H3 (1:1000, Abcam) diluted in blocking buffer over night at 4°C. After primary antibody incubation the membranes were washed with TBS-T, two times short, one time 15 min and two times 5 min, followed by incubation with suitable secondary antibodies coupled to horse radish peroxidase (Dianova) for one hour at room temperature. Membranes were washed with TBS-T as described above and detected using ECL-reagent and Hyperfilm (Amersham Biosciences). To compare levels of phospho-rylated versus total MAP2, blots reacted with phospho-specific antibodies were stripped by incubation in Restore WB Stripping buffer (Pierce) for 20 min, reblocked in blocking buffer for one hour and reacted with an anti MAP2 antibody as described above. Efficient removal of phospho-specific antibodies was confirmed by incubation with ECL reagent and film exposure after stripping. To detect laminin-γ1, GCM was spun down (5 min at 15800g) to remove cell debris and then mixed with sample buffer with or without β-mercaptoethanol. Immunoblots of GCM were reacted with a chain-specific monoclonal antibody (1:1000, cat.# MAB1920, Chemicon). Concentration of laminin-γ1 was determined by comparison to purified laminin-1 (BD Biosciences) on the same gel.

2.10 Radioactive labeling and lipid analysis

RGCs were cultured at 200 cells/mm^2 under control conditions for seven days before cells were washed with D-PBS and cultured for another two days in NB$^+$ where B27 was omitted, in the presence of 0.8 µCi 1-^{14}C-Acetate (13 nmol, Sigma). After 48 hours of incubation with the radioactive precursor, culture medium was removed and cells were washed with PBS and scraped off in water using a rubber policeman. For pulse-chase labeling, cells were washed with D-PBS before adding fresh NB$^+$ medium (without B27) and additional treatment as given in Tab. 5. After further 68 hours culture medium was removed for lipid extraction, cells were washed with PBS and scraped off in water.

Table 5: Treatment of RGCs after pulse labeling (added to 2 ml of NB$^+$ without B27).

Condition	Treatment
Control	1,7 ml DMEM$^+$, 1 µl BDNF (50 µg/ml, final conc. 27 ng/ml)
GCM treatment	1,7 ml GCM, 1 µl BDNF (50 µg/ml, final conc. 27 ng/ml)
Cholesterol treatment	1,7 ml DMEM$^+$, 1 µl BDNF (50 µg/ml, final conc. 27 ng/ml), 3.7 µl Cholesterol in EtOH (5 mg/ml, final conc. 5 µg/ml)

For sample adjustment, DNA content was determined from cell homogenate. Cells were homogenized by syringe with 27G cannula and sonication for 10 min and 3 x 5 µl were taken off from each sample for DNA content measurement. Cell homogenate (5 µl) was mixed with 194.5 µl TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA) and 0.5 µl PicoGreen reagent (Molecular Probes) and incubated for at least 5 min at room temperature. DNA content was determined by measuring sample fluorescence at an excitation wavelength of 480 nm and an emission wavelength of 526 nm using a spectrometer (Photon Technology International), and calculated in correlation to a standard curve. For each sample, three probes were determined together with a blank value, which was subtracted from the mean sample value.

For lipid extraction, sample volumes of cell suspension and culture medium were adjusted to equivalent DNA content. Lipids from cell suspension were extracted by hexan/isopropanol as described by Hara & Radin (1978). Cell suspension was mixed with 20 fold sample volume of hexan/isopropanol (3:2) and incubated for 30 min at 4°C, interrupted by shaking all 10 min. Suspensions were centrifuged (5 min at 15800g) and supernatants were collected in glass-tubes. Lipids from culture medium were extracted by chloroform/methanol as described by Oram (1986). First, medium was centrifuged (3 min

at 15800 g) to remove cell debris and mixed with 5-fold volume of chloroform/methanol (2:1) by strong shaking. After centrifugation for 2 min at 1500 g, the upper aqueous phase was removed and the lower organic phase was mixed 1:1 with water. The suspension was again centrifuged (5 min at 1500 g) and the organic phase was convicted to a new glass-tube using a Pasteur pipette. Solvents from cell and medium extraction were vaporized by a nitrogen stream and extracted lipids were solved in small volumes of chloroform for transfer to thin layer chromatography (TLC)-plates.

Lipids were separated by TLC using pebble-gel-plates (60 Å, F254, Merck) and a combination of two different solvent systems. Prior to use, plates were heated for 30 min at 120°C. After cooling to room temperature samples were absorbed to the plates using glass micropipettes (Hirschhorn Laborgeräte). For the later identification of separated lipids, radioactive labeled and unlabeled lipids were absorbed on the same plate in line with samples. The following lipid standards were used: ^{3}H-cholesterol; cholesteryl [1-^{14}C] oleat; L-3-phosphatidylcholine,1,2-di [1-^{14}C] palmitoyl (Amersham Biosciences); cholesterol; cholesterololeate; phosphatidylcholine; 1,2-diacyl-sn-glycero-3-phosphatidylethanolamine; sphingomyelin and 24-OH-cholesterol (Sigma). Separation was performed by two runs, the first in chloroform/methanol/water (60:30:5) until the plate middle, followed by drying and the second run in hexan/diethylether/acetic acid (80:10:1.5) until the upper end of the plate. Labeled lipids were detected by autoradiography, using X-ray films (BioMax MS, Kodak) in combination with an intensifying screen (BioMax TranScreen, Kodak). Total amounts of lipids were visualized by detection with iodine (Mangold, 1960). Therefore, TLC-plates were reacted for several hours with iodine steam, and staining was documented using a digital camera (Powershot G3, Canon).