Neuronal Ca^{2+} sensor protein VILIP-1 affects cGMP signalling of guanylyl cyclase B by regulating clathrin-dependent receptor recycling in hippocampal neurons

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Summary

The family of neuronal Ca^{2+} sensor (NCS) proteins is known to influence a variety of physiological and pathological processes by affecting signalling of different receptors and ion channels. Recently, it has been shown that the NCS protein VILIP-1 influences the activity of the receptor guanylyl cyclase GC-B. In transfected cell lines, VILIP-1 performs a Ca^{2+}-dependent membrane association, the reversible Ca^{2+}-myristoyl switch of VILIP-1, which leads to an increase in natriuretic peptide-stimulated cGMP levels. In this study, we have investigated the effect of VILIP-1 on cGMP signalling in C6 cells and in primary hippocampal neurons, where VILIP-1 and GC-B are co-expressed in many but not all neurons and partially co-localize in the soma and in dendrites. Our data indicate that VILIP-1 modulates GC-B activity by influencing clathrin-dependent receptor recycling. These data support a general physiological role for VILIP-1 in membrane trafficking in the intact hippocampus, where the NCS protein may affect processes, such as neuronal differentiation and synaptic plasticity e.g. by influencing cGMP-signalling.

Key words: Ca^{2+}-binding protein, Guanylyl cyclase B, Hippocampus, Myristoylation, Receptor desensitization, Receptor recycling

Introduction

The family of neuronal Ca^{2+} sensor (NCS) proteins, including NCS-1, DREAM/KChIP-3 (downstream regulatory element antagonist modulator/potassium channel interacting protein) or VILIP-1 (Visinin-like-protein-1), has recently been implicated in a variety of physiological processes ranging from synaptic plasticity (Genin et al., 2001; Sippy et al., 2003; Braunewell et al., 2003), pain modulation (Cheng et al., 2002), neuropathological processes (Kabbani et al., 2002; Bahi et al., 2003; Schnurra et al., 2001; Bernstein et al., 2002; Bernstein et al., 2003) to cancer (Mahloogi et al., 2003). At the molecular level, NCS proteins have been shown to play multiple key roles in controlling neuronal function (Braunewell and Gundelfinger, 1999; Burgoyne et al., 2004). Recently some of the molecular mechanisms by which NCS proteins modulate neuronal signalling cascades have been explored in detail.

NCS proteins can translocate to subcellular membrane compartments by a molecular mechanism termed Ca^{2+}-myristoyl switch, which is dependent on Ca^{2+}-binding and N-terminal myristoylation of the proteins. Recent work has revealed differences in Ca^{2+}-sensitivity and in translocation kinetics of NCS proteins to subcellular membranes (O'Callaghan et al., 2002; Spilker et al., 2002a). In contrast to NCS-1, which seems to be Ca^{2+}-independently associated with Golgi membranes (O'Callaghan et al., 2002), VILIP-1 shows a stimulus-dependent, reversible translocation to Golgi membranes and yet undefined specializations in neuronal dendrites (Spilker et al., 2002a; Spilker and Braunewell, 2003). These data indicate that the Ca^{2+}-myristoyl switch of NCS proteins can provide a fast signalling mechanism to influence membrane-associated receptor systems in a Ca^{2+}-dependent or independent manner.

Consistent with this notion, VILIP-1 was shown to modulate various membrane-localized receptors, including the receptor guanylyl cyclases GC-A and -B (Braunewell et al., 2001; Spilker et al., 2002b). Similarly, the highly homologous protein neurocalcin δ was shown to modulate the activity of homologous guanylyl cyclases, like retGC1 expressed in retinal neurons (Krishnan et al., 2004) and ONE-GC expressed in the olfactory neuroepithelium (Duda et al., 2004). In C6 and PC12 cells an influence of VILIP-1 on cGMP-signalling is observed following activation of GC-A and -B by the atrial natriuretic peptide (ANP) and the C-type natriuretic peptide (CNP), respectively. In primary cerebellar cultures, VILIP-1 was found to affect neuronal GC-B specifically but not glial GC-A (Braunewell et al., 2001). Thus, it is likely that VILIP-1 is a physiological modulator of the neuronal CNP signalling pathway, the receptor GC-B and the second messenger cGMP, and therefore, may influence cGMP-dependent neuronal processes, including neuroprotective/neurotoxic cascades (Fiscus et al., 2002), different forms of synaptic plasticity (Schuman and Madison, 1991; Monfort et al., 2002) and learning and memory (Telegdy, 1994).

It was previously shown that NCS proteins control membrane

traffic and surface expression of ion channels and receptors (An et al., 2000; Weiss et al., 2000; Zhao et al., 2001), and that VILIP-1 increases the cell surface expression of the α4-subunit of the nicotinic acetylcholine receptor (Lin et al., 2002b). To investigate whether VILIP-1 has a general role for membrane trafficking, we have studied whether the VILIP-1 effect on GC-B activity in C6 cells is also dependent on cell surface expression and receptor recycling. To validate these findings further and to test for a possible physiological significance of the VILIP-1 effect on GC-B in the hippocampus, we have examined the effect of VILIP-1 on GC-B signalling in primary hippocampal neurons. In this study we will provide evidence that VILIP-1 modulates receptor guanylyl cyclase function in hippocampal neurons, and that the underlying molecular mechanism resulting in enhanced surface expression and activity of GC-B is a general effect on clathrin-dependent receptor trafficking.

Materials and Methods

Materials

CNP, PAO, monensin and wortmannin for cell stimulation experiments were obtained from Sigma (St Louis, MO) and Calbiochem (San Diego, CA). Biotinylated CNP was purchased from Phoenix Pharmaceuticals (Bellmont, CA). Cell culture reagents were obtained from Gibco-Invitrogen (San Diego, CA). Unless otherwise specified, all other reagents were purchased from Sigma and Roth (Karlsruhe, Germany).

Antibodies

Rat and rabbit polyclonal antibodies, raised against recombinant VILIP-1 fusion protein, were affinity-purified on corresponding glutathion-S-transferase (GST)-tagged fusion proteins immobilized on N-hydroxysuccinimide ester coupled agarose columns (Bio-Rad, Hercules, CA) as previously described (Braunewell et al., 2001). Rabbit polyclonal antibodies against GC-B were raised against the C-terminal 14 aa of GC-B and affinity purified as described (Braunewell et al., 2001). Monoclonal antibodies used in this study comprised anti-MAP-2 (Sigma). Polyclonal clathrin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Cy3 and Alexa Fluor™ 488 labelled secondary antibodies were purchased from Dianova (Hamburg, Germany) and Molecular Probes (Eugene, OR).

Cell culture and transfection of hippocampal neurons

Hippocampal cultures were prepared as previously described (Spilker et al., 2002a). Briefly, hippocampi from fetal wistar rat brains (embryonic day 18) were dissociated by enzyme digestion with 0.1% trypsin at 37°C for 20 minutes followed by trituration through two different sized syringes. Cells were plated onto poly-D-lysin coated 12 well culture plates (12 mm in diameter) at a density of 120,000 cells for transient transfection experiments and in 24 well culture plates with glass coverslips at a density of 60,000 cells for immunocytochemistry in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and antibiotics. 24 hours after plating culture medium was exchanged for Neurobasal medium (Invitrogen) supplemented with 1×B27, 0.5 mM L-glutamine and antibiotics and cells were maintained in a humidified 37°C atmosphere containing 5% CO_2. Hippocampal neurons were transfected with cDNA coding for GFP, VILIP-1- or VILIP-3-GFP after 6 days in culture essentially as described (Spilker et al., 2002a; Spilker et al., 2002b; Spilker and Braunewell, 2003) using the lipofectamin transfection method (Invitrogen).

SDS-PAGE and western blot analysis

Parental C6 cells, C6-V1 cells stably expressing VILIP-1 cDNA and C6-V1M cells stably expressing the myristoylation mutant cDNA of VILIP-1 [amino acid mutation at position 2 (G2A) to prevent N-terminal myristoylation] in an inducible lacSwitch vector system (Stratagene, La Jolla, CA), were cultured to confluency in 12 well tissue culture dishes (Falcon) with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine and penicillin and streptomycin (100 mg ml^{-1}) in a humidified 95% air, 5% CO_2 incubator essentially as described previously (Braunewell et al., 1997). In C6-V1 and C6-V1M cells, VILIP-1-expression was induced overnight with 5 mM IPTG and, by virtue of leaky expression of VILIP-1 in C6-V1 and C6-V1M cells, these cells were compared with parental C6 cells. Cultures of C6, C6-V1 and C6-V1M cells, hippocampal cultures transfected with control vector (pOPR) and VILIP-1-transfected cultures (pOPR-V1) were homogenized in an appropriate volume homogenization buffer (25 mM Tris, 150 mM NaCl, pH 7.5), containing the protease inhibitors benzamidine (1 mM), phenylmethylsulfonylfluoride (0.1 mM). Nuclei and debris were removed by centrifugation at 1000 ***g*** for 5 minutes and equal protein concentrations (in μg protein ml^{-1} buffer) of the resulting cell homogenates were applied to 5-20% gradient SDS-PAGE. To investigate the expression levels of VILIP-1 and GC-B in the cells the previously described antibodies were used for western blot analysis (Braunewell et al., 1997). Protein concentrations were measured by the BCA assay (Pierce, Rockford, IL).

Determination of cell surface expression of GC-B in C6 cells using biotinylated CNP

GC-B surface expression was measured using an ELISA-system with subconfluent C6 and C6-V1 glioma cell cultures. In brief, C6 and C6-V1 cells were incubated for different periods of time with biotinylated CNP (0.2 μM). After fixation with paraformaldehyd [2% in ice cold phosphate buffered saline (PBS, pH 7.4) for 10 minutes], the cells were washed four times with PBS. Receptor-bound biotinylated CNP located on the cell surface was detected by incubation with streptavidin-coupled horseradish peroxidase for 45 minutes. After repeated washing with PBS, the cells were incubated with peroxidase substrate (ABTS, Roche, Mannheim) at 37°C for 1 hour. The reaction was stopped by addition of SDS (1% in PBS) and the supernatant was measured at 405 nm in a Dynex MR II ELISA-reader.

Determination of clathrin-dependent membrane transport via measuring the content of clathrin in membrane and cytosolic fractions following GC-B stimulation

Clathrin membrane association studies were done according to Beattie et al. (Beattie et al., 2000). C6 and C6-V1 cells were cultured as described above and stimulated with or without CNP (0.2 μM) for 10 minutes. The cells were washed once with cold PBS (4°C), harvested and pelleted in 1.5 ml of ice cold (4°C) fractionation buffer (50 mM Tris, 150 mM NaCl, 25 mM HEPES, pH 7.4) containing the phosphatase inhibitors NaF (5 mM), $NaVO_3$ (1 mM) and 1×complete© protease inhibitor cocktail (Roche, Mannheim, Germany). Membranes were disrupted by three cycles of freezing, followed by thawing. After each cycle a 25 gauge needle was used to disrupt the material further. Samples were spun at 1000 ***g*** for 5 minutes to remove nuclei and intact cells, and the supernatant was centrifuged at 100,000 ***g*** for 40 minutes using a sucrose gradient (fractionation buffer containing 0.32 M sucrose). The resulting pellet (P2) contained small and large fragments of the plasma membrane, internal membranes derived from the plasma membranes, other cellular membranes, and organelles such as mitochondria and ribosomes; S2 contained the cytosol. P2 was resuspended in the fractionation buffer and the supernatant (S2) was lysed 1:1 (v/v) in 0.5 M Tris buffer including 1% Triton X-100. Equal amounts of protein from the lysed P2 and S2 fractions, respectively, were subjected to SDS-PAGE, transferred to nitrocellulose, and immunostained with clathrin antibodies.

Determination of transferrin internalization and transferrin receptor recycling

Parental C6 cells were cultured on glass coverslips as described above. Transient transfection was carried out overnight on subconfluent C6 cultures with a vector containing GFP or VILIP-1-GFP cDNA essentially as previously described (Spilker et al., 2002a; Spilker et al., 2002b). For internalization and recycling of Rhodamine-labelled human transferrin (Tetramethyl-rhodamine labelled transferrin, Molecular Probes, Eugene, OR), C6 cells were first incubated in fresh medium for 30 minutes at 37°C and then incubated in medium containing 30 μg ml^{-1} rhodamine-labelled transferrin for 5 minutes. After this pulse period, the medium was replaced by culture medium without rhodamine-labelled transferrin. Following an incubation of 8 minutes, the cells were washed twice with ice-cold PBS, fixed with 4% paraformaldehyde, and analyzed by fluorescence microscopy. As control, the experiments were performed at 4°C to block all membrane transport processes and receptor endocytosis.

Stimulation experiments and determination of intracellular cGMP in intact cells

C6, C6-V1 and C6-V1M cells were cultured to confluency in 12-well tissue culture dishes (Falcon) and expression of VILIP-1 was induced overnight with 5 mM IPTG. Hippocampal neurons were plated at a density of 120,000 cells per well in 12-well tissue culture dishes and were transfected after 6 days in vitro as described above. Before the experiments the cells were washed with DMEM medium containing 20 mM Hepes, pH 7.4 and pre-incubated with DMEM containing 20 mM Hepes, pH 7.4, 1 mM IBMX for 30 minutes. The concentrations and incubation time of the effector CNP was 0.2 μM for 20 minutes at 37°C. Wortmannin was pre-incubated for 1 h at a concentration of 100 nM, monensin at 10 μM for 45 minutes, and PAO at 10 μM for 15 minutes. Measuring of intracellular cGMP concentrations from ethanolic (60%) cell extracts was performed with radioligand assay kits (TRK 500, Amersham, Piscataway, NJ) or with a cGMP enzyme immunoassay kit (RPN 226, Amersham).

Immunocytochemistry

Following fixation of hippocampal neurons with 4% paraformaldehyde in PBS, pH 7.4 for 20 minutes at room temperature, cells were washed twice with 25 mM glycine in PBS to quench background staining due to free reactive groups of paraformaldehyde. Subsequently the cells were permeabilized and blocked in 0.2% Triton X-100, 3% bovine serum albumine, 10% horse serum in PBS (blocking solution) for 30 minutes. Cells were incubated with primary antibodies diluted in blocking solution at 4°C overnight. In background controls the first antibodies were omitted. After washing three times with PBS secondary antibodies diluted in blocking solution without Triton X-100 were applied to the neurons for 1 hour at room temperature. After removal of unbound antibodies coverslips were mounted on slides with Mowiol (Calbiochem) including 1,4-diazobicyclo-[2.2.2]-octane (Merck, Darmstadt, Germany) to reduce fading. GFP and Cy3 fluorescence were visualized using a laser scanning microscope (Leica TCS SP2; Wetzlar, Germany) with Argon-ion (488 nm) and Helium-Neon (543 nm) laser excitation. The excitation light was coupled in via the main dichroic beam splitters (RSP500 for GFP and DD488/543 for Cy3 fluorescence). The emitted light was collected in sequential scans in the range of 505 nm to 550 nm (GFP) and 550 nm to 600 nm (Cy3), respectively. The pinhole was adapted between 1 (GFP) and 3 (Cy3) airy units. For the experiments, a Leica HCX APO L 63×/0.9 water immersion objective was used at the upright microscope. The spatial resolution was 0.37 μm per pixel (in the *x*- and *y*-axes) for the aquired 512×512 pixel images. To avoid any photodamage, the lowest laser intensity necessary for an adequate signal-to-noise ratio was used. Conventional fluorescence microscopy was performed on a Leica DMR fluorescence microscope. Images were recorded digitally and processed using the Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA) and the NIH image 1.62 software (National Institutes of Health, Bethesda, MD) (available at http://rsb.info.nih.gov/nih-image/).

Results

Examination of the time course of GC-B activation by VILIP-1 and examination of the expression level of GC-B in C6 and VILIP-1-transfected C6-V1 cells

In C6 cells GC-B was stimulated with CNP (0.2 μM) for 20 minutes (Fig. 1A). The activation of the receptor cyclase was monitored by measuring the cellular cGMP contents in the presence of the phosphodiesterase inhibitor IBMX. Other inhibitors, i.e. zaprinast showed similar results (data not shown). Non-transfected C6 cells show an increase in cGMP levels following CNP stimulation (Fig. 1A, C6). In C6 cells that had been stably transfected with the VILIP-1-cDNA a higher reactivity towards CNP was observed as compared with non-transfected control cells (Fig. 1A, C6-V1). In kinetic studies the comparison of non-transfected (C6) with VILIP-1-transfected C6 cells (C6-V1) revealed that after CNP-stimulation of GC-B the VILIP-1 effect reaches maximal strength and a significant difference 20 minutes after receptor stimulation (Fig. 1B). Since cGMP accumulation was enhanced by using IBMX the decrease of cGMP levels at later time points is explained by export of cGMP into the medium which was controlled in parallel (data not shown). The myristoylation mutant of VILIP-1 (Fig. 1C, C6-V1M), although being strongly overexpressed (data not shown), was only able to stimulate GC-B to a much lower extent than wild type VILIP-1. This observation is reminiscent of what was previously observed in PC12 cells (Braunewell et al., 2001). Western blot analysis shows that no VILIP-1 expression is observed in non-transfected C6 cells (Fig. 1D, lane 1) but can be detected as a 22 kDa protein in stably transfected C6-V1 cells (Fig. 1D, lane 2). C6 cells express only GC-B but not GC-A (Braunewell et al., 2001) and the amount of GC-B protein (Fig. 1D, lanes 3, 4) does not significantly differ between non-expressing (Fig. 1D, lane 3) and VILIP-1-expressing cells (Fig. 1D, lane 4). Therefore, rather than changing receptor levels VILIP-1 seems to modulate receptor activity by other means, possibly involving the desensitization process of the receptor which occurs maximally 15-20 minutes following CNP activation (Pandey et al., 2002).

Expression of VILIP-1 increases the surface expression of GC-B receptor following ligand stimulation in C6 cells

It has been previously shown that a desensitization-dependent internalization of GC-A occurs (Rathinavelu and Isom, 1991) and that the reduction of surface expression of GC-A reaches a maximum level approximately 15-20 minutes after receptor stimulation (Pandey et al., 2002). By contrast, at this time point the receptor response is not desensitized in VILIP-1-transfected cells, but a rather strong increase in cGMP accumulation can be noticed compared with non-transfected cells (Fig. 1B). To test whether VILIP-1 may interfere with desensitization and directly change surface expression of GC receptors, non-transfected (C6) and VILIP-1-transfected (C6-V1) C6 cells were incubated with biotinylated CNP for 20 seconds and the surface expression of CNP-bound GC was measured using a streptavidin-coupled ELISA assay (Fig. 2). Interestingly, although the total content of

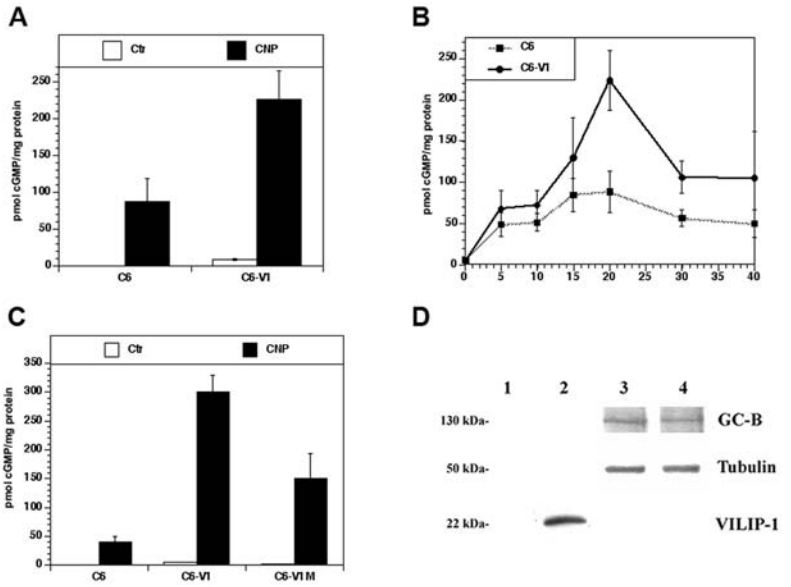

Fig. 1. (A) Enhancement of membrane cGMP levels following CNP-stimulation of natriuretic peptide receptor GC-B in untransfected and VILIP-1-transfected neural C6 cells. In VILIP-1-transfected glioma cells (C6-V1) and non-transfected C6 control cells (C6) cGMP accumulation was measured in unstimulated (Ctr) and CNP-stimulated (CNP, 0.2 μM) cells in the presence of the PDE inhibitor IBMX. (B) Time course of cGMP accumulation in non-transfected and VILIP-1-transfected C6 cells. Kinetic analysis of cGMP accumulation of non-transfected C6 cells (C6) and VILIP-1-transfected C6 cells (C6-V1) after CNP stimulation for various time points in the presence of IBMX. (C) Comparison of membrane cGMP levels following CNP-stimulation of natriuretic peptide receptor GC-B in untransfected, wild-type VILIP-1-, and myristoylation mutant-VILIP-1-transfected neural C6 cells. In wild-type VILIP-1-transfected glioma cells (C6-V1), myristoylation mutant VILIP-1-transfected (C6-V1M) and nontransfected C6 control cells (C6) cGMP accumulation was measured in unstimulated (Ctr) and CNP-stimulated (CNP, 0.2 μM) cells in the presence of the PDE inhibitor IBMX. (D) Expression of VILIP-1 and GC-B in non-transfected C6 and VILIP-1-transfected C6-V1 cells. Western blot analysis shows that no VILIP-1 expression can be detected in non-transfected C6 cells (lane 1), but can be detected as a 22 kDa protein in stably transfected C6-V1 cells (lane 2). The amount of GC-B protein with a M_r of 130 kDa (lanes 3, 4) does not differ between C6 cells (lane 3) and C6-V1 cells (lane 4) when compared with tubulin (50 kDa) as control. Mean values±s.d. are from two experiments for A and one representative experiment out of two for B and C carried out in triplicate.

GC-B proteins does not differ between C6 and C6-V1 cells (see Fig. 1D), VILIP-1-transfected C6 cells (C6-V1) show an increased surface binding of biotinylated CNP when compared with non-transfected C6 cells (Fig. 2A), indicating an increased surface expression of GC receptors in VILIP-1-expressing cells already under basal (unstimulated) conditions. Incubation of cells with biotinylated CNP at different time points (1, 5, 10, 15, 20 and 30 minutes) revealed an effect of VILIP-1 on the recycling of GC receptors. In C6 cells following CNP-stimulation a strong decrease in the level of surface expression of GCs was observed reaching a maximum of decrease at 10 minutes and then returned to background levels after 30 minutes (Fig. 2B, C6). By contrast, in VILIP-1-transfected cells no decrease in surface expression occurred and only after 20 minutes a slow reduction of surface expression is seen (Fig. 2B, C6-V1). These data indicate that VILIP-1 expression attenuates CNP-induced receptor recycling and thus receptor desensitization.

Expression of VILIP-1 changes the subcellular distribution of clathrin in C6 cells following CNP stimulation indicating changes in clathrin-dependent membrane transport of GC receptors

To investigate whether the observed VILIP-1 effect on cGMP accumulation and cell surface expression of GC-B is dependent on clathrin-mediated receptor recycling, subcellular fractionation experiments were performed. Induction of endocytosis is known to lead to the accumulation of clathrin in a surface and microsomal membrane fraction (P2 fraction), when separated from the remaining cytosolic S2 fraction (Beattie et al., 2000). Interestingly, western blot analysis with clathrin antibodies showed a changed clathrin distribution in the P2 and S2 fraction upon CNP stimulation in non-transfected (C6) and VILIP-1-transfected (C6-V1) C6 cells (Fig. 3A,B). In untransfected C6 cells an increased association with the P2 membrane fraction is observed after CNP stimulation (+CNP) (Fig. 3A, C6, compare –/+CNP). VILIP-1-transfected C6-V1 cells show already a slightly increased basal association of clathrin with the P2 fraction without stimulation, and following CNP-stimulation no more significant change in the association of clathrin with the P2 fraction is observable (Fig. 3A, C6-V1, compare with C6 and compare –/+CNP). In the S2 fraction the opposite effect was seen (data not shown). Quantification of the subcellular distribution of clathrin from western blot analysis (n=7) in non-transfected C6 cells revealed a significant (80%) CNP-induced increase in the amount of clathrin in the P2 membrane fraction, whereas in C6-V1 cells no change or even a small decrease in the amount of clathrin in the P2 fraction occured in cells which had been stimulated with CNP (Fig. 3B). These data indicate that VILIP-1-expression modulates not only CNP-dependent GC activity but also basal and CNP-induced, clathrin-dependent processes, most likely representing GC receptor recycling.

Transient expression of VILIP-1-GFP alters clathrin-mediated receptor trafficking of the transferrin receptor in C6 cells

To prove that the effect of VILIP-1 on CNP-induced clathrin re-distribution represents a clathrin-dependent membrane trafficking process, we studied trafficking of the transferrin receptor, a marker for clathrin-dependent receptor recycling, in transiently transfected C6 cells. C6 cells transfected with VILIP-1-GFP were incubated with Rhodamine-labelled transferrin and afterwards the subcellular localization of transferrin (Fig. 3C,D, red channel) was examined by fluorescence microscopy. The expression of VILIP-1-GFP was shown by green fluorescence of the GFP fusion protein (Fig. 3D, green). In cells not expressing GFP-VILIP-1 (compare Fig.

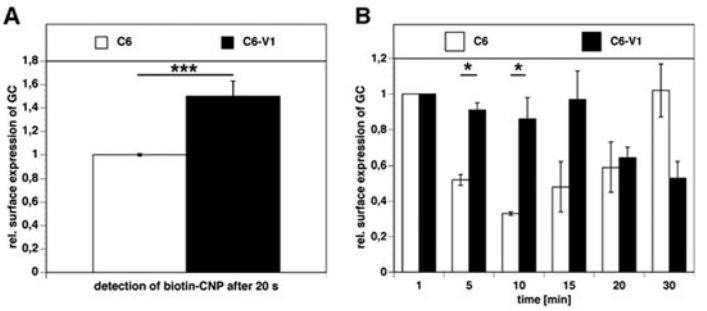

Fig. 2. (A) Surface expression of receptor GCs as shown by binding of biotinylated CNP in non-transfected and VILIP-1-transfected C6 cells. Non-transfected (C6) and VILIP-1-transfected (C6-V1) C6 cells were incubated with biotinylated CNP (0.2 μM for 20 seconds) and the relative surface expression of CNP-bound GC was measured using a streptavidin ELISA assay. (B) Time course of surface expression of GC receptors following ligand stimulation with biotinylated CNP in non-transfected and VILIP-1-transfected C6 cells. Non-transfected (C6) and VILIP-1-transfected (C6-V1) C6 cells were incubated with biotinylated CNP (0.2 μM) for a time period of 1, 5, 10, 15, 20 and 30 minutes and the surface expression of CNP-bound GC was measured in a streptavidin ELISA. Values were normalized to the 1 minutes value in C6 and C6-V1 cells, respectively. Mean values±s.e.m. are from five experiments in A and three in B. Asterisks (*,***) mark significant differences (P<0.05 with n=3, P<0.005 with n=5, Student's t-test).

3C,D) a clear intracellular localization of rhodamine-labelled transferrin was observed, which was concentrated around the nucleus, a clear sign for transferrin receptor internalization (van Dam et al., 2002). By contrast, in GFP-VILIP-1-expressing C6 cells the nuclear localization was not apparent, but a rather cell surface associated localization became evident (compare Fig. 3C,D), indicating that VILIP-1-expression affected transferrin receptor recycling. In non-expressing and GFP-expressing (Fig. 3F, green) C6 cells no difference in transferrin receptor recycling was observed (compare Fig. 3E,F). The difference in localization of the transferrin receptor (Fig. 3C,E, red channel) from two experiments was quantified (Table 1) as number of cells showing perinuclear localization in non-transfected C6 versus GFP- or VILIP-1-transfected C6 cells (Fig. 3D,F, green fluorescence). Expression of VILIP-1 led to a reduction of perinuclear localization of the transferrin receptor (19%) compared with C6 (67%) or C6-GFP (81%) controls, indicating that receptor recycling was altered. In summary, these results in C6 cells clearly show that VILIP-1 influences clathrin-dependent membrane trafficking, most likely affecting the recycling of receptors from and to the cell surface.

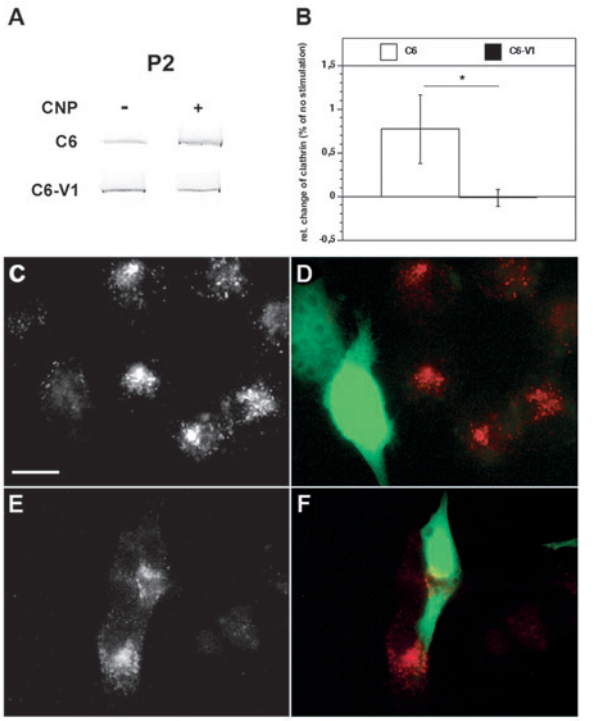

Fig. 3. Subcellular distribution of clathrin in non-transfected and VILIP-1-transfected C6 cells following CNP-stimulation as a measurement for membrane transport of GC receptor. (A) Western blot analysis using clathrin antibodies was performed with (+CNP) or without (–CNP) previous CNP-stimulation of non-transfected (C6) and VILIP-1-transfected (C6-V1) C6 cells and after separation of the P2 membrane fraction from the cytosolic fraction. (B) Quantification of the subcellular distribution of clathrin in non-transfected and VILIP-1-transfected C6 cells following CNP-stimulation. In non-transfected (C6) and VILIP-1-transfected (C6-V1) cells the association of clathrin with the P2 membrane fraction following CNP-stimulation was quantified using western blot analysis as shown in A. Mean values±s.e.m. are from seven experiments in B. Asterisks (*) mark a significant difference (P<0.05, n=7, Student's t-test). (C-F) Effect of GFP or VILIP-1-GFP expression on the subcellular localization of the transferrin receptor. C6 cells were transfected with GFP (E,F) or VILIP-1-GFP (C,D) and incubated with Rhodamine-transferrin for 13 minutes. After fixation the subcellular localization of Rhodamine-labelled transferrin (C-F, red channel) was observed by fluorescence microscopy and compared with VILIP-1-GFP (D, compare red and green channel) and GFP-expression (F, compare red and green channel). Bar in C=40 μm.

GC-B and VILIP-1 co-localize in a large subpopulation of hippocampal neurons and are both distributed throughout the neuron with a punctate staining pattern in dendritic processes

GC-B and VILIP-1 show a prominent expression in brain, especially in the hippocampus (Herman et al., 1996; Braunewell and Gundelfinger, 1999). Thus, it was interesting to investigate whether the proteins are both co-localized in hippocampal neurons and whether they co-distribute in neuronal compartments. These results can give insight whether the modulation of GC-B by VILIP-1 might also occur in hippocampal neurons and therefore might play a physiological role in the hippocampus. Therefore, to verify a possible functional interaction of VILIP-1 with GC-B in living hippocampal neurons, we performed co-localization studies. Indirect immunofluorescence staining of hippocampal cell cultures (14 days in vitro) with specific antibodies against VILIP-1 (rat) and GC-B (rabbit) reveal co-expression of the two proteins in most hippocampal neurons in culture as shown by co-staining of VILIP-1 and MAP-2 (Fig. 4A), GC-B and MAP-2 (Fig. 4B) and VILIP-1 and GC-B (Fig. 4C). However, there are also examples for a non-overlapping expression in a

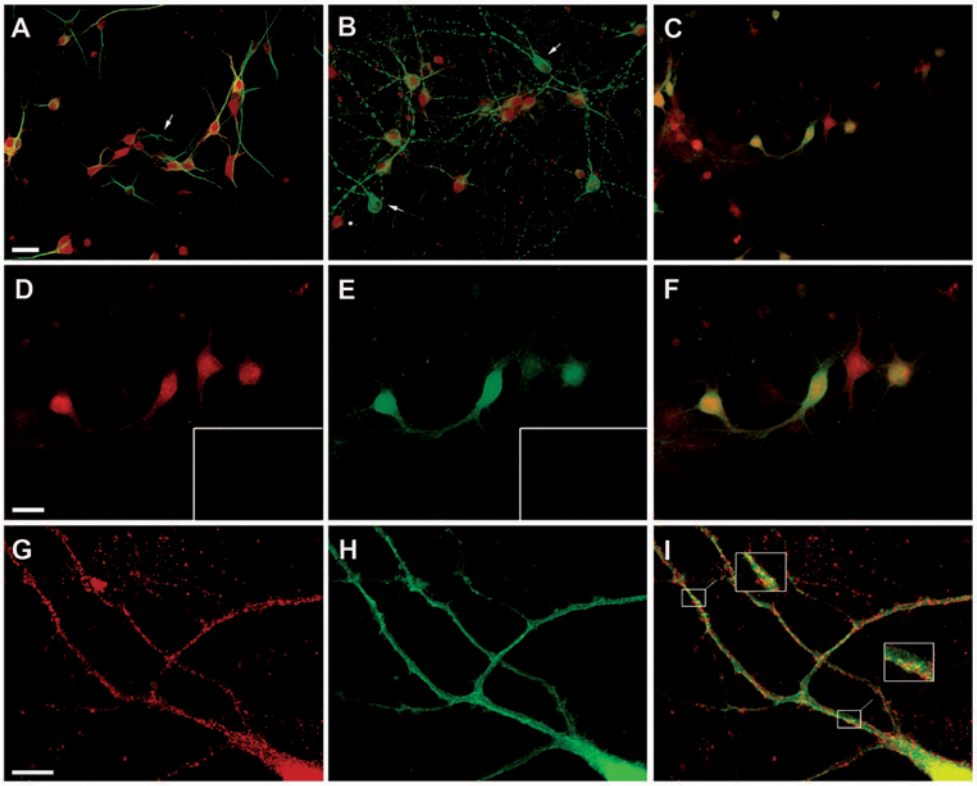

Fig. 4. Co-localization of GC-B and endogenous VILIP-1 in hippocampal neurons. Hippocampal cell cultures (14 days in vitro) were labelled by indirect immunofluorescence using affinity-purified rat and rabbit polyclonal antibodies against VILIP-1 and GC-B, monoclonal MAP-2 and secondary Cy3 antibodies (red) or secondary Alexa Fluor™ 488 antibodies (green). (A) Co-localization of VILIP-1 (red) and MAP-2 (green). (B) Co-localization of GC-B (red) and MAP-2 (green). Note, some MAP-2-positive neurons in A and B do not express VILIP-1 or GC-B (arrows). (C) Co-localization of VILIP-1 (green) and GC-B (red). (D) Magnification of hippocampal neurons in C expressing GC-B (red). (E) Magnification of hippocampal neurons in C expressing VILIP-1 (green). (F) Merged images of D and E show co-localization of GC-B and VILIP-1 (yellow) at higher magnification. (G) Localization of GC-B (red) in dendrites of a hippocampal neuron. (H) Localization of VILIP-1 (green) in dendrites of a hippocampal neuron. (I) Merged images of G and H show co-localization (yellow) of GC-B with VILIP-1 in hippocampal dendrites. Insets in D and E show background staining with secondary antibodies only. Bars in A=30 μm, in D=20 μm and in G=5 μm.

neuronal subpopulation as shown by co-staining of VILIP-1 and GC-B (Fig. 4D-F). The overlay of red and green fluorescence (Fig. 4F) shows neurons that are expressing GC-B (Fig. 4D) but that are devoid of VILIP-1 (Fig. 4E). Specificity of the GC-B and VILIP-1 staining was shown by background controls with secondary antibodies only (Fig. 4D,E, insets). At higher magnification the subcellular distribution of VILIP-1 (Fig. 4H) and GC-B (Fig. 4G) in dendritic processes can be observed. The overlay of red and green fluorescence reveals strong co-expression in distinct punctate structures (Fig. 4I), indicating a high likelihood for a functional role of VILIP-1-modulated GC-B signalling in neuronal dendrites.

Table 1. Quantification of the effect of VILIP-1-GFP expression on the subcellular localization of the transferrin receptor

	No. of cells showing perinuclear localization of labelled transferrin	%
C6	34 of 51	66.67
C6-V1-GFP	7 of 37	18.92
C6-GFP	29 of 36	80.56

Number of cells showing perinuclear staining of labelled transferrin in untransfected C6 cells, GFP-transfected C6 cells and VILIP-1-GFP-transfected C6 cells.

For C6 cells and VILIP-1-GFP-transfected C6 cells n=3; for GFP-transfected C6 cells n=2.

VILIP-1 and GC-B are both expressed at high levels in primary hippocampal neurons and VILIP-1 affects cGMP accumulation evoked by CNP-stimulation of membrane-bound GC-B in transiently VILIP-1-transfected hippocampal neurons

VILIP-1 has been shown to affect cGMP accumulation in different stably transfected neuronal cell lines and to interact with the catalytic domain of GC-B, therefore we were interested whether VILIP-1 may influence cyclase activity in transiently transfected primary hippocampal neurons overexpressing the VILIP-1 cDNA. The expression of endogenous VILIP-1 (Fig. 5A, lane 1) and GC-B (Fig. 5A, lane 2) in hippocampal cultures was verified with specific antibodies reacting with protein bands of a molecular mass of 22 and 130 kDa, respectively, in western blots. Transfection of hippocampal neurons with pOPR-V1 vector containing the VILIP-1 cDNA led to enhanced VILIP-1 expression (Fig. 5A, compare lane 3 and lane 4), but tubulin (lane 4) and GC-B (lane 6) expression did not change, compared with cells transfected with the empty vector (lanes 3 and 5).

In neurons that were transfected with the empty vector (Fig. 5B, pOPR), CNP-stimulation led to enhanced cGMP accumulation indicating functional expression of GC-B. In VILIP-1-transfected hippocampal neurons (Fig. 5A, pOPR-V1) a highly significant two-fold increase in cGMP-production following CNP stimulation was detected compared with vector controls (Fig. 5A, compare pOPR and pOPR-V1). The observed modulatory effect of transiently expressed VILIP-1 on GC-B activity in hippocampal neurons is similar to the effect observed

in stably transfected cell lines (Fig. 1A-C), ruling out the possibility that the effect is restricted to some clonal cell lines. These experiments clearly document that VILIP-1 is able to affect cGMP accumulation following transient transfection of primary neuronal cultures.

Effect of VILIP-1 on membrane transport processes such as recycling of guanylyl cyclase receptor in primary hippocampal neurons

To find out whether the effect of VILIP-1 on surface expression of receptor GCs may be linked to the functional effect on GC-B receptor activity in hippocampal neurons we investigated whether pharmacological interference with distinct membrane transport phenomena can affect VILIP-1-dependent cGMP accumulation. Control-transfected (pOPR) and VILIP-1-transfected (pOPR-V1) hippocampal neurons were stimulated with CNP for 20 minutes in the presence or absence of the membrane transport inhibitors monensin, wortmannin and phenyl arsinide (PAO). The late endosome inhibitor monensin which affects clathrin-dependent exocytosis, and PAO, which affects clathrin-dependent endocytosis, led to a significant decrease of CNP-stimulated cGMP accumulation in control-transfected neurons, indicating that for CNP-induced desensitization of GC-B, receptor recycling on the level of exo- and endocytosis may play a role (Fig. 5C, pOPR). In VILIP-1-transfected hippocampal neurons monensin and PAO strongly decreased the VILIP-1-mediated effect on cGMP accumulation back to levels observed in control-transfected cells (Fig. 5C, pOPR-V1). Wortmannin, known to interfere with phosphoinositide 3-kinase (PI3K) activity and thereby influencing fast receptor recycling (van Dam et al., 2002), did not significantly influence cGMP accumulation and even showed a slight increase in control-transfected and in VILIP-1-transfected cells. These results show that receptor recycling is important for functional activity and desensitization of receptor GC-B. VILIP-1 likely affects clathrin-dependent receptor recycling of GC-B via a monensin-sensitive and wortmannin-insensitive mechanism in hippocampal neurons.

VILIP-1 but not VILIP-3 expression attenuates CNP-induced internalization and receptor recycling of GC-B in primary hippocampal neurons

To prove the effect of VILIP-1 on CNP-induced internalization, receptor recycling and thus, desensitization of GC-B, we performed confocal immunofluorescence studies in transfected primary hippocampal neurons. A closely related NCS-family member, VILIP-3, served as a negative control because VILIP-

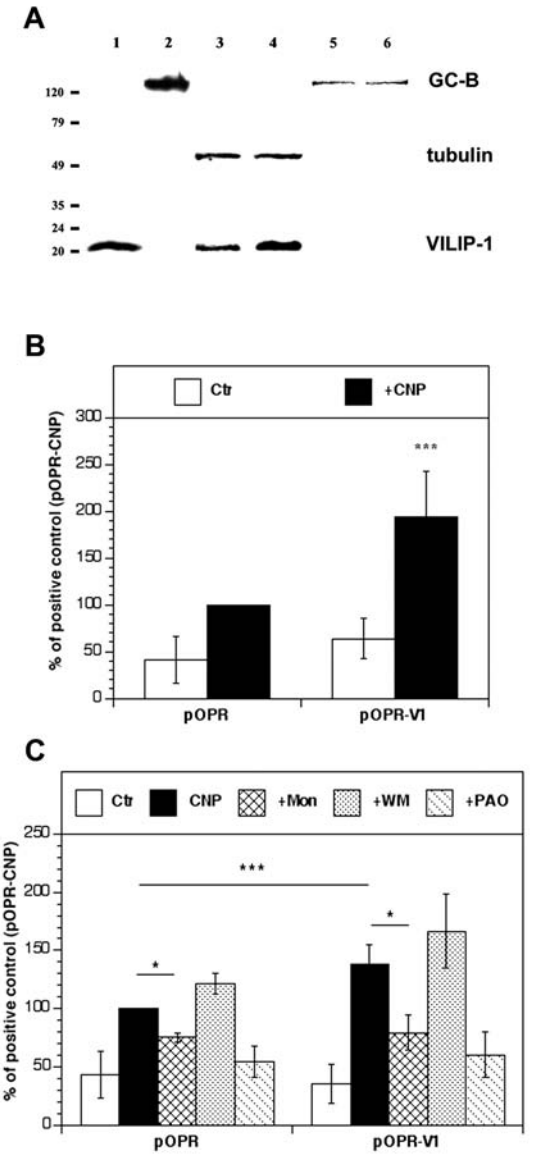

Fig. 5. Protein expression of GC-B and of endogenous and heterologously expressed VILIP-1 in hippocampal neurons. (A) Homogenates of hippocampal cells were analyzed by western blot analysis using specific antibodies against VILIP-1 (lanes 1, 3 and 4) and GC-B (lanes 2, 5 and 6). VILIP-1-expression is shown in untransfected (lane 1), control-transfected (lane 3, pOPR) and VILIP-1-transfected (lane 4, pOPR-V1) hippocampal neurons. GC-B-expression is shown in untransfected (lane 2), control-transfected (lane 5, pOPR) and VILIP-1-transfected (lane 6, pOPR-V1) hippocampal neurons. Sizes of marker proteins are indicated at the left margin. 30 μg of protein was loaded in lanes 1 and 2, 10 μg in lanes 3-6. (B) Enhancement of membrane cGMP levels following CNP-stimulation of natriuretic receptor GC-B in untransfected and VILIP-1-transfected hippocampal neurons. CNP-stimulated cGMP accumulation in hippocampal neurons transiently transfected with control vector (pOPR) and vector containing VILIP-1-cDNA (pOPR-V1). Mean values±s.d. are from seven experiments carried out in triplicate. Asterisks (***) mark a significant difference between CNP-stimulated pOPR and pOPR-V1-transfected cells (P<0.005, n=7, Student's t-test). (C) Effect of different inhibitors of membrane transport on CNP-stimulated cGMP accumulation in control-transfected and VILIP-1-transfected hippocampal neurons. Control-transfected (pOPR) and VILIP-1-transfected (pOPR-V1) hippocampal neurons were stimulated with CNP for 20 minutes in the presence or absence of monensin (+Mon) or wortmannin (+WM), which inhibit receptor recycling at different stages; or in the presence of the endocytosis inhibitor PAO (+PAO). CNP-stimulated cGMP accumulation in control-transfected (pOPR) hippocampal neurons was set to 100% and all other values were expressed as % of control (CNP, pOPR). Mean values±s.d. are from at least three experiments, except PAO (two experiments), and were carried out in triplicate. Asterisks (*,***) mark significant differences (P<0.05, P<0.005, n=<3, Student's t-test).

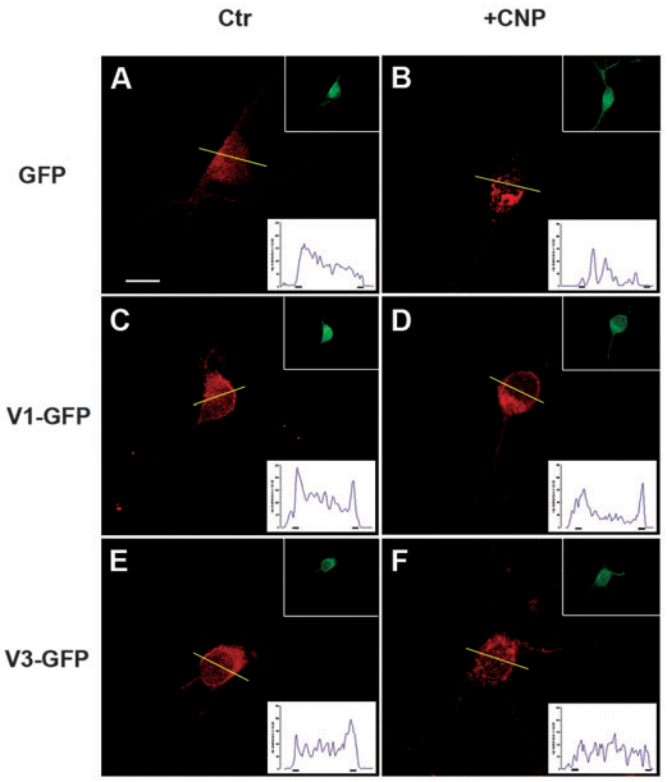

Fig. 6. Effect of heterologous VILIP-1 and VILIP-3 expression on the subcellular localization of GC-B protein in hippocampal neurons with and without CNP-stimulation. Hippocampal cell cultures (24 hours after transfection) were analyzed by confocal immunofluorescence microscopy using affinity-purified polyclonal antibodies against GC-B and secondary Cy3 anti-rabbit antibodies (red). GFP, VILIP-1-GFP and VILIP-3-GFP expression (green) was detected as green fluorescence of the GFP-fusion protein following transient transfection of the vector constructs. Subcellular localization of GC-B (red) was detected with (A,C,E) or without CNP-stimulation (B,D,F) in GFP-transfected (A,B), VILIP-1-GFP-transfected (C,D) and VILIP-3-GFP-transfected (E,F) hippocampal neurons. Upper insets show co-localization of GC-B with VILIP-1-GFP, VILIP-3-GFP or GFP expression (A-F, green). The lower insets in A-F show the quantification of the pixel values (relative GC-B fluorescence intensity) from a cross section of the shown neurons as indicated with the yellow bars. Black bars in the insets indicate the localization of the cell surface membrane. Bar in A=20 μm.

3 does not influence cGMP accumulation in transfected cell lines (Spilker et al., 2002b).

When GFP control-transfected hippocampal neurons (insets, green fluorescence) were stimulated with CNP the subcellular localization of GC-B (red fluorescence) changed from a combined cell surface membrane and intracellular localization to a more pronounced localization in intracellular membrane compartments (compare Fig. 6A and B), indicating that receptor internalization takes place. The heterologous expression of VILIP-1-GFP (insets, green fluorescence) led to an increased surface localization already without stimulation and additionally interfered with the CNP-induced GC-B-internalization (compare Fig. 6C with D, and A with B). By contrast, when VILIP-3-GFP was expressed the internalization of GC-B receptor still occured upon CNP-stimulation, similar to the GFP control-transfected neurons (compare Fig. 6E and F with 6A and B), showing the specificity of the VILIP-1 effect. The differences in the subcellular GC-B localization before and after CNP-stimulation in control-transfected and VILIP-1-transfected hippocampal neurons were categorized as intracellular, combined intracellular/cell surface or cell surface expression of GC-B and were quantified from 4 experiments (Table 2). In GFP control-transfected neurons the cell surface expression of GC-B decreases following CNP-stimulation from 20% to 0%, indicating internalization of the receptor. In VILIP-1-GFP-transfected neurons the cell surface localization is increased from 20% to 53% already without CNP-stimulation, whereas the intracellular localization is decreased from 33% to 0% when compared with GFP control-transfected cells. Following CNP-application the number of cells with cell surface localization slightly increases to 57%, showing that VILIP-1 interfered with receptor recycling under both, basal and CNP-stimulated, conditions. By contrast, VILIP-3-GFP did not interfere with internalization. In summary, VILIP-1 expression increases cell surface expression of GC-B by interfering with receptor recycling. These data are in line with the results obtained with C6/C6-V1 cells (Fig. 2B). Thereby, VILIP-1 influences the desensitization process of GC-B, a mechanism that most likely accounts for the effect of VILIP-1 on GC-B activity in primary hippocampal neurons.

Discussion

Previous results suggested the NCS protein VILIP-1 as a modulator of several neuronal signalling cascades: it has been implicated in modulation of adenylyl cyclase activity (Braunewell et al., 1997; Lin et al., 2002a; Mahloogi et al., 2003) and related cyclase enzymes, including the soluble and the membrane-localized GCs (Braunewell et al., 2001; Spilker et al., 2002b). Interestingly, these data were recently supported by findings that the related protein neurocalcin δ was found to interact with and directly influence related guanylyl cyclases, namely the retinal and the olfactory GCs (Krishnan et al., 2004; Duda et al., 2004). However, we have not found the same direct activation mechanism, although VILIP-1 can interact in a Ca^{2+}-dependent manner with the cytosolic catalytic domains of the cell surface receptors GC-A and GC-B in vitro (Braunewell et al., 2001). Moreover, VILIP-1 also interacts with a cytosolic domain of another membrane receptor, the α4β2 nAChR (Lin et al., 2002b). As in the case with GC-B, VILIP-1 influences surface expression and activity of the receptor. However, the precise role of the interaction of VILIP-1 with these two unrelated membrane-localized receptors for the effect on their surface expression and activity has still to be elucidated.

One of the mechanisms by which VILIP-1 modulates different neuronal signalling cascades seems to be the Ca^{2+}-myristoyl switch. Ca^{2+}-binding is necessary for the membrane localization of the protein (Braunewell et al., 2001), which is in line with the Ca^{2+}-myristoyl switch model of VILIP-1 signalling as a prerequisite for activation of receptor signalling systems (Braunewell et al., 2001; Spilker et al., 2002a; Lin et al., 2002b). Following a Ca^{2+} stimulus VILIP-1 translocates to trans-Golgi membranes and to cell surface membranes, e.g. in dendritic structures in hippocampal neurons and has thus been postulated to serve as a cellular signalling mechanism in living cells (Spilker et al., 2002a; Spilker and Braunewell, 2003).

Table 2. Quantification of the effect of VILIP-1-GFP and VILIP-3-GFP expression on the subcellular localization of GC-B in hippocampal neurons

	W/o stim			+CNP		
	GC-B cell surface	Both	GC-B intracellular	GC-B cell surface	Both	GC-B intracellular
GFP (15/33)	20.00	46.67	33.33	0	27.27	72.73
V1-GFP (17/21)	52.94	47.06	0	57.14	38.10	4.76
V3-GFP (13/9)	15.38	46.15	38.46	11.11	32.33	55.56

Number of cells with cell surface, combined cell surface/intracellular and cell surface localization of GC-B in GFP-transfected, VILIP-1-GFP- and VILIP-3-GFP-transfected hippocampal neurons with (+CNP) or without (w/o stim) CNP-stimulation. In parentheses the number of cells counted for the conditions w/o stim and CNP-stimulation, respectively, are shown (n=4).

Interestingly in this context, in PC12 cells it was shown that the effect of VILIP-1 greatly depends on the subcellular localization. Wild-type VILIP-1, which is localized in the cytosol and at the surface membrane, acts stronger at membrane-localized guanylyl cyclase A, whereas the myristoylation mutant VILIP-1, which is mainly localized in the cytosol more strongly enhances soluble guanylyl cyclase activity (Braunewell et al., 2001). We have made similar observations for GC-B in C6 cells (Fig. 1C). Moreover, the Ca^{2+}-dependent membrane association also seem to be important for the effect on another membrane receptor, the α4β2 nAChR. Mutants lacking the myristoylation or the calcium-binding sites have no effect on surface expression of the receptor (Lin et al., 2002b).

To further characterize the molecular mechanisms of the VILIP-1 effect on GC-B we have investigated the possible role of the modulation of cell surface expression for functional activity. Sustained stimulation of GCs by an agonist induces loss of functional receptors at the cell surface, leading to a reduction in responsiveness to subsequent stimulation. Internalization of two closely related receptors, GC-A and the enterotoxin receptor GC-C, has been shown to be a mechanism of desensitization (Pandey et al., 2002; Ghanekar et al., 2003), which, besides phosphorylation, regulates the activity of natriuretic peptide receptors (Lucas et al., 2000). VILIP-1 expression was shown to enhance cell surface expression of nAChRs (Lin et al., 2002b), thus, a particularly interesting possibility is that VILIP-1 may influence GC-B activity by affecting GC-B surface expression. Endocytosis, receptor recycling and associated desensitization of GC-A (Pandey, 2001; Pandey et al., 2002) show a similar kinetic profile as the VILIP-1 effect on guanylyl cyclase activity (this study), raising the possibility that the VILIP-1 effect is linked to receptor recycling. In our experiments VILIP-1 not only increased CNP-induced cGMP accumulation but clearly changed the surface expression of GCs upon ligand stimulation, i.e. more ligand bound to surface membranes of VILIP-1-expressing C6 cells. In parallel, VILIP-1 also influences the subcellular distribution of clathrin following CNP stimulation in C6 cells, indicating that clathrin-regulated membrane trafficking plays a role for GC-B function. Moreover, the transient expression of VILIP-1 in C6 cells also affected transferrin receptor recycling. The transferrin receptor has been used as prototype marker for clathrin-dependent receptor recycling (Maxfield and McGraw, 2004). Therefore, we conclude that in C6 cells VILIP-1 affects cGMP-signalling via a general effect of altering clathrin-dependent membrane trafficking of the GC-B receptor.

This notion is further supported by a circumstantial line of evidence. Various NCS proteins can interact with molecules connected to membrane trafficking, including interactions with α- and β-clathrin and β-2-adaptin (Ivings et al., 2002; Palmer et al., 2001). Furthermore, NCS proteins like the KChIPs and NCS-1 have already been shown to influence surface expression of Ca^{2+} and potassium channels (An et al., 2000; Weiss et al., 2000; Zhao et al., 2001). NCS-1 interacts with ARF1 to control trans-Golgi network-plasma membrane trafficking (Haynes et al., 2005). For VILIP-1 it was shown that it can interact with the cortical actin cytoskeleton (Lenz et al., 1996; Spilker et al., 2002a), which is also involved in the endocytosis process; VILIP-1 influences rhoA activity, a modulator of the cortical cytoskeleton (Mahloogi et al., 2003), and finally VILIP-1 is able to increase cell surface expression of the α4β2 nicotinic acetylcholine receptor (Lin et al., 2002b). A general role for VILIP-1 in membrane transport and receptor trafficking is supported by its localization in raft membranes (Orito et al., 2001) and at the Golgi (Spilker et al., 2002a), membrane compartments known to be involved in endocytosis (Herreros et al., 2001). Recently, VILIP-1 was found to be associated with clathrin-coated vesicles from rat brain, suggesting an involvement in clathrin-dependent trafficking (Blondeau et al., 2004). Therefore, it appears more than likely that VILIP-1, directly or indirectly, affects general membrane trafficking in neurons.

Thus, in primary hippocampal neurons, a more physiological environment, the relevance of the effect of VILIP-1 on GC-B surface expression was further investigated. VILIP-1 co-localized with GC-B in many but not all cultured hippocampal neurons, and also significantly enhanced CNP-stimulated cGMP accumulation following transient transfection with the VILIP-1 cDNA. Moreover, heterologous VILIP-1-expression led to an increased surface expression and attenuation of GC-B internalization, which was not observed with the highly homologous protein VILIP-3. It has been previously shown that in the case of GC-A following ligand activation about 40-50% of internalized ligand-receptor complexes recycle back to the plasma membrane with an apparent half life, $t_{1/2}$, of 8 minutes (Pandey et al., 2002). It is conceivable that within the 20 minutes time span of our cGMP-measurement an effect on changed membrane transport and recycling may account for the VILIP-1 effect on GC activity. We examined the role of membrane transport in hippocampal neurons and asked whether VILIP-1, for example by influencing endocytosis (PAO) or receptor recycling (monensin, wortmannin), may prevent desensitization of GC-B. The application of the general inhibitor of receptor-mediated endocytosis PAO as well as the sodium ionophore monensin, an agent known to prevent receptor recycling, significantly reduced the GC-B activity and the effect of VILIP-1 on GC-B activity. Monensin, by raising the pH within the recycling endosomal compartment, disrupts trafficking

pathways, but leaves endocytosis unaffected (Mollenhauer et al., 1990). As revealed by the pharmacological inhibition of membrane trafficking we could show that the effect of influencing receptor recycling is sufficient to explain a modulatory effect on cGMP accumulation, such as the VILIP-1 effect on GC-B activity.

The inhibition of endocytosis by PAO and thus, the increased surface expression, lead to a reduced GC-B activity in normal and VILIP-1-overexpressing neurons. A similar effect has been described earlier (Gray et al., 2001), where the increased surface expression of a serotonin receptor resulting from the inhibition of endocytosis by PAO, was associated with receptor desensitization and thus, reduced activity, and additionally blocked the possibility for recycling of new resensitized receptor. Monensin does the opposite, it prevents receptor recycling and thus, decreases surface expression. It has the same effect as PAO, namely a decrease in GC-B activity, by means of blocking the possibility for recycling of newly resensitized receptors. Another agent interfering with receptor recycling, wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor, had no significant effect on cGMP accumulation in non-transfected and in VILIP-1-transfected cells. Recent studies on transferrin recycling suggest that two distinct recycling pathways exist (Sheff et al., 1999; van Dam et al., 2002; van Dam and Stoorvogel, 2002). Transferrin partially recycles via a monensin-sensitve pathway through the recycling endosomes (slow pathway, $t_{1/2}$=10-15 minutes), whereas another part of internalized transferrin takes a fast, wortmannin (PI3K)-sensitive recycling route from the sorting endosomes directly back to the plasma membrane (fast recycling, $t_{1/2}$=2-3 minutes) (Sheff et al., 1999). The fast route that bypasses recycling endosomes seems to strongly depend on PI3K since it is inhibited by wortmannin leaving the slow pathway via recycling endosomes unaffected (van Dam et al., 2002). Similar data obtained using the drugs monensin and wortmannin have been found for the epidermal growth factor receptor, where the application of monensin, but not wortmannin inhibited receptor recycling (Chen and Wang, 2001). Given that monensin, but not wortmannin inhibits the effect of VILIP-1 on cGMP accumulation via GC-B, it is likely that VILIP-1 exerts its effect via a wortmannin (PI3K)-insensitive, but monensin-sensitve mechanism of receptor trafficking. Thus, the GC-B desensitization mechanism via receptor recycling seems to depend on a slow pathway (10-15 minutes) via the recycling endosomes. These results fit to the kinetics of the VILIP-1 effect on GC activity (this study) and the observed receptor half-life during desensitization of the related receptor GC-A (Pandey et al., 2002). Moreover, a very similar effect of monesin on the recycling of GC-A, where monensin blocked the reappearance of internalized receptors on the cell surface, was described in PC12 cells (Rathinavelu and Isom, 1991).

In summary, heterologous expression of VILIP-1 clearly increases basal and CNP-stimulated surface expression of GC-B, which is directly linked to the modulation of basal and CNP-induced GC-B activity. The co-localization of VILIP-1 with GC-B in hippocampal neurons and in the soma and dendritic membranes suggests that VILIP-1 may also influence GC activity under physiological conditions in the hippocampus in vivo. In the context with the Ca^{2+}-dependent localization of VILIP-1 at the trans-Golgi network and dendritic membrane specializations in hippocampal neurons (Spilker et al., 2002a) it is conceivable that GC-B and VILIP-1 may influence neuronal signalling in dendrites of hippocampal neurons in a Ca^{2+}- and cGMP-dependent manner. As shown in detail for the example of GC-B, VILIP-1 appears as a general regulator of clathrin mediated events throughout the cell. VILIP-1 effects include modulation of surface expression of the nAChR (Lin et al., 2002), receptor recycling of GC-B and the transferrin receptor (this study) and most likely modulation of a set of other neuronal receptors can be expected. Through this global mechanism on receptor trafficking VILIP-1 might contribute to processes of synaptic plasticity under physiological and pathological conditions in the hippocampus.

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References

An, W. F., Bowlby, M. R., Betty, M., Cao, J., Ling, H. P., Mendoza, G., Hinson, J. W., Mattsson, K. I., Strassle, B. W., Trimmer, J. S. et al. (2000). Modulation of A-type potassium channels by a family of calcium sensors. *Nature* **403**, 553-556.

Bahi, N., Friocourt, G., Carrie, A., Graham, M. E., Weiss, J. L., Chafey, P., Fauchereau, F., Burgoyne, R. D. and Chelly, J. (2003). IL1 receptor accessory protein like, a protein involved in X-linked mental retardation, interacts with Neuronal Calcium Sensor-1 and regulates exocytosis. *Hum. Mol. Genet.* **12**, 1415-1425.

Beattie, E. C., Howe, C. L., Wilde, A., Brodsky, F. M. and Mobley, W. C. (2000). NGF signals through TrkA to increase clathrin at the plasma membrane and enhance clathrin-mediated membrane trafficking. *J. Neurosci.* **20**, 7325-7333.

Bernstein, H.-G., Braunewell, K.-H., Spilker, C., Danos, P., Baumann, B., Diekmann, S., Gundelfinger, E. D. and Bogerts, B. (2002). Hippocampal expression of the calcium sensor protein VILIP-1 in schizophrenia. *NeuroReport* **23**, 393-396.

Bernstein, H.-G., Becker, A., Keilhoff, G., Gorcyca, W. A., Braunewell, K.-H. and Grecksch, G. (2003). Ketamine induced changes of the expression of neuronal calcium sensor proteins VILIP-1, -3 and hippocalcin in a partial schizophrenia model in rats. *Neurosci. Lett.* **339**, 95-98.

Blondeau, F., Ritter, B., Allaire, P. D., Wasiak, S., Girard, M., Hussain, N. K., Angers, A., Legendre-Guillemin, V., Roy, L., Boismenu, D. et al. (2004). Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. *Proc. Natl. Acad. Sci. USA* **101**, 3833-3838.

Braunewell, K.-H., Spilker, C., Behnisch, T. and Gundelfinger, E. D. (1997). The neuronal calcium-sensor protein VILIP modulates cAMP accumulation in stably transfected C6 glioma cells: amino-terminal myristoylation determines functional activity. *J. Neurochem.* **68**, 2129-2139.

Braunewell, K.-H. and Gundelfinger, E. D. (1999). Intracellular neuronal calcium sensor proteins: a family of EF-hand calcium-binding proteins in search of a function. *Cell Tissue Res.* **295**, 1-12.

Braunewell, K.-H., Brackmann, M., Schaupp, M., Spilker, C., Anand, R. and Gundelfinger, E. D. (2001). Intracellular neuronal calcium sensor (NCS) protein VILIP-1 modulates cGMP signalling pathways in transfected neural cells and cerebellar granule neurones. *J. Neurochem.* **78**, 1277-1286.

Braunewell, K. H., Brackmann, M. and Manahan-Vaughan, D. (2003). Group I mGlu receptors regulate the expression of the neuronal calcium sensor protein VILIP-1 in vitro and in vivo: implications for mGlu receptor-dependent hippocampal plasticity? *Neuropharmacology.* **44**, 707-715.

Burgoyne, R. D., O'Callaghan, D. W., Hasdemir, B., Haynes, L. P. and Tepikin, A. V. (2004). Neuronal Ca(2+)-sensor proteins: multitalented regulators of neuronal function. *Trends Neurosci.* **27**, 203-209.

Chen, X. and Wang, Z. (2001). Regulation of epidermal growth factor receptor endocytosis by wortmannin through activation of Rab5 rather than inhibition of phosphatidylinositol 3-kinase. *EMBO Rep.* **2**, 842-849.

Cheng, H. Y., Pitcher, G. M., Laviolette, S. R., Whishaw, I. Q., Tong, K. I., Kockeritz, L. K., Wada, T., Joza, N. A., Crackower, M., Goncalves, J. et al. (2002). DREAM is a critical transcriptional repressor for pain modulation. *Cell* **108**, 31-43.

Duda, T., Fik-Rymarkiewicz, E., Venkataraman, V., Krishnan, A. and Sharma, R. K. (2004). Calcium-modulated ciliary membrane guanylate

cyclase transduction machinery: constitution and operational principles. *Mol. Cell. Biochem.* **267**, 107-122.

Fiscus, R. R. (2002). Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells. *Neurosignals* **11**, 175-190.

Genin, A., Davis, S., Meziane, H., Doyere, V., Jeromin, A., Roder, J., Mallet, J. and Laroche, S. (2001). Regulated expression of the neuronal calcium sensor-1 gene during long-term potentiation in the dentate gyrus in vivo. *Neuroscience* **106**, 571-577.

Ghanekar, Y., Chandrashaker, A. and Visweswariah, S. S. (2003). Cellular refractoriness to the heat-stable enterotoxin peptide is associated with alterations in levels of the differentially glycosylated forms of guanylyl cyclase C. *Eur. J. Biochem.* **270**, 3848-3857.

Gray, J. A., Sheffler, D. J., Bhatnagar, A., Woods, J. A., Hufeisen, S. J., Benovic, J. L. and Roth, B. L. (2001). Cell-type specific effects of endocytosis inhibitors on 5-hydroxytryptamine(2A) receptor desensitization and resensitization reveal an arrestin-, GRK2-, and GRK5-independent mode of regulation in human embryonic kidney 293 cells. *Mol. Pharmacol.* **60**, 1020-1030.

Haynes, L. P., Thomas, G. M. and Burgoyne, R. D. (2005). Interaction of neuronal calcium sensor-1 and ADP-ribosylation factor 1 allows bidirectional control of phosphatidylinositol 4-kinase {beta} and trans-Golgi network-plasma membrane traffic. *J. Biol. Chem.* **280**, 6047-6054.

Herman, J. P., Dolgas, C. M., Rucker, D. and Langub, M. C., Jr (1996). Localization of natriuretic peptide-activated guanylate cyclase mRNAs in the rat brain. *J. Comp. Neurol.* **369**, 165-187.

Herreros, J., Ng, T. and Schiavo, G. (2001). Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. *Mol. Biol. Cell* **12**, 2947-2960.

Ivings, L., Pennington, S. R., Jenkins, R., Weiss, J. L. and Burgoyne, R. D. (2002). Identification of Ca2+-dependent binding partners for the neuronal calcium sensor protein neurocalcin delta: interaction with actin, clathrin and tubulin. *Biochem. J.* **363**, 599-608.

Kabbani, N., Negyessy, L., Lin, R., Goldman-Rakic, P. and Levenson, R. (2002). Interaction with neuronal calcium sensor NCS-1 mediates desensitization of the D2 dopamine receptor. *J. Neurosci.* **22**, 8476-8486.

Krishnan, A., Venkataraman, V., Fik-Rymarkiewicz, E., Duda, T. and Sharma, R. K. (2004). Structural, biochemical, and functional characterization of the calcium sensor neurocalcin delta in the inner retinal neurons and its linkage with the rod outer segment membrane guanylate cyclase transduction system. *Biochemistry* **43**, 2708-2723.

Lenz, S. E., Henschel, Y., Zopf, D., Voss, B. and Gundelfinger, E. D. (1992). VILIP, a cognate protein of the retinal calcium binding proteins visinin and recoverin, is expressed in the developing chicken brain. *Brain Res. Mol. Brain Res.* **15**, 133-140.

Lin, L., Braunewell, K.-H., Gundelfinger, E. D. and Anand, R. (2002a). Functional analysis of calcium-binding EF-hand motifs of Visinin-like Protein-1. *Biochem. Biophys. Res. Commun.* **296**, 827-832.

Lin, L., Jeanclos, E. M., Treuil, M., Braunewell, K.-H., Gundelfinger, E. D. and Anand, R. (2002b). The calcium sensor protein Visinin-like Protein-1 modulates the surface expression and agonist-sensitivity of the α4β2 nicotinic acetylcholine receptor. *J. Biol. Chem.* **277**, 41872-41878.

Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P. and Waldman, S. A. (2000). Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol. Rev.* **52**, 375-414.

Mahloogi, H., Gonzalez-Guerrico, A. M., de Cicco, R. L., Bassi, D. E., Goodrow, T., Braunewell, K.-H. and Klein-Szanto, A. J. P. (2003). Graduate decrease of VILIP-1 Expression during mouse skin tumor progression and its role in regulating tumor cell invasive behaviour. *Cancer Res.* **63**, 4997-5004.

Maxfield, F. R. and McGraw, T. E. (2004). Endocytic recycling. *Nat. Rev. Mol. Cell. Biol.* **5**, 121-132.

Mollenhauer, H. H., Morre, D. J. and Rowe, L. D. (1990). Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta* **1031**, 225-246.

Monfort, P., Munoz, M. D., Kosenko, E. and Felipo, V. (2002). Long-term potentiation in hippocampus involves sequential activation of soluble guanylate cyclase, cGMP-dependent protein kinase, and cGMP-degrading phosphodiesterase. *J. Neurosci.* **22**, 10116-10122.

O'Callaghan, D. W., Ivings, L., Weiss, J. L., Ashby, M. C., Tepikin, A. V. and Burgoyne, R. D. (2002). Differential use of myristoyl groups on neuronal calcium sensor proteins as a determinant of spatio-temporal aspects of Ca2+ signal transduction. *J. Biol. Chem.* **277**, 14227-14237.

Orito, A., Kumanogoh, H., Yasaka, K., Sokawa, J., Hidaka, H., Sokawa, Y. and Maekaw, S. (2001). Calcium-dependent association of annexin VI, protein kinase C alpha, and neurocalcin alpha on the raft fraction derived from the synaptic plasma membrane of rat brain. *J. Neurosci. Res.* **64**, 235-241.

Palmer, C. L., Burbidge, S. A. and Henley, J. M. (2001). Hippocalcin and its interaction with β2 adaptin. *Abstr. Soc. Neurosci.* **27**, 528.

Pandey, K. N. (2001). Dynamics of internalization and sequestration of guanylyl cyclase/atrial natriuretic peptide receptor-A. *Can. J. Physiol. Pharmacol.* **79**, 631-639.

Pandey, K. N., Nguyen, H. T., Sharma, G. D., Shi, S. J. and Kriegel, A. M. (2002). Ligand-regulated internalization, trafficking, and down-regulation of guanylyl cyclase/atrial natriuretic peptide receptor-A in human embryonic kidney 293 cells. *J. Biol. Chem.* **277**, 4618-4627.

Rathinavelu, A. and Isom, G. E. (1991). Differential internalization and processing of atrial-natriuretic-factor B and C receptor in PC12 cells. *Biochem. J.* **276**, 493-497.

Schnurra, I., Bernstein, H. G., Riederer, P. and Braunewell, K. H. (2001). The neuronal calcium sensor protein VILIP-1 is associated with amyloid plaques and extracellular tangles in Alzheimer's disease and promotes cell death and tau phosphorylation in vitro: a link between calcium sensors and Alzheimer's disease? *Neurobiol. Dis.* **8**, 900-909.

Schuman, E. M. and Madison, D. V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* **254**, 1503-1506.

Sheff, D. R., Daro, E. A., Hull, M. and Mellman, I. (1999). The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions, *J. Cell Biol.* **145**, 123-139.

Sippy, T., Cruz-Martin, A., Jeromin, A. and Schweizer, F. E. (2003). Acute changes in short-term plasticity at synapses with elevated levels of neuronal calcium sensor-1. *Nat. Neurosci.* **6**, 1031-1038.

Spilker, C., Dresbach, T. and Braunewell, K.-H. (2002a). Reversible translocation and activity-dependent localization of the calcium-myristoyl switch protein VILIP-1 to different membrane compartments in living hippocampal neurons. *J. Neurosci.* **22**, 7331-7339.

Spilker, C., Gundelfinger, E. D. and Braunewell, K.-H. (2002b). Different properties of the intracellular neuronal calcium sensor proteins VILIP-1 and VILIP-3: from subcellular localization to cellular function. *Proteins Proteomics* **1600**, 118-127.

Spilker, C. and Braunewell, K. H. (2003). Calcium-myristoyl switch, subcellular localization, and calcium-dependent translocation of the neuronal calcium sensor protein VILIP-3, and comparison with VILIP-1 in hippocampal neurons. *Mol. Cell Neurosci.* **24**, 766-778.

Telegdy, G. (1994). The action of ANP, BNP and related peptides on motivated behavior in rats. *Rev. Neurosci.* **5**, 309-315.

van Dam, E. M. and Stoorvogel, W. (2002). Dynamin-dependent transferrin receptor recycling by endosome-derived clathrin-coated vesicles. *Mol. Biol. Cell* **3**, 169-182.

van Dam, E. M., Ten Broeke, T., Jansen, K., Spijkers, P. and Stoorvogel, W. (2002). Endocytosed transferrin receptors recycle via distinct dynamin and phosphatidylinositol 3-kinase-dependent pathways. *J. Biol. Chem.* **277**, 48876-48883.

Weiss, J. L., Archer, D. A. and Burgoyne, R. D. (2000). NCS-1/Frequenin functions in an autocrine pathway regulating Ca2+ channels in bovine adrenal chromaffin cells. *J. Biol. Chem.* **275**, 40082-40087.

Zhao, X., Varnai, P., Tuymetova, G., Balla, A., Toth, Z. E., Oker-Blom, C., Roder, J., Jeromin, A. and Balla, T. (2001). Interaction of neuronal calcium sensor-1 (NCS-1) with phosphatidylinositol 4-kinase beta stimulates lipid kinase activity and affects membrane trafficking in COS-7 cells. *J. Biol. Chem.* **276**, 40183-40189.