III. Materials and methods

1. Antibodies

Rabbit polyclonal antibodies against VILIP-1 and hippocalcin raised against recombinant His-tagged VILIP-1 or hippocalcin fusion proteins and affinity-purified as previously described (Braunewell et al., 2001; Bernstein et al., 2003) were used. Other antibodies were purchased from indicating companies:

Chicken polyclonal antibody against NCS-1	Calbiochem Temecula, USA
mAb arg3.1/arc	Santa Cruz Biotechnology, USA
Mouse anti-GFAP	DAKO, USA
Rabbit anti-Parvalbumin PV	Swant, Switzerland
Rabbit anti-CR	Swant, Switzerland
Rabbit anti-Calbindin-D28k	Swant, Switzerland
mAb299 nAChR	Covance, USA
Mouse anti NeuN	Chemicon, Germany
Guinea pig anti-α4 nAChR polyclonal antibody	Chemicon, Germany
Mouse anti GAD67	Chemicon, Germany
Mouse anti-Tubulin	Sigma, USA

2. *In vivo* application of drugs

Drug application (Tocris Cookson, Bristol, UK) was carried out via a cannula implanted in the ipsilateral cerebral ventricle of male Wistar rats (8- to 10-week old). The group I mGluR agonist DHPG (18.3 µg in 5 µl) and/or group I mGluR antagonist 4CPG (19.52 µg in 5 µl) or vehicle for controls (5 µl of 0.9% NaCl) were injected. Ipsilateral hippocampi were removed 2, 4, 8, and 24 h following DHPG or vehicle application. The drug application and implantation procedure was equivalent to our previously reported method where mGluR agonist application induced slow-onset potentiation which was recorded after implantation of stimulating and recording electrodes into the Schaffer collaterals and stratum radiatum of the CA1 region, or alternatively in the medial perforant path and the granule cell layer of the dentate gyrus (DG), respectively (Braunewell et al., 2003; Manahan-Vaughan et al., 1996; Manahan-Vaughan and Braunewell, 1999). All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

3. Western blotting and immunodetection

Rat tissue homogenates were lysed with 1% SDS, 1% Triton X-100 in phosphate-buffered saline (PBS, pH 7.6) including a protease inhibitor cocktail (Boehringer–Mannheim, Germany). Protein concentrations were determined using a BCA kit (Pierce, Rockfort, IL, USA). For all examined peripheral tissues 50 µg protein (except for testis 10 µg) and as control 20 µg of brain tissue (hippocampus) were separated on a 5–20% gradient SDS–polyacrylamide gel using the Laemmli buffer system (Laemmli, 1970) and blotted onto PVDF. After blocking of unspecific binding sites for 2 h with blocking buffer (5% low-fat milk powder, 0.1% Tween 20 in TBS) the membranes were incubated overnight at 4 °C with rabbit polyclonal anti-VILIP-1 antibody, or chicken polyclonal anti-NCS-1 antibody, respectively. The immunoreactivity was visualized using HRP-coupled goat anti-rabbit or anti-chicken antibodies (Dianova, Hamburg, Germany) and the ECL chemiluminescence detection system (Amersham Biosciences, Freiburg, Germany).

4. Western blot analysis of rat hippocampi

Homogenates of hippocampi which had been treated without DHPG (vehicle control), with DHPG (18.3 µg in 5 µl), with DHPG/4CPG (18.3 and 19.52 µg in 5 µl) and with 4CPG alone (19.52 µg in 5 µl) for 24 h, or DHPG-treated (18.3 µg in 5 µl) ipsilateral hippocampi which were removed 2, 4, 8, and 24 h after experimental manipulations were applied to 5–20% gradient SDS–PAGE and Western blotting was performed as described. The blots were incubated overnight at 4 °C with affinity-purified rabbit polyclonal antibodies against VILIP-1 (Braunewell et al., 1997; Spilker et al., 2002) chicken polyclonal antibodies against NCS-1 (Chemicon, Temecula, USA), arg3.1/arc (Santa Cruz Biotechnology, Santa Cruz USA) (Guthrie et al., 2000) and tubulin (Sigma, St. Louis, USA) followed by secondary antibodies coupled to alkaline phosphatase. The immunoreactivity was visualized with nitroblue-tetrazolium-chloride and 5-bromo-4-chloro-3-indoyl-phosphate. Alternatively immunoreactivity was visualized using HRP-coupled goat anti-rabbit or goat anti-chicken secondary antibodies (Dianova, Hamburg, Germany) and the ECL detection system (Amersham Biosciences, Freiburg, Germany). Protein concentrations were measured with the BCA-Kit (Pierce, Rockfort, USA).

5. Western blot data analysis

Quantification of the Western blots was done by densitometric analysis using the NIH Image program v1.61 (National Institutes of Health, Bethesda, MD, available at http://rsb.info.nih.gov/nih-image/). The ratio of the densities for VILIP-1, NCS-1, and tubulin in controls was set to 100%. The relative expression level of NCS-1/VILIP-1 was expressed as the ratio of the densities for NCS-1/VILIP-1 and tubulin in the experimental conditions as a percentage of the control ratio. The relative expression levels in percentage of control are

expressed as mean values ± standard deviations (SD). Where appropriate the statistical significance of the differences was determined by Student's *t* test or for the time course experiments by ANOVA with post hoc Fisher's PLSD. Values of $p < 0.05$ were considered as statistically significant (*).

6. Immunofluorescence and fluorescence imaging

Five adult Wistar rats (180–200 g) were anesthetized with diethyl ether and decapitated. Brains were removed rapidly and 400-µm-thick horizontal hippocampal slices were sectioned using a vibroslice (Campden MA752). Sections were fixed with 4% paraformaldehyde at 4 °C overnight and then cryoprotected in a 30% sucrose solution at room temperature. 40 µm thick frozen sections were prepared again (Leica microtome RM2055).

The free-floating 40 µm horizontal sections were rinsed with PBS, preincubated with 3% normal goat serum for 30 minutes, and then incubated at 4 °C for overnight with rabbit or rat anti-VILIP-1 (1:1,000), (Braunewell et al., 2001, 1997), with Mouse anti NeuN (1:1000 Chemicon), GFAP (1:1000 DAKO), rabbit anti-Parvalbumin (PV) (1:1,000; Swant), rabbit anti-CR (1:1,000, Swant), rabbit anti-Calbindin-D28k (1:1,000, Swant), Antibodies against the α-subunit of the nAChR were mAb299 (Covance, Berkeley, CA, USA) and Guinea pig anti-α4 nAChR polyclonal antibody (Chemicon, Germany), respectively with 0.3% Triton X-100 in PBS, except mouse anti GAD67 (1:1,000 Chemicon) were incubated with rabbit anti-VILIP-1 at room temperature for 3 days. After washing the sections were incubated at room temperature with corresponding fluorescent, secondary antibodies used, which were Alexa 488-conjugated or Cy3 (all 1:1000; Jackson Laboratories, distributor: Dianova) diluted 1:1000 in 3% normal goat serum. For immunochemistry, sections were rinsed thoroughly in PBS and were stained by using the peroxidase method (ABC system with diaminobenzidine as chromogen, Vector Laboratories). All sections were mounted on slides with Mowiol (Calbiochem), including 1,4-diazobicyclo-[2.2.2]-octane (Merck, Darmstadt, Germany) to reduce fading.

Conventional fluorescence microscopy was performed on a Leica DMR fluorescence microscope for visualizing Alexa488, and Cy3 fluorescence. Alexa488, Cy3, and Cy5 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. To avoid any photodamage, the lowest laser intensity necessary for an adequate signal-to-noise ratio was used. Images were recorded digitally and processed

using the Adobe Photoshop 7 (Adobe Systems, San Jose, CA) and the NIH image J1.36 software (National Institutes of Health, Bethesda, MD) (available at http://rsb.info.nih.gov/).

7. Cell culture, transient transfection and stable human α4β2 nAChR transfected HEK293 cell

The stably expressed human α4β2 nAChR (Hα4β2L$^-$/1) with the following denotations: H, human; α4β2 nAChR subtype composed of α4, β2 subunits; L$^-$, HEK-293 cells that do not express the $L\alpha_{1C\text{-}b}Ca^{2+}$ channel 1, clone 1, generously donated by Dr. A Maelicke, was used in the experiments. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 13% fetal calf serum (FCS) and maintained in an incubator at 37°C, 10% CO_2. To keep the cells in the exponential phase of growth, they were harvested every 3 days and plated at a density of 1.5×10^4 cells/cm^2.

8. Primary hippocampal neurons culture and transient transfection

Neuronal cultures were prepared from hippocampi of 18-day-old fetal Wistar rats essentially following the protocol of Goslin and Banker (Goslin and Banker, 1998). Briefly, hippocampi were dissociated by enzyme digestion with 0.1% trypsin at 37°C for 20 min, followed by triturating through two different-sized syringes. Cells were plated onto poly-D-lysine-coated glass coverslips (12 mm in diameter) at a density of 60×10^3 cells for transient transfection experiments and 10-20 $\times 10^3$ cells for immunocytochemistry in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Twenty-four hours after plating, the culture medium was exchanged for Neurobasal medium (Invitrogen) supplemented with 2% B27, 0.5 mM L-glutamine, antibiotics, and cells were maintained in a humidified 37°C atmosphere containing 5% CO_2. The standard transfection protocol of Lipofectmine2000, as mentioned before, was applied in different experiments as follows:

Table 1 **Transfection protocol for patch clamp experiments**

Age of neuron / Experiment	Age of hippocampal neuron when performed transfection	Age of hippocampal neuron performed patching clamp	Constructs ratio
α4β2 nAChR current measurement	DIV 7	DIV 9	α4:β2:NSE-GFP/ VILIP-1-GFP 10:10:1
IPSCs measurements	DIV7	DIV14-15	NSE-GFP/ VILIP-1-GFP
Intrinsic properties measurement	DIV14	DIV 15-16	NSE-VILIP-1-GFP

9. Immunocytochemistry for cultured hippocampal neurons

Hippocampal neurons were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 minutes at room temperature. Before permeabilization, cells were washed twice in PBS. Subsequently, the

cells were permeabilized and blocked in 0.3% Triton X-100, 3% goat serum in PBS (blocking solution) for 30 min. Cells were incubated with primary antibodies diluted in blocking solution at 4°C overnight. After washing three times with PBS, secondary antibodies diluted in blocking solution, without Triton X-100, were applied to the neurons for 1 hr 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.

10. Electrophysiological measurements

10. 1 Measurement of α4β2 nAChR currents in HEK293 cells and hippocampal neurons

Whole-cell current recordings from stably transfected cells were performed 12 to 24 h after transfection, according to patch-clamp techniques using an EPC-7 patch-clamp system (HEKA Elektronik, GmbH, Germany). The bathing solution was composed of (mM) 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose and 10 mM HEPES (pH 7.3; 300 Osm), and the internal pipette solution contained 140 mM CsCl (equilibrated with CsOH), 11mM EGTA, 10 mM HEPES and 1 mM $MgCl_2$ (pH 7.3; 300 Osm). In patch recordings of hippocampal neurons, whole-cell current recordings from stably transfected cells were performed 48 h after transfection. Sodium channels, NMDA, AMPA, $GABA_A$ receptors and α7 nAChR were blocked by 0.5 μM TTX (Tetrodotoxin citrate, Tocris UK), 30μM D-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid, Sigma), 5μM bicuculline (Sigma), 30μM CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione, Sigma), 1nM MLA (methylallylaconitine, Sigma), which were included in Artificial cerebro-spinal fluid (ACSF). ACSF containing 129 mM NaCl, 21 mM $NaHCO_3$, 3 mM KCl, 1.6 mM $CaCl_2$, 1.8 mM $MgSO_4$, 1.25 mM NaH_2PO_4 and 10 mM glucose (pH 7.4). The internal solution contained the following (in mM): potassium gluconate 136.5, KCl 17.5, NaCl 9, $MgCl_2$ 1, HEPES 10, EGTA 0.2, Mg-ATP 2, GTP, 0.3, pH 7.20.The microelectrodes were pulled from borosilicate glass (external diameter 2.0 mm), and the resistance of the pipettes was 2-5 MΩ using the external and internal solutions described above. After formation of a high-resistance seal with the cell under investigation, capacitance transients were minimized using the C-Fast facility of the system. No compensation was made for series resistance. Whole-cell currents were induced by fast application of the test substances by means of a single patch pipette or home made multiple micropipettes with a joined tip, positioned near the cell. The cells were superfused with the bathing solution at a rate of 1.5-2.0 ml/min. All experiments were done at room temperature. Atrophine (1 μM) was included in the bathing solutions in order to inhibit intrinsic muscarinic response. Signals were low-pass (Bessel) filtered at 3 kHz (whole-cell measurements) analyzed on a PC using the TIDA software package version3 (HEKA Elektronik, GmbH, Germany). All data are presented as mean + standard error.

10. 2 Measurement of inhibitory postsynaptic currents (IPSCs) in hippocampal neurons

Hippocampal neurons transfected with GFP alone or GFP-NSE-VILIP-1 for 5-6 days, were visualized by fluorescence microscopy and whole cell model patching clamp was performed. To record the 10μM ACh by bath application evoked IPSCs mediated via non-α7 nAChR, 1μM atrophine, 30μM D-AP5, 30μM CNQX, 5nM MLA were included in external solution (ACSF) to block muscarinic acetylcholine, NMDA, AMPA, and α7nACh receptors. The internal solution contained the following (in mM): potassium gluconate 136.5, KCl 7.5, NaCl 9, $MgCl_2$ 1, HEPES 10, Mg-ATP 2, GTP, 0.3, pH 7.20. Data was analyzed using the Minianalysis software.

10. 3 Measurement of intrinsic properties in hippocampal neurons

Whole-cell current recordings from transient VILIP-1-GFP transfected cells were performed 1-2 days after transfection, according to patch-clamp techniques using an EPC-7 patch-clamp system (HEKA Elektronik, GmbH, Germany). ACSF as extracellular buffer (ACSF containing 129 mM NaCl, 21 mM $NaHCO_3$, 3 mM KCl, 1.6 mM $CaCl_2$, 1.8 mM $MgSO_4$, 1.25 mM NaH_2PO_4 and 10 mM glucose (pH 7.4). The internal solution contained the following (in mM): potassium gluconate 136.5, KCl 17.5, NaCl 9, $MgCl_2$ 1, HEPES 10, EGTA 0.2, Mg-ATP 2, GTP, 0.3, pH 7.20 flows 2ml/min. The microelectrodes were pulled from borosilicate glass (external diameter 2.0 mm), and the resistance of the pipettes was 2-5 MΩ using the external and internal solutions described above. After formation of a high-resistance seal with the cell under investigation, capacitance transients were minimized using the C-Fast facility of the system. No compensation was made for series resistance. Whole-cell currents were induced by fast application of the test substances by means of a single patch pipette or home made multiple micropipettes with a joined tip, positioned near the cell. The cells were superfused with the bathing solution at a rate of 1.5-2.0 ml/min. All experiments were done at room temperature. Signals were low-pass (Bessel) filtered at 3 kHz (whole-cell measurements) analyzed on a PC using the TIDA software package version3 (HEKA Elektronik, GmbH, Germany). All data are presented as mean ± standard deviation.