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Neuronal Ca²⁺ sensor VILIP-1 leads to the upregulation of functional $\alpha 4\beta 2$ nicotinic acetylcholine receptors in hippocampal neurons

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Abstract

The neuronal Ca²⁺-sensor protein VILIP-1, known to affect clathrin-dependent receptor trafficking, has been shown to interact with the cytoplasmic loop of the $\alpha 4$ -subunit of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChR), which is the most abundant nAChR subtype with high-affinity for nicotine in the brain. The $\alpha 4\beta 2$ nAChR is crucial for nicotine addiction and the beneficial effects of nicotine on cognition. Its dysfunction has been implicated in frontal lobe epilepsy, Alzheimer's disease and schizophrenia. Here we report that overexpression of VILIP-1 enhances ACh responsiveness, whereas siRNA against VILIP-1 reduces $\alpha 4\beta 2$ nAChR currents of hippocampal neurons. The underlying molecular mechanism likely involves enhanced constitutive exocytosis of $\alpha 4\beta 2$ nAChRs mediated by VILIP-1. The two interaction partners co-localize in a Ca²⁺-dependent manner with syntaxin-6, a Golgi-SNARE protein involved in trans-Golgi membrane trafficking. Thus, we speculate that regulation of VILIP-1-expression might modulate surface expression of ligand-gated ion channels, such as the $\alpha 4\beta 2$ nAChRs, possibly comprising a novel form of physiological up-regulation of ligand-gated ion channels.

Introduction

Neuronal Ca²⁺-sensor (NCS) proteins are involved in a variety of Ca²⁺-dependent signal transduction processes, including exocytosis, modulation of receptor-, ligand-gated ion channel- and signal effector function (Ikura and Ames, 2006; Burgoyne, 2007). VILIP-1 (visinin-like protein-1, *VSNLI*), belonging to the subfamily of VILIPs, has been shown to affect

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several signaling pathways, such as cAMP- as well as cGMP-signaling. It has also been implicated in clathrin-dependent receptor trafficking of the transferrin receptor and the receptor guanylyl cyclase GC-B (Braunewell and Gundelfinger, 1999; Brackmann et al., 2005). More recently, VILIP-1 was identified in a functional protein complex with glutamate receptors of the kainate subtype GluR6 (Coussen et al., 2005), and form a signaling complex with P2X2 receptors in which it regulates surface expression of the receptor and its sensitivity and peak response to ATP (Chaumont et al., 2008). Moreover, VILIP-1 is a modulator of another ligand-gated ion channel, the $\alpha 4\beta 2$ nicotinic acetylcholine receptors (nAChRs) that increases their surface expression levels and agonist sensitivity in oocytes (Lin et al., 2002). The nAChRs are activated by the endogenous neurotransmitter acetylcholine (ACh) and are cation-permeable channels. Pentameric nAChRs arise from eight subunits ($\alpha 2$ -4, 6-10) which form the ACh binding site, and four subunits ($\alpha 5$, $\beta 2$ -4) which have a structural function but also contribute to the binding site. The $\alpha 4\beta 2$ is the most abundant nAChR subtype in the brain, which in rodents forms the high-affinity binding site for the agonist nicotine (Le Novère et al., 2002; Hogg et al., 2003). Like most brain nAChRs, $\alpha 4\beta 2$ receptors appear to be localized primarily presynaptically where they facilitate the release of various neurotransmitters (Dani et al., 2001). The $\alpha 4\beta 2$ nAChR has been implicated in nociception, nicotine dependence and learning and memory (Mansvelder et al., 2005; Tapper et al., 2004; Maskos et al., 2005). An implication in attention deficit hyperactivity syndrome (ADHD, Levin et al., 2006) and in schizophreniform diseases (Bresse et al., 2000; De Luca et al., 2006) have been documented, and losses in numbers of $\alpha 4\beta 2$ nAChRs in the cortex and the hippocampus in Alzheimer's disease have been correlated with cognitive deficits (Gotti and Clementi, 2004).

Studies with nAChR knockouts for the $\alpha 4$ and the $\beta 2$ subunits and the $\alpha 4$ knock-in mouse, which is hypersensitive to nicotine, identify $\alpha 4\beta 2$ nAChR subunits as responsible for the essential features of nicotine-addiction (Tapper et al., 2004; Maskos et al., 2005; Piciotti et al., 1998). Chronic exposure of $\alpha 4\beta 2$ receptors to nicotine results initially in receptor desensitization (Giniatullin et al., 2005), followed by subsequent functional up-regulation of high affinity nicotine binding sites (Schwartz and Kellar, 1983), thus most likely enhancing release of dopamine in the mesolimbic rewarding system (Dani et al., 2001). The molecular mechanisms of the nicotine-induced functional up-regulation relevant for addiction are under investigation (Buisson and Bertrand, 2002), and may depend on high affinity receptor stabilization, intracellular maturation, enhanced trafficking or reduced endocytosis (Peng et al., 1994; Harkness et al., 2002; Vallejo et al., 2005; Sallette et al., 2005; Pakkanen et al., 2005; Darsow et al., 2005). However, nicotine-induced up-regulation has not only been found in brain reward areas, but also in several learning and memory related brain areas such as the hippocampus (Hernandez and Terry, 2005). Interestingly, agonists for the $\alpha 4\beta 2$ receptor produce long-lasting cognitive effects in animal models, which can outlast the actual presence of the agonists for several weeks. The basis for this discrepancy may reside in mechanisms similar to the long-lasting changes following nicotine-dependent induction of long-term potentiation and/or long-term depression (LTP, LTD) (Buccafusco et al. 2005). Since the neuronal Ca^{2+} sensor VILIP-1 is up-regulated during hippocampal LTP induction (Braunewell et al., 2002; Brackmann et al., 2004), is a functional modulator of $\alpha 4\beta 2$ nAChRs (Lin et al., 2002), and is co-expressed with $\alpha 4\beta 2$ nAChRs in subpopulations of hippocampal neurons (Zhao and Braunewell, 2008; Gierke et al., 2008), the up-regulation of $\alpha 4\beta 2$ nAChRs by endogenous modulators such as VILIP-1 might be a physiological mechanism relevant for hippocampal plasticity. Here we describe the existence of VILIP-1-dependent up-regulation of functional $\alpha 4\beta 2$ nAChRs in hippocampal neurons, and the initial analysis of the underlying molecular mechanisms.

Results

Expression of VILIP-1 enhances cell surface expression of endogenous and co-transfected $\alpha 4\beta 2$ nAChR in hippocampal neurons

To investigate whether the neuronal Ca^{2+} sensor VILIP-1 act as modulator of $\alpha 4\beta 2$ nAChR in hippocampal neurons leading to up-regulation of functional receptors, we transfected hippocampal cell cultures with VILIP-1-cDNA. In ELISA using monoclonal antibody specific for the $\alpha 4$ subunit of the nAChR (mAb299) the relative amount of receptor at the cell surface was determined. The enhanced protein expression of VILIP-1 led to an enhanced surface expression of endogenous $\alpha 4\beta 2$ receptor in primary neurons (Fig. 1A, left panel). Similarly, when VILIP-1 was co-expressed with $\alpha 4\beta 2$ -flag-cDNA ($\alpha 4\beta 2$ cDNA ratio 1:10 to yield high affinity receptor) a 1.8-fold enhancement of surface expression was measured (Fig. 1A, right panel), which is of comparable magnitude as the VILIP-1 effect previously observed in the oocyte expression system and tsA 201 cells (Lin et al., 2002). In controls, when hippocampal neurons co-transfected with flag-tagged $\alpha 4\beta 2$ nAChR and GFP (Fig. 1B) were examined by immunofluorescence microscopy, no or very weak surface expression of the receptor was noticed (Fig. 1C). However, when the receptor was co-transfected with VILIP-1-GFP cDNA (Fig. 1D) instead of GFP a significant surface expression was noticeable (Fig. 1E). When the exact number of neurons expressing $\alpha 4\beta 2$ nAChR at the surface was quantified in the co-transfected cultures a nearly threefold increase in surface-expressing neurons from 25% to 74% was observed (Fig. 1F). Similar numbers of neurons were used in control and VILIP-1-transfected cultures, no changes in cell number after transfection occurred, and in neurons transfected with $\alpha 4\beta 2$ nAChR cDNA an effect of VILIP-1 on gene expression can be ruled out. Thus, these results suggest that increased protein expression of VILIP-1 leads to strong up-regulation of cell surface expression of endogenous and transfected $\alpha 4\beta 2$ nAChR in primary hippocampal neurons.

Expression of VILIP-1 enhances cell surface expression of transiently and stably transfected $\alpha 4\beta 2$ nAChR in HEK cells

To set up an eukaryotic expression system which is useful for biochemical studies and for testing whether VILIP-1 can affect functional up-regulation of the $\alpha 4\beta 2$ nAChR, we examined HEK cells transiently or stably transfected with $\alpha 4\beta 2$ nAChR (Fig. 2). VILIP-1-GFP was co-transfected with flag-tagged $\alpha 4\beta 2$ receptor and compared to co-transfection with GFP or without co-transfection (Fig. 2A–F). When $\alpha 4\beta 2$ nAChR subunits were transfected alone only little surface expression of the receptor was observed by immunofluorescence staining (Fig. 2A). However, under permeabilizing conditions in the presence of the detergent TX-100 strong intracellular staining was detected, indicating the presence of receptor protein inside the cells (Fig. 2B). In GFP control transfected HEK cells (Fig. 2C) surface expression of $\alpha 4\beta 2$ nAChR was weak (Fig. 2D), whereas co-expression with VILIP-1-GFP (Fig. 2E) led to a significant increase in surface expression as shown by immunofluorescence surface staining (Fig. 2F). It has been previously described that $\alpha 4\beta 2$ nAChR although expressed at significant levels within transfected HEK cells, often need additional treatment in form of a temperature shift from 37° to 30 °C to reach the cell surface (Lin et al., 2002). Interestingly, VILIP-1 expression alone seems to be sufficient to lead to surface expression without a temperature shift, although a big fraction of the receptor most likely remains in the ER and Golgi (Fig. 2B). Counting the number of cells with surface expression (Fig. 2K) a significant increase of cells with surface expression was observed in VILIP-1-GFP co-transfected cells, 64% of the cells, in comparison to 25% in GFP alone co-transfected HEK cells. Finally we examined whether the effect also occurs in HEK cells stably transfected with human cDNA of $\alpha 4\beta 2$ nAChR (Samochocki et al., 2003). In this case, compared to the effect of GFP control expression (Fig. 2G) on receptor expression (Fig. 2H), we observed a pronounced effect of VILIP-1-GFP expression (Fig. 2I) on the magnitude of surface expression of the receptor (Fig. 2J). Most cultured cells showed surface

expression due to the nature of stable expression (Samochocki et al., 2003), therefore the magnitude of surface expression of nAChR in the stably transfected cells was quantified from the immunostainings with mAb299 against the $\alpha 4$ subunit using the ImageJ program. A significant increase in membrane-associated surface expression of the receptor was observed in VILIP-1-GFP-transfected compared to GFP transfected cells (Fig. 2L). Again in these experiments with transfected $\alpha 4\beta 2$ nAChR cDNA an effect of VILIP-1 on gene expression can be ruled out. Thus, our data suggest that VILIP-1 not only increases the number of cells showing surface expression in HEK cells transiently transfected with rat $\alpha 4\beta 2$ nAChR, but also increases the amount of receptor at the surface in HEK cells stably transfected with rat and human $\alpha 4\beta 2$ nAChRs.

Molecular mechanisms of the VILIP-1 induced upregulation of functional $\alpha 4\beta 2$ nicotinic acetylcholine receptor in hippocampal neurons

VILIP-1 performs a Ca^{2+} -myristoyl switch which enhances membrane association of VILIP-1 in an ionomycin/ Ca^{2+} -dependent manner through the exposition of a hydrophobic myristoyl residue (Spilker et al., 2002). When we stimulated hippocampal neurons with carbachol or ionomycin (data not shown), which both can increase intracellular Ca^{2+} concentrations, we detected, in comparison to controls (Fig. 3A, G), translocation of VILIP-1 to the cell surface (Fig. 3D, J). When in the same experiment the Ca^{2+} -dependent co-localization with the ER was examined (Fig. 3A–F), VILIP-1 (in red, Fig. 3A, D) and the ER marker calnexin (in green, Fig. 3B, E) did not co-localize (Fig. 3C, F, VILIP-1: red, calnexin: green), irrespective of Ca^{2+} stimulation. However, when co-staining with the Golgi marker syntaxin-6 was performed (Fig. 3G–L), VILIP-1 (in red; Fig. 3G, J) was found to show co-localization with syntaxin-6 (in green; Fig. 3H, K) even under unstimulated conditions (Fig. 3I, co-localization in yellow). Following carbachol treatment (Fig. 3L) a large increase in co-localization was observed, which is perfectly in agreement with the previously observed Ca^{2+} -dependent co-localization of VILIP-1 with the trans-Golgi marker syntaxin6 (Spilker et al., 2002). Our results on the apparent lack of ER co-localization render the ER compartment as a less likely place for modulation of nAChR trafficking by VILIP-1. These findings, however, indicate that VILIP-1, which localizes to the Golgi as well as to the cell surface membrane, may regulate $\alpha 4\beta 2$ nAChRs trans-Golgi trafficking.

Co-localization of $\alpha 4\beta 2$ nAChR with ER and Golgi marker and Ca^{2+} -dependent co-localization of VILIP-1-GFP, $\alpha 4\beta 2$ nAChR and Golgi marker syntaxin-6 in hippocampal neurons

To simultaneously track the localization of $\alpha 4\beta 2$ nAChR within the intracellular membranes of the ER and Golgi, we performed triple staining experiments (Fig. 4). When hippocampal neurons transfected with flag-tagged $\alpha 4\beta 2$ nAChR were stained with an anti-flag antibody and antibodies against syntaxin-6 (Fig. 4A–C) and against calnexin (Fig. 4D–F), we found a significant co-localization of the $\alpha 4\beta 2$ nAChR with the ER marker calnexin, seen in purple in the merged image (Fig. 4C), similar to the previously described data (Ren et al., 2005). In the case of the trans-Golgi marker syntaxin-6, we observed less, but still consistent co-localization with the receptor in yellow (Fig. 4F). As expected, no co-localization of the three markers together in white was noticeable in the merged image (data not shown).

Thus, results in Fig. 3 and 4 clearly show that both interaction partners can be detected at Golgi membranes and point to the Golgi as a possible place of interaction of VILIP-1 with $\alpha 4\beta 2$ nAChRs. To directly test the novel hypothesis that VILIP-1 following Ca^{2+} stimulation can interact with $\alpha 4\beta 2$ nAChR at intracellular membranes of the Golgi, we performed triple co-localization experiments to detect $\alpha 4\beta 2$ nAChR, VILIP-1 and the Golgi marker syntaxin-6 simultaneously (Fig. 4GH). Even without stimulation occasional co-localization of the three markers was observed as white co-staining in the merged picture (Fig. 4G, upper inset: VILIP-1, red, middle inset: $\alpha 4\beta 2$ green, lower inset: syntaxin-6, blue). In carbachol treated

cultures, an enhanced association of VILIP-1 with the membranes was noticeable (compare Fig. 4G and H, upper inset: VILIP-1, red) similar to the observation in Fig. 3. More importantly, a substantial increase in co-localization of all three markers was observed as white co-staining in distinct spots within the hippocampal neurons (Fig. 4H), as highlighted in the magnified box, representing the place of highest level of white co-localization of the three markers. To verify this assumption, we quantified the number of white spots showing co-localization (Fig. 4I), which revealed a significant ~fivefold increase in the number of spots per cells from an average of 9 in vehicle-treated versus 44 in carbachol-treated cultures.

Thus, data from Fig. 3 and 4 collectively show that the ER might not be the likely place where $\alpha 4\beta 2$ nAChR and VILIP-1 co-localize and interact. However, from the triple staining data (Fig. 4) the novel notion arises that the two interaction partners $\alpha 4\beta 2$ nAChR and VILIP-1 also partially, probably transiently, co-localize at the level of trans-Golgi membranes. This co-localization of VILIP-1 with syntaxin-6, a SNARE involved in membrane trafficking processes in the trans-Golgi and the endocytotic compartment is strongly enhanced in a Ca^{2+} -dependent manner following carbachol treatment.

VILIP-1 affects membrane transport of the $\alpha 4\beta 2$ nAChR at the level of exocytosis in transfected HEK cells

We have previously shown that VILIP-1 affects clathrin-dependent trafficking and receptor recycling of the natriuretic peptide receptor GC-B and the transferrin receptor at the level of endo- and/or exocytosis (Brackmann et al., 2005). We now wanted to directly address whether VILIP-1 can affect Golgi exit and therefore membrane trafficking at the level of constitutive exocytosis. We aimed for biochemical proof using biotinylation assays with HEK cells co-transfected with $\alpha 4\beta 2$ nAChR and VILIP-1 (Fig. 5A). Input controls (Fig. 5A, left panel) showed that in cells co-transfected with vector or with VILIP-1 and $\alpha 4\beta 2$ nAChR similar amounts of flag-tagged $\alpha 4\beta 2$ nAChR can be found in whole cell precipitates. Next, we confirmed the effect of VILIP-1 on enhanced surface expression by surface biotinylation of proteins and detection of flag-tagged $\alpha 4\beta 2$ nAChR in streptavidin-precipitates from the cell extracts. Under these conditions the expression of VILIP-1, compared to vector controls, leads to increased staining intensity of flag-immunoreactive band at ~65 on Western blots, representing enhanced surface expression of α receptor subunit (Fig. 6A, middle panel). We next used the α receptor subunit as biochemical marker for the surface expression of the $\alpha 4\beta 2$ nAChR. When surface proteins were enzymatically digested before the biotinylation, the observed reappearance of the α -subunit signal (~65kDa) of the nAChR, which showed the strongest signal, in the streptavidin-precipitates is a measure of the transport from the Golgi to the surface over time. When the re-appearance of the α -subunit was compared in VILIP-1 versus empty vector transfected HEK cells, it became obvious that more receptor re-appeared and therefore a higher rate of exocytosis occurred within 10 minutes of measurement in VILIP-1-transfected cells, compared to vector control (Fig. 5A, right panel). Quantification of the Western blots (Fig. 5A, middle and right panel) showed a significant 50% increase in surface expression (Fig. 5B) and a 2.5 fold increase in constitutive exocytosis (Fig. 5) of $\alpha 4\beta 2$ nAChR in VILIP-1-transfected HEK cells (pOPR-V1) compared to pOPR vector controls. The biotinylation experiments indicate that VILIP-1 did not influence the expression level of the receptor, but most likely at the level of the syntaxin-6-positive Golgi and/or transGolgi/endosomal compartment, can affect $\alpha 4\beta 2$ nAChR surface expression by influencing trafficking between Golgi and surface, namely constitutive exocytosis, in transfected HEK cells.

VILIP-1 affects responsiveness of $\alpha 4\beta 2$ nAChR to ACh in transiently and stably transfected HEK cells and in transfected hippocampal neurons

To test whether the effect of VILIP-1 on surface expression of $\alpha 4\beta 2$ nAChR also leads to functional up-regulation of the expressed receptor we performed whole-cell patch-clamp

studies (Fig. 6). First, recordings from $\alpha 4\beta 2$ nAChR and GFP or VILIP-1-GFP-cotransfected HEK cells and from GFP or VILIP-1-GFP-transfected HEK cells stably expressing the human $\alpha 4\beta 2$ nAChR (cell line Ha4b2Lx/1) were analyzed. ACh evoked whole-cell responses at saturating concentration of 3mM, which could be completely blocked by 100nM DH β E, an antagonist of $\alpha 4\beta 2$ receptors, indicating the presence of functional $\alpha 4\beta 2$ receptor in the Ha4b2Lx/1 cell lines ectopically expressing the human $\alpha 4\beta 2$ nAChR. Following antagonist wash out the current could be recovered (Fig. 6A, upper panel). These data are comparable to those previously reported (Samochocki et al., 2003). Similar results were obtained using transiently transfected HEK293 cells (data not shown). Under the same saturation conditions (3 mM ACh) the response to ACh was significantly increased in amplitude in the VILIP-1-GFP co-transfected cells compared to the responses in GFP-transfected HEK cells expressing human $\alpha 4\beta 2$ nAChR (Fig. 6A, compare representative traces in red, VILIP-1-GFP, and black, GFP), and in the transiently expressing HEK cells (data not shown).

Next, we looked at $\alpha 4\beta 2$ nAChR and GFP or VILIP-1-GFP-cotransfected primary hippocampal neurons in which nicotinic currents had been isolated by blocking GABAergic, glutamatergic and muscarinergic transmission with bicuculline, CNQX, TTX, APV and atropine. Again at saturation conditions (3 mM ACh) the response to ACh could be blocked by the receptor antagonist DH β E (Fig. 6B, upper panel). To reduce possible side effects of desensitization, only the first current obtained from each cell was plotted for further statistic analysis. Only healthy looking neurons moderately expressing VILIP-1-GFP were used and showed a significant increase in the amplitude of ACh currents compared to healthy GFP-control neurons of comparable size and morphology (Fig. 6B, compare representative traces in red, VILIP-1-GFP, and black, GFP). When siRNA against VILIP-1 was co-transfected with GFP, we could observe a downregulation in VILIP-1 immunoreactivity in the GFP co-transfected cells, when compared to neurons co-transfected with GFP and scrambled siRNA (compare Fig. 7A, B to C, D). When hippocampal neurons were transfected with siRNA against VILIP-1 and later co-transfected with EGFP and $\alpha 4\beta 2$ nAChR a reduced activation of $\alpha 4\beta 2$ nAChR currents was observed (Fig. 7E), which reached highly significant levels. These results support the notion that enhanced VILIP-1 expression not only increases surface expression, but at the same time increase the responsiveness of cells expressing the rat and human $\alpha 4\beta 2$ nAChR for its ligand ACh. In contrast, application of siRNA specific for VILIP-1 leading to the knockdown of endogenous VILIP-1 expression, was able to significantly attenuate the ACh-induced currents. The average $\alpha 4$ nicotinic current amplitude of 140 pA was strongly reduced to about 34 pA following co-transfection of $\alpha 4\beta 2$ nAChRs with VILIP-1 siRNA. Thus, we conclude that VILIP-1 is able to up-regulate functional $\alpha 4\beta 2$ nAChRs in hippocampal neurons.

Discussion

Neuronal Ca²⁺-sensor protein VILIP-1 enhances surface expression leading to upregulation of functional $\alpha 4\beta 2$ nAChR in hippocampal neurons

In addition to nicotine-induced functional up-regulation (Buisson and Bertrand, 2002) a physiological mechanisms for up-regulation of functional $\alpha 4\beta 2$ nAChRs seem to exist. VILIP-1 is a myristoylated neuronal Ca²⁺-sensor protein which was identified as an interaction partner of $\alpha 4\beta 2$ nAChRs in a yeast-two-hybrid interaction screen using the large intracellular loop of the $\alpha 4$ subunit as bait. Initial characterization in oocytes showed that co-expression of VILIP-1 with recombinant $\alpha 4\beta 2$ nAChR up-regulated surface expression two-fold and increased the agonist-sensitivity to ACh three-fold. The modulation of the recombinant $\alpha 4\beta 2$ nAChR by VILIP-1 was attenuated with myristoylation mutants or EF-hand mutants not able to bind Ca²⁺. We have put forward the hypothesis that VILIP-1, possibly via the mechanism of the Ca²⁺-myristoyl switch (Ames et al., 1997; Spilker et al., 2002), may serve as a modulator

of $\alpha 4\beta 2$ nAChR (Lin et al., 2002). Interestingly, the application of nicotine to hippocampal cultures leads to the Ca^{2+} -dependent and reversible membrane-translocation of VILIP-1 via activation of $\alpha 7$ - but not $\alpha 4$ -containing nAChRs. This may provide a novel functional crosstalk between $\alpha 4$ - and $\alpha 7$ -containing nAChRs (Zhao et al., 2008). Here we examine the potential physiological importance of the effect. VILIP-1 enhances surface expression of flag-tagged $\alpha 4\beta 2$ nAChRs in hippocampal neurons leading to increased ACh evoked currents, thus, functionally up-regulating these $\alpha 4\beta 2$ nAChRs in hippocampal neurons. Thus, we postulate that VILIP-1 is part of a novel physiological mechanism for up-regulation of functional $\alpha 4\beta 2$ nAChRs in the brain.

Neuronal Ca^{2+} -sensor protein VILIP-1 is excluded from ER, but localizes to Golgi, indicating a role in $\alpha 4\beta 2$ nAChR trafficking via the Golgi compartment in hippocampal neurons

An ER export motif, important for cargo selection into COPII-coated vesicles that transport proteins from the ER to the Golgi, was identified within the cytoplasmic loop of both subunits and are critical structural determinants for ER export of fully assembled nAChRs (Ren et al., 2005). One possible hypothesis to explain the effect of VILIP-1 would be that it interacts with the ER export motif and accelerates exit of the $\alpha 4\beta 2$ nAChR from the ER. To test this hypothesis, we have performed co-localization studies in hippocampal neurons in culture. VILIP-1 did not localize to the ER in the presence or absence of Ca^{2+} , which makes it less likely that VILIP-1 influences ER export. One remaining possibility could be that Ca^{2+} release causes rapid association between VILIP-1 and $\alpha 4\beta 2$ nAChR in the ER, but that once this association occurs $\alpha 4\beta 2$ nAChR are rapidly released from the ER. However, we were able to show localization of VILIP-1 with markers of the trans-Golgi network, which was strongly enhanced by increasing the intracellular Ca^{2+} level. Thus, we postulate that VILIP-1 plays a role in modulating exit from the Golgi rather than the ER, thereby leading to enhanced surface expression of the receptor in primary hippocampal neurons.

The Ca^{2+} -myristoyl switch of VILIP-1 and co-localization with $\alpha 4\beta 2$ nAChR in syntaxin-6 positive Golgi structures define a novel signalling mechanism

We have previously postulated that the Ca^{2+} -myristoyl switch of VILIP-1 is part of a signalling mechanism which enables a receptor system to respond to a Ca^{2+} stimulus in living hippocampal neurons (Spilker et al., 2002; Brackmann et al., 2005), and thereby possibly acts as a coincidence detection complex. The molecular mechanism underlying coincidence detection in such a protein complex seems not to involve direct and fast Ca^{2+} -dependent activation of the receptor at the cell surface, but depending on activity of the neuron and intracellular Ca^{2+} -levels appears to rely on the slow modulation of receptor trafficking, occurring in the minute range (Lin et al., 2002; Brackmann et al., 2005). This model is supported by previous reports showing that VILIP-1 interacts with membrane transport intermediates isolated from rat brain, such as synaptic vesicles and clathrin-coated vesicles (CCV) (Blondeau et al., 2004; Morciano et al., 2005), and the fact that VILIP-1 affects clathrin-dependent membrane trafficking of the transferrin receptor and the receptor guanylyl cyclase B (Brackmann et al., 2005). Receptor trafficking is mediated by a complex machinery of trafficking and sorting proteins. CCVs and the *trans*-Golgi network (TGN) represent key sorting sites for many transmembrane proteins (Bonifacio and Traub, 2003). In this study we have found co-localization of VILIP-1 and $\alpha 4\beta 2$ nAChR with one component of the transport machinery, the trans-Golgi and endosomal SNARE syntaxin-6. The primary role of SNAREs (soluble NSF attachment receptor) is to mediate fusion of cellular transport vesicles with a target compartment. In hippocampal neurons the SNARE syntaxin-6 is present in complexes with SNAPs (soluble NSF attachment protein) and is primarily found on the trans-Golgi network, where it partially co-localizes with the adapter protein AP-1 on clathrin-coated membranes (Bock et al., 1997). Moreover, syntaxin-6 is part of a SNARE complex which is enriched on clathrin-coated and synaptic vesicles in rat brain (Kreykenbohm et al., 2002).

Syntaxin-6 is also found in endosomes and regulates the delivery of microdomain-associated lipids and proteins to the cell surface, which are required for caveolar endocytosis (Choudhury et al., 2006). VILIP-1 is able to affect the clathrin-dependent receptor recycling of a set of receptors including the transferrin receptor, the receptor guanylyl cyclase GC-B (Brackmann et al., 2005). These findings support an involvement of VILIP-1 in clathrin-dependent transport of syntaxin-6-positive vesicles from the trans-Golgi network to the cell surface and/or in endosomal trafficking. Moreover, VILIP-1 was identified to interact with several ligand-gated ion channels, including the $\alpha 4\beta 2$ nAChR (Lin et al., 2002), glutamate receptors of the kainate subtype GluR6 (Coussen et al., 2005) and P2X2 receptors (Chaumont et al., 2008). VILIP-1 forms a signaling complex with P2X2 receptors and proteins such as N-ethylmaleimide-sensitive factor (NSF), tubulin α , vesicle amine transport protein 1 homolog (VAT1), glutamic acid decarboxylase (GAD) and synapsin IIb (Chaumont et al., 2008), indicating that VILIP-1 might play a general role in trafficking of these ligand-gated ion channels via cellular transport vesicles.

Biochemical experiments reveal that the neuronal Ca^{2+} -sensor protein VILIP-1 affect $\alpha 4\beta 2$ receptor trafficking at the level of constitutive exocytosis

Based on these literature data the co-localization of VILIP-1 and $\alpha 4\beta 2$ nAChR with the trans-Golgi SNARE syntaxin-6 is functionally linked to membrane transport via post-Golgi pathways. To prove this assumption we have performed biotinylation assays, which together with enzymatic cleavage of newly synthesized receptors at the cell surface allowed us to precisely address the constitutive exocytotic pathway in cells co-transfected with flag-tagged $\alpha 4\beta 2$ nAChR and VILIP-1. These results clearly demonstrated biochemically that not only VILIP-1 did significantly enhance surface protein expression of $\alpha 4\beta 2$ nAChR, but that within 10 minutes following complete removal of all surface receptors, the re-appearance of surface expression of the tagged receptor was strongly promoted in VILIP-1 transfected cells. These findings support an involvement of VILIP-1 in clathrin-dependent transport of syntaxin-6-positive vesicles from the trans-Golgi network to the cell surface and possibly in endosomal trafficking. Other members of the NCS protein family seem to perform related functions in receptor trafficking, but at different trafficking compartments. KChIP stimulates traffic of Kv4 K⁺ channels from a post-ER but pre-Golgi vesicular compartment (Hasdemir et al., 2005). In contrast, NCS-1, like VILIP-1, stimulates constitutive traffic from the TGN (Haynes et al., 2005).

Possible implications for hippocampal network activity and synaptic plasticity: a working hypothesis

The nAChRs are known to enhance GABAergic neurotransmission in different brains structures, underlying the cholinergic modulation of synaptic plasticity and network activity (Couey et al., 2007; Nashmi et al., 2007). A variety of evidence has been provided for a link between nicotinic AChR enhancement, cognitive function (Mansvelder et al., 2005) and disease (Hogg et al., 2003; Gotti and Clement, 2004). Amino acid mutations in the human $\alpha 4\beta 2$ nAChR associated with autosomal dominant nocturnal frontal lobe epilepsy, which when are engineered in the corresponding $\alpha 4\beta 2$ nAChR in mice, lead to disturbances of inhibitory synchronization of cortical networks by GABAergic interneurons (Klaassen et al., 2006). Since VILIP-1 is misregulated in hippocampal pyramidal neurons and in CA1 and dentate gyrus interneurons in schizophrenic brains (Bernstein et al., 2002, 2003), and is up-regulated following induction of hippocampal LTP *in vivo* (Braunewell et al., 2004; Brackmann et al., 2004), we postulate that VILIP-1 in conjunction with the $\alpha 4\beta 2$ receptor plays an important role in modulating network activity in healthy and diseased hippocampus (Braunewell, 2005). When looking for the possible functional impact of pathologically increased VILIP-1 expression, we have observed nAChR-dependent enhancement in IPSCs in rat hippocampal cell culture transfected with VILIP-1-GFP, likely mediated by the $\alpha 4\beta 2$ nAChR (Gierke et al.,

2008). This is supported by the fact that functional expression of the high-affinity nicotine-binding $\alpha 4\beta 2$ nAChR appears to be restricted to certain interneuron populations (Alkondon and Albuquerque, 2001; Alkondon and Albuquerque, 2002). The $\alpha 4\beta 2$ nAChRs predominantly contribute to IPSCs generated by interneurons of stratum oriens and stratum radiatum in the CA1 region of rat hippocampal slices *in vitro* (Alkondon and Albuquerque, 2001; Alkondon and Albuquerque, 2002) and in rat hippocampal cultures, where 65% of IPSCs were identified to be dependent on $\alpha 4\beta 2$ nAChR (Braga et al., 2004). From these different sets of data it appears that VILIP-1 in cultured hippocampal neurons induces an enhancement in surface expression of $\alpha 4\beta 2$ nAChRs and, in turn, enhancement of ACh-induced currents via $\alpha 4\beta 2$ nAChR. Thus, we can put forward a hypothesis (Fig. 8) for the molecular mechanism of VILIP-1-mediated up-regulation of $\alpha 4\beta 2$ nAChR in hippocampal neurons, which might serve as a novel physiological mechanism underlying plasticity of nicotinic neurotransmission.

Experimental Methods

Materials

Nicotine, brefeldin A, carbachol, monensin for cell stimulation experiments were purchased from Sigma (St Louis, MO). Cell culture reagents were obtained from Gibco-Invitrogen (San Diego, CA). Unless otherwise specified, all other reagents were purchased from Sigma and Roth.

Antibodies

Rat polyclonal antibody, raised against recombinant VILIP-1 fusion protein, was 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 (Spilker et al., 2002). Antibodies against the α -subunit of the nAChR were mAb299 (Covance, Berkeley, CA, USA) and Guinea pig anti- $\alpha 4$ nAChR polyclonal antibody (Chemicon, Germany). Further polyclonal antibodies used in this study comprised rabbit anti-calnexin (Sigma and Stressgene, Victoria, BC, Canada), rabbit and goat anti-flag (Sigma, Abcam, Cambridge, UK) and mouse anti-syntaxin6 (Sigma). Cy3, Alexa Fluor™ 488 and Cy5 labeled secondary antibodies were purchased from Dianova (Hamburg, Germany), Molecular Probes (Eugene, OR) and Abcam.

Cell culture and transfection of HEK 293 cells and hippocampal neurons

Human embryonic kidney cells 293 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10 % fetal bovine serum, 2mM L-glutamine and antibiotics at 37°C. Cells were maintained in a humidified 95% air containing 5% CO₂. H $\alpha 4\beta 2$ L-/1 (Samochocki et al., 2003) cells stably expressing the human $\alpha 4\beta 2$ nAChR (H, human; $\alpha 4\beta 2$; L-, HEK-293 cells that do not express the L 1C-bCa²⁺ channel 1, clone 1) were grown in DMEM containing 13 % fetal calf serum in an incubator at 37°C, 10% CO₂. 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/cm². For transient transfection the HEK cells were plated in 24-well dishes and 12 h later transfected using Lipofectamine 2000 (Invitrogen). To increase the transfection efficiency cells were incubated for 4–6 h in unsupplemented DMEM. $\alpha 4\beta 2$ surface expression was detected after incubation at 37°C for 48 h. Hippocampal cultures were prepared from fetal wistar rat brains (E18) as previously described (Spilker et al., 2002). Cells were plated onto poly-D-lysine 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 1x B27, 0.5 mM L-glutamine and antibiotics. Hippocampal neurons were transfected with cDNA or siRNA after

10–14 days in culture using the lipofectamin transfection method (Invitrogen). The cDNAs coding for GFP, VILIP-1-GFP were essentially as previously described Spilker et al., 2002. Rat $\alpha 4$ -flag and $\beta 2$ -flag nAChR subunit cDNA was as described (Ren et al., 2005). Carbachol (0.1mM) and ionomycin (1 μ M) stimulation was applied 20 h after transfection for 2 minutes.

Enzyme-linked immunoassay for quantitating cell surface $\alpha 4\beta 2$ nAChRs

Cell surface $\alpha 4\beta 2$ nAChRs were measured in hippocampal neurons plated in 12-well dishes. Cells were washed once in PBS and fixed with 3% paraformaldehyde for 5 minutes at room temperature. After 4 washes with PBS, the cells were blocked with 3% BSA in PBS for 30 minutes, and then incubated for 1h at room temperature with a mAb against the $\beta 2$ nAChR subunit (mAb 295) whose epitope is located in the extracellular domain of the subunit. After 6 washes with PBS, the cells were incubated with horseradish peroxidase-conjugated goat anti-rat secondary Ab for 1 h, washed 8 times with PBS, and finally incubated with the HRP substrate 3, 3', 5, 5'-tetramethylbenzidine (Sigma) for 30minutes. The absorbance of the supernatant was then measured at 405nm in a Beckmann spectrophotometer. The nonspecific background binding of Abs to cells was determined using nontransfected cells and was typically <0.5% of the total binding observed for wild type $\alpha 4\beta 2$ nAChRs.

Biotinylation assay for quantitating exocytosis of $\alpha 4\beta 2$ nAChRs

To test the effect of VILIP-1 on surface expression, HEK cells were co-transfected with $\alpha 4\beta 2$ nAChR and pOPR-V1 or as control with pOPR for 48h and were afterwards biotinylated using Sulfo-NHS-SS-Biotin (Pierce Rockford, IL.; 0.15 mg/ml) in PBS for 10 minutes on ice. Cells were washed three times with TBS (Tris 25 mM, pH 7.4, NaCl 137 mM, KCl 5 mM, CaCl₂ 2.3 mM, MgCl₂ 0.5 mM, Na₂HPO₄ 0.143 g/l), lysed 10–30 minutes in extraction buffer (Tris-HCl 10 mM pH 7.5, EDTA 10 mM, Triton X-100 1%, SDS 0.1% and protease inhibitor cocktail 1x). For input controls, cell were first lysed and then biotinylated. After centrifugation supernatants containing equal amount of protein were incubated with streptavidin beads (Pierce Rockford, IL) for 1–2h, washed four times in extraction buffer (without detergents) and subjected to SDS-PAGE. Western Blot analysis using anti-flag antibodies was performed to detect $\alpha 4\beta 2$ nAChR. To specifically test the effect on exocytosis cell surface was stripped before biotinylation using trypsin digestion (0.1 mg/ml) at 4°C for 15minutes. After a chase period of 10minutes at 37°C, biotinylation was done at 4°C. Following washing, cell lysis, precipitation with streptavidin beads as above Western Blot analysis was performed to detect $\alpha 4\beta 2$ nAChR which had been exocytosed using anti-flag antibodies.

Immunocytochemistry

Following fixation of hippocampal neurons with 3% paraformaldehyde in PBS, pH 7.4 for 5 minutes at room temperature (adapted from Ren et al., 2005), cells were washed twice with 25 mM glycine in PBS to quench background staining. The cells were blocked for surface staining in 3% bovine serum albumine, 10% horse serum in PBS (blocking solution) or blocked and permeabilized with 0.1% Triton X-100 added to blocking solution for 30 minutes, respectively. 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 cells for 1 h 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, Alexa Green 488, Cy3 and Cy5 fluorescence were visualized using a Leica SP laser scanning microscope (Leica DM LFSA or DM 2500; Wetzlar, Germany) with Argon-ion (488 nm), Helium-Neon (543 nm) and Helium-Neon (633 nm) or solid-state (488, 532, 635 nm) laser excitation, respectively. The excitation light was coupled in via the main dichroic beam splitters (RSP500 for GFP and DD488/543

for Cy3 or DD488/635 for GFP, Alexa Green 488, Cy5 and DD405/532 for Cy3 fluorescence, respectively). The emitted light was collected in sequential scans in the range of 505 nm to 550 nm (GFP, Alexa Green 488), 550 nm to 600 nm (Cy3) and 650 nm to 750 nm (Cy5), respectively. The pinhole was set airy 1. For the experiments, Leica HCX PL APO 63x/1.4 and 100x/1.4 oil immersion objectives were used at the upright microscope. The spatial resolution was 0.37 $\mu\text{m}/\text{pixel}$ (in the x- and y-axes) for the acquired 512×512 and 1024×1024 pixel images. To avoid photo damage, 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 7.0 (Adobe Systems, San Jose, CA) and the ImageJ 1.37 software (available at <http://rsb.info.nih.gov/ij/>).

Co-transfection of hippocampal neurons with siRNA and EGFP

To demonstrate the knock down of VILIP-1 using siRNA, 1 μl Lipofectamin-2000 (Invitrogen) was preincubated with 25 μl Opti-MEM I reduced serum medium (Gibco, Carlsbad, CA) at room temperature (20°C) for 5 min. Meanwhile, either 1 μl (20 μM) control siRNA or a mixture of three VILIP-1 antisense siRNAs in combination with 0.1 μl EGFP vector was diluted to 25 μl Opti-MEM I reduced serum medium (Gibco) in a ratio of 10: 1. Allstars negative control siRNA and three different VILIP-1 siRNA sequences were obtained from (Qiagen, Hilden, Germany). The VILIP-1 sequences are: rat Vsnl1-5 sense r(AGC CGU UAG UCU GAA UUA A)dTdT, antisense r(UUA AUU CAG ACU AAC GGC U) dTdT, Vsnl1-6 sense r(CAA AGA UGA CCA GAU UAC A)dTdT, antisense r(UGU AAU CUG GUC AUC UUU G)dTdT, Vsnl1-8 sense r(GUG CGA CAU UCA GAA AUG A)dTdT, antisense r(UCA UUU CUG AAU GUC GCA C)dTdT. Lipofectamin 2000 and plasmid dilution were mixed and incubated at room temperature for 20 min. 50 μl DNA and Lipofectamin 2000 complexes were added to each well of the 7-day-old hippocampal cells that had been cultured in 0.5 ml growth medium. 72 hours after transfection, the cells were used for subsequent experiments. To measure the $\alpha 4$ nAChR current after siRNA transfection, a second transfection was performed as follows: 1 μl Lipofectamin-2000 (Invitrogen) was preincubated with 25 μl Opti-MEM I reduced serum medium (Gibco, Carlsbad, CA) at room temperature (20°C) for 5 min. 0.1 μg EGFP, 1 μg $\alpha 4$, and 5 μg $\beta 2$ nAChR vector were diluted to 25 μl Opti-MEM I reduced serum medium (Gibco). Then the Lipofectamin 2000 and plasmid dilution were mixed and incubated at room temperature for 20 min. 50 μl DNA and Lipofectamin 2000 complexes were added to each well. 72 hours after transfection, the cells were used for patching experiments.

Patch clamp analysis

Whole-cell current recordings from stably transfected HEK cells were performed 12 to 24 h after transfection 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 mOsm), and the internal pipette solution contained 140 mM CsCl (equilibrated with CsOH), 11 mM EGTA, 10 mM HEPES, and 1 mM MgCl_2 (pH 7.3; 300 mOsm). In patch recordings of hippocampal neurons, whole-cell currents from transiently transfected cells were recorded 48 h after transfection. Cells of comparable size and morphology were used for the different groups (GFP vs VILIP-1-GFP). Sodium channels, NMDA, AMPA, and GABA_A receptors were blocked by 0.5 μM TTX, 30 μM AP-5, 30 μM CNQX, 5 μM bicuculline, 1 nM MLA. ACSF (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) was used as extracellular buffer. 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 7.2. Microelectrodes (borosilicate glass) had an external diameter 2.0 mm, and the pipette resistance was 2–5 M Ω . Following formation of a high-resistance seal, capacitance transients in cells were minimized using the C-Fast facility. 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/minutes. In all experiments, atropine (1 μ M) was included in the bathing solutions in order to inhibit intrinsic muscarinic responses. Signals were low-pass (Bessel) filtered at 3 kHz (whole-cell measurements) and analyzed on a PC using the TIDA software (Version3, HEKA Elektronik, GmbH, Germany). All data are presented as mean $\pm$ SEM.

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Abbreviations

ACh	acetylcholine
NCS	neuronal Ca^{2+} sensor
nAChR	nicotinic acetylcholine receptor
VSNL1	visinin-like protein-1

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Fig. 1. Expression of VILIP-1 enhances surface expression of endogenous and co-transfected $\alpha 4\beta 2$ nAChR in hippocampal neurons

A. VILIP-1 cDNA (pOPR-V1) or control vector (pOPR) was transfected alone (left) or co-transfected with flag-tagged $\alpha 4\beta 2$ nAChR (right) into two week old primary hippocampal cultures. 16h following transfection, where no changes in cell numbers were observed, the surface expression of the endogenous $\alpha 4\beta 2$ nAChR (left) or of the flag-tagged $\alpha 4\beta 2$ nAChR (right) was quantified using mab299 against the $\alpha 4$ -subunit (left) or an anti-flag antibody (right) in ELISA. **B–E.** Control GFP vector (B, C) or VILIP-1-GFP cDNA (D, E) were co-transfected with flag-tagged $\alpha 4\beta 2$ nAChR (B–E) into 2 week old primary hippocampal cultures. After 40h in culture surface expression of the flag-tagged $\alpha 4\beta 2$ nAChR (C, E) was monitored with anti-flag antibody by confocal immunofluorescence microscopy. Bar in E is 20 μ m. **F.** Quantification of the number of cells expressing flag-tagged $\alpha 4\beta 2$ nAChR at the cell surface in GFP (C) and VILIP-1-GFP (E) co-transfected hippocampal neurons. Mean values $\pm$ S.D. are from five experiments for A and three experiment for F carried out in triplicate.

Fig. 2. Expression of VILIP-1 enhances surface expression of transiently transfected $\alpha 4\beta 2$ nAChR in HEK cells and of human $\alpha 4\beta 2$ nAChR in stably transfected HEK cells. A–F Flag-tagged $\alpha 4\beta 2$ nAChR was transfected alone (A, B) or co-transfected with GFP (C, D), or with VILIP-1-GFP cDNA (E, F) into HEK cells. After 48h in culture only little surface expression of the flag-tagged $\alpha 4\beta 2$ nAChR was monitored (A), but the receptor was strongly expressed intracellularly when stained in the presence of TX100 (B). Surface expression in GFP (C, D) and VILIP-1-GFP (E, F) co-transfected HEK cells was monitored with anti-flag antibody by confocal immunofluorescence microscopy (D, F). Bar in F is 20 μ m. **G–J.** GFP (G, H) or VILIP-1-GFP cDNA (I, J) was transfected into HEK cells stably expressing the human $\alpha 4\beta 2$ nAChR. After 48h in culture surface expression of human $\alpha 4\beta 2$ nAChR (H, J)

was monitored by confocal immunofluorescence microscopy in GFP (G, H) and VILIP-1-GFP (I, J) transfected cells with mAb299 antibody against the α -subunit of nAChR (H, J). Cross-bleeding of channels was avoided by sequential analysis of the red and green channel. Bar in J is 20 μ m. **K.** Quantification of the number of cells expressing flag-tagged $\alpha 4\beta 2$ nAChR in GFP (D) and VILIP-1-GFP (F) co-transfected HEK cells. No differences in cell numbers between GFP- and GFP-VILIP-1-transfected cells was observed. **L.** Quantification of the surface expressing of human $\alpha 4\beta 2$ nAChR in GFP (H) and VILIP-1-GFP (J) transfected HEK cells in mAb299 stained cultures using the NIH ImageJ program. Mean values $\pm$ S.D. are from three experiments carried out in triplicate.

Fig. 3. Ca^{2+} -dependent localization of VILIP-1 to cell surface and the intracellular membranes of Golgi and ER in hippocampal neurons

A–L. Hippocampal neurons were unstimulated (A–C, G–I) or stimulated with carbachol (D–F, J–L), which increases intracellular Ca^{2+} concentrations to enhance the membrane association of VILIP-1 (compare A to D and G to J), as described in detail previously for ionomycin treatment (28) **A–F.** Co-localization with the ER marker calnexin (B, E) could not be found in the merged images (C, F, VILIP-1: red, calnexin: green). **G–L.** Co-staining with the trans-Golgi marker syntaxin-6 (H, K) was observed in yellow in the merged images (I, L), and was enhanced after carbachol treatment to increase intracellular calcium (L), as previously

described in detail for ionomycin treatment (Spilker et al., 2002). Representative stainings out of at least three experiments carried out in triplicate are shown. Bar in M is 20 μm .

Fig. 4. Co-localization of $\alpha 4\beta 2$ nAChR, ER and Golgi marker in a triple co-stained hippocampal neuron

A–F. Hippocampal neurons cultured for two weeks transfected with flag-tagged $\alpha 4\beta 2$ nAChR were simultaneously stained with anti-flag antibody and antibodies against syntaxin-6 and calnexin. **A–C.** Co-localization of $\alpha 4\beta 2$ nAChR (A) with the ER marker calnexin (B) is seen in purple in the merged picture (C). **D–F.** Occasional co-localization of $\alpha 4\beta 2$ nAChR (D) with the Golgi marker syntaxin-6 (E) is seen in yellow in the merged picture (F). Note, that no co-localization of Golgi marker syntaxin-6 can be found with $\alpha 4\beta 2$ nAChR and the ER marker calnexin which would be observed as white in merged pictures (data not shown). Bar in F is 20 μm . Ca^{2+} -dependent co-localization of VILIP-1-GFP, $\alpha 4\beta 2$ nAChR and the Golgi marker syntaxin-6 in a triple co-stained hippocampal neuron. **G, H.** Hippocampal neurons cultured for two weeks were transfected with flag-tagged $\alpha 4\beta 2$ nAChR and VILIP-1-GFP and simultaneously stained with anti-flag antibody and antibodies against syntaxin-6. **G.** Co-localization of VILIP-1-GFP/ $\alpha 4\beta 2$ nAChR with the Golgi marker syntaxin-6 is seen

occasionally (arrow) without stimulation as white co-staining in the merged picture (G, upper inset: VILIP-1, middle inset: $\alpha 4\beta 2$ nAChR, lower inset: syntaxin-6, white box shows the magnified area above). **H.** In carbachol treated cultures, substantial co-localization of all three markers is observed as white co-staining in distinct spots within the hippocampal neuron (H, arrows point at white co-localization spots, white box shows the magnified area with highest density of co-localization). Bar in A, B is 5 μ m. **I.** Quantification of the co-localization of VILIP- 1-GFP, $\alpha 4\beta 2$ nAChR and syntaxin-6 as number of white spots in control (G) carbachol-treated (H) triple co-stainings. Mean values $\pm$ S.D. are from three experiments carried out in duplicate.

Fig. 5. Expression of VILIP-1 affects surface expression via accelerating exocytosis of the $\alpha 4\beta 2$ nAChR in double-transfected hippocampal neurons and in HEK cells

A. In HEK cells co-transfected with flag-tagged $\alpha 4\beta 2$ nAChR and pOPR-VILIP-1 or pOPR vector alone, biotinylation of cellular proteins and of surface proteins was performed and the flag-tagged $\alpha 4\beta 2$ nAChR was detected in Western blot analysis of streptavidin-precipitates from the cell extracts. For input controls (left panel) following lysis and biotinylation, or for surface protein determination (middle panel) following biotinylation and then lysis, the flag-immunoreactive bands in streptavidin-precipitates at about 70 and 55kDa representing $\alpha 4$ and $\beta 2$ subunits are monitored by Western blot analysis of control vector (pOPR) and VILIP-1-transfected (pOPR-V1) HEK cell extracts. Exocytosis was determined after enzymatic digest of surface proteins and subsequent biotinylation and lysis. The re-appearance of the $\alpha 4$ -subunit signal in the streptavidin-precipitates was measured by biotinylation 10min after enzymatic

treatment (right panel). **B, C.** Quantification of the Western blot in A, middle panel, indicates **(B)** a significant 50% increase in relative surface expression and **(C)** a 2,5 fold increase in relative level of exocytosis, in A, right panel, of the $\alpha 4$ -subunit of the nAChR in VILIP-1-transfected HEK cells.

Fig. 6. Potentiation of ACh-induced whole-cell currents by expression of VILIP-1-GFP in HEK293 cells stably expressing human $\alpha 4 \beta 2$ nAChR (A) and in hippocampal neurons transfected with rat $\alpha 4 \beta 2$ nAChR (B)

A. Whole-cell responses to ACh in the absence and presence of 100nM DH β E recorded from a single cell of the Ha4b2Lx/1 cell line, which was held at -60mV. The ACh concentration applied (3mM) was in the range of the saturation response. ACh evoked current was blocked completely by 100nM of the $\alpha 4 \beta 2$ nAChR antagonist DH β E (second trace). The current recovered after DH β E wash out. Representative traces of whole-cell responses following ACh stimulation in GFP (black trace) and GFP-VILIP-1 (red trace) transfected HEK cells are shown in the lower left panel. The response to ACh in VILIP-1-GFP transfected cells (lower right panel, n=33, from two experiments) is highly significant increased in amplitude (p<0.01)

compared to the responses to ACh in GFP-transfected cells (n=18, from two experiments).

B. Whole-cell responses to ACh, in the absence and presence of 100nM DH β E recorded from a single cultured hippocampal neuron which was held at -60mV. The evoked current partially recovered after DH β E wash out. Representative traces of whole-cell responses following ACh stimulation in GFP (black trace) and GFP-VILIP-1 (red trace) transfected neurons are shown in the left panel. The response to ACh in the VILIP-1-GFP transfected cells (lower right panel, n=13, from two experiments) shows highly significant increase in amplitude (p<0.05), as compared to GFP-transfected neurons (n=14, from three experiments).

Fig 7. SiRNA knockdown of VILIP-1 in GFP co-transfected hippocampal neurons (A–D) leads to reduction of ACh-induced whole-cell $\alpha 4\beta 2$ nAChR currents

Representative confocal image showing control siRNA and GFP co-transfected hippocampal neuron (A) and expression of endogenous VILIP-1 (B). VILIP-1-siRNA/GFP-coexpressing neurons (C) show strongly reduced expression of endogenous VILIP-1 (D). In the representative experiment shown an average of 13 out of 15 co-transfected neurons per dish showed strongly reduced VILIP-1 expression levels, whereas 29 out of 33 neurons in the control siRNA group did not show changes in expression. Bar in A, B is 30 μ m, bar in C, D is 15 μ m. E. In hippocampal neurons co-transfected with $\alpha 4\beta 2$ nAChRs and control siRNA (Ctr siRNA) the average $\alpha 4$ nicotinic current amplitude was strongly reduced following co-

transfection with VILIP-1 siRNA (VILIP-1 siRNA). Data are presented as mean $\pm$ SEM; ** $p < 0.01$ compared with control group.

Fig. 8. Hypothesis for a novel physiological mechanism of up-regulation of functional $\alpha 4 \beta 2$

The cartoon shows the neuronal Ca^{2+} -sensor VILIP-1, is activated following stimulation of a neuron by a signal, such as glutamate, carbachol or nicotine, which is acting on receptors increasing the intracellular Ca^{2+} level (1). VILIP-1 shuttles to cell surface and Golgi membranes as well as clathrin-coated vesicles, where it co-localizes with $\alpha 4 \beta 2$ nAChR and with syntaxin-6, a SNARE implicated in clathrin-dependent transport mechanisms in the trans-Golgi network. VILIP-1 causes enhanced exocytosis and surface transport of $\alpha 4 \beta 2$ nAChR (2), consequently increasing the surface expression and finally the sensitivity of the neuron towards ACh (3). The nicotine or glutamate induced translocation of VILIP-1 to cellular membranes (1, Zhao et al., 2008), and in turn the up-regulation of functional $\alpha 4 \beta 2$ nAChRs (2, Lin et al., 2002, this study) may contribute to plasticity of nicotinic neurotransmission in principal neurons and/or in interneurons in the hippocampus, since the expression of VILIP-1 in conjunction with the $\alpha 4 \beta 2$ nAChR enhances the frequency of inhibitory postsynaptic currents (IPSCs) in hippocampal cultures (3, Gierke et al., 2008). Thereby, the physiological up-regulation of $\alpha 4 \beta 2$ nAChR by VILIP-1 might modulate hippocampal network activity and synaptic plasticity.