V. Discussion

1. The expression of NCS proteins in peripheral tissues

The peripheral tissue distribution of members of the NCS protein family including VILIP-1, Hippocalcin, and NCS-1 was investigated experimentally by Western blot analysis. All NCS proteins showed major expression in the nervous system. In peripheral tissues VILIP-1 is detected mainly in heart, liver, lung and testis in human and rat but also stomach and skin in rat. Furthermore, the presence of VILIP-1 in heart and colon was recently shown by RT-PCR (Ohya and Horowitz, 2002), the presence in skin was shown by immunohistochemistry (Mahloogi et al., 2003), thus, reconfirming our results. Therefore, from these data, it can be concluded that VILIP-1 is mainly a neuronal protein, however, significant expression levels can also be found in some peripheral tissues in a developmentally regulated manner. In contrast to the other NCS proteins, Hippocalcin showed a very restricted protein expression pattern exclusively in brain tissues. A wide distribution of NCS-1 protein was observed in most peripheral organs of human and rat which is in line with previous findings for heart and the gastrointestinal system (Decher et al., 2001; Ohya and Horowitz, 2002). Differences in the degree of expression were observed for VILIP-1 and NCS-1 especially in heart, liver, lung and kidney in human and lung, kidney, testis and skin in rat. Whereas in lung and skin VILIP-1 showed high and NCS-1 low expression levels it was vice versa in kidney, possibly indicating organ specific functions of the two NCS proteins. The peripheral distribution of NCS proteins in relation to brain expression was much higher in embryonic tissue compared to adult rat tissues, indicating a developmental restriction of NCS protein expression. The developmental changes in the strength of NCS protein expression occurred as a decrease in expression in liver, lung, kidney, spleen, pancreas and colon and may additionally suggest to a specific function of defined NCS proteins during the development of organs (Gierke et al., 2004). Thus, it is likely that NCS proteins do not only play a role in the development and physiology of peripheral organs but also under certain pathophysiological conditions. This has recently been shown for VILIP-1 in the form of an involvement in growth and invasiveness of head/neck tumors (Mahloogi et al., 2003).

2. The distribution of VILIP-1 in rat hippocampus

In rat hippocampus, neuronal calcium-sensor proteins have been detected in hippocampal cells. For example, hippocalcin and NCS-1 have been found in hippocampal pyramidal cells (Kobayashi et al., 1992; Schaad et al., 1996). The neuronal visinin-like Ca^{2+}-binding protein 2 (VILIP-2) has been found in dentate granule cells and hippocampal pyramidal cells (Saitoh et al., 1994; Lenz et al., 1996a), and neurocalcinδ, highly homologous to VILIP-3 and hippocalcin,

has been found exclusively in hippocampal interneurons (Martinez-Guijarro et al., 1998).

VILIP-1 expression in principal neurons and interneurons as seen in rat hippocampus, is similar to the distribution in chick brain (Lenz et al., 1996a), but slightly different to the expression profile in human hippocampus, in which only a few VILIP-1-IR neurons were found in the CA2 and CA4 subfields (Bernstein et al., 2002). On the mRNA level, hippocalcin, another calcium binding protein, was also reported at a high rate of expression in rat hippocampus. As reported previously, the hippocalcin gene is expressed heterogeneously in pyramidal cells (Grant et al., 1996), and the VILIP-1 gene is expressed uniformly in pyramidal cells, but expressed in putative GABAergic interneurons (stratum radiatum and stratum oriens) of both CA1 and CA3 sectors (Lenz et al., 1996a). This was confirmed on the protein level by immunofluoresence staining. Different expression profiles for VILIP-1 and hippocalcin were seen in rat hippocampus. Most intense hippocalcin-IR was found in the somata and dendritic trees of principal neurons in CA1, CA3 (data not shown). Interestingly, immunofluoresence staining showed striking abundant dendritic expression of VILIP-1 in Schaffer collateral fibers of CA1, the stratum radiatum, molecular layers of CA3 and the internal molecular layer of the dentate gyrus.

GABAergic non-pyramidal cells in the hippocampus are known to be morphologically and physiologically heterogeneous and were shown to contain different kinds of calcium binding proteins, such as calbindin-D28k, Parvalbumin (PV) and calretinin (CR) (Celio, 1986; Katsumaru et al., 1988; Demeulemeester et al., 1989; Danos et al., 1991). GAD67, a marker of GABAergic neurons, was not detectable in all interneurons compared with the result of GDA67/65 mRNA hybridization in rat hippocampus (data not shown), and only partial co-localization of VILIP-1-IR and GAD67 in nonprincipal neurons was found. This might be due to the relatively low expression level of GAD67 and low content of the GAD67 in the some somata of hippocampal interneurons. Therefore, I further characterized the VILIP-1-IR nonprincipal neurons by immunofluoresence staining with three calcium binding proteins, which are well defined interneuron markers: PV, calbindin-D28k and CR.

Only a few VILIP-1-IR neurons containing PV were seen in the stratum oriens of the CA1 region of rat hippocampus, this is similar to the finding in the PV-GFP transgenic mouse (data not shown) and matches the observation of 10% co-localization in human brain (Bernstein et al., 1999). Most VILIP-1-IR nonprincipal neurons contain either Calbindin-D28k or CR. This was seen in various subfields of the CA1, CA3 and dentate gyrus regions. VILIP-1-IR nonprincipal neurons co-localized with Calbindin-D28k were found in the stratum radiatum of CA1, CA3 and the border of the hilus, because most Calbindin-D28k interneurons were located in those subfields. In rat hippocampus, the most abundant co-localization of VILIP-1-IR interneurons with CR was found in various layers of the CA1, CA3 and dentate gyrus, especially at the

border between the hilus and the granular layer of the dentate gyrus. Additional VILIP-1 and PSA-NCAM, CR and PSA-NCAM staining experiments were also performed in mouse hippocampal slices. Big mossy cells with weak CR-IR were seen in the deep hilus, CR-positive cells localized in the SGZ were of a small size compared to interneurons in the border area, which were PSA-NCAM immunopositive, but VILIP-1 immunonegative indicating those newly born cells indeed contain CR but not VILIP-1 in mouse hippocampus. This result further confirmed our finding that the newly born cells do not contain either CR or VILIP-1 in rat hippocampus， which is in contrast to that moderate or weak VILIP-1-IR was found in mature granule cells in the rat dentate gyrus in present study (Fig 3) consistent with previous study of Bernstein et al (Bernstein et al., 2003).

Pyramidal shaped VILIP-1-IR interneurons were often seen in the border of the hilus. In the dentate gyrus, most VILIP-1/Calbindin-D28k or CR double-labeled interneurons corresponded to pyramidal-like cells located in the hilar border. In addition, some horizontal, fusiform VILIP-1/Calbindin-D28k cells located in the hilar border of the granule cell layer were also found. However, they are always negative for PV, but 50% of them are CR-IR. In contrast, I have found that about 30% of the pyramidal-like VILIP-1-IR cells in the dentate gyrus also contain Calbindin-D28k. The morphology of the pyramidal-like cells suggests that they may be activated in both a feedforward manner by the entorhinal cortex and the commissural-associational pathway or even CA3 pyramidal cells and in a feed-back manner by mossy fiber collaterals (Ribak and Peterson, 1991; Kneisler and Dingledine, 1995).

There are at least six different mGluR1α positive types of interneuron in rat hippocampus, including O-LM cells and oriens bistratified cells in the stratum oriens, which mainly contained VILIP-1, but showed a relatively weak immunoreactivity compared with the interneurons in the border of the hilus. Additionally, it is likely that those VILIP-1-IR interneurons in the alveus and stratum oriens of CA1 partially coexisted with somatostatin or NPY (data not shown). Interestingly, partial co-localization of VILIP-1 and α4 nAChR was also found in some interneurons located in the stratum oriens, stratum radiatum of CA1, and the hilus of the dentate gyrus, which agreed with the previous finding that α4 nAChR is mainly present in certain interneurons, based on the data of immunofluoresence, single cell RT-PCR, and patch clamp studies (Gahring et al., 2004; Sudweeks and Yakel, 2000; McQuiston and Madison, 1999; Alkondon and Albuquerque, 2001, 2002).

In general, the functional meaning for coexistence of VILIP-1 and so-called ‘‘buffer type,’’ neuronal calcium binding proteins, for example, Calbindin-D28k and CR in an interneuron is as yet unknown, however, according to their various calcium-binding affinities, structural and

functional characters they might be involved in different calcium mediated events (Ikura, 1996). Other neuronal calcium-sensor proteins different from VILIP-1 have been found in the hippocampus, including NCS-1, and hippocalcin (Kobayashi et al., 1992). Deisseroth suggested that the critical Ca^{2+} signal must be transmitted to a downstream effector within 1–2 μm of sites of Ca^{2+} entry during LTD/LTP. The existence of a Ca^{2+}/myristoyl switch has been shown within living cells for hippocalcin, VILIP-1, and VILIP-3 (Spilker et al., 2002; O'Callaghan et al., 2002). VILIP-1 has a calcium-myristoyl switch, which allows VILIP-1 to bind Golgi and cell membranes in a calcium-dependent manner because of its high affinity for calcium (Spilker et al., 2002). In contrast, NCS-1 has a distinct, predominantly perinuclear localization (similar to trans-Golgi markers) at both resting and raised levels of Ca^{2+}, due to the myristoyl tail of NCS-1 exposed at resting condition, allowing membrane association independent of calcium (O'Callaghan et al., 2002). Thus, different neuronal calcium sensor proteins might serve different functions in particular neuronal subtypes, such as in principal cells and interneurons, or different calcium sensor proteins might take part in a defined function depending on their affinities for calcium, and molecular characters.

Regardless of what the precise function of VILIP-1 is, the heterogeneous expression in different neuronal subpopulations as well as the different expression level in hippocampal neurons suggests that different types of neurons, for example, interneurons, containing this calcium-sensor protein are likely to express specific functional properties. Electrophysiological characterization and correlative morphological and immunocytochemical studies will have to be used to clarify the particular functional properties of VILIP-1 in hippocampal neurons.

3. Redistribution of endogenous VILIP-1 triggered via nAChR in hippocampal neurons

Hippocampal neuron culture represents a simple and useful model system to study the localization and trafficking of heterologously expressed proteins. Functionally, nAChRs are ligand-gated cationic channels with the capacity to elicit local changes in cytoplasmic Ca^{2+} levels, and different subtypes of neuronal nAChRs are known to be differentially permeable to Ca^{2+} (Bertrand et al., 1993; Séguéla et al., 1993; Fucile, 2004). Although much evidence indicates that nAChRs can directly regulate synaptic plasticity in the hippocampus (Fujii and Sumikawa, 2001; Ji et al., 2001; McGehee, 2002), the cellular mechanisms involved are not fully understood, but likely to involve Ca^{2+}. The influx of Ca^{2+} through nAChRs has implications not only for synaptic plasticity, but also for the release of neurotransmitters for those presynaptic terminals that express Ca^{2+}-permeable nAChRs (Dajas-Bailador and Wonnacott, 2004; Fucile, 2004). In the hippocampus, Gray et al. (Gray et al., 1996) found that activation of α7-containing nAChRs increased intraterminal Ca^{2+} levels and facilitated glutamatergic release. Calcium ions can activate and regulate a variety of signal transduction

cascades, and play a key role in the short- and long-term regulation of nAChRs (Quick and Lester, 2002; Dajas-Bailador and Wonnacott, 2004; Fucile, 2004). Because of the importance of nAChR function in the hippocampus in relation to cognition and synaptic plasticity, and the fact that diverse subtypes of α7- and non-α7-containing nAChRs are preferentially expressed in hippocampal interneurons, I investigated the effect of VILIP-1 via nAChR activation in hippocampal neurons.

For VILIP-1, I could show that an increase in intracellular calcium concentration, by different means, such as glutamate receptor activation or nAChR activation, induces a translocation of myristoylated VILIP-1 to specialized sites of the plasma membrane, thus suggesting that VILIP-1 containing the myristoyl tail is located in the cytosol in the calcium-free state, and calcium binding leads to translocation to the membrane compartment and subsequent activation of the target molecule. The demonstration of reversible translocation of VILIP-1 by applying EGTA further suggests that VILIP-1 can reversibly shuttle calcium signals from or to cellular membranes, for example, to sites close to the cortical actin cytoskeleton (Spilker et al., 2002; Lenz et al., 1996b). I could show that a selective blockade of α7 nAChR by preincubation of MLA, a specific antagonist of α7 nAChR, dramatically decreased VILIP-1 translocation compared to the nicotinic effect mediated via activiation of all nAChRs. Further inhibition of the α4 nAChR subunit by preincubation of 100nM DHβE only slightly decreased the effect of nicotine, suggesting that both α7 and α4 nAChR contribute to the rise of intracellular Ca^{2+} leading to the redistribution of VILIP-1, but the α7 subunit is predominant in increasing Ca^{2+}. This might be due to not only a small subpopulation of interneurons containing both α4β2 nAChR and VILIP-1 as suggested by the previous immunofluoresence data of hippocampal slices, but also due to the fact that the Ca^{2+} permeability of α4 nAChR is relatively lower than α7 nAChR (Fucile, 2004; Ragozzino et al., 2002).

Castro and Alkondon et al. (Castro and Albuquerque, 1995; Alkondon et al., 1996) demonstrated that approximately 5.6% of the ACh triggered α7 nAChR current is carried by Ca^{2+} whereas approximately 9% of NMDA-evoked current is carried by Ca^{2+}. Considering that mean open time and the kinetics of inactivation of α7 nAChR are much faster than those of the NMDA receptor, the Ca^{2+} influx through the NMDA receptor should be longer lasting than that through the α7 nAChR (Alkondon et al., 1996; Albuquerque et al., 1997). However, 5μM nicotine is efficient in increasing intracellular Ca^{2+} mainly via α7 nAChR leading to VILIP-1 redistribution to the membrane, although 5μM nicotine concentration does not reach the EC_{50} of α7 nAChR (27μM in hippocampal neuron (Alkondon and Albuquerque, 1993, 1995) contrasted with 7.8μM in *Xenopus* oocytes (Gerzanich et al., 1994), furthermore about 57% of cells showed VILIP-1 distribution whereas about 48% of cells showed VILIP-1 distribution via

activation of NMDA receptors evoked by 100 μM glutamate/10 μM glycine. This effect, in contrast to the theoretical Ca^{2+} permeability of two receptors might be due to the wide range and abundant expression of α7 nAChR in cultured hippocampal neurons (more than 90% of cultured hippocampal neurons contain α7 nAChR mRNA (Alkondon et al., 1994) and predominant functional α7 nAChR occurs in hippocampal culture (93%) (Alkondon et al., 1993), additionally, functional α7 nAChR current but small amplitude (about 12pA) was detectable in most pyramidal neurons of the hippocampus CA1 in young mice, which can be inhibited by MLA mostly (Ji et al., 2001), and Ca^{2+}, Na^{+} influx via activation of α7 nAChR might further activate Ca^{2+} release from internal Ca^{2+} store, voltage operated calcium channels or facilitate synaptic transmission, respectively (Alkondon and Albuquerque, 1993).

MLA or MLA/DHβE blocked the redistribution of VILIP-1 evoked by nicotine, but not in the group evoked by glutamate/glycine in the current study, suggesting nAChR and NMDAR can contribute to the rise of Ca^{2+} in hippocampal neurons. Furthermore, the character of the inward rectification of α7 nAChR mediated currents and the outward rectification of NMDA induced currents suggests that although the NMDA receptors and α7 nAChR with high Ca^{2+} permeability are apparently located on similar areas of hippocampal neurons, it is likely that NMDA receptors and α7 nAChR are involved in different Ca^{2+} signaling (Alkondon et al., 1996). Thus, one can expect that in some synaptic terminals containing α7 nAChR and NMDA receptors, the focal increase Ca^{2+} of α7 nAChR activated by ACh will lead to a local depolarization to remove Mg^{2+} from the NMDA receptor. Another complicated pathway was demonstrated by Welsby et al. (Welsby et al., 2006) that nicotinic activation of the Ca^{2+} -permeable α7 nAChRs fills RyR Ca^{2+} stores, and release of Ca^{2+} from such stores by high-frequency stimulation via Ca^{2+} -induced Ca^{2+} release and activation of mGluRs induces an additional component of LTP. Thus, nAChR particularly α7 subunit may act in coaffecting with the NMDARs to regulate neuronal excitability and synaptic plasticity in hippocampal neurons.

4. Neuronal Ca^{2+}-sensor protein VILIP-1 is a physiological modulator for functional up-regulation of α4β2 nAChR: possible link to synaptic plasticity

To clarify whether the up-regulation of surface expression reflects a functional up-regulation of α4β2 nAChR in hippocampal neurons, I performed whole cell patch clamp studies in stably transfected HEK cells and in hippocampal neurons. ACh evoked currents in HEK cells stably transfected with human α4β2 nAChR and in two week old neuronal cultures, transiently transfected with rat α4β2 nAChR, were almost completely blocked by the α4β2 receptor antagonist DHβE indicating that following transfection, most of the current recorded was through expressed α4β2 nAChRs. VILIP-1-GFP, but not GFP expression caused significant increase in the amplitude of these currents. Interestingly, there was some variation observed in

the magnitude of the responses. Plotting amplitude against the deviation of average identified a group of neurons as high ACh responders with about 2.5 fold higher amplitude current than the second group of low responders (data not shown). The group of high responders might indicate a strong basal expression of α4β2 receptor for example in certain types of interneurons in the hippocampal formation (Alkondon and Albuquerque, 2001, 2004). Indeed, our immunohistochemical studies in the rat hippocampal formation clearly indicated co-localization of VILIP-1 in a discrete subpopulation of hippocampal neurons, which according to specific localization and morphology were clearly characterized as interneurons. In the hilus region of the dentate gyrus as well as in the stratum oriens and moleculare of the hippocampal CA1 regions these cells apparently represent interneurons.

There is generally a strong nAChR expression in the hippocampus (Wada et al., 1989), which arises from the cholinergic innervation of the medial septum and the diagonal band (Woolf, 1991). Furthermore, nAChRs are localized on GABAergic interneurons where they are described as enhancing or diminishing synaptic plasticity and network activity (Alkondon and Albuquerque, 2001, 2004). The *in vivo*-application of nicotinic antagonists within the hippocampus impairs memory performance (Bancroft and Levin, 2000) and the application onto hippocampal brain slices *in vitro* influences synaptic plasticity such as LTP (Hamid et al., 1997; Fujii et al., 1999). Presynaptic nAChRs enhance the release of glutamate in the hippocampus (Radcliffe and Dani, 1998), and thereby enhance the induction of plasticity (Ji et al., 2001; Mansvelder and McGehee, 2000). Depending on the timing of nicotinic activity and presynaptic stimulation postsynaptic nAChRs contribute to the induction of LTP or LTD (Ji et al., 2001; Ge and Dani, 2005). Moreover, mutants in the human α4β2 nAChR associated with autosomal dominant nocturnal frontal lobe epilepsy, which are engineered in mice, lead to disturbances of inhibitory synchronization of cortical networks by GABAergic interneurons (Klaassen et al., 2006).

5. VILIP-1 enhances inhibitory GABAergic transmission to pyramidal neurons

Hippocampal interneurons can coordinate the activity of principal cells, and interneurons therefore play a role in the regulation of hippocampal output. Interneurons are thought to be responsible for regulating the hippocampal network such as oscillations (Jones and Yakel, 1999). Considering that one interneuron might contain different subtype nAChRs (Sudweeks and Yakel, 2000), contribution of other nAChRs to IPSCs evoked by ACh application might be possible, Since increased IPSCs evoked by ACh can be completely blocked by MLA in combination with DHβE, it seems that α4 and α7 predominately contribute to the enhancement of IPSCs induced by ACh in hippocampal neuron culture. Therefore, the contribution of other nAChR subunits for IPSCs in cultured hippocampal neuron is likely to be weak even though they might be present.

The observed the enhancement of IPSCs triggered by activation of α4 nAChRs in rat hippocampal cell culture transfected with VILIP-1-GFP seems to contradict the previous finding that α7 nAChR activation is predominant for the translocation of VILIP-1 to cell surface membranes triggered by nicotine. The α7 nAChR contributes most of the Ca^{2+} influx following nicotine application due to a wide range of expression of α7 nAChR in rat hippocampal neurons (including principal neurons) and due to its high Ca^{2+} permeability. In contrast, functional expression of α4 nAChR appears to be restricted to certain interneuron population, as was demonstrated in the current study (see Fig. 9), and has been reported by other groups (Gahring et al., 2004; Alkondon and Albuquerque, 2001, 2002). Although both α4 and α7 nAChR contribute to the enhancement of IPSCs evoked by ACh in rat hippocampal neurons, Alkondon and Albuquerque (Alkondon and Albuquerque, 2001, 2002) convincingly demonstrated that α4 nAChR predominately contributes to PSCs in interneurons of the stratum oriens, and radiatum in the CA1 region of rat hippocampal slices *in vitro*. Additionally, in rat hippocampal cultures a 65% proportion of IPSCs was identified to be dependent on α4 nAChRs (Braga et al., 2004). This predominant proportion of IPSCs contributed by α4 nAChRs might be due to the localization of α4 nAChR in both, the axonal and dendritic domains (Alkondon et al., 1996; Xu et al., 2006), although with a low Ca^{2+} permeability character (Fayuk and Yakel, 2005). In contrast, α7 targets were preferentially found in the somatodendritic compartment (Xu et al., 2000; Khiroug et al., 2003). The expression of the interacting partner α4 nAChR seems to be restricted to interneurons; therefore VILIP-1 is able to influence the functional activity of interneurons. From the current data it appears that the overexpression of VILIP-1 in cultured hippocampal neurons, as in the interneurons containing endogenous α4 nAChR, can induce an enhancement of ACh triggered IPSCs to neighboring cells.

Alkondon and Albuquerque (Alkondon and Albuquerque, 2001, 2002) suggested that the function of pyramidal neurons could be modulated via nAChR in different ways: facilitation of GABAergic transmission via activation of either somatodendritic nAChRs or presynaptic/preterminal nAChRs of the interneurons can exert an inhibitory effect on pyramidal neurons. Thus cholinergic nicotinic inhibition could suppress weak excitatory signals arriving at the pyramidal neuron dendrites; therefore only strong signals would be propagated. This offers a filter mechanism for extraneous signals and increases the attentional function, which α4β2 nAChRs predominately contribute to this effect. This was supported by the finding of Ji et al. that the enhanced IPSC mediated by locale nicotine application blocked short term potentiation (STP) and diminished long term potentiation (LTP) in the CA1 of rat hippocampus *in vitro* (Ji et al., 2001). A similar result was also found in the prefrontal cortex, that nicotine enhanced inhibitory GABAergic transmission to pyramidal neurons in layer V. The nicotine mediated IPSCs can be partially inhibited by MLA, suggesting that MLA-sensitive nAChRs do contribute

to the effect of nicotine on IPSCs (Couey et al., 2007), which is similar to the finding in cultured hippocampal neurons (Alkondon and Albuquerque, 2001, 2002). On the other hand, nAChR-mediated GABA release can cause neuronal hyperpolarization, which in turn, affects neuronal function. For example, hyperpolarization removes inactivation of inward currents (Cobb et al., 1995), and this action resets the membrane potential of the neurons such that a subsequent excitatory input will be more effective in eliciting action potentials. This mechanism can increase the efficiency of the neuron network as a whole.

In the future, the analysis of the effects of VILIP-1 on synaptic transmission in the context of fully functional neuronal networks that are modulated by nAChR is essential for the understanding of how VILIP-1 alters overall neuronal activity in the hippocampus. Further work is needed for the detailed molecular and electrophysiological characterization of the VILIP-1 and α4β2 nAChR positive interneurons we have identified, their precise role for synaptic plasticity and their relationship to the observed misregulation of VILIP-1 expression in several neurological diseases (Braunewell, 2005).

6. Time-dependent upregulation of VILIP-1 and NCS-1 expression following application of DHPG in the hippocampus *in vivo*

The permanent storage of memory is dependent on newly synthesized proteins as is the late phase of various plasticity phenomena in the hippocampus (Braunewell et al., 2000; Richter-Levin and Yaniv, 2001; Abraham and Williams, 2003). Recently it has been shown that hippocampal LTP *in vivo* can last for up to one year (Abraham, et al., 2002). In order to characterize this late protein-dependent phase of synaptic plasticity in more detail in this study, we have examined protein expression in a simple model of chemically induced plasticity in the rat hippocampus *in vivo*. We have compared the expression of two novel candidate plasticity genes, the neuronal calcium sensor proteins NCS-1 and VILIP-1, which most likely are functionally involved in processes of synaptic plasticity, and we have analyzed the time-dependent regulation of expression following DHPG-induction.

Application of DHPG in a concentration which has previously been shown to result in induction of a form of LTP called slow-onset potentiation in the rat hippocampus *in vivo* (Manahan-Vaughan and Reymann,1997; Braunewell, et al., 2003) led to an enhancement in the expression of two neuronal calcium sensor proteins, NCS-1 and VILIP-1, measured 24 h following drug administration. The effect of the group I mGluR agonist DHPG was specific, since the antagonist 4CPG was able to attenuate the effect on VILIP-1 (Braunewell et al., 2003) and NCS-1 expression (this study). We could show that both proteins showed significant enhancement 24 h following plasticity induction which may favour a role for these proteins as

factors for late-LTP. Although LTP measurements *in vitro* last for only up to 4–8 h the 24 h time point seems to be relevant for *in vivo* measurements, where late-LTP has been shown to last for days and even to exist up to several months (Abraham et al., 2002). Interestingly, inhibition of the group I mGluR5 in the hippocampus *in vivo* leads to attenuation of LTP which starts being significant by 2.5 h and is still observed 24 h after induction of LTP. In this study it was also shown that the mGluR5 antagonism leads to an impairment of working and reference memory which became evident 5 days after drug treatment and which most likely depends on inhibition of protein synthesis (Naie and Manahan-Vaughan, 2004)

We could also show that increases in expression are visible at earlier time points, although not at significant levels, starting at 2 h following induction of plasticity. Since in Western blot analysis only the overall expression of the proteins in the whole hippocampus can be estimated it is tempting to speculate that significantly enhanced expression levels may exist in defined subpopulations of neurons at these earlier time points and thus, NCS proteins may also contribute to the normal late phase of LTP starting at 1–2 h following LTP induction. It is conceivable that at the cellular level different NCS proteins show strong regulation and are differentially expressed in subpopulations of neurons. Furthermore, since DHPG and group I mGluRs differentially contribute to the expression of LTP and LTD *in vivo* (Kulla et al.,1999; Manahan-Vaughan, 1997), for review see (Braunewell and Manahan-Vaughan, 2001), also different localization- and time-dependent regulation of NCS-proteins may be envisaged for the different forms of plasticity.

Our results support a role for NCS-1 and VILIP-1 in the molecular changes underlying protein synthesis-dependent hippocampal plasticity *in vivo*. Activation of mGluRs may function as a trigger for the regulation of hippocampal NCS-1 and VILIP-1 expression. NCS proteins in turn may lead to long-term changes in neuronal signaling during synaptic plasticity, possibly leading to an enhancement of long-term memory as implied by data from *C. elegans* (Gomez et al., 2001). The interaction between mGluRs and NCS proteins could thus correspond to an important mechanism linked to the long-term consolidation of hippocampal LTP and LTD, possibly also influencing long-term memory.

7. Intrinsic electrophysiological properties of VILIP-1 transfected hippocampal neurons

In hippocampal neuron culture, more than 4 subtypes of neurons including basket cell (interneurons) could be identified by morphological criteria or electrophysiological properties and immunohistochemical methods. However, hippocampal neurons prepared from E18 are mainly comprised of pyramidal neurons. The proportion of cells within the entire hippocampus that show GABA-like immunoreactivity has been reported to be between 7% and 11%, although

this may be an underestimation due to false negative staining and would be better estimated with *in situ* hybridization for glutamate decarboxylase, for example. To perform patch clamp recording in the current study, cells were often eliminated from the study for membrane potentials of less than -40 mV. It may be that this population of "rejected" cells had a high proportion of pyramidal cells. However, only pyramidal-like neurons were selected. Therefore, most recorded cells were likely to have been pyramidal neurons

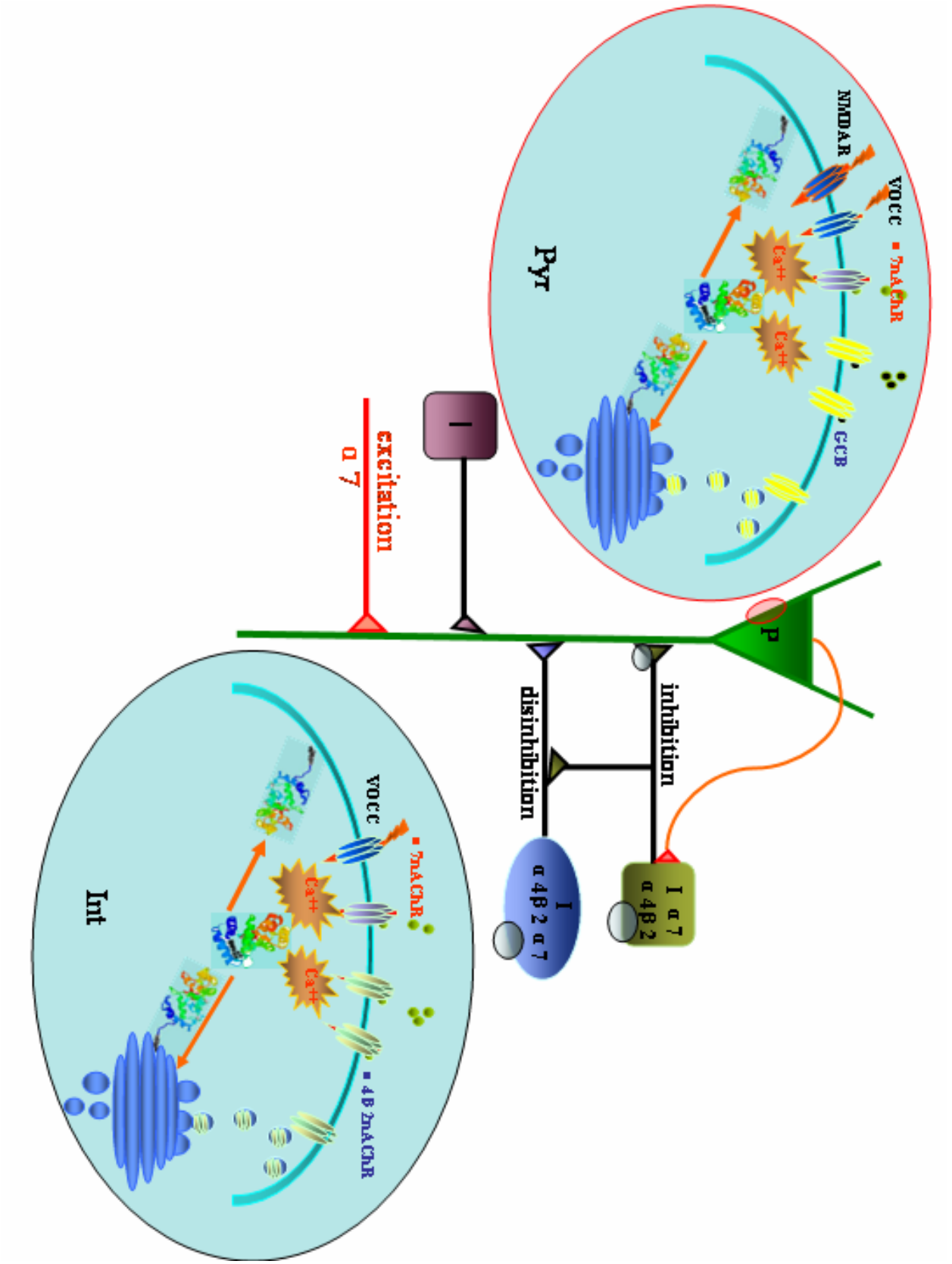
All the intrinsic properties of cultured hippocampal neurons including action potential durations, are similar to the findings of other groups (Evans et al., 1998), however, the side effect of transfection and overexpression could not be ruled out in the current study, since the control group cells were not transfected. GFP alone construct will be included as a proper control in future experiments. Additionally, afterhyperpolarization measurement was omitted in the current study, since we found that afterhyperpolarization current randown quickly due to conventional whole cell recording, which quite often disappeared within 1-2 minutes of establishing whole cell conditions similar to the finding from other groups (Shah and Haylett, 2000). Therefore, perforated patch recording will be performed to minimize afterhyperpolarization rundown in the future.

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

Direct evidence for a link between nicotinic enhancement, cognitive function (Mansvelder and McGehee, 2000; Mansvelder et al., 2006; Couey et al., 2007) and disease (Jones et al., 1999; Hogg et al., 2003; Gotti and Clementi, 2004) have been provided by various studies. The α4β2 nAChR is known to enhance GABAergic neurotransmission in different brain structures, leading to the modulation of network activity (Jones and Yakel, 1997; Alkondon et al., 1997; McQuiston and Madison, 1999; Couey et al., 2007). It has been reported that intrinsic cholinergic interneurons can pattern network activity via nAChRs in rat hippocampal slices. Since VILIP-1 is misregulated in hippocampal pyramidal neurons and interneurons in brains of schizophrenic patients (Bernstein et al., 2002, 2003), in Alzheimer patient brains (Braunewell et al., 1999; Schnurra et al., 2001), and following induction of LTP in the rat hippocampus *in vivo* (Braunewell et al., 2003; Brackmann et al., 2004), together with enhancement of functional α4β2 nAChR in certain interneurons *in vitro* leading to enhancement of IPSCs triggered by ACh, we postulate that VILIP-1 in conjunction with the α4β2receptor plays an important role in modulating network activity in healthy and diseased hippocampus. Based on these previous findings and the current results we want to put forward a hypothesis: sharing similar molecular mechanism, modulation of VILIP-1 might modulate synaptic plasticity or other neuronal activities in physiological and pathological conditions via modulation of α4β2 nAChR in interneurons, which

might serve as a long-term coincidence detection mechanism for plasticity of nicotinergic neurotransmission, or via modulation of other partners in pyramidal neurons, for example, receptor guanylyl cyclase B (GC-B), which might affect processes, such as neuronal differentiation and synaptic plasticity (Brackmann et al., 2005) (Fig. 20). The Ca^{2+}-myristoyl switch of neuronal Ca^{2+} sensor VILIP-1 shuttles the protein in a Ca^{2+}-dependent manner to the cell surface and trans-Golgi membranes. Recent work of our laboratory demonstrates that in the trans-Golgi network VILIP-1 is part of a complex with α4β2 nAChR, clathrin and the SNARE syntaxin-6, indicating a functional involvement in receptor trafficking from the Golgi (Fig. 20 black bulb). In line with these findings VILIP-1 causes enhanced surface transport of α4β2 nAChR via the constitutive exocytotic pathway which, within minutes after a Ca^{2+} stimulus, increases surface expression and sensitivity of the neuron towards cholinergic input (Fig. 20 cartoon of network). This undescribed form of functional up-regulation of α4β2 nAChR might constitute a novel physiological mechanism of Ca^{2+}-dependent long-term plasticity in hippocampal neurons. The α4β2 nAChRs activation can excite GABAergic interneurons in the hippocampus therefore activation of interneurons via nAChR offers inhibition or disinhibition to the nearby principal cells because the subset of interneurons containing nAChR can innervate both principal neurons and interneurons. On the other hand, previous work of our laboratory demonstrated that VILIP-1 might interact with other partners, for example, GC-B enhancing the surface expression of GC-B by influencing clathrin-dependent receptor recycling in hippocampal neurons (Brackmann et al., 2005) (Fig. 20 red bulb). Additionally, recent work of our lab demonstrates that activation of GC-B via CNP enhances LTP in rat subiculum suggesting that VILIP-1 might modulate synaptic plasticity or other functions, for example, by influencing cGMP-signaling (Brackmann et al., unpublished data). Further elucidation of the regulatory mechanism underlying receptor export trafficking is needed and may also provide an important foundation for developing new therapeutic strategies for treating neurological diseases with cognitive disturbances.

Figure 20 Hypothesis for a novel physiological mechanism of functional up-regulation of nAChR which might modulate the impact of nAChR-mediated GABAergic transmission.
The cartoon shows the neuronal Ca^{2+} sensor VILIP-1, which is activated following stimulation of a neuron by depolarization, glutamate/glycine or ACh, acting on receptors increasing the intracellular Ca^{2+} level in both pyramidal neurons and interneurons of hippocampal CA1. In interneurons, VILIP-1 shuttles to the cell surface and also to Golgi membranes, where it co-localizes with α4β2 nAChR. VILIP-1 causes enhanced exocytosis and surface transport of α4β2 nAChR consequently increasing the surface expression and finally the sensitivity of the neuron towards ACh (black bulb). The functional up-regulation of α4β2 nAChR, which occurs within minutes may contribute to the plasticity of nicotinergic neurotransmission in principal neurons as well as in interneurons in the hippocampus, since the α4β2 nAChR is known to enhance GABAergic neurotransmission, thus both an inhibition or disinhibition effect on pyramidal cells is possible (network cartoon). In pyramidal neurons, similar VILIP-1 signaling may occur, but different partners of VILIP-1 may be involved. VILIP-1 may cause surface expression of those partners, for example, GC-B (red bulb); thereby modulating the synaptic plasticity or other functions, for example, by influencing cGMP-signaling. Thus, the physiological functional up-regulation of α4β2 nAChR in interneurons or GC-B in pyramidal cells by VILIP-1 is likely to modulate hippocampal network activity.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