I. Introduction

1. Neuronal Ca^{2+} homeostasis and signaling

Ca^{2+} plays an important role in many neuronal processes including neuronal development, neuronal differentiation, axon guidance, neuronal migration, cell cycle, apoptosis, gene expression, and synaptic plasticity (Bliss and Collingridge, 1993; Berridge, 1993; Trump and Berezesky, 1995). These processes occur over a wide time range--from submilliseconds to days, like the triggering of neurotransmitter release or inducing gene expression during various forms of brain plasticity (Augustine et al., 2003). Furthermore, due to the extreme complexity of neuronal morphology, many different Ca^{2+} mediated processes can occur simultaneously within the same cell (Hausser et al., 2000). The complex geometry and functions of the neuron make the study of neuronal Ca^{2+} signals particularly intriguing and challenging.

Calcium entry

Calcium influx from the extracellular space

The basal cytosolic Ca^{2+} concentration is maintained at 10-100nM in most neurons (Burnashev and Rozov, 2005), while the concentration in the surrounding extracellular space and in the intracellular stores is about 1-2 mM. Therefore, a large electrochemical gradient for Ca^{2+} exists across the membrane. During stimulation, the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) elevates rapidly reaching several μM in the bulk cytosol. This increase in $[Ca^{2+}]i$ frequently expresses complex temporal and spatial patterns (Augustine et al., 2003).

In most neurons of the central nervous system there are two major classes of Ca^{2+} channels: the voltage operated Ca^{2+} channels (VOCCs) (Catterall, 2000) and ligand operated Ca^{2+} permeable receptor channels, i.e. NMDA type of glutamate receptors (NMDARs), the neuronal nicotinic acetylcholine receptor (nAChRs), the type3 serotonin receptor (5HT3R), and the Ca^{2+}-permeable subtype of AMPA and kainate glutamate receptors (Burnashev, 1998). In the first type, an electrical signal (depolarization) generally triggers the opening of VOCC (Perez-Reyes and Schneider, 1995) in the plasma membrane allowing the Ca^{2+} to enter the cytosol from the outside. In the second case, the specific agonist i.e. acetylcholine (ACh) or glutamate binds the nAChR, and NMDAR respectively leading to Ca^{2+} influx (Figure 1). Interestingly, NMDAR is a ligand and voltage gated channel. Opening the NMDA channel requires agonist binding coincidently with depolarization of the membrane to remove the Mg^{2+} block from the channel pore (Kuner and Schoepfer, 1996).

Calcium release from internal stores

In the cytoplasm Ca^{2+} is mainly stored in the endoplasmic reticulum (ER) and mitochondria (Meldolesi, 2001). Mitochondria are able to accumulate large amounts of Ca^{2+} along the steep electrochemical gradient via the Ca^{2+} uniporter. Ca^{2+} is released from mitochondria either via a sodium dependent (Na^{+}/Ca^{2+} exchanger) or a sodium independent way (H^{+}/Ca^{2+} exchanger) (Nicholls, 2005). The mitochondrial Ca^{2+} cycle is involved in cytosolic Ca^{2+} regulation under physiological and pathological conditions. On the other side, it represents a mechanism for coupling neuronal and metabolic activity (Chan, 2006; Kann and Kovacs, 2007).

ER can be considered as a Ca^{2+} sink or source containing 100-800μM Ca^{2+} under resting conditions. ER forms a nanoscopic Ca^{2+} tunnel allowing long range Ca^{2+} transport in neurons (Petersen et al., 2001). This is of particular importance as the diffusion rate of free (unbound) Ca^{2+} in the cytosol is very low. Propagating Ca^{2+} signals, travel along ER via a regenerative mechanism (Coronado et al., 1994; Ogawa, 1994). Ryanodine receptors (RyR) at the ER membrane are Ca^{2+} stimulated / putative cyclic ADP-ribose stimulated Ca^{2+} channels (Meszaros et al., 1993). Thus, locally released Ca^{2+} will stimulate the opening of nearby RyRs leading to a release of more Ca^{2+} from the ER into the cytosol (positive feedback) (Bootman and Berridge, 1995). Although Ca^{2+} stimulates Ca^{2+} release, a high local Ca^{2+} concentration might be inhibitory, limiting Ca^{2+} release through a single store receptor in time. Alternatively, Ca^{2+} can be released from ER via IP3 receptor (IP3R) channels (Figure 1). In addition, but not necessarily linked to Ca^{2+} store (ER) depletion, IP3 is able to activate the transient receptor potential channels (TRPC) in the cell member, which is a nonselective cation channel, leading to the influx of cation including Ca^{2+}. Similar to RyR, the IP3 Receptor is also influenced by Ca^{2+}. Moreover the release of Ca^{2+} from one of these receptors can facilitate the opening of RyR receptors, propagating spatially the Ca^{2+} signal. In some neurons, both types of Ca^{2+} store receptors/channels are present allowing the generation of complex calcium signal patterns (Irving et al., 1992; Louvet and Collin, 2005). During the rise of Ca^{2+} in certain subcellular domains, a small proportion of the Ca^{2+} binds to the effectors that are responsible for stimulating numerous Ca^{2+}-dependent processes.

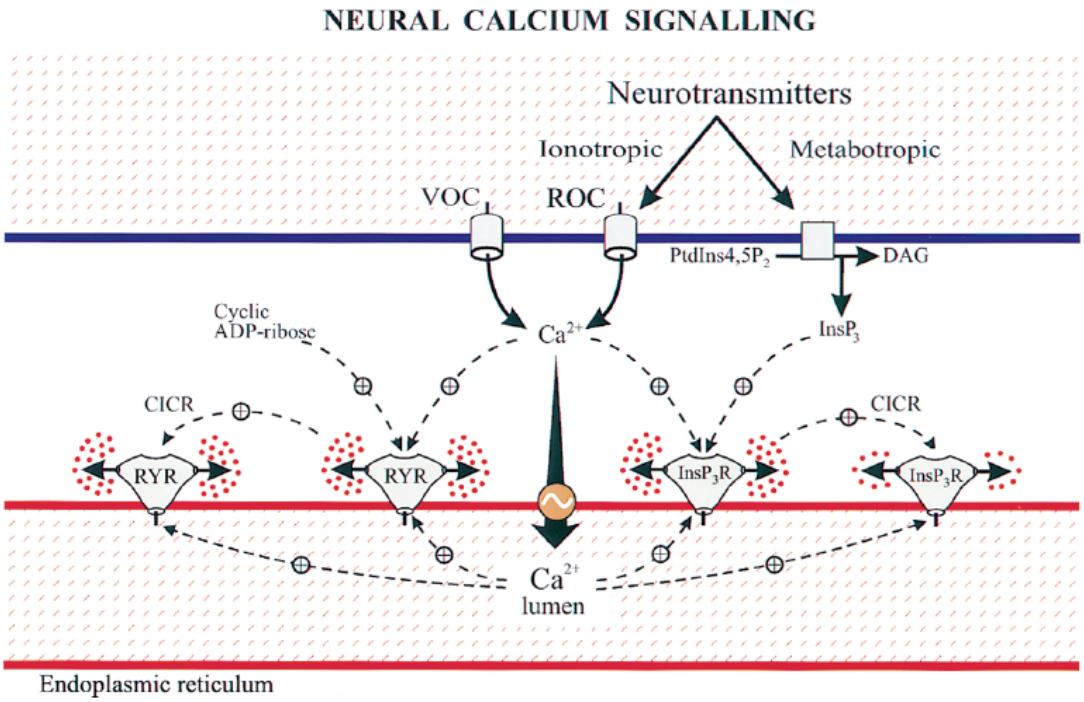

Figure 1 Neuronal Calcium Signaling (Berridge, 1998)

The entry of Ca^{2+} from the extracellular source is regulated by voltage-operated channels (VOCC) or by ligand-operated channels controlled by the ionotropic neurotransmitters. Metabotropic neurotransmitters stimulate the formation of inositol 1,4,5-trisphosphate (IP3), which acts on InsP3 receptors (IP3R) to release Ca^{2+} from the endoplasmic reticulum. The latter also has ryanodine receptors (RYR) sensitive to cyclic ADP ribose. Both the IP3Rs and the RYRs are also sensitive to Ca^{2+}, and this process of calcium-induced calcium release can set up propagated Ca^{2+} waves. Uptake of Ca^{2+} into the lumen primes these receptors by enhancing their Ca^{2+} sensitivity.

Neuronal Ca^{2+} homeostasis

In any case, to use Ca^{2+} as an intracellular signal, cells must keep resting cytosolic Ca^{2+} at a low level to take advantage of the gradient as a driving force to generate rapid $[Ca^{2+}]i$ transients. Basal $[Ca^{2+}]i$ is maintained by a complex homeostatic mechanism (Miller, 1991). The level of intracellular Ca^{2+} is determined by a balance between the effect that introduces Ca^{2+} into the cytoplasm and the opposite effect through which this signal is removed by the combined action of buffers, pumps and exchangers. The key processes/organelles involved in lowering $[Ca^{2+}]i$ are: (1) Mitochondria; (2) Organelles associated with the endoplasmic reticulum; (3) Ca^{2+} binding proteins; (4) Na^{+}/Ca^{2+} exchange; (5) Ca^{2+}/H^{+} exchange (6) Ca^{2+} pumps (Berridge et al., 2003; Miller, 1991). All eukaryotic cells have Ca^{2+} pumps in their plasma membrane that transport Ca^{2+} out of the cytosol at the expense of ATP hydrolysis. Nerve cells have an additional Ca^{2+} pump that couples the efflux of Ca^{2+} to the influx of 3 Na^{+} (Na^{+}/Ca^{2+} exchanger) thus exploiting the electrochemical gradient for sodium. Due to its low affinity for Ca^{2+}, the Na^{+}/Ca^{2+} exchanger, operates only when cytosolic Ca^{2+} levels rise above 1µM, as occurs after repeated nerve stimulation. There is another Ca^{2+}-ATPase pump in the ER membrane which enables the ER to take up and store Ca^{2+}, thus lowering $[Ca^{2+}]i$ and allowing ER to function as a

Ca^{2+} source (Miller, 1991; Berridge, 2002). Once the stimulation is over, the release of Ca^{2+} from internal store stops, the remaining "excess" of cytosolic Ca^{2+} is pumped out of the cell and also taken back into the internal Ca^{2+} stores (Friel, 2000; Verkhratsky, 2004; Mata and Sepulveda, 2005).

Normally, even during stimulation, global intracellular Ca^{2+} levels never go beyond 5μM, although local Ca^{2+}, for example, spine Ca^{2+} can reach more than 10μM during titanic stimuli to induce LTP (Petrozzino et al., 1995). If Ca^{2+} removal mechanisms are impaired, the free Ca^{2+} concentration can reach toxic levels (>10mM). Overloading $[Ca^{2+}]i$ due to a defect in Ca^{2+} "metabolism" is considered as the main cause of cell death (apoptosis) (Richter, 1993), which might play a role in various nerve system diseases including Huntington's disease, Parkinson's disease, Alzheimer's disease, epilepsy etc (Fendyur et al., 2004; Kann and Kovacs, 2007).

The main functions of neuronal Ca^{2+} signaling

Aside from the function of calcium in neuronal development and in the nucleus, Ca^{2+} has the ability to modulate neuronal excitability by altering membrane potential. Ca^{2+} is the key regulator in a broad spectrum of intracellular events, including changes in membrane potential, transmitter release, synaptic plasticity, ion channel activity, receptor phosphorylation, enzyme activation, metabolic activation, gene transcription etc.

Besides the membrane potential changes due to direct Ca^{2+} influx via VOCC, there are ion channels modulated by Ca^{2+}, like the Ca^{2+} dependent potassium channels responsible for the variations in membrane potential, which are either controlled by Ca^{2+} entering through voltage operated channels or Ca^{2+} released from internal stores during afterhyperpolarizations (AHPs) or depolarizing after potentials (DAPs), which are commonly found phenomena in most neurons, for instance hippocampal neurons (Storm, 1987). In addition, Ca^{2+} is a critical signaling messenger in neurotransmission and synaptic plasticity. Ca^{2+} influx to the cytoplasm space via voltage/ligand operated channels or internal stores triggers (at least to some degree) the transmitter release in the presynaptic terminal. Accumulating evidence suggests that not only Ca^{2+} through NMDA receptors or through voltage-operated channels is a major Ca^{2+} source, but internal stores, which can be Ca^{2+} sources or sinks, may contribute to long term potentiation (LTP) and long term depression (LTD)(Malenka, 1991; Teyler et al., 1994). For instance, metabotropic glutamate receptors (mGluRs) coupled with phosphoinositide signaling and cholinergic receptors play a role in hippocampal LTD and LTP, respectively (Harney, 2006; Welsby et al., 2006). Finally, Ca^{2+} rises at synapses trigger Ca^{2+} waves or other signal cascades including MAKP, PKA or calmodulin kinase pathway (Berridge et al., 2003). Then Ca^{2+} signals may propagate to the nucleus directly or indirectly and further modulate gene expression, which

is necessary for long term plasticity in the brain (Berridge et al., 2003; Carrasco, 2006).

2. Calcium binding proteins

Many of the effects of Ca^{2+} are carried out via binding to specific proteins, called Calcium-Binding Proteins (CaBPs). Different types of CaBPs are involved in the responses to the rise of Ca^{2+} in the cytosol. Those CaBPs are calcium dependent enzymes, channels, or calcium dependent modulating proteins. By contrast, other CaBPs serve as Ca^{2+} buffers and do not participate in signal transduction by themselves. To bind Ca^{2+}, proteins possess different Ca^{2+} binding structures, for example, the EF-hand, which is about thirty amino acids long and consists of an alpha-helix (E), loop, and second alpha-helix (F), was first defined in parvalbumin containing a repeated helix-loop-helix motif. Calcium binding proteins contain from two to twelve copies of the EF-hand domain (Kretsinger and Nockolds, 1973; Persechini et al., 1989). More than 120 classified EF hand proteins subfamilies are known today (http://www.ensembl.org). The same Ca^{2+} binding motif, EF hand, can often be found, in both sensor and buffer proteins (Ikura, 1996).

CaBP are functionally divided in two categories:

Calcium Buffer proteins: Ca^{2+} buffer proteins in the cytosol and in the intracellular Ca^{2+} stores (for example, parvalbumin, calretinin, calbindin-D28k) help to stabilize the intracellular Ca^{2+} concentration, allowing proper Ca^{2+} signal generation, and protecting cells from a Ca^{2+} overload (Baimbridge, 1992). Binding of Ca^{2+} to these proteins is generally characterized by a slow kinetics and a relatively high affinity (and capacity). Upon calcium binding they generally show no exposure of hydrophobic surfaces, and do not interact with target proteins.

Calcium Sensor proteins: Calcium dependent enzymes (for example, kinases, phosphatases, lipases and proteases), channels (for example, Ca^{2+} gated channels, IP3R, RyR) or calcium dependent modulators (activators, inhibitors) of the activity of target proteins belong to the category of calcium sensors. Calcium sensors are proteins that transduce the Ca^{2+} signal to other cellular components. Bound Ca^{2+} induces a conformational change of the calcium sensing proteins resulting in the exposure of hydrophobic surface(s), and allowing for interactions with other target proteins.

2.1 Neuronal calcium sensors: a conserved calcium-binding protein family

An emerging branch of the EF-hand CaBP superfamily is the family of nervous-system specific CaBP modulators: Neuronal Calcium Sensors (NCS). More than 40 members have already been identified in the NCS family (Burgoyne, 2001). They were cloned from different eukaryotic

species from yeast to human. Based on sequence similarities they were grouped in 6 subfamilies as depicted in the phylogenetic tree in Figure 2 (Braunewell and Gundelfinger, 1999). The members of the NCS family possess a high degree of homology sharing between 37 and 100% identity at the amino acid level. They are small acidic proteins of 185 to 265 residues with a molecular weight of 22 to 24kDa (Braunewell and Gundelfinger, 1999; Burgoyne, 2001). All contain 4 putative EF-hand motifs although the first EF-hand is degenerated and not able to bind Ca^{2+}. Many share a consensus sequence for N-terminal myristoylation. Ca^{2+} affinities ranging from low nM to low μM concentrations are well poised to bind physiological Ca^{2+}. Ca^{2+} binging can be cooperative or not (Burgoyne, 2001; O'Callaghan and Burgoyne, 2003). Their exact *in vivo* function remains generally elusive. They are thought to be implicated in the fine modulation of neuronal sensitivity and excitability (Burgoyne et al., 2004; Burgoyne, 2007; Braunewell, 1999, 2005)

We are interested in the Visinin like proteins (VILIPs); therefore, I will briefly introduce the members of VILIPs, and focus on VILIP-1.

VILIP-2

VILIP-2 (rat) which is closely related to (rat/chick) VILIP-1 (89% a. a. identity) seems to be mainly expressed in the brain (cortex, hippocampus, hypothalamus, midbrain, olfactory bulb) except the cerebellum and pons (Kajimoto et al., 1993). The highest amount of VILIP-2 mRNA is observed in the hippocampus. Only a small amount is detected in the cerebellum (Saitoh et al., 1994; Paterlini et al., 2000).

VILIP-3

VILIP-3 from rat has 97% of amino acid identity with its human homologue, hlp2 (both proteins are therefore likely to be orthologues), and 69% with VILIP-1. From phylogenetic studies VILIP-3 seems to be closer to neurocalcin than to VILIP-1 (see Figure 3). VILIP-3 seems to be expressed mostly in the cerebellum; it is also found in the pons plus medulla oblongata, and hippocampus, but not in other regions of the brain (Kajimoto et al., 1993; Saitoh et al., 1995).

Hippocalcin

Orthologues of hippocalcin were cloned form the rat and human (Kobayashi et al., 1992; Takamatsu et al., 1994). Hippocalcin is most highly expressed in hippocampal pyramidal neurons in contrast to VILIP-3 which is mostly expressed in cerebellar Purkinje cells.

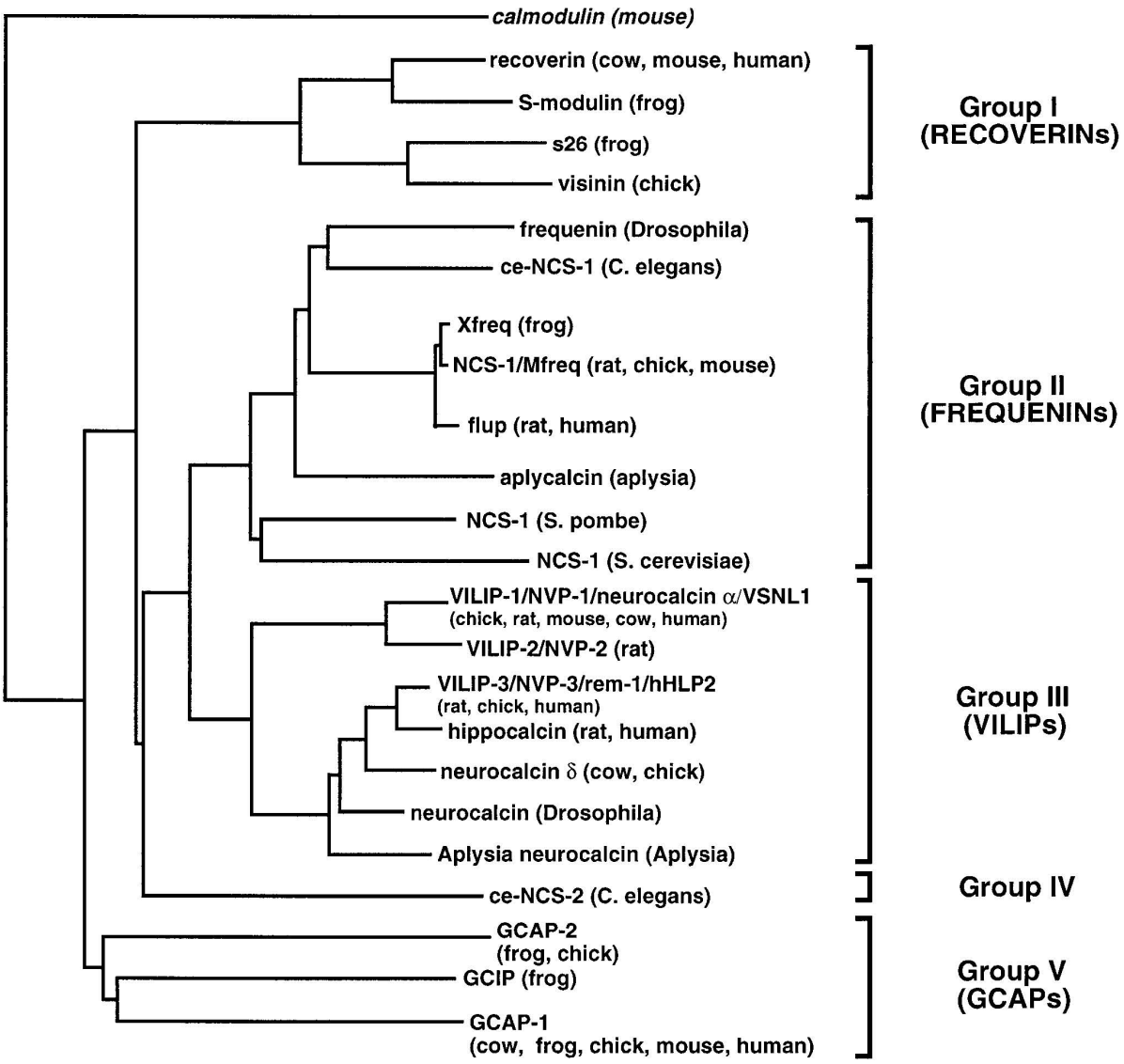

Figure 2 Dendrogram showing the relationship between the members of the family of intracellular NCS proteins based on amino acid similarity. Calmodulin is used as a reference member of the superfamily of EF-hand calcium binding proteins (Braunewell and Gundelfinger, 1999).

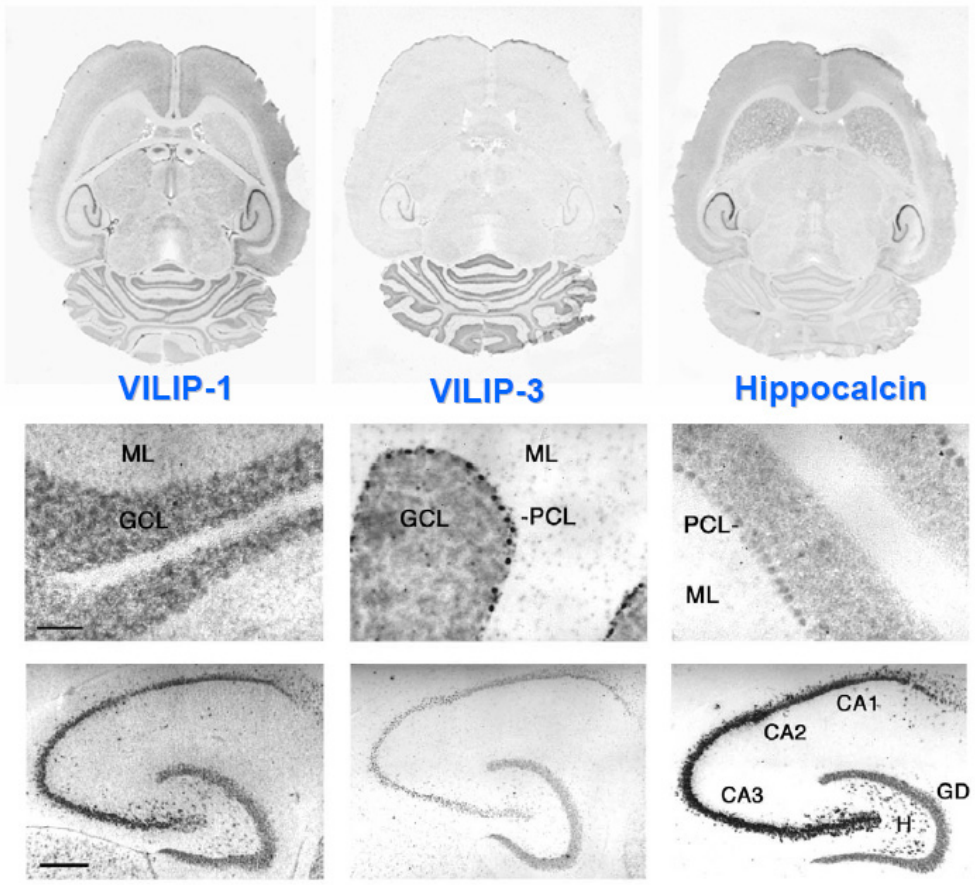

Figure 3 *in situ* distribution pattern of VILIPs in rat brain (Spilker et al., 2000)

Structure and biochemical properties of VILIP-1

The gene encoding VILIP-1 protein has been cloned in several species such as chicken, mouse, rat and human (Lenz et al., 1992; Kuno et al., 1992; Kato et al., 1998). Sequence alignments reveal that VILIP-1 is extremely well conserved through evolution (Figure 4). VILIP-1 is involved in a variety of Ca^{2+}-dependent signal transduction processes, including exocytosis, modulation of receptors, ion channels, signal effector protein function as well as membrane trafficking (Lin et al., 2002; Brackmann et al., 2005; Dai et al., 2006; Burgoyne, 2007). VILIP-1, with widespread distribution and multiple functions in many neuronal cell types, has been implicated in the pathology of a variety of CNS diseases (Braunewell, 2005), such as Alzheimer disease (Braunewell et al., 1999; Schnurra et al., 2001) and schizophrenia (Bernstein et al., 2002, 2003; Zhao et al., submitted). There is also some indication of the involvement of VILIP-1 in learning and memory processes. The expression of VILIP-1 in the hippocampus is regulated via mGluRs (Braunewell et al., 2003; Brackmann et al., 2004; Zhao et al., submitted), which are known to be important for certain forms of plasticity and learning (Braunewell and Manahan-Vaughan, 2001; Manahan-Vaughan and Braunewell, 2005).

Beside their different expression pattern, the calcium binding affinity of VILIP proteins is also different (Table 1). Thus multiple functions of VILIPs (or calcium sensor proteins) can be implicated in a certain type of neuron or a certain kind of neuronal activity (Figure 5) (Burgoyne, 2001).

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V1_Homo_sapiens.    MGKQNSKLAPEVMEDLVKSTEFNEHELKQWYKGFLKDCPS     40
V1_Rattus_norveg    MGKQNSKLAPEVMEDLVKSTEFNEHELKQWYKGFLKDCPS     40
V1_Mus_musculus.    MGKQNSKLAPEVMEDLVKSTEFNEHELKQWYKGFLKDCPS     40
V1_Gallus_gallus    MGKQNSKLAPEVMEDLVKSTEFNEHELKQWYKGFLKDCPS     40
V1_Xenopus_tropi    MGKQNSKLAPEVMEDLVKSTEFNEHELKQWYKGFLKDCPS     40
Consensus           mgkqnsklapevmedlvkstefnehelkqwykgflkdcps

V1_Homo_sapiens.    GRLNLEEFQQLYVKFFPYGDASKFAQHAFRTFDKNGDGTI     80
V1_Rattus_norveg    GRLNLEEFQQLYVKFFPYGDASKFAQHAFRTFDKNGDGTI     80
V1_Mus_musculus.    GRLNLEEFQQLYVKFFPYGDASKFAQHAFRTFDKNGDGTI     80
V1_Gallus_gallus    GRLNLEEFQQLYVKFFPYGDASKFAQHAFRTFDKNGDGTI     80
V1_Xenopus_tropi    GRLNLDEFQQLYVKFFPYGDASKFAQHAFRTFDKNGDGTI     80
Consensus           grlnl efqqlyvkffpygdaskfaqhafrtfdkngdgti

V1_Homo_sapiens.    DFREFICALSITSRGSFEQKLNWAFNMYDLDGDGKITRVE    120
V1_Rattus_norveg    DFREFICALSITSRGSFEQKLNWVFNMYDLDGDGKITRVE    120
V1_Mus_musculus.    DFREFICALSITSRGSFEQKLNWAFNMYDLDGDGKITRVE    120
V1_Gallus_gallus    DFREFICALSITSRGSFEQKLNWAFNMYDLDGDGKITRVE    120
V1_Xenopus_tropi    DFREFICALSITSRGSFEQKLNWAFNMYDLDGDGKITRVE    120
Consensus           dfreficalsitsrgsfeqklnw fnmydldgdgkitrve

V1_Homo_sapiens.    MLEIIEAIYKMVGTVIMMKMNEDGLTPEQRVDKIFSKMDK    160
V1_Rattus_norveg    MLEIIEAIYKMVGTVIMMKMNEDGLTPEQRVDKIFSKMDK    160
V1_Mus_musculus.    MLEIIEAIYKMVGTVIMMKMNEDGLTPEQRVDKIFSKMDK    160
V1_Gallus_gallus    MLEIIEAIYKMVGTVIMMKMNEDGLTPEQRVDKIFSKMDK    160
V1_Xenopus_tropi    MLEIIEAIYKMVGTVIMMKMNEDGLTPEQRVDKIFSKMDK    160
Consensus           mleiieaiykmvgtvimmkmnedgltpeqrvdkifskmdk

V1_Homo_sapiens.    NKDDQITLDEFKEAAKSDPSIVLLLQCDIQ              190
V1_Rattus_norveg    NKDDQITLDEFKEAAKSDPSIVLLLQCDIQ              190
V1_Mus_musculus.    NKDDQITLDEFKEAAKSDPSIVLLLQCDIQ              190
V1_Gallus_gallus    NKDDQITLDEFKEAAKSDPSIVLLLQCDIQ              190
V1_Xenopus_tropi    NKDDQITLDEFKEAAKSDPSIVLLLQCDIQ              190
Consensus           nkddqitldefkeaaksdpsivlllqcdiq
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Figure 4 Amino acid sequence alignment of VILIP-1 of 5 species. The consensus indicates the amino acids conserved in all the sequences.

Table.1. Ca^{2+} affinities of NCS proteins (Burgoyne, 2001)

Protein	Assay	Ca^{2+} affinity (μM)	Co-operativity (Hill coefficient)
NCS-1	Ca^{2+}-binding	0.3	Yes (2)
NCS-1*	Ca^{2+}-dependent increase in tryptophan fluorescence	<0.1	?
NCS-1	Phosphodiesterase activation	0.1	?
VILIP-1 (neurocalcin α)	Ca^{2+}-binding	1	No
VILIP-3 (neurocalcin δ)*	Ca^{2+}-binding	≈ 0.6	Yes (>2)
	Ca^{2+}-dependent increase in tryptophan fluorescence	1.3–1.7	Yes (1.8)
	Ca^{2+}-dependent membrane binding	0.75	Yes (2.3)
Hippocalcin*	Ca^{2+}-dependent membrane binding	0.6	?
Recoverin*	Ca^{2+}-dependent membrane binding	2.1	Yes (2.38)
GCAP1*	GC activity	0.26	Yes (?)
GCAP2*	GC activity	0.25	Yes (1.7–1.9)
GCAP3*	GC activity	0.25	Yes (?)

*Data from myristoylated protein.

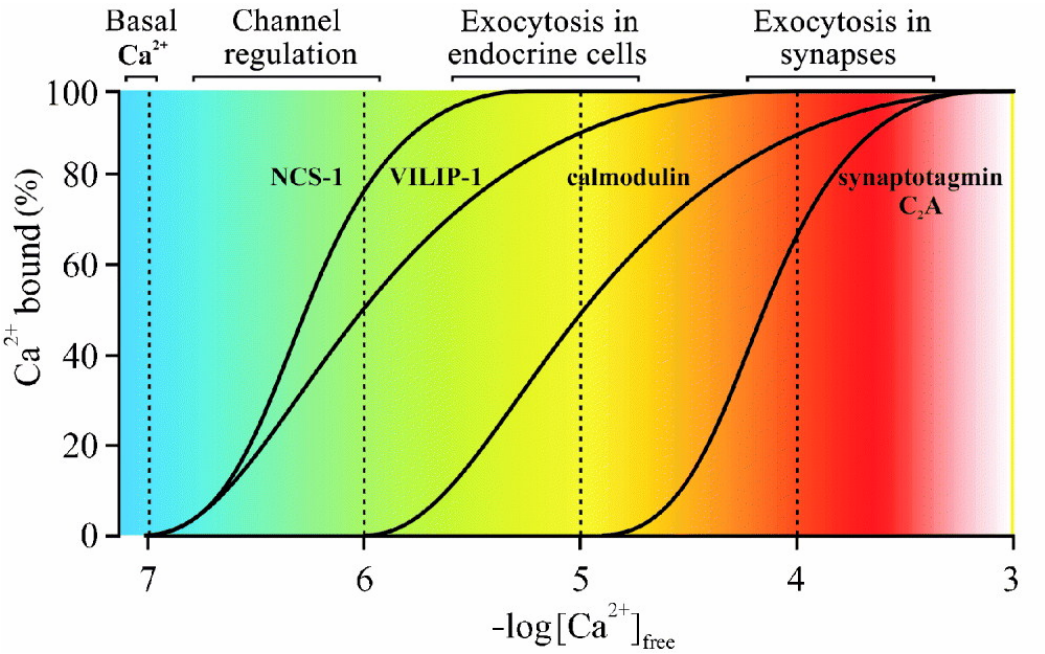

Figure 5 Comparison of the Ca^{2+}-binding affinities for various Ca^{2+}-binding proteins expressed in neurons (Burgoyne, 2001)**.**

3. Effect of VILIP-1 on the α4β2 nicotinic acetylcholine receptor (nAChR)

3. 1 Brief introduction of acetylcholine receptors

Acetylcholine receptors (Lindstrom, 1996; Hogg et al., 2003) consist of 2 major subtypes, the muscarinic-activated metabotropic receptors (second messenger coupled) and the fast-ionotropic cationic nicotine-activated channel receptors, both of which are activated by the endogenous neurotransmitter acetylcholine. Nicotine affects the nicotinic acetylcholine receptors (nAChRs), which constitute a family of ligand-gated ion channels widely expressed in the nervous system. Receptors of the nicotinic subclass can be distinguished further as “muscle” or “neuronal”. While the muscle and neuronal nicotinic receptors exhibit similar sensitivity to gating by acetylcholine, the

muscle receptor is much less sensitive to nicotine. The mammalian nAChR family (for review see Lindstrom 1996, 2003; Hogg et al., 2003) is composed of multiple subunits including 7 subunits that harbor the principal components of the ligand binding site (α2, α3, α4, α6, α7, α9, and α10) and 4 structural subunits (α5, β2, β3, and β4) that impart unique functional and pharmacological properties to the receptors. Pentameric nicotinic receptor subtypes arise from eight subunits (a2-4, 6-10) which form the acetylcholine binding site, and four subunits (α5, β2-4) which have a structural function but also contribute to the binding site (Figure 6) (Lindstrom, 1996).

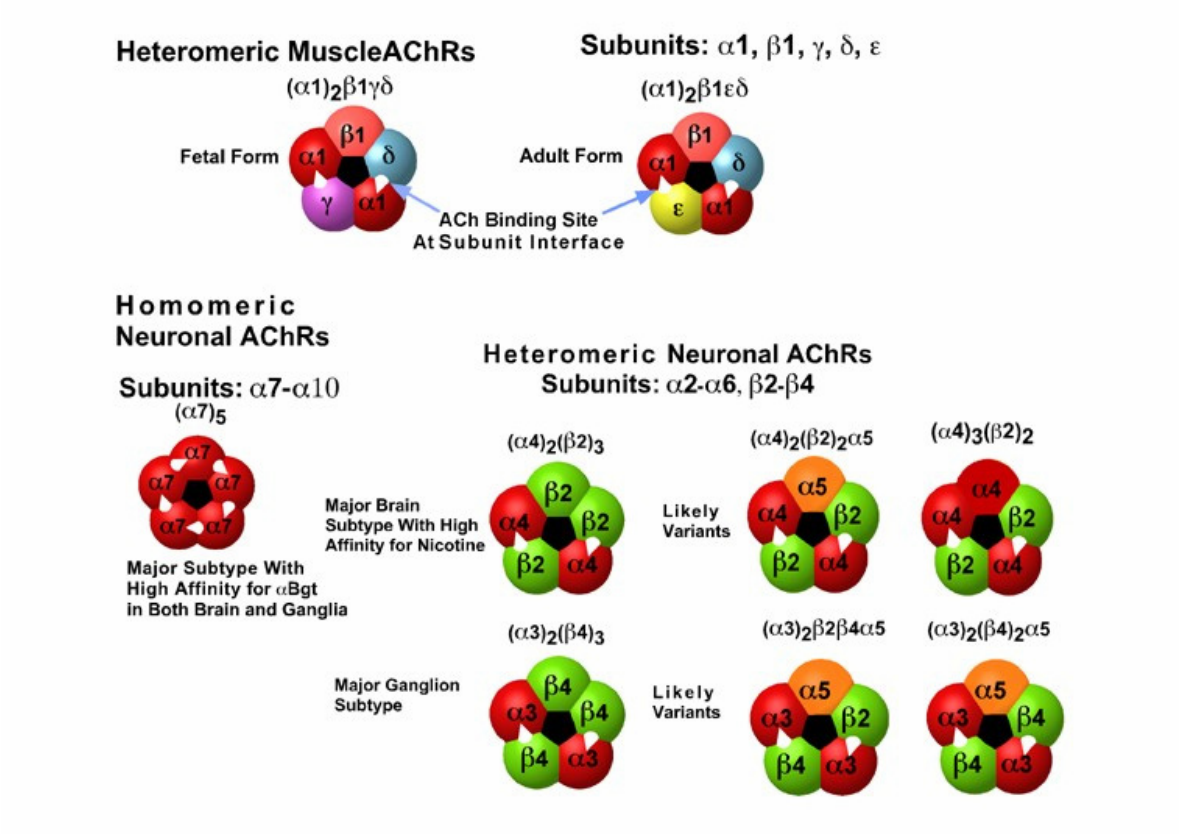

Figure 6 Structure of nicotinic acetylcholine receptors (Lindstrom, 1996)

From the physiological perspective, nAChRs fall into 3 major subgroups, the high-affinity nicotine binding receptors harboring the nAChR α4 subunit, α-bungarotoxin binding proteins composed of nAChR α7 and the receptors of the autonomic nervous system composed of nAChRα3/β4 subunits. A particularly interesting aspect of nAChRs is that despite being fast-excitatory ion channels, they are localized in many parts of the cell including aggregates in the cell body (soma), presynaptic terminals (where they contribute to modulation of neurotransmitter release), and in or adjacent to the postsynaptic density. Because of these diverse locations, their participation in neurotransmission can indirectly affect the amount of neurotransmitter released. This modulatory role directly contributes to the establishment and maintenance of the balance between the excitatory and inhibitory systems (Alkondon and Albuquerque, 2001, 2004; Alkondon et al., 1996; Hasselmo et al., 2002; Ji and Dani, 2000;

Ji, 2001; McGehee, 2002; Shao and Yakel, 2000).

In rodents, 90% of the high-affinity nicotine binding sites in the brain contain α4 and β2 subunits (Whiting and Lindstrom, 1988). The α4β2 nicotinic subunits are abundantly found in the neocortex, hippocampus, septum, thalamus, midbrain, and brainstem (Wada et al., 1989). Hence, α4β2 receptors are the neuronal nicotinic receptor subtype most likely to participate in nicotine addiction. Previous molecular biological attempts to assess the contribution of nAChRs to the physiology and behaviour of nicotine addiction revealed that particularly α4β2 receptors influence the brain reward system. Studies with nAChR knockouts for the α4 and the β2 subunits and a α4 knock-in mouse, which is hypersensitive to nicotine, identify α4β2 nAChRs subunits as responsible for the essential features of nicotine-addiction, including reward, tolerance, sensitization, and point to hypersensitivity as the mechanism by which nicotine produces its addictive effects (Picciotto et al., 1999; Maskos et al., 2005; Tapper et al., 2004).

Chronic exposure of α4β2 receptors to nicotine, as occurs in smokers, 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), leading to enhanced release of dopamine in the mesolimbic rewarding system (Dani and Heinemann, 1996; Dani et al., 2001). Up-regulation is observed in the brains of human smokers (Breese et al., 1997) as well as in chronically nicotine-treated animals (Marks et al., 1992) and in cultured cells heterologously expressing nAChRs (Whiteaker et al., 1998). Nicotine-induced up-regulation of α4β2 nAChR has been found in brain areas related to learning and memory like the hippocampus as well as in the mesolimbic reward system (Hernandez and Terry, 2005). These data suggest that "functional up-regulation" is a physiologically relevant mechanism comprising an important biological mechanism for the effect of nicotine on addiction as well as on memory.

The molecular mechanisms of the "functional up-regulation" relevant to addiction and memory are currently under investigation. Since no increase in nAChR subunit mRNA levels and no dependence on protein synthesis have been observed, a post-translational mechanism has been postulated (Buisson and Bertrand, 2002). The receptor exists in a high and low affinity state for nicotine binding and chronic nicotine treatment might lead to stabilization of the high affinity state. Alternatively, the chronic exposure to nicotine could either slow down receptor endocytosis or increase membrane trafficking of presynthesized high affinity receptors (Buisson and Bertrand, 2001, 2002). There are recent data showing that nicotine exposure stabilizes α4β2 receptors in a high-affinity state (Vallejo et al., 2005). Similarly, nicotine can act intracellularly on early maturation steps of the receptor which leads to an increase in high-affinity binding sites (Sallette et al., 2005). On the other hand, it was shown that increased nicotine binding following

chronic nicotine treatment reflects membrane trafficking of the receptor (Pakkanen et al., 2005), a decreased surface turnover (Peng et al., 1994) or increased receptor trafficking to the surface (Harkness and Millar, 2002). Recently, an increase in the receptor number at the cell surface was found to depend on an exocytotic mechanism (Darsow et al., 2005). Thus, the precise mechanisms and the cellular machinery required for nicotine-induced up-regulation still remain unclear.

3. 2 Effect of VILIP-1 on the α4β2 nAChR

In a yeast-two-hybrid protein-protein interaction screen, using the large intracellular loop of the α4 subunit as bait, VILIP-1 was found as an interaction partner (Lin et al., 2002). VILIP-1 binds to a 30 amino acid region in the loop encompassing the identified ER export motif (Ren et al., 2005). Co-expression of VILIP-1 with recombinant α4β2 nicotinic acetylcholine receptor in oocytes upregulated the surface expression levels two-fold and increased the agonist-sensitivity to acetylcholine three-fold (Lin et al., 2002). The modulation of the recombinant α4β2 nicotinic acetylcholine receptor by VILIP-1 was attenuated with VILIP-1 myristoylation mutants or EF-hand mutants not able to bind Ca^{2+}. These results suggest that VILIP-1 represents a novel modulator of the α4β2 nicotinic acetylcholine receptor which increases the surface expression level and agonist sensitivity of the receptor in response to changes in the intracellular levels of Ca^{2+} (Lin et al., 2002). Therefore we hypothesise that VILIP-1 might have a key role in "functional up-regulation" of nAChR.

One of the mechanisms by which VILIP-1 modulates different neuronal signalling cascades is the Ca^{2+}-dependent membrane association (Braunewell and Gundelfinger, 1999), which has been well described for different NCS proteins on a biochemical and protein structure level (Zozulya and Stryer, 1992; Ames et al., 1997). Ca^{2+}-binding of NCS proteins leads to exposure of the hydrophobic myristic acid moiety, followed by membrane association of the protein, the so called calcium-myristoyl switch. In cell lines and hippocampal neurons the Ca^{2+}-myristoyl switch of VILIP-1 occurs within seconds and is reversible (Spilker et al., 2002, Spilker and Braunewell, 2003). Increasing $[Ca^{2+}]i$ in hippocampal neurons by glutamate or KCl application results in translocation of VILIP-1 to the plasma membrane, as shown by co-localization with the membrane-associated actin cytoskeleton. Moreover, a Ca^{2+}-dependent co-localization of VILIP-1 with syntaxin6 occurs, which is a marker for the trans-Golgi network and is part of a signalling complex involved in Golgi-to-surface membrane trafficking (Spilker et al., 2002; Spilker and Braunewell, 2003). Thus, the reversible localization of signalling molecules such as VILIP-1 to distinct signalling compartments, like surface receptors, cytoskeletal structures or intracellular membranes and adaptor complexes for membrane transport, might comprise a cellular signalling mechanism critical for the selective activation of downstream signalling

cascades (Spilker et al., 2002; Teruel and Meyer, 2000).

The Ca^{2+}-myristoyl switch is responsible for the effect of VILIP-1 on cAMP- and cGMP signalling (Braunewell et al., 1997, 2001; Lin et al., 2002; Spilker et al., 2000; Mahloogi et al., 2003; Brackmann et al., 2005) since Ca^{2+}-binding- or myristoylation mutants of VILIP-1 are not able to influence adenylyl cyclase activity (Braunewell et al., 2001; Lin et al., 2002) and the myristoylation mutant of VILIP-1 shows strongly reduced functional activity (Braunewell et al., 2001). Thus, Ca^{2+}-binding is not only necessary for the membrane localization of the protein (Braunewell et al., 2001), but is also a prerequisite for activation of receptor signalling systems (Braunewell et al., 2001, Spilker et al., 2002, Lin et al., 2002). The molecular mechanisms of the functional activity of VILIP-1 have been analyzed in more detail for guanylyl cyclase B (GC-B). VILIP-1 interacts with the catalytic domain of GC-B and it is a physiological modulator of GC-B function in cerebellar neurons (Braunewell et al., 2001). VILIP-1 associates with clathrin-coated vesicles isolated from the rat brain, suggesting an involvement in clathrin-dependent trafficking (Blondeau et al., 2004). In line with this assumption, VILIP-1 modulates GC-B activity in primary hippocampal neurons by influencing clathrin-dependent receptor recycling (Brackmann et al., 2005). Additionally, the transient expression of VILIP-1 in C6 glioma cells also affected recycling of the transferrin receptor, which is a prototype marker for constitutive clathrin-dependent receptor recycling (Maxfield and McGraw, 2004). Therefore, VILIP-1 plays a general role in membrane transport and receptor trafficking in hippocampal neurons (Brackmann et al., 2005). Similarly, VILIP-1 also affects other receptors such as α4β2 nAChR. VILIP-1-expression in oocytes increased the agonist-sensitivity of co-expressed α4β2 nAChR to acetylcholine three-fold and upregulated the surface expression levels two-fold. This effect depends on the Ca^{2+}-myristoyl switch as revealed by studies with the myristoylation mutant (Lin et al., 2002). **However, whether the effect of VILIP-1 on α4β2 nAChR occurs in hippocampal neurons or not, the underlying molecular mechanisms and the functional consequences for neuronal excitability are presently unknown.**

4. Potential role of NCS proteins in hippocampal synaptic plasticity in health and disease

Recently, NCS proteins have become a focus of interest as plasticity candidate genes. The NCS protein VILIP-1 is expressed at high levels in the rat hippocampus and shows increased expression following electrical and chemical induction of long-term potentiation (LTP) in the hippocampal formation (Brackmann et al., 2004). In cultured hippocampal neurons VILIP-1 shows mGluR-dependent expression, and stimulus-dependent and reversible translocation to Golgi and cell surface membranes. The calcium-myristoyl switch of VILIP-1 might provide a fast signaling mechanism to influence membrane-associated signaling effectors (Spilker et al., 2002). In rat hippocampal cell cultures, increase of NCS-1 expression can switch paired-pulse depression to facilitation without

altering basal synaptic transmission or initial neurotransmitter release probability. In addition, facilitation also persisted during high-frequency trains of stimulation, suggesting that NCS-1 can recruit 'dormant' vesicles (Sippy et al., 2003). In comparison, activation of hippocalcin, another calcium sensor protein leads to the formation of a complex with AP-2, part of the clathrin-mediated endocytic machinery. This complex binds to the GluR2 subunit of the AMPA receptor, displacing N-ethylmaleimide-sensitive fusion protein (NSF) and recruiting clathrin, thus initiating internalization of AMPA receptors. Therefore hippocalcin is involved in hippocampal LTD (Palmer et al., 2005). Furthermore, hippocalcin-/- mice, had worse performance in the Morris water maze test than hippocalcin+/+ mice and also showed impairments in a discrimination learning task (Kobayashi et al., 2005). Thus, NCS proteins likely participate in a variety of learning and memory activities including LTD and LTP, the main phenomena of plasticity. As is the case for most Ca^{2+} signaling pathways (Carafoli, 2002, 2004), in addition to their role in physiological signaling, NCS proteins have been implicated in various diseases for example, Alzheimer’s disease (AD) schizophrenia, and in cancer (Braunewell, 2005).