Interaction of the Neuronal Gap Junction Protein Connexin 36 with Alpha Calcium/Calmodulin-Dependent Protein Kinase II

by

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The abovementioned statement was made as a solemn declaration. I conscientiously believe and state it to be true and declare that it is of the same legal significance and value as if it were made under oath.

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Bochum, September 2012
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List of Abbreviations

α-CaMKII alpha-calcium/calmodulin-dependent protein kinase II
aa amino acid
AB after bleaching
AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP adenosine triphosphate
BB before bleaching
C carboxy
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
CNS central nervous system
CL cytoplasmic loop
CLB cytoplasmic loop binding
CT carboxy terminus
CTB carboxy terminus binding
Cx connexin
Cx36 connexin 36
Da dalton
D_post intensity of the donor after acceptor photobleaching
D_previous intensity of the donor before acceptor photobleaching
DIV days in vitro
ECFP enhanced cyan fluorescent protein
EGFP enhanced green fluorescent protein
EL extracellular loop
EYFP enhanced yellow fluorescent protein
FRET fluorescence resonance energy transfer
FRET_{eff} fluorescence resonance energy transfer efficiency
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>FRIL</td>
<td>freeze-fracture replica immunogold labeling</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GJs</td>
<td>gap junctions</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>h</td>
<td>human</td>
</tr>
<tr>
<td>Inx</td>
<td>innexin</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LSM</td>
<td>laser scanning microscope</td>
</tr>
<tr>
<td>LTD</td>
<td>long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>m</td>
<td>mouse</td>
</tr>
<tr>
<td>mCx36</td>
<td>mouse Cx36</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>amino</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl D-aspartate receptor</td>
</tr>
<tr>
<td>NT</td>
<td>amino terminus</td>
</tr>
<tr>
<td>ORN</td>
<td>olfactory receptor neurons</td>
</tr>
<tr>
<td>Panx</td>
<td>pannexin</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pS</td>
<td>picosiemens</td>
</tr>
<tr>
<td>PSD</td>
<td>post synaptic density</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>STP</td>
<td>short term potentiation</td>
</tr>
<tr>
<td>r</td>
<td>rat</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>TRN</td>
<td>thalamic reticular nucleus</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Abstract

Gap junctions are the structural equivalents of electrical synapses in the central nervous system (CNS), which can be formed by one or more types of connexin (Cx) proteins. Connexin 36 (Cx36) is a major component of electrical and mixed synapses in the CNS. It is supposed to provide a target for activity-dependent changes occurring during molecular processes underlying functional plasticity at sites of electrical synapses. Recent studies imply that Cx36 is a putative partner for several binding proteins, including alpha Ca$$^{2+}$/Calmodulin Dependent Kinase II (α-CaMKII). Two cytoplasmic domains of Cx36 (carboxy terminal and cytoplasmic loop) carry binding and phosphorylation motifs for α-CaMKII. In the first part, to evaluate the physical interaction between Cx36 and α-CaMKII, fluorescence resonance energy transfer (FRET) protocol was applied in N2A cells, which were transfected with different Cx36, and α-CaMKII constructs. Experiments for FRET assessment were performed after photo-inactivation of Cx36-EYFP with a 514 nm laser in Ca$$^{2+}$/ionomycin treated and Ca$$^{2+}$/ionomycin untreated cells. The results indicate that the increase in donor fluorescence (α-CaMKII-ECFP) was inversely proportional to the decrease of the acceptor fluorescence (Cx36-EYFP) in Ca$$^{2+}$/ionomycin treated N2A cells. These results suggest a physical interaction of Cx36 and α-CaMKII when the cells were exposed to Ca$$^{2+}$/ionomycin. N2A cells, which were transfected with Cx36 and α-CaMKII mutants lacking the two α-CaMKII binding sites did not show any significant interaction after Ca$$^{2+}$/ionomycin treatment. Similarly, we found that a Cx36 mutant lacking the cytoplasmic loop-binding site of α-CaMKII, also did not interact with α-CaMKII, while a Cx36 mutant lacking the carboxy-terminal binding site of α-CaMKII interacted with α-CaMKII. From these experiments it is concluded that the cytoplasmic loop of Cx36 is essential for α-CaMKII binding. To explore a possible effect of autophosphorylation of α-CaMKII on binding efficacy the same FRET protocol was applied to N2A cells using a constitutive autophosphorylated α-CaMKII-ECFP-T286D mutant and a mutant that lacks phosphorylation: α-CaMKII-ECFP-T286A. It was founded that neither of these α-CaMKII mutants interacts with Cx36 in Ca$$^{2+}$/ionomycin treated N2A cells.

The second part of the project, aimed to clarify whether binding of α-CaMKII to Cx36 is subject of activity-dependent mechanisms such as translocation after stimulation. For this purpose, α-CaMKII-ECFP and Cx36-EYFP co-transfected N2A cells and primary hippocampal neurons were taken into live cell imaging under confocal microscopy.

In α-CaMKII-ECFP-WT and Cx36-EYFP-WT co-transfected N2A cells the ratio of α-CaMKII-ECFP-WT to Cx36-EYFP-WT in gap junction plaques increased by time after stimulation with Ca$$^{2+}$/ionomycin. This significant increase indicated a translocation/accumulation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT containing gap junction domains in the heterologous expression system.

The ratio of α-CaMKII-ECFP to Cx36-EYFP-WT does not increase significantly when Cx36 lacks the binding sites for α-CaMKII-ECFP. In addition to these findings, in N2A cells, the autophosphorylation deficient mutant α-CaMKII-ECFP-T286A did not translocate to Cx36-EYFP domains, while the
constitutively active mutant α-CaMKII-ECFP-T286D was saturated in Cx36 gap junction plaques. These results show that autophosphorylation of α-CaMKII may be a key step in the translocation process of this enzyme to Cx36 expressing sites.

In the third part, Cx36-EYFP-WT expression and possible translocation of α-CaMKII-ECFP-WT was studied in neurons. Translocation of α-CaMKII-ECFP to Cx36-EYFP-WT expressing sites after applying different stimulation paradigms was investigated. Confocal images obtained from co-transfected neurons demonstrated no significant translocation of α-CaMKII-ECFP to Cx36-EYFP expressing sites after glu/gly (100 µM/10 µM) application or electrical stimulation.

The negative results obtained from the neuronal translocation analyses raised the question of the exact localization of Cx36-EYFP-WT in neurons, and if it is part of synaptic complexes, since the lack of translocation could be explained by false targeting (non-synaptic). Therefore, immunofluorescence techniques were used to identify co-localization of Cx36-EYFP-WT with makers for chemical synapses, which provide a possible target for Cx36 trafficking to form mixed synapses. It was found that at least a fraction of Cx36-EYFP-WT co-localizes with sites of synaptophysin immunoreactivity and with FM 4-64 labeling.
1 Introduction

1.1 Gap junctions and gap junction proteins

Cell to cell communication is vital for multicellular organisms. A plethora of intramembrane mechanisms enable cells to communicate with each other and gap junctions (GJs) constitute one of those tools.

GJ’s are specialized intercellular channels, which allow the exchange of molecules [generally < 1000 Da] including ions (Ca\(^{2+}\) and K\(^{+}\)), second messengers (cAMP, cGMP, and inositol 1,4,5-triphosphate (IP3)), short peptides, amino acids (aa) and metabolites (glutamate [glu], glucose, adenosine, AMP, ADP, ATP and glutathione) (Goldberg et al., 2002), between adjacent cells. Today, it is strongly agreed that GJs are more than simple intercellular channels, but contribute to plenty of additional functions such as organizing speed and synchrony of signal transmission, metabolic cooperation in form of bystander effects, regulation of growth control and migration.

GJs contribute to cell-to-cell communication to increase or decrease the speed of signal transmission in the cardiovascular or central nervous system (CNS) where synchrony among cell populations is amenable. GJs in the nervous system take part in interneuronal communication in form of electrical synapses and provide neuronal pathways, where maximal speed, synchronous neuronal firing and cooperative oscillatory events is required (for reviews see: Rozental et al., 2000, Söhl et al., 2005).

In non-excitable cells, such as the lens fibers of the eye, GJs allow symbiotic interactions between highly differentiated, functionally compromised cells and more active, renewable cells, which perform cellular functions for the weaker cells (for review see: Simon et al., 1998).

GJs also act to rescue somatic cell mutations in key metabolic and signaling enzymes; normal cells deliver junction-permeable intermediaries, permitting the survival of mutant cells by the blockage of cellular pathways (Hirschi, et al., 1996).

In addition GJs also are suggested to perform functions in tumor suppression mechanisms and are thought to be involved in differentiation, cell growth, development and morphogenesis (Cina et al., 2007; Kihara et al., 2010; Sultana et al., 2008).
Gap junctions constitute conduits between two adjoining cells and are formed by two connexons (hemichannels), which consist of six connexin subunits. Each connexon joins end-to-end with a connexon in the opposing membrane of an adjacent cell to provide a direct aqueous pathway between the cytoplasms of the coupled cells. Connexons form a central pore, which allows ions, messenger molecules, and metabolites to pass between neighboring cells. GJ channels span a small gap (3.5 nm) between the cell membranes and connect the cytoplasm of neighboring cells. (A). Topological model of a connexin protein. Each connexin contains four transmembrane domains: M1-M2-M3-M4 (B), two highly conserved extracellular loops (E1 and E2), one intracellular loop and highly variable intracellular carboxyl (CT) and amino (NT) termini. While the length of the NT is conserved among Cxs, the sequence of the carboxyl-terminus is variable depending on the type of connexin.

GJs connect two adjacent cells with each other via intercellular channels (Figure 1A). An extended area of these channels is called a GJ plaque (Figure 1A). Each connexon (hemichannel) is comprised of six protein subunits (Figure 1B), which are oriented perpendicular to the cell membranes to form a central pore. This central pore serves as a conduit for ions and low-molecular-mass molecules with a cut-off limit of approximately 1 kDa (Figure 1A).
GJs of vertebrates and invertebrates are similar in both structure and function, the former are composed of the Cx family (Eiberger et al., 2001; Willecke et al., 2002), while the latter are composed of members of a topologically similar, but sequentially non-homologous family called the innexins (Inxs) (for review see: Phelan et al., 2001). In 2000, in silico screening lead to the discovery of a third putative gap junctional protein family, which are called pannexins (Panx) (Panchin et al., 2000). All three known pannexin genes display distant homology to the innexins, but no sequence similarity to connexins.

1.1.1 Connexins

The topology of Cx proteins consists of four hydrophobic transmembrane regions (M1-M2-M3-M4), two extracellular loops (EL) and three cytoplasmic domains: the carboxy terminal (CT), amino terminal (NT) and cytoplasmic loop (CL) domains (Figure 1B). The transmembrane domains and extracellular loops are highly homologous among Cxs and each extracellular loop contains three conserved cysteine residues. In Cxs the NT, shows homology across Cxs where CT and CL domains are less homologous. Cytoplasmic domains contribute to channel gating, voltage sensitivity, pH sensitivity and to the assembly and trafficking of Cxs to the membrane by carrying binding sites for cytoskeletal interacting proteins (Giepmans et al., 2001; Helbig et al., 2010; Xin et al., 2010).

At present, 21 Cx genes in the human genome and 20 Cx genes in the mouse genome have been identified (Eiberger et al., 2001). The common way of annotating Cxs is to classify the proteins according to their molecular weights. In the literature, the accepted way to label Cxs is the placing of the putative molecular weight after the Cx abbreviation. (i.e., a Cx, with a molecular mass of 36 kDa is named Cx36). The molecular weights of Cxs vary between 25-62 kDa.

Cxs exhibit tissue and cell specific expressions, and most cells express more than one type of Cx. Some isoforms of Cxs are ubiquitously distributed in various types of cells (like Cx43 or Cx32), while other Cxs are organ, tissue and cell specific. Examples for tissue specificity are Cx50, which is exclusively expressed in lens fibers, or Cx30.2 that has been found expressed in the retina only (Schütte et al., 1998).

Under basal physiological conditions, connexons are closed (Quist et al., 2000). However, changes in the extracellular and intracellular environment can lead to opening of connexon hemichannels, and allow release of intracellular signaling molecules into the extracellular environment. It is important to note that responses to certain extracellular or intracellular cues differ between different connexons.

Cxs in humans have been linked to several congenital human syndromes, i.e. cardiovascular and neuron-related diseases, cataracts, oculodentodigital dysplasia and various types of skin diseases (for review see: Zoidl and Dermietzel 2010, Pfenniger et al., 2011).
1 Introduction

1.1.2 Innexins

GJ structure and functions were mainly investigated in the vertebrates, where they were thought to be formed solely by Cxs. Studies in Drosophila melanogaster and Caenorhabditis elegans, which possess no Cx genes, revealed that invertebrate GJs were composed of a different gene family, the innexins (Inxs, invertebrate analogues of connexins). Inx proteins are not homologous to Cxs in terms of primary sequence, however GJ channels comprised by Inxs share functional similarities with intercellular channels made by Cxs. In Inxs the four transmembrane domains are conserved as well as the extracellular loops. Unlike Cxs, each extracellular loop of Inxs carries only two conserved cysteine residues (for review see: Phelan et al., 2005), and there is a notable difference in the length of the two extracellular loops as compared to Cxs. In general, extracellular sites are significantly longer in Inxs, although there is considerable variation among family members. The differences are assumed to results in a wider gap between the apposed membranes at many invertebrate junctions as compared to the vertebrate GJs.

1.1.3 Pannexins

Panxs constitute a further group of channel forming proteins, which exhibit homology to Inxs. They lack primary sequence homology with the vertebrate Cxs, although they share the same membrane topology with Cxs and Inxs. It was hypothesized that Panx also participate in GJ formation. However, except one experimental evidence, in Xenopus laevis oocyte expression system (Bruzzzone et al., 2003), there are no empirical data, confirming the contribution of Panx to GJ formation.

Panxs are expressed in various tissues including kidney, skin, eye, neuron and glia (for review see: Panchin et al., 2005; Barbe et al., 2006, Prochnow et al., 2008). The Panxs consist of only three members in mammals: Panx1 [426 amino acids (aa), 47.6 kDa], Panx2 [664 aa, 73.3 kDa], and Panx3 [392 aa, 44.7 kDa].

The current opinion is that Panxs form particular types of channels, which provide functions in signaling and releasing of molecules between the cytoplasm and the extracellular environment, for instance in Ca^{2+} wave propagation, vasodilation through ATP release, initiation of inflammatory responses by IL-1 beta (Lai et al., 2007; Woehrle et al., 2010; Huang et al., 2007; D’hondt et al., 2010; Zappalà et al., 2007).

1.2 Connexin 36: the most abundant neuronal connexin

Cx36 is the major GJ forming protein of neurons in the CNS. Its expression has been thought to be largely limited to neurons, lately it is shown that Cx36 contributes in coupling of microglia as well (Dobrenis et al., 2005), and was found between insulin secreting beta cells of the exocrine pancreas.
The mouse Cx36 (mCx36) gene was mapped on chromosome 2 in the position F3, in a region that is syntenic to human chromosome 15q14, while the human ortholog Cx36 is located on chromosome 15q14 (Teubner et al., 2000; Al–Ubaidi et al., 2000; Gulisano et al., 2000; Belluardo et al., 1999). mCx36 is the mammalian ortholog of Cx35.5, a connexin first cloned from the retina of skate fish (Raja erinacea) (O’Brien et al., 1996).

Cx36 consists of 321 amino acids and shows the typical features of members of the Cx family (Condorelli et al., 1998; Söhl et al., 2000). The intracellular loop of human and rodent Cx36 shows a glycine-rich tract of 18 amino acids, which is absent in the corresponding region of fish and skate proteins. Most of the differences between Cx35.5 and Cx36 are located in the putative intracellular loop.

It is shown that in transfected PC12 (adrenal medulla cells) and N2A (Neuroblastoma 2A cells) Cx36 containing GJs have a small conductance (10–15 pS) and they are weakly voltage-sensitive with a half-inactivation voltage of ± 75 mV (Srinivas et al., 1999). Cxs may also form hexameric hemichannels, or connexons, which serve non-junctional functions (for review see: Goodenough & Paul 2003; Saez et al., 2003, Scemes et al., 2007).

1.3 Activity dependent regulation of electrical synapses

Until the 1950’s it was accepted that cellular communication among neurons is only mediated chemically. However, this dogma changed in 1959 by the demonstration of electrical transmission in crayfish (Furshpan et al., 1959). Later, several groups provided evidence for the presence of electrical coupling in the mammalian brain as well as in cardiac tissue and between smooth muscle cells. The discovery of co-localized electrical and chemical components in the “large myelinated club endings” of Mauthner cells in Goldfish (Carassius auratus), was reason to term these structures “mixed synapses” (Furshpan et al., 1964, Nakajima et al., 1974), which is not specific for the goldfish brain, but rather common in diverse regions of the mammalian CNS (Rash et al., 2001).

Over a decade ago, a debate had started whether electrical synapses show activity dependent plasticity similar to chemical synapses. Aids in proof for activity-dependent behavior of electrical synapses came from electrophysiological studies on the Mauthner cells, indicating that by applying tetanic stimulation protocols, potentiation at postsynaptic sites of hippocampal neurons is possible (Yang et al., 1990). Obviously, junctional conductance at electrical synapses is modifiable and prone to generate both short and long-term potentiation (STP & LTP) as well as long-term depression (LTD) (Pereda et al., 1995; Yang et al., 1990; Yang et al., 1991).
1.3.1 Connexin 36 carries two binding sites and three phosphorylation sites for α-CaMKII

Tetanic stimulation elicits LTP and STP in club endings of Mauthner cells similar to LTP and STP generated at chemical synapses in rodent hippocampal slices. These activity dependent changes in synaptic efficacy depend of N-methyl-D-aspartate receptors (NMDAR), which lead to an increase in postsynaptic Ca\(^{2+}\) efficacy and the subsequent activation of α-CaMKII.

Additionally, LTP and STP at club endings can be blocked by ketamine (NMDAR antagonist), BAPTA (Ca\(^{2+}\) chelator), or KN-93 (α-CaMKII inhibitor) after postsynaptic injections (Pereda et al., 1996).

The consequence from these studies suggests that Ca\(^{2+}\) and α-CaMKII are also involved in short and long-term changes of electronic coupling. It became clear that Ca\(^{2+}\) signals are responsible for the activity-dependent potentiation of GJs in mixed synapses of Mauthner cells, which were recently shown to contain Cx35 (the ortholog of Cx36, see above). The increase in efficacy of GJ electrical transmission depends on Ca\(^{2+}\) influx through NMDARs (although there could be other sources), followed by an increase of intracellular postsynaptic [Ca\(^{2+}\)]\(_i\), which leads to activation of α-CaMKII (Pereda et al., 1996). α-CaMKII, which is a well-recognized multifunctional kinase that transduces numerous neuronal [Ca\(^{2+}\)]\(_i\) signals, is also one of the most abundant kinases in neurons highly enriched in postsynaptic densities (PSD). Likely targets of α-CaMKII are AMPARs (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and GJ proteins (Lampe et al., 2004). It is suggested that α-CaMKII also plays a central role in modulating postsynaptic efficacy of electrical synapses, as it is the case in chemical synapses. There are remarkable mechanistic parallels between the long-term potentiation of mammalian glutamatergic synapses (Stein et al., 2003) and those operating in electrical synapses of the goldfish. Cx36, as one of the major components of electrical and mixed synapses in the CNS, may provide a functional substrate for changes occurring during processes involved in neuronal plasticity as well. Recent studies imply that Cx36 is a putative partner for binding proteins, including α-CaMKII, which has been demonstrated to interact with the CT of Cx36. Additionally, both cytoplasmic sites of Cx36 carry binding as well as phosphorylation sites for α-CaMKII (Alev et al., 2008).

In silico analyses and pull down assays reported that Cx36 has two juxtamembrane sequences that serve as potential binding sites for α-CaMKII within the cytoplasmic domain. There are further three potential amino acid residues providing phosphorylation sites for α-CaMKII: S110 and T111 within the cytoplasmic loop and S315 at the carboxyl-terminal domain (Alev et al., 2008).

These binding sites reside at the cytoplasmic loop (CLB= Cytoplasmic Loop Binding, amino acids 175-195) between the second and third transmembrane domains of Cx36, and at the carboxyl terminal domain (CTB= Carboxy Terminal Binding, amino acids 272–292). Interestingly, the sequence of the
autoinhibitory domain of α-CaMKII shows similarity of CTB and CLB sites of Cx36 (Figure 2A), where CLB site of Cx36 is also highly similar with NR2B subunit of NMDAR (Alev et al., 2008). Similar results were obtained for Cx34.7, the perch Cx36 homolog (Figure 2C) (O’Brien et al., 1998). The sequence homology between Cx36 and the NR2B subunits of the NMDAR led (Figure 2C) a model for Cx36 interaction with α-CaMKII, which is similar to the interaction of NR2B and α-CaMKII. The model proposes that Cx36 CLB first interacts with the T-site of the catalytic domain of α-CaMKII, like the NR2B subunit. Binding of Ca^{2+}/calmodulin separates the autoinhibitory domain from the catalytic domain and T-site, and enables the CLB region to bind. After α-CaMKII autophosphorylation, the S-site of the kinase is ready to bind the CTB region of the Cx36 (Alev et al., 2008) (Figure 2D).
2 Aims of the thesis

Especially, calcium elevation initiates a biochemical cascade through activation of α-CaMKII and it is shown that this enzyme involves in mechanisms of activity-dependent plasticity in chemical synapses (Fink and Meyer, 2002; Schulman et al., 2004; Merrill et al., 2005; Wayman et al., 2008).

However, little is known about interactions of α-CaMKII with Cx proteins in regulation of the GJ plaques. It is found that α-CaMKII has binding sites on Cx36 (Alev et al., 2008).

In this thesis work it is therefore first aimed to elucidate the physical interaction between Cx36 and α-CaMKII proteins. This was performed using fluorescence resonance energy transfer (FRET) protocols in a heterologous expression system by means of the N2A neuroblastoma cell line transfected with different Cx36 and α-CaMKII constructs.

Secondly, it was tried to clarify, whether binding of α-CaMKII is subject of activity dependent mechanisms such as translocation after stimulation. For this purpose, cell lines and primary hippocampal neurons were used and different stimulation paradigms were applied to elicit increase of cellular activity.

Thirdly, the localization of Cx36-EYFP proteins in transfected primary hippocampal neurons were also investigated with different immunofluorescence techniques, in order to understand if in, which types of synapses Cx36 protein is located.

To sum up, the work presented here, aimed to prove the hypothesis derived from in vitro experiments that Cx36 is subject of activity dependent interaction with α-CaMKII.
3 Materials and Methods

3.1 Organisms

3.1.1 Animals

Sprague Dawley rats were kept under standard housing conditions with a 12 h light/dark cycle (light on at 8 am) in a temperature and humidity controlled room and with free access to water and food. Animal handling was performed according to the standards of the German animal protection law and licensed by the regional country admission council [32 115-5/99]. All experiments including animal tissues were performed with authorization.

3.1.2 Eukaryotic cell lines

N2A cells derived from Mus musculus (Klebe and Ruddle, 1969) were kindly provided by Prof. Dr. David Spray (Albert Einstein College, NY, USA). Handling and storage of the cells were performed in the S1 laboratories of Department of Neuroanatomy and Molecular Brain Research, Ruhr University Bochum.

3.2 Chemicals and reagents

All chemicals and reagents used for the applications are mentioned below in Table 1.

<table>
<thead>
<tr>
<th>Chemicals and reagents</th>
<th>Company</th>
<th>Chemicals and reagents</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Sigma Aldrich</td>
<td>hydrochloric acid</td>
<td>J.T Baker</td>
</tr>
<tr>
<td>APV</td>
<td>Sigma Aldrich</td>
<td>Immersol</td>
<td>Carl Zeiss</td>
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</tbody>
</table>
Table 1: (Continued) List of chemicals and reagents.

<table>
<thead>
<tr>
<th>Chemicals and reagents</th>
<th>Company</th>
<th>Chemicals and reagents</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Normapur</td>
</tr>
<tr>
<td>Biocidal</td>
<td>Wak Chemie</td>
<td>KN-93</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Biocytin Alexa Fluor 594</td>
<td>Invitrogen</td>
<td>liquid nitrogen</td>
<td>RUB</td>
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<tr>
<td>bovine serum albumin</td>
<td>PAA</td>
<td>methanol</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>bromphenol blue</td>
<td>Riedel-de Haën</td>
<td>magnesium chloride</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>carbon dioxide</td>
<td>RUB</td>
<td>minimum essential media</td>
<td>Sigma Aldrich</td>
</tr>
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<td>calcium chloride</td>
<td>J.T.Baker</td>
<td>non essential amino acids</td>
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<td>Invitrogen</td>
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<td>Tocris science</td>
<td>normal goat serum</td>
<td>PAN Biotech</td>
</tr>
<tr>
<td>dimethyl sulfoxide</td>
<td>Sigma-Aldrich</td>
<td>neurobasal medium</td>
<td>Gibco</td>
</tr>
<tr>
<td>Dulbecco's modified eagle medium</td>
<td>Gibco</td>
<td>paraformaldehyde</td>
<td>Sigma-Aldrich</td>
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<td>Effectene transfection reagent</td>
<td>Qiagen</td>
<td>poly- L-lysine</td>
<td>Invitrogen</td>
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<tr>
<td>ethylenediaminetetraacetic acid</td>
<td>Merck</td>
<td>potassium chloride</td>
<td>Riedel-de Haën</td>
</tr>
<tr>
<td>ethylene glycol tetraacetic acid</td>
<td>Sigma Aldrich</td>
<td>potassium dihydrogenphosphate</td>
<td>J.T.Baker</td>
</tr>
<tr>
<td>ethanol</td>
<td>J.T.Baker</td>
<td>ProLong Gold antifade reagent</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>fetal calf serum</td>
<td>HyClone, Perbio</td>
<td>sodium chloride</td>
<td>J.T.Baker</td>
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<tr>
<td>Fura-2 AM</td>
<td>Invitrogen</td>
<td>sodium dodecyl sulfate</td>
<td>AppliChem</td>
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<tr>
<td>FM 4-64</td>
<td>Invitrogen</td>
<td>sodium pyruvate</td>
<td>PAA Laboratories</td>
</tr>
<tr>
<td>glutamax</td>
<td>Invitrogen</td>
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</table>
3 Materials and Methods

<table>
<thead>
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<th>Chemicals and reagents</th>
<th>Company</th>
<th>Chemicals and reagents</th>
<th>Company</th>
</tr>
</thead>
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<tr>
<td>glucose</td>
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<td>sodium hydrogen phosphate</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>L-glutamine</td>
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<td>tetramethylethlenedi-amine</td>
<td>AppliChem</td>
</tr>
<tr>
<td>glycerol</td>
<td>J.T Baker</td>
<td>tris(hydroxymethyl)aminomethane</td>
<td>AppliChem</td>
</tr>
<tr>
<td>glycine</td>
<td>AppliChem</td>
<td>trypsin-EDTA</td>
<td>PAA</td>
</tr>
<tr>
<td>Hank’s buffered salt solution</td>
<td>Gibco</td>
<td>trypton</td>
<td>BioChemica,</td>
</tr>
<tr>
<td>+/+-</td>
<td></td>
<td></td>
<td>AppliChem</td>
</tr>
</tbody>
</table>

3.3 Compositions of cell culture media, buffers, solutions, antibodies and peptides

3.3.1 Solutions for cell culture

Growth medium for N2A

D-MEM (+4.5 g/l glucose, + L-glutamine, - pyruvate)

1 % L-glutamine

1 % non-essential amino acids (NEA)

1 % penicillin/streptomycin (10,000 U/ml, 10 mg/ml)

1 % Na-pyruvate (1mM)

5 % fetal calf serum (FCS)

Phosphate buffered saline (PBS)

138 mM NaCl

2.8 mM KCl

10 mM Na2HPO4

1.8 mM KH2PO4

pH 7.4

Neurobasal medium

+ 1 % glutamax

+ 2 % B-27 supplement

+ 1 % N-2 supplement

+ 0.5 mg/ml gentamycin

Poly-L-lysine solution

1 % PLL in water

Paraformaldehyde (PFA) 4 %

PFA is diluted in water by heating and adding drops of NaOH. Later on the solution cooled and filtered
3 Materials and Methods

3.3.2 Solutions for live cell imaging

**Live cell imaging**
- HBSS +/-, 1 % glucose

**Increasing Ca\(^{2+}\) concentration for neurons**
- 100 µM glutamate, 10 µM glycine

**To increase Ca\(^{2+}\) concentration for N2A cells**
- 10 µM ionomycin, 2 mM CaCl\(_2\)

3.3.3 Solutions for immunocytochemistry

**Blocking solution**
- 10 % NGS, 0.1 % Triton X-100 in PBS

**PBS-T**
- PBS, 0.1 % Tween 20

3.3.4 Antibodies

All antibodies used for the applications are mentioned below in Table 2 and Table 3.

### Table 2: List of primary antibodies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Company</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-CaMKII</td>
<td>mouse</td>
<td>Sigma</td>
<td>α-CaMKII</td>
</tr>
<tr>
<td>Cx36</td>
<td>mouse</td>
<td>Sigma</td>
<td>Cx36</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>mouse</td>
<td>Abcam</td>
<td>synaptophysin</td>
</tr>
</tbody>
</table>

31
Table 3: List of secondary antibodies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Company</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-mouse IRDye 680</td>
<td>Goat</td>
<td>Licor Biosciences</td>
<td>Against mouse with IRDye 680 tag</td>
</tr>
<tr>
<td>Alexa Fluor 594 anti-mouse</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>Against mouse with Alexa Fluor 594 tag</td>
</tr>
</tbody>
</table>

3.3.5 Plasmids, proteins and peptides

Plasmids, proteins and peptides (United Peptide Cooperation) that have been used during this thesis are mentioned below in Table 4 and Table 5. Figure 3 shows an illustration of Cx36-WT and mutant proteins.

Table 4: List of plasmids.

<table>
<thead>
<tr>
<th>List of the plasmids</th>
<th>Content of the plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx36-WT-EYFP</td>
<td>Plasmid containing WT Cx36 fused to EYFP (Figure 3A)</td>
</tr>
<tr>
<td>Cx36ΔCLB-EYFP</td>
<td>Plasmid containing lack of cytoplasmic loop binding site of α-CaMKII at Cx36 fused to EYFP (Figure 3C)</td>
</tr>
</tbody>
</table>
### Table 4: (Continued) List of plasmids.

<table>
<thead>
<tr>
<th>List of the plasmids</th>
<th>Content of the plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx36-ΔCTB-EYFP</td>
<td>Plasmid containing lack of carboxy terminal binding site of α-CaMKII at Cx36 fused to EYFP (Figure 3D)</td>
</tr>
<tr>
<td>Cx36-ΔCLB-ΔCTB-EYFP</td>
<td>Plasmid containing lack of cytoplasmic loop binding and carboxy terminal binding site of α-CaMKII at Cx36 fused to EYFP (Figure 3B)</td>
</tr>
<tr>
<td>α-CaMKII-WT-ECFP</td>
<td>Plasmid containing WT α-CaMKII fused to ECFP</td>
</tr>
<tr>
<td>α-CaMKII-T286A-ECFP</td>
<td>Plasmid containing α-CaMKII where threonine 286 is exchange for alanine</td>
</tr>
<tr>
<td>α-CaMKII-T286D-ECFP</td>
<td>Plasmid containing α-CaMKII where threonine 286 is exchanged for aspartate</td>
</tr>
</tbody>
</table>

![Illustration](image)

**Figure 2: Illustration of Cx36-WT protein and the mutants.**

Illustration represents Cx36-WT protein, which has two binding sites for α-CaMKII (A). Black segments represent the deletion of CLB and/or CTB. (B) Figure depicts Cx36ΔCLB-ΔCTB protein, which lacks both binding sites of α-CaMKII. (C) Black segment represent the deletion of CLB. Figure depicts Cx36ΔCLB protein, which lacks CLB site of α-CaMKII. (D) Black segment represent the deletion of CTB. Figure depicts Cx36ΔCTB protein, which lacks CTB site of α-CaMKII.
Table 5: List of proteins and peptides.

<table>
<thead>
<tr>
<th>Names of the Proteins or Peptides</th>
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<tbody>
<tr>
<td>CaM-KIIN</td>
<td>United Peptide Cooperation</td>
</tr>
</tbody>
</table>

3.3.6 Antibiotics

Gentamycin (Life Technologies), Ionomycin (Sigma-Aldrich), Penicillin/Streptomycin (PAA)

3.4 Equipments

3.4.1 Plastic and glassware

All plasticware and glassware are summarized in Table 6.

Table 6: List of plastic and glasswear.

<table>
<thead>
<tr>
<th>Ware</th>
<th>Company</th>
<th>Ware</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml safe seal tubes</td>
<td>Sarstedt</td>
<td>gloves</td>
<td>Medittrade Gentle Skin</td>
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<tr>
<td>5-15-50 ml polypropylene tubes</td>
<td>Becton Dickinson</td>
<td>microliter pipette tips</td>
<td>Sarstedt</td>
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<tr>
<td>24-well plates</td>
<td>Becton Dickinson</td>
<td>microloader pipette tips</td>
<td>Eppendorf</td>
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3.4.2 Laboratory equipment

All laboratory equipment is summarized in Table 7.

Table 7: List of laboratory equipment.

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<th>Equipment</th>
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<th>Equipment</th>
<th>Company</th>
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</thead>
<tbody>
<tr>
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<td>Thermoscientific,</td>
<td>magnetic stirrer</td>
<td>Ika Laboratory Equipment</td>
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<tr>
<td>balance</td>
<td>Sartorius</td>
<td>light microscope</td>
<td>Olympus CK2</td>
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<td>centrifuge</td>
<td>Sigma Laborcentrifugen</td>
<td>microwave</td>
<td>Samsung</td>
</tr>
<tr>
<td>centrifuge rotor</td>
<td>Sorvall</td>
<td>objectives</td>
<td>Acroplan; C. Zeiss</td>
</tr>
<tr>
<td>computer</td>
<td>Apple</td>
<td>pipette</td>
<td>Eppendorf</td>
</tr>
<tr>
<td>confocal microscope</td>
<td>Carl Zeiss LSM 510 META</td>
<td>pH-Meter</td>
<td>Fisher Brand</td>
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</table>
### Table 7: (Continued) List of laboratory equipment.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
<th>Equipment</th>
<th>Company</th>
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</thead>
<tbody>
<tr>
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<td>Integra Biosciences</td>
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<td>54</td>
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</tr>
<tr>
<td>fluorescence microscope</td>
<td>Axiocert 220M, Carl Zeiss</td>
<td>pump filter</td>
<td>Sartorius Midisart</td>
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<td>Carl Zeiss</td>
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<td>2000</td>
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<td></td>
<td>Eclipse TE2000-S; Nikon, Japan</td>
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</tr>
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<td>refrigerator</td>
<td>Liebherr Premium</td>
</tr>
<tr>
<td>freezer -80 °C</td>
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<td>shaker</td>
<td>Ika Vibrax Vxr</td>
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<tr>
<td>glass pipette puller</td>
<td>Heka</td>
<td>stereoscope</td>
<td>Olympus SZX9</td>
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<td>IkaMag, Janke Kunkel</td>
<td>sterile bench</td>
<td>Heraus Heraquard</td>
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<td></td>
<td>Ikalabortechnik</td>
<td></td>
<td>and Holten Laminair</td>
</tr>
<tr>
<td>ice machine</td>
<td>Scotsman</td>
<td>timer</td>
<td>Oregon</td>
</tr>
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<td>stereoscope</td>
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</table>

#### 3.4.3 Software

Adobe Photoshop 7 (Adobe), AxioVision Release 4.6 (Carl Zeiss), LSM 510 META Software (Carl Zeiss), Metaflur software (Universal Imaging, West Chester, PA), Microsoft Excel and Word (Microsoft), GraphPad Prism 5 (GraphPad Software Inc.).
3.5 Cell culture

3.5.1. N2A cells

3.5.1.1 Maintenance of N2A cells

N2A cells were incubated in tissue culture dishes (10 cm Ø; Becton Dickinson, Heidelberg, Germany) in high glucose (4.5 mg/l) Dulbecco’s Modified Eagles medium (Gibco BRL Life Technologies, Karlsruhe, Germany) with 5% fetal calf serum (PAA Laboratories GmbH), 1% non-essential amino acid (PAA Laboratories GmbH), 2 mM L-glutamine, (PAA Laboratories GmbH), 1 mM sodium pyruvate (PAA Laboratories GmbH), penicillin (100 U/ml) and streptomycin (100 µg/ml; PAA Laboratories GmbH). Cells were kept in an incubator (HeraCell, Heraeus, Hanau, Germany) at 37 °C, with humidity control and 5% CO₂ carbogen gas.

3.5.1.2 Passaging of N2A cells

N2A cells were passaged routinely two times per week. For this purpose cells were washed with PBS at 37 °C. After incubation with trypsin-EDTA for 5 min at 37 °C, 5 ml of fresh medium was added to the cells and dissociated cells were centrifuged at 200 g for 5 min in 12 ml falcon tubes at RT. Later, supernatants were discarded and cells were suspended in 5 ml of fresh medium. 0.5 ml of the cell suspension (~1.5x10⁶ cells) was added to 10 ml fresh growth medium and cultivation was continued in a 10 cm culture dish.

3.5.1.3 Preparation of frozen N2A cells

For storage of N2A cells, frozen samples were prepared. For this purpose medium was aspirated and the cells were washed with 37 °C PBS. After the incubation with trypsin-EDTA for 5 min at 37 °C, 5 ml of fresh medium was added to the cells and dissociated cells were centrifuged at 200 g for 5 min in 12 ml Falcon tubes at RT. After the centrifugation, supernatants were discarded and the cells were suspended carefully in freezing medium, consisting of 80% medium, 10% FBS, and 10% DMSO (Sigma D2650), in 1.5 ml Eppendorf cups (Eppendorf AG, Hamburg, Germany) and stored at -80 °C overnight. These frozen vials were placed into a liquid nitrogen tank (-196 °C) for long-term storage.
3 Materials and Methods

3.5.1.4 Reconstitution of N2A cells

Frozen N2A cells were placed into water bath at 37 °C, and 10 ml warm DMEM (without antibiotics) containing 20 % fetal calf serum (FCS). After the centrifugation at 900 rpm for 5 min at RT, cells were triturated gently in 10 ml medium. N2A cells were then seeded into 10 cm plates at the desired density. One day after thawing, the medium was changed.

3.5.1.5 Transient transfection of N2A cells

For imaging experiments, N2A cells were seeded on poly-L-lysine coated glass bottom culture dishes (MatTek Corporation, Ashland, USA) and placed in the incubator. After 24 h, 200-400 ng plasmid DNA was supplemented by using Effectene transfection reagent (QIAGEN, Hilden, Germany) according to the manufacturer’s guidelines. The medium was changed 24 h after transfection.

3.5.2 Primary rat hippocampal neurons

3.5.2.1 Isolation of primary rat hippocampal neurons

One day before the preparation, 10 ml of FCS was inactivated in 56 °C for 30 min and stored at 4 °C. On the next day, P0-P1, Sprague Dawley rats were sacrificed and the heads were placed onto clean paper towel to get rid of excess blood. Skins of the heads were carefully removed and scalps were opened. Hemispheres of each pup were placed into a plastic dish containing cold PBS by help of a blunt spatula. Under the binocular, hippocampi were dissected and stored into cold HBSS+/- solution. Dissected hippocampi were removed from HBSS+/-, and cells were dissociated in 10 ml 0.05 % trypsin solution for 25 min at RT on a shaker under slow mode. After the incubation, hippocampi were removed from the trypsin solution and placed into 10 % FBS and HBSS solution. Cells were suspended 6-7 times with a 10 ml sterile pipette and then filtered through a 100 µm disposable filter (Partec Cell-Tric, Görlitz, Germany) to another falcon tube, which contained 10 % FBS and HBSS solution.

After centrifugation for 4 min at 1200 rpm, supernatant was removed; cells were suspended in the neurobasal medium and distributed onto poly-L-lysine coated glass bottom culture dishes (MatTek Corporation).
3.5.2.2 Maintenance of primary rat hippocampal neurons

Primary rat hippocampal neurons in glass bottom culture dishes were kept at 37 °C in a humidified atmosphere with 5 % CO₂ and medium changed at DIV 7.

3.5.2.3 Transfection of primary rat hippocampal neurons

For transfection of primary rat hippocampal neurons, Effectene transfection reagent was used at DIV 13 according to the manufacturer’s recommendation.

3.6 Detection of protein expression with immunocytochemistry

Immunocytochemistry of transfected neurons was performed two days after the transfection. Cells were fixed with 4 % fresh PFA at RT for 15 min and gently rinsed with PBS. Cell membranes were permeabilized with 1 % Triton X-100 in PBS for 10 min. To block unspecific binding, cells were incubated in 10 % non-immune goat serum and thereafter, primary antibodies were added into the blocking solution in a 1:200 dilution. Incubation with the primary antibody was performed overnight at 4 °C. The next day, cells were rinsed for three times in PBS-T for 10 min, and then incubated for 1 h at RT with species specific secondary antibodies, diluted in PBS-T (1:1000), coupled with Alexa Fluor fluorescent dyes (Invitrogen, Karlsruhe, Germany). Finally, cells were rinsed with PBS-T and coverslips mounted using ProLong Gold antifade reagent (Molecular Probes, Invitrogen, Eugene, OR, USA).

3.7 Digital image acquisition

3.7.1 Time laps imaging via confocal microscopy

Time laps imaging of N2A cells and hippocampal neurons were performed with a Zeiss Laser Scanning Microscope 510 META. Image acquisition was performed with a fully automated LSM-510 using a Plan Neofluar 63× oil objective and incubator for the best temperature control. Using LSM software, multichannel and time-lapse experiments were set up and images were acquired for a total time of 30-60 min. The time lapse images were further analyzed using the following LSM 510 modules: time, area percentage, area sum, mean density and density standard deviation, intensity and background subtraction. Automatic measurement was done with this setting, creating a data table that indicates these values per observed field. The data were imported into Microsoft Excel and GraphPad prism (GraphPad Software, California, USA) to create a plot of intensity of the region of interest and time.
3.7.2 Fluorescence resonance energy transfer (FRET) and acceptor photobleaching protocol with confocal microscopy (general considerations)

To measure FRET between two proteins, proteins should be tagged with donor and acceptor fluorophores, respectively.

EYFP/EGFP, EYFP/ECFP, Cy3/EGFP, Cy3/Cy5, TRITC/FITC are accepted as reliable acceptor-donor pairs because in each donor-acceptor fluorophore pair, the “donor,” has an emission spectrum overlapping the excitation spectrum of the second fluorophore, the “acceptor” (Figure 4A).

FRET can be detected in living or fixed samples by different approaches. One of the approaches to measure FRET is the acceptor photobleaching protocol. This protocol, involves measuring of the intensity of the donor fluorescence in the presence of the acceptor fluorescence. This can be done by comparing donor fluorescence intensity in the same sample before and after destroying the acceptor fluorescence by photobleaching.
Figure 3: The spectra of CFP and YFP proteins as FRET pairs and principles of the acceptor photobleaching method.

FRET pairs are always chosen from fluorophores where the donor emission overlaps acceptor excitation. This setting is also applicable for cyan and yellow fluorescence proteins. (A) Here, the graph shows that emission of CFP overlaps the excitation of YFP. (B) In case of energy transfer between the donor CFP and the acceptor YFP, photobleaching of the acceptor leads an increase in the emission of the donor, CFP. Here also time-course analysis of fluorescence intensity before and after photobleaching in the presence or absence of a protein-protein interaction is shown. Blue and yellow curves indicate the levels of CFP and YFP fluorescence before photobleaching (BB) and after photobleaching (AB), respectively. In case of FRET, bleaching of the acceptor molecule leads to an increase in donor fluorescence. In the absence of interaction between proteins, CFP levels before and after the bleach do not change. (The artwork is taken from http://microscopy.berkeley.edu/courses/tlm/fluor_techniques/fret.html, http://www.plantmethods.com/content/2/1/12/figure/F3).

In this thesis work, the acceptor protocol was applied to fixed samples. α-CaMKII-ECFP-(donor) and Cx36-EYFP-(acceptor) proteins were co-expressed in N2A cells for 24-48 h and cells were fixed with 4 % PFA and taken to confocal microscope.
Dbb (donor before bleaching) and Abb (acceptor before bleaching) images were acquired with a Carl Zeiss LSM 510 META, which was equipped with correct filter sets and excitation lasers. After the acquisition of Dbb and Abb images, the acceptor was photo-inactivated with a 514 nm laser line, and increase/decrease in Dab (donor after bleach) and Aab (acceptor after bleach) were recorded.

3.7.3 Fluorescence resonance energy transfer (FRET) analysis of Cx36 and α-CaMKII interaction (specific experimental setting)

To show physical interaction between Cx36 and α-CaMKII proteins, N2A cells were plated on coverslips and transfected with Cx36 (WT or α-CaMKII binding site mutants) and α-CaMKII (WT or threonine mutants) as summarized in Table 8. After 48 h of transfection, translocation of α-CaMKII, to Cx36 expressing GJ plaques was activated by exposing the cells to Ca\(^{2+}\)/ionomycin (2 mM/10 µM) (Bayer et al., 2001) followed by fixation in 4 % PFA. In control experiments, transfected N2A cells were exposed to solutions that lack Ca\(^{2+}\)/ionomycin and subsequently fixed with 4 % PFA.

After bleaching of the acceptor (in our case Cx36-EYFP at GJ plaques), using the 514 nm laser line, the change in intensity of donor fluorescence (in our case α-CaMKII-ECFP) was quantified by comparing pre-bleached and post-bleached images. FRET can be detected if intensity of donor fluorescence α-CaMKII-ECFP increases significantly. This effect indicates a physical interaction between Cx36-EYFP and α-CaMKII-ECFP at the location of the photobleached site, i.e. GJ plaques.

Table 8: Combination of plasmids used in transfecting N2A cells 48 h prior to FRET experiments.

<table>
<thead>
<tr>
<th>Combinations of plasmids in N2A cells</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Cx36-EYFP-WT</td>
<td>α-CaMKII-ECFP-WT</td>
</tr>
<tr>
<td>Cx36-EYFP-∆CLB</td>
<td>α-CaMKII-ECFP-WT</td>
</tr>
<tr>
<td>Cx36-EYFP-∆CTB</td>
<td>α-CaMKII-ECFP-WT</td>
</tr>
<tr>
<td>Cx36-EYFP-∆CLB-∆CTB</td>
<td>α-CaMKII-ECFP-WT</td>
</tr>
<tr>
<td>Cx36-EYFP-WT</td>
<td>α-CaMKII-ECFP-T286A</td>
</tr>
<tr>
<td>Cx36-EYFP-WT</td>
<td>α-CaMKII-ECFP-T286D</td>
</tr>
</tbody>
</table>
3.7.4 Ca\(^{2+}\) Imaging

N2A cells were plated on glass bottom culture dishes and loaded with Fura-2 AM (10 µM; Molecular Probes), for 45 min, at 37 °C. Afterwards, cells were rinsed with PBS and were imaged on an epifluorescence microscope (Eclipse TE2000-S; Nikon, Japan). Changes in Fura-2 fluorescence were recorded by Metafluor software before and after Ca\(^{2+}\)/ionomycin stimulation (Universal Imaging, West Chester, PA).

3.7.5 Imaging of active presynaptic sites

To show the localization of active presynapses in neuron cultures, we used FM4-64 staining. Cells in DIV 13 were exposed to high extracellular KCl (50 mM) and FM4-64 (15 µM) for 3 minutes inside the CO\(_2\) incubator. At the end of the KCl-FM4-64 incubation, cells washed gently and the localization of the presynapses were detected with confocal microscopy.

3.8 Electrical Stimulation

Electrical stimuli were generated with a pulse generator; trains of 0.5 ms square pulses were delivered between 10-100 Hz using constant current mode through glass micropipettes that were placed near the imaged neuron with 10–70 µA currents.

3.9 Statistics

Graph Pad Prism 5.0 C (for Macintosh) was used to perform the statistical analysis. Results in this thesis are shown in mean values ± SE (standard error). Statistical significance of the results were checked by Student’s T-test. Significant values were demonstrated with asterisks: *** indicate \(p < 0.001\), ** indicate \(0.001 \leq p < 0.01\), * indicates \(0.01 \leq p < 0.05\).
4 Results

4.1 Interaction between Cx36 and α-CaMKII

4.1.1 Effects of changes of \([\text{Ca}^{2+}]\), in Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected N2A cells

In the CNS, spatial and temporal changes in intracellular \(\text{Ca}^{2+}\) levels control a multitude of cellular functions. In neurons, a local rise in intracellular calcium concentration \([\text{Ca}^{2+}]\) is a key event for triggering translocation of α-CaMKII to its substrates (Thalhammer 2006). The same phenomenon has been shown in α-CaMKII transfected Human Embryonic Kidney 293 cells (HEK cells) after \(\text{Ca}^{2+}/\text{ionomycin} (2 \text{ mM}/10 \text{ µM})\) exposure (Bayer et. al, 2001). Moreover, \(\text{Ca}^{2+}\) also promotes binding of CaMKII to Cx36 binding sites as it has been described previously in \textit{in vitro} pull down experiments (Alev et al., 2008).

Before investigating the interaction of α-CaMKII-ECFP-WT with Cx36-EYFP-WT in N2A cells, calcium imaging was used to confirm the expected increase of intracellular calcium in real-time before and after the application of \(\text{Ca}^{2+}/\text{ionomycin} (2 \text{ mM}/10 \text{ µM})\) solution.

To address this issue, Fura-2 AM was used to monitor intracellular \(\text{Ca}^{2+}\) levels. Fura-2 AM is a cell-permeable acetoxymethyl (AM) ester of Fura-2, which can bind free \(\text{Ca}^{2+}\). During incubation, cells take up Fura-2 AM. Subsequently, the esterified Fura-2 AM form is cleaved by intracellular esterases into active Fura-2. The spectral characteristics of Fura-2 AM are the same as those of unbound Fura-2, but only Fura-2 is sensitive to \(\text{Ca}^{2+}\) ions and can not re-enter into the cell membrane. A higher concentration of \(\text{Ca}^{2+}\) increases the fluorescence of Fura-2 when excited at 340 nm and decreases the fluorescence when excited at 380 nm. Monitoring the decreasing signal generated by the 380 nm excitation indicates the extent of cleavage of Fura-2 AM and thereby binding to \(\text{Ca}^{2+}\) ions. Ratio values of Fura-2 fluorescence intensities emitted at two excitation wavelengths (340 nm and 380 nm) were obtained using the Metaflour software (Universal Imaging, West Chester, PA).

In α-CaMKII-ECFP-WT and Cx36-EYFP-WT co-transfected N2A cells, ionomycin was applied to mediate a \([\text{Ca}^{2+}]\) rise by inducing a bulk influx across the membrane. In Figure 5A, pseudocolored real time images depict the α-CaMKII-ECFP-WT and Cx36-EYFP-WT transfected N2A cells, before and after the application of \(\text{Ca}^{2+}/\text{ionomycin} (2 \text{ mM}/10 \text{ µM})\). Intracellular calcium levels of the cells rise to high levels from resting levels after the application of \(\text{Ca}^{2+}/\text{ionomycin} (2 \text{ mM}/10 \text{ µM})\). The graph in Figure 5B shows a 4× increase in \([\text{Ca}^{2+}]\) after the application of a \(\text{Ca}^{2+}/\text{ionomycin} (2 \text{ mM}/10 \text{ µM})\) solution.
Figure 4: Intercellular Ca\(^{2+}\) change in Cx36-EYFP-WT and α-CaMKII-ECFP-WT transfected cells in response to Ca\(^{2+}\)/ionomycin (2 mM/10 µM).

(A) In α-CaMKII-ECFP-WT and Cx36-EYFP-WT transfected N2A cells, ionomycin mediates [Ca\(^{2+}\)] amplitification, inducing the Ca\(^{2+}\) influx across the cell membrane. Here pseudocoloured real time images are shown before and after the application of Ca\(^{2+}\)/ionomycin (2 mM/10 µM) indicating that intracellular calcium levels of the cells rise to high levels from resting levels. Images were acquired in 1 sec intervals after the stimulation solution was applied to the cells (Scale bar = 100 µM, n = 3).

(B) The graph depicts the intracellular Ca\(^{2+}\) change induced by Ca\(^{2+}\)/ionomycin (2 mM/10 µM) in Fura-2 AM loaded N2A cells. The black arrow at t = 35 s indicates the application time of Ca\(^{2+}\)/ionomycin (2 mM/10 µM).

4.1.2 Cx36 protein interacts with α-CaMKII

Protein-protein interaction requires close associations of molecules, which take place at almost any level of cell function and are fundamental for most biological processes. For α-CaMKII, which is present in high quantities in the central nervous system, several interaction partners have been described.

Despite the our general knowledge that interaction of α-CaMKII with target proteins induces synaptic plasticity at sites of chemical synapses, plasticity of electrical synapses has also been shown to play a central role in modulating electrical coupling (Pereda et al., 2003), in particular in the Mauthner club endings.

The physical interaction of α-CaMKII with the neuronal GJ protein Cx36, was primarily analyzed by \textit{in silico} analysis. Two potential consensus motifs for α-CaMKII binding within the cytoplasmic domains of Cx36 were discovered. The first consensus motif is at the juxtamembrane position of the cytoplasmic loop (CLB, amino acids 175–195) whereas the second consensus motif lies at the
juxtamembrane position of the carboxyl terminus (CTB, amino acids 272–292). In addition to the predicted CLB and CTB binding sites, four putative α-CaMKII phosphorylation motifs were identified in two regions in the cytoplasmic loop [Alev et al., 2008].

Later, the physical interaction of α-CaMKII with Cx36 was shown with pull-down assays. GST fusion proteins, which contain either the cytoplasmic loop site (GST-Cx36CL, amino acids 100–201) or the carboxy-terminus (GST-Cx36CT, amino acids 272–321) were incubated with 35S-labeled CaMKII protein under different conditions, where Ca²⁺ and CaM were either present or absent. CaMKII was found to bind to GST-Cx36CL and GST-Cx36CT proteins in these assays and Ca²⁺/CaM acted as the promoter of this binding [Alev et al., 2008].

According to these in vitro data, we hypothesized that α-CaMKII is one of the interaction partners of the major electrical synapse protein Cx36 and Ca²⁺ may promote in vivo interaction of these two proteins in the GJ plaques of N2A cells. To prove this concept, we performed FRET analyses after photobleaching (see Material and Methods).

In transfected N2A cells Cx36-EYFP-WT protein was found to locate at sites of the plasma membrane in form of typical GJ plaques between two adjacent cells, while α-CaMKII-ECFP-WT protein is distributed evenly in the cytoplasm.

Figure 6A and Figure 6E indicate α-CaMKII-ECFP-WT expression before and after photobleaching of Cx36-EYFP-WT in N2A cells. The arrows depict the α-CaMKII-ECFP-WT accumulation in the region of GJ plaques. In Figure 6B, the arrow depicts the Cx36-EYFP-WT plaque before photobleaching, where the arrow in Figure 6F indicates the photobleached GJ plaque. After performing the photobleaching protocol (Figure 6F), the intensity of the α-CaMKII-ECFP-WT in the GJ plaque region was increased. This increase in donor fluorescence, α-CaMKII-ECFP-WT, is proportional to the decrease of the acceptor fluorescence (Cx36-EYFP-WT), thus indicating physical interaction between Cx36 and α-CaMKII in fixed N2A cells that were exposed to Ca²⁺/ionomycin.
Figure 5: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-WT in Ca\(^{2+}\)/ionomycin stimulated N2A cells, before and after acceptor photobleaching.

Acceptor and donor proteins are shown before (A-B-C-D) and after photobleaching (E-F-G-H). Cx36-EYFP-WT (acceptor) protein assembles in forms of GJ plaques between two adjacent cells (B: white arrow), while α-CaMKII-ECFP-WT (donor) protein is distributed in the cytoplasm (A). In this experiment, Cx36-EYFP-WT (B) was bleached with 514 nm laser and becomes invisible in (F). (C) and (G) show the merged images of Cx36-EYFP-WT and α-CaMKII-ECFP-WT before and after acceptor photobleaching, respectively. (D) and (H) show detailed view of pseudocolored intensity plots of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-WT. The increase in the intensity of α-CaMKII-ECFP-WT after bleaching is visible in (H); this increase in the intensity of α-CaMKII-ECFP-WT indicates FRET between the two proteins. (Scale bar - 5 µm and images are pseudocolored).

To explore whether this effect is Ca\(^{2+}\) dependent, the contribution of Ca\(^{2+}\) for FRET between Cx36-EYFP-WT and α-CaMKII-ECFP-WT was evaluated. Therefore, N2A cells were treated with a control solution without Ca\(^{2+}\)/ionomycin. Figure 7 shows N2A cells that were subjected to this form of control experiments. Figure 7A and Figure 7E show α-CaMKII-ECFP-WT before and after the photobleaching of Cx36-EYFP-WT. In Figure 7B, the arrow depicts the Cx36-EYFP-WT plaque before photobleaching where the Cx36-EYFP-WT plaque is not visible anymore (Figure 7F) after bleaching. No significant increase in the intensity of α-CaMKII-ECFP-WT (Figure 7H) after the photobleaching of Cx36-EYFP-WT in the region of GJ plaques is evident.

This result supports the above hypothesis that Ca\(^{2+}\)/ionomycin stimulation induces FRET between α-CaMKII-ECFP-WT and Cx36-EYFP-WT in GJ plaques of N2A cells.
Figure 6: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-WT in N2A cells stimulated with solution containing no Ca²⁺/ionomycin, before and after acceptor photobleaching.

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In this control experiment Cx36-EYFP-WT (B) was bleached with a 514 nm laser line and becomes invisible in (F). (C) and (G) show the merged images of Cx36-EYFP-WT and α-CaMKII-ECFP-WT before and after acceptor photobleaching, respectively. (D) and (H) show the detailed view of the intensity of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-WT. There is no change in the intensity of α-CaMKII-ECFP-WT after photobleaching (H). FRET does not occur between two proteins when the solution lacks Ca²⁺/ionomycin. (Scale bar = 2 µm and images are pseudocolored) (experiment was performed 4 times in triplicate, n=12)

In order to quantify the apparent interaction, FRET efficiencies \( F_{\text{eff}} \) and the distance between two proteins were calculated for Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected N2A cells.

\[ F_{\text{eff}} = \left( D_{\text{post}} - D_{\text{previous}} \right) / D_{\text{post}} \]

FRET efficiency depends strongly (sixth-power relationship) on the distance between the donor and acceptor fluorophores. Also it depends on the donor-to-acceptor separation distance \( R \) with an inverse 6th order law due to the dipole-dipole coupling mechanism:

\[ F_{\text{eff}} = R_0^6 / (R_0^6 + r^6) \]

"R₀" is the distance at which 50 % energy transfer takes place and "r" represents the distance. "R₀" is the fluorophore constant that is determined by the amount of overlap between the excitation and emission spectra of the donor and acceptor. "R₀" also depends on the relative orientation of the donor and acceptor. "R₀" value for the donor-acceptor CFP/YFP FRET pair is 4.92 nm while other fluorophore pairs yield "R₀" values in the range of 2 nm to 6 nm.

By using these two equations below, the distance between Cx36-EYFP-WT and α-CaMKII-ECFP-WT were calculated (Figure 8).
Figure 7: Equations used to calculate the FRET efficiencies of the previous experiments.

(A) Fret_{eff} was calculated, using the formula \( FRET_{eff} = (D_{\text{post}} - D_{\text{pre}})/D_{\text{post}} \), where \( D_i \) is the CFP average intensity at the \( i^{th} \) time point after background subtraction. FRET efficiency depends strongly (sixth-power relationship) on the distance between the donor and acceptor fluorophores and depends on the formula stated at Figure 8B. (B) The amount of FRET, or its efficiency \( FRET_{eff} \), is given by \( FRET_{eff} = R_0^6/(R_0^6 + r^6) \), where \( r \) is the distance between the two fluorophores and \( R_0 \) is the distance at which 50% energy transfer takes place.

FRET efficiencies for Cx36-EYFP-WT and \( \alpha\)-CaMKII-ECFP-WT proteins in co-transfected N2A cells were calculated for both, experiment and control conditions [Table 9]. The highest FRET efficiency between Cx36-EYFP-WT and \( \alpha\)-CaMKII-ECFP-WT was measured in GJ plaques of N2A cells that were exposed to \( \text{Ca}^{2+}/\text{ionomycin} \) 3.4 % ± 0.534 (experiment was performed 4 times in triplicate, \( n=12 \)). As reference measurements, Cx36-EYFP-WT fluorescence outside GJ plaques were chosen (Reference ROIs) and the FRET efficiency in these regions were calculated 0.9 % ± 0.526, when the cells were stimulated with \( \text{Ca}^{2+}/\text{ionomycin} \) solution (Table 9).

### Table 9: FRET efficiencies of Cx36-EYFP-WT and \( \alpha\)-CaMKII-ECFP-WT in the presence and absence of \( \text{Ca}^{2+}/\text{ionomycin} \) solution.

<table>
<thead>
<tr>
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<th>Cells stimulated with ( \text{Ca}^{2+}/\text{ionomycin} ) solution</th>
<th>Cells stimulated with control solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROI (GJ plaques)</td>
<td>Reference ROI</td>
</tr>
<tr>
<td>Cx36-EYFP-WT and ( \alpha)-CaMKII-ECFP-WT</td>
<td>3.4 % (± 0.534)</td>
<td>0.9 % (± 0.526)</td>
</tr>
</tbody>
</table>

In control experiments lacking \( \text{Ca}^{2+}/\text{ionomycin} \) stimulation, FRET efficiency of the two proteins was also calculated (experiment was performed 4 times in triplicate, \( n=12 \)). In GJ plaques FRET efficiency is equal to 0.2 % ± 0.03, where FRET efficiency in Reference ROIs is 0.3 % ± 0.04 (Table 9).

Thus, the FRET efficiency of \( \alpha\)-CaMKII-ECFP-WT and Cx36-EYFP-WT in \( \text{Ca}^{2+}/\text{ionomycin} \) stimulated N2A cells, is significantly increased in GJ domains compared to Reference ROIs (\( p < 0.001 \)) of the same samples, and to Reference ROI (\( p < 0.001 \)) and GJ plaques of \( p < 0.001 \) N2A cells in control solutions (Figure 9).
FRET efficiencies for Cx36-EYFP-WT and α-CaMKII-ECFP-WT were calculated in transfected N2A cells, which were exposed to Ca^{2+}/ionomycin solution or control solution. The highest FRET efficiency between Cx36-EYFP-WT and α-CaMKII-ECFP-WT is observed in GJ plaques of cells that were exposed to Ca^{2+}/ionomycin. Asterisks indicate significant differences, *** indicates $p < 0.001$ (experiment was performed 4 times in triplicate, n=12).

FRET between two proteins only occurs when the distance between two proteins is smaller than 10 nm of distance. In Table 10, the distance between Cx36-EYFP-WT and α-CaMKII-ECFP-WT is calculated by using the equation in Figure 8B.

Table 10: Distance between Cx36-EYFP-WT and α-CaMKII-ECFP-WT were calculated in the presence and absence of Ca^{2+}/ionomycin solution.

<table>
<thead>
<tr>
<th></th>
<th>Cells stimulated with Ca^{2+}/ionomycin solution</th>
<th>Cells stimulated with control solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROI (GJ plaques)</td>
<td>Reference ROI</td>
</tr>
<tr>
<td>Cx36-EYFP-WT and α-CaMKII-ECFP-WT</td>
<td>8.41 nm (± 1.05)</td>
<td>10.5 nm (± 1.33)</td>
</tr>
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</table>

Distances mentioned in Table 10 show that cells exposed to Ca^{2+}/ionomycin revealed a distance between the two proteins (Cx36-EYFP-WT and α-CaMKII-ECFP-WT) equal to 8.41 nm (± 1.05) (Table 10) (experiment was performed 4 times in triplicate, n=12). However, the cells lacking Ca^{2+}/ionomycin treatment were exposed to only control solution, the distance between two proteins increases to 13.4 nm (± 1.36) (experiment was performed 4 times in triplicate, n=12). These two proteins do not show FRET in the Reference ROIs under Ca^{2+}/ionomycin solution or control solution exposure, and they reveal 12.6 nm (± 1.07) and 10.5 nm (± 1.33) distances, respectively, which is above the cut off limit of FRET interaction [Herman et al., 1989].
4.1.3 Cytoplasmic loop binding site (CLB) dominates over carboxyl terminal binding site (CTB) in Cx36 and α-CaMKII interaction

The results described in the previous chapter raised the question regarding the possible contribution of the binding sites of Cx36 to the interaction with α-CaMKII-ECFP-WT. As described before, Cx36 carries two consensus motives for α-CaMKII binding. The first motive is in the juxtamembrane position of the cytoplasmic loop (CLB, amino acids: 175–195), where the second consensus motive is found in the juxtamembrane position of the carboxyl terminus (CTB, amino acids: 272–292).

Here, it was hypothesized that FRET between Cx36 and α-CaMKII cannot take place when the binding sequences for α-CaMKII are deleted.

To prove this hypothesis, a Cx36 protein mutant Cx36-EYFP-ΔCLB-ΔCTB was generated (Figure 3B). Cx36-EYFP-ΔCLB-ΔCTB is a Cx36 fusion protein that lacks the two binding sites for α-CaMKII. N2A cells transfected with Cx36-EYFP-ΔCLB-ΔCTB and α-CaMKII-ECFP-WT were exposed to Ca²⁺/ionomycin or to control solution without Ca²⁺/ionomycin (experiment was performed 4 times in triplicate, n=12). Cx36-EYFP-ΔCLB-ΔCTB protein also assembles in form of plasma membrane-bound GJ plaques like the Cx36-EYFP-WT protein (Figure 10B), while α-CaMKII was found to be evenly distributed in the cytoplasm (Figure 10A).
Figure 9: Display of the Cx36-EYFP-ΔCLB-ΔCTB (double site binding mutant) and α-CaMKII-ECFP-WT in Ca²⁺/ionomycin stimulated N2A cells.

The figure shows the acceptor and donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-ΔCLB-ΔCTB (acceptor protein) also assembles GJ plaques between two adjacent cells (B: white arrow), while α-CaMKII-ECFP-WT (donor) protein is distributed in the cytoplasm and in the Cx36-EYFP-ΔCLB-ΔCTB expressing GJ plaques (A). In this experiment, Cx36-EYFP-ΔCLB-ΔCTB (B) is bleached with the 514 nm laser and becomes invisible (F). (C) and (G) show the merged images of Cx36-EYFP-ΔCLB-ΔCTB and α-CaMKII-ECFP-WT before and after acceptor photobleaching, respectively. (D) and (H) show a detailed view of the intensity of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-ΔCLB-ΔCTB. The intensity of α-CaMKII-ECFP-WT after bleaching did not change (arrow in H). This indicates that no FRET takes place between the two proteins. (Scale bar - 3 µm and images are pseudocolored).

FRET analyses between these two proteins were calculated in two different ROIs as indicated in the previous chapter. First ROI was Cx36-EYFP-ΔCLB-ΔCTB carrying GJ plaque and the second ROI was the reference point where Cx36-EYFP-ΔCLB-ΔCTB proteins do not show visible GJ assemblies. Figure 10A and Figure 10E indicate the expression of α-CaMKII before and after photobleaching of Cx36-EYFP-ΔCLB-ΔCTB. No significant increase in the intensity of α-CaMKII-ECFP-WT after photobleaching (Figure 10H) was visible, indicating that FRET does not take place between Cx36-EYFP-ΔCLB-ΔCTB and α-CaMKII-ECFP-WT when both binding sites are missing in the Cx36 protein (experiment was performed 4 times in triplicate, n=12).

Cx36-EYFP-ΔCLB-ΔCTB and α-CaMKII-ECFP-WT co-transfected N2A cells were also exposed to control solution (Figure 11). There was no FRET measured between Cx36-EYFP-ΔCLB-ΔCTB and α-CaMKII-ECFP-WT proteins (experiment was performed 4 times in triplicate, n=12).
Figure 10: Display of the Cx36-EYFP-ΔCLB-ΔCTB (double site binding mutant) and α-CaMKII-ECFP-WT in control solution stimulated N2A cells, before and after acceptor photobleaching.

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-ΔCLB-ΔCTB (acceptor) protein also assembles to GJ plaques between two adjacent cells (B: white arrow), while α-CaMKII-ECFP-WT (donor) protein is distributed in the cytoplasm (A) and also in the GJ plaques. In this experiment Cx36-EYFP-ΔCLB-ΔCTB (B) is bleached with the 514 nm laser and not visible anymore (F). (C) and (G) show the merged images of Cx36-EYFP-ΔCLB-ΔCTB and α-CaMKII-ECFP-WT before and after acceptor photobleaching respectively. (D) and (H) show the detailed view of the intensity of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-ΔCLB-ΔCTB. The intensity of α-CaMKII-ECFP-WT after bleaching did not change in (H), indicating that FRET does not take place between the two proteins. (Scale bar - 4 µm and images are pseudocolored)

FRET efficiency is negative for GJ plaques in Ca²⁺/ionomycin stimulated N2A cells and control solution stimulated N2A cells. On the other hand, the FRET efficiency is equal to 0.4 % (± 0.05) and the distance between two proteins are 12.03 nm (± 1.00) in the Reference ROI of Ca²⁺/ionomycin stimulated N2A cells.

These data clearly illustrate that deletion of the two binding sites of Cx36, prevents the interaction between Cx36 and α-CaMKII, indicating that the cytoplasmic binding sites are crucial for the generation of FRET signals.

These results obtained from FRET analyses raised two important questions: (i) are both binding sites of Cx36 needed for interaction with α-CaMKII, and (ii) if only one binding site may be needed, which site is the key player for α-CaMKII interaction?

In order to address these questions single site mutants of Cx36 protein were generated and exposed to the same stimulation paradigm as described above. The first Cx36 mutant lacks the CLB site (Cx36-EYFP-ΔCLB) (Figure 3C) and the second mutant lacks the CTB site (Cx36-EYFP-ΔCTB) (Figure 3D).
To unravel if α-CaMKII has any binding preference for one of the binding sites FRET experiments were applied in N2A cells, which were co-transfected with α-CaMKII and the Cx36 binding site mutants (Figure 12 and Figure 13).

In order to show the effect of deletion of the CTB site on FRET between two proteins, N2A cells were co-transfected with Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT plasmids and then stimulated with Ca\(^{2+}\)/ionomycin (2 mM/10 µM) (Figure 12) (experiment was performed 4 times in triplicate, n=12).
Figure 13 depicts Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT co-transfected N2A cells after exposure to control solution (experiment was performed 4 times in triplicate, n=12).

Quantification of FRET efficiency of Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT proteins was in the range of 1.4 % (± 0.43) (Table 11) and the distance between the two proteins was calculated to be 9.7 nm (± 1.11) (Table 12), indicating that in the presence of CLB binding of α-CaMKII to Cx36 is still possible.

When Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT co-transfected N2A cells are exposed to control solution no positive FRET efficiency was calculated.
Figure 12: Display of Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT in N2A cells stimulated with solution containing no Ca$^{2+}$/ionomycin, before and after acceptor photobleaching.

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In this experiment Cx36-EYFP-ΔCTB (B) was bleached with the 514 nm laser and becomes invisible in (F). (C) and (G) shows the merged images of Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT before and after acceptor photobleaching, respectively. (D) and (H) show the detailed view of the intensity of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-ΔCTB. There is no change in the intensity of α-CaMKII-ECFP-WT after acceptor photobleaching (H). FRET does not occur between two proteins when stimulation solution lacks Ca$^{2+}$/ionomycin. (Scale bar - 2 µm and images are pseudocolored).
Table 11: FRET efficiencies of Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT in presence and absence of Ca\(^{2+}\)/ionomycin solution.

<table>
<thead>
<tr>
<th>Cells stimulated with</th>
<th>ROI (GJ plaques)</th>
<th>Reference ROI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})/ionomycin solution</td>
<td>1.4 % (± 0.43)</td>
<td>0.2 % (± 0.02)</td>
</tr>
<tr>
<td>Control solution</td>
<td>0.12 % (± 0.04)</td>
<td>0.14 % (± 0.01)</td>
</tr>
</tbody>
</table>

Table 12: Distance between Cx36-EYFP-ΔCTB and α-CaMKII-ECFP-WT were calculated in presence and absence of Ca\(^{2+}\)/ionomycin solution.

<table>
<thead>
<tr>
<th>Cells stimulated with</th>
<th>ROI (GJ plaques)</th>
<th>Reference ROI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})/ionomycin solution</td>
<td>9.7 nm (± 1.11)</td>
<td>13.8 nm (± 1.19)</td>
</tr>
<tr>
<td>Control solution</td>
<td>14.9 nm (± 1.20)</td>
<td>14.7 nm (± 1.11)</td>
</tr>
</tbody>
</table>

To elucidate the effect of CLB site deletion for α-CaMKII binding Cx36-EYFP-ΔCLB mutants were used to perform FRET analyses.

Figure 14A and Figure 14E show the expression of α-CaMKII-ECFP-WT protein before and after photobleaching of N2A cells. Cx36-EYFP-ΔCLB is still able to form GJ plaques like the Cx36-EYFP-WT and the Cx36-EYFP-ΔCTB mutants (Figure 14B). Arrow in Figure 14F points to the photobleached, Cx36-EYFP-ΔCLB containing GJ plaque. Figure 14D and Figure 14H depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale before and after photobleaching of Cx36-EYFP-ΔCLB. The intensity of α-CaMKII-ECFP-WT does not change when Cx36-EYFP-ΔCLB GJ plaque was bleached. Therefore, unlike the situation with the CTB mutant, no FRET takes place between both proteins (experiment was performed 4 times in triplicate, n=12).
Figure 13: Display of Cx36-EYFP-ΔCLB and α-CaMKII-ECFP-WT in Ca^{2+}/ionomycin stimulated N2A cells, before and after acceptor photobleaching.

Shown are the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-ΔCLB (acceptor) protein also assembles to GJ plaques between two adjacent cells (B, white arrow), while α-CaMKII-ECFP-WT (donor) protein is distributed in the cytoplasm (A). In this experiment Cx36-EYFP-ΔCLB (B) is bleached with 514 nm laser line and is not visible in (F). (C) and (G) show the merged images of Cx36-EYFP-ΔCLB and α-CaMKII-ECFP-WT before and after acceptor photobleaching, respectively. (D) and (H) show detailed views of the intensity of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-ΔCLB. The intensity of α-CaMKII-ECFP-WT after bleaching did not change in (H). This indicates that no FRET takes place between two proteins. (Scale bar - 3 µm and images are pseudocolored)

Similarly, no FRET signal was captured when Cx36-EYFP-ΔCLB and α-CaMKII-ECFP-WT co-expressing cells were exposed to control solution without Ca^{2+}/ionomycin (experiment was performed 4 times in triplicate, n=12) (Figure 15). After photobleaching of Cx36-EYFP-ΔCLB (Figure 15B), there is no increase in the intensity of α-CaMKII-ECFP-WT (Figure 15F). These results indicate that Cx36-EYFP-ΔCLB and CaMKII-ECFP-WT proteins are not in close proximity and FRET does not take place between the two proteins in the presence or absence of Ca^{2+}. The only justifiable interpretation of these data is that CLB is essential for CaMKII and seems to dominate CTB binding.
Figure 14: Display of Cx36-EYFP-ΔCLB and α-CaMKII-ECFP-WT in N2A cells stimulated with solution contains no Ca^{2+}/ionomycin, before and after acceptor photobleaching.

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-ΔCLB (acceptor) protein also assembles GJ plaques between two adjacent cells (B: white arrow), while α-CaMKII-ECFP-WT (donor) protein is distributed in cytoplasm (A). In this experiment Cx36-EYFP-ΔCLB (B) was bleached with 514 nm laser line and becomes invisible in (F). (C) and (G) show the merged images of Cx36-EYFP-ΔCLB and α-CaMKII-ECFP-WT before and after acceptor photobleaching, respectively. (D) and (H) show the detailed view of the intensity of α-CaMKII-ECFP-WT fluorescence. (H) depicts the α-CaMKII-ECFP-WT expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-ΔCLB. The intensity of α-CaMKII-ECFP-WT after bleaching did not change in (H) indicating that no FRET takes place between both proteins. (Scale bar = 3 µm and images are pseudocolored)

Quantification of FRET efficiency of Cx36-EYFP-ΔCLB and α-CaMKII-ECFP-WT proteins was in the range of 0.06 % (± 0.06) and the distance between the two proteins was calculated to be 16.8 nm (± 1.01) indicating that there is no FRET between α-CaMKII-ECFP-WT and Cx36-EYFP-ΔCLB in GJ plaques.

4.1.4 Autophosphorylation of α-CaMKII does not effect the interaction with Cx36

Admittedly, one of the characteristics that are special for the α-CaMKII enzyme is its autophosphorylation feature. This feature is crucial for its interaction with target receptor proteins (Colbran et al., 1993).

Pull down assays showed that autophosphorylation of α-CaMKII was less effective at the cytoplasmic loop site (CLB) of Cx36 in terms of binding and phosphorylation in contrast to the carboxy-terminal segment, which exhibited higher efficacy toward both effects (Alev et al., 2008).

In the following chapter, the attention is focused on whether the autophosphorylation of α-CaMKII effects the interaction of the enzyme with Cx36. For these investigations, two α-CaMKII mutants were
generated. The first mutant is a constitutively active α-CaMKII form (α-CaMKII-T286D) where threonine in position 286 is substituted by an aspartate. Therefore, the kinase mimics auto-phosphorylation and renders constitutively active.

FRET protocols were applied to N2A cells, which were co-transfected with Cx36-EYFP-WT and α-CaMKII-T286D. Since the autonomous activity of α-CaMKII-ECFP-T286D is known to be Ca\(^{2+}\)-independent, α-CaMKII-ECFP-T286D does not require to be stimulated with Ca\(^{2+}/\)ionomycin. However, all cells were treated equally and N2A cells were exposed to both Ca\(^{2+}/\)ionomycin and control solutions (Figure 16 and Figure 17).

![Figure 15: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-T286D in Ca\(^{2+}/\)ionomycin stimulated N2A cells, before and after acceptor photobleaching.](image)

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-WT (acceptor) protein also assembles to GJ plaques between two adjacent cells (B: white arrow), while α-CaMKII-ECFP-T286D (donor) protein is distributed in cytoplasm and assembles at GJ plaque region. The intensity of α-CaMKII-ECFP-T286D after bleaching did not change in (H), and this indicates that no FRET takes place between domains (A). After Cx36-EYFP-WT (B) is bleached with the 514 nm laser and becomes invisible in (F), (C) and (G) show the merged images of Cx36-EYFP-WT and α-CaMKII-ECFP-T286D before and after acceptor photobleaching, respectively. (D) and (H) show the detailed view of intensity of α-CaMKII-ECFP-T286D fluorescence. (H) depicts the α-CaMKII-ECFP-T286D expression in the two proteins. (Scale bar = 3 µm and images are pseudocolored).

Like α-CaMKII-ECFP-WT the T286D mutant is evenly distributed in the cytoplasm (Figure 16A and Figure 16E) and assembles at sites of GJ domains. Arrow in 16B depicts the Cx36-EYFP-WT before photobleaching. After Cx36-EYFP-WT is bleached (Figure 16F), there is no increase in the intensity of α-CaMKII-ECFP-T286D (Figure 16H). This is demonstrated in Figure 16H, where α-CaMKII-ECFP-T286D expression is magnified and shown in rainbow display (experiment was performed 4 times in triplicate, n=12).
Co-transfected α-CaMKII-ECFP-T286D and Cx36-EYFP-WT N2A cells (Figure 17) were also exposed to control solution, which lacked Ca$^{2+}$/ionomycin (experiment was performed 4 times in triplicate, n=12). Analysis in these cells showed that no change in FRET efficiency after acceptor photobleaching occurs. This allows two interpretations: either no interaction takes place between α-CaMKII-ECFP-T286A and Cx36-EYFP-WT proteins or the highly phosphorylated form shows already saturation at baseline conditions, making a change in FRET efficiency after Ca$^{2+}$/ionomycin impossible.
Figure 16: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-T286D in N2A cells stimulated with solution containing no Ca\(^{2+}\)/ionomycin, before and after acceptor photobleaching.

The figure depicts acceptor and donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-WT (acceptor) protein also assembles into GJ plaques between two adjacent cells (B: white arrow). This experiment Cx36-EYFP-WT (B) is bleached with 514 nm laser line and is not visible anymore in (F). (C) and (G) show the merged images of Cx36-EYFP-WT and α-CaMKII-ECFP-T286D before and after acceptor photobleaching, respectively. (D) and (H) show the detailed view of the intensity of α-CaMKII-ECFP-T286D fluorescence. (H) depicts the α-CaMKII-ECFP-T286D expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-WT. The intensity of α-CaMKII-ECFP-T286D after bleaching did not change (H), indicating that no FRET takes place between the two proteins. (Scale bar = 3 µm and images are pseudocolored).

Quantification of FRET efficiency of Cx36-EYFP-WT and α-CaMKII-ECFP-T286D proteins was in the range of 0.15 % (± 0.01) and the distance between the two proteins was calculated to be 14.4 nm (± 1.01), indicating that there is no FRET between α-CaMKII-ECFP-T286D and Cx36-EYFP-WT in GJ plaques.

The second α-CaMKII mutant endows an inactive kinase (α-CaMKII-ECFP-T286A), where threonine 286 is exchanged with alanine and the kinase is lacking autophosphorylation. In the subsequent experiments N2A cells were co-transfected with α-CaMKII-ECFP-T286A and the Cx36-EYFP-WT constructs and were subjected to FRET experiments (experiment was performed 4 times in triplicate, n=12).

The α-CaMKII-ECFP-T286A mutant is also distributed in the cytoplasm like α-CaMKII-ECFP-WT protein (Figure 18A and Figure 18E). The results show that after the bleaching of Cx36-EYFP-WT (Figure 18B and Figure 18F), in the regions of GJ plaques there is no increase in the intensity of α-CaMKII-ECFP-T286A (Figure 18H). This result excludes FRET between α-CaMKII-ECFP-T286A and Cx36-EYFP-WT proteins after Ca\(^{2+}\)/ionomycin stimulation.
Figure 17: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-T286A in Ca\(^{2+}\)/ionomycin stimulated N2A cells before and after acceptor photobleaching.

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In N2A cells, Cx36-EYFP-WT (acceptor) protein also assembles to GJ domains between two adjacent cells (B: white arrow), while α-CaMKII-ECFP-T286A (donor) protein is distributed in cytoplasm (A). In this experiment Cx36-EYFP-WT (B) is bleached with 514 nm laser and not visible (F). (C) and (G) show the merged images of Cx36-EYFP-WT and α-CaMKII-ECFP-T286A before and after acceptor photobleaching respectively. (D) and (H) show the detailed view of the intensity of α-CaMKII-ECFP-T286A fluorescence. (H) depicts the α-CaMKII-ECFP-T286A expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-WT. The intensity of α-CaMKII-ECFP-T286A after bleaching did not change in (H), indicative for a lack of FRET between the two proteins. (Scale bar = 3 µm and images are pseudocolored).

Similar results were obtained in cells, which were subjected to control solution (Figure 19D and Figure 19H), when Cx36-EYFP-WT and α-CaMKII-ECFP-T286A were co-transfected and exposed to the control solution that lacks Ca\(^{2+}\)/ionomycin. No FRET takes place after bleaching of Cx36-EYFP-WT (Figure 19B and Figure 19H).
Figure 18: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-T286A in N2A cells stimulated with solution contains no Ca\(^{2+}\)/ionomycin, before and after acceptor photobleaching.

The figure shows the acceptor and the donor proteins before (A-B-C-D) and after photobleaching (E-F-G-H). In this experiment Cx36-EYFP-WT (B) is bleached with the 514 nm laser and is not visible anymore (F). (C) and (G) show the merged images of Cx36-EYFP-WT and α-CaMKII-ECFP-T286A before and after acceptor photobleaching. (D) and (H) show a detailed view of the intensity of α-CaMKII-ECFP-T286A fluorescence. (H) depicts the α-CaMKII-ECFP-T286A expression in the GJ plaque region in rainbow scale after photobleaching of Cx36-EYFP-WT. The intensity of α-CaMKII-ECFP-T286A after bleaching did not change in (H), this indicates that no FRET takes place between the two proteins. (Scale bar = 3 µm and images are pseudocolored).

Quantification of FRET efficiency of Cx36-EYFP-WT and α-CaMKII-ECFP-T286A proteins was in the range of 0.25 % (± 0.03) and the distance between the two proteins was calculated to be 13.3 nm (± 1.23), indicating that there is no FRET between α-CaMKII-ECFP-T286A and Cx36-EYFP-WT in GJ plaques.

4.2 Translocation of α-CaMKII to Cx36 expressing gap junction plaques

Recent improvements in molecular, structural and electrophysiological techniques allow direct monitoring of activity-dependent changes of CaMKII in chemical synapses. Treatment of hippocampal neurons with NMDA or glutamate/glycine (glu/gly) causes the translocation of the kinase to PSDs along the dendrites via Ca\(^{2+}\) mediated mechanism (Shen and Meyer et al., 1999). The translocation of the kinase has been postulated to be important for the regulations of the synaptic functions. In contrast, very little is known about the dynamics of the CaMKII in electrical synapses.

Not only the FRET data presented in this thesis but also co-localization of α-CaMKII and Cx36 (Alev et al., 2008) in the inferior olive and co-localization/association of CaMKII with the fish homolog connexin35 at mixed synapses (Flores et al., 2010) provided evidence that electrical synapses constitute an assembly of proteins where connexins dynamically interact with CaMKII.
To further uncover these dynamic events we focused on the following questions:

i) Can translocation of α-CaMKII in regions of GJ plaques be initiated by Ca\(^{2+}\)/ionomycin treatment?

ii) Do the binding sites of Cx36, contribute to the translocation and the accumulation of α-CaMKII after Ca\(^{2+}\)/ionomycin treatment?

To provide answers to these questions N2A cells were co-transfected with different α-CaMKII and Cx36-EYFP constructs and subjected to live cell imaging. In each experiment an initial 400 sec of baseline recordings were followed by Ca\(^{2+}\)/ionomycin application. A subsequent 30 min recording was performed to evaluate the influence of Ca\(^{2+}\)/ionomycin.

In Figure 20A, a line graph belonging to the ratio of α-CaMKII-ECFP-WT fluorescence to Cx36-EYFP-WT fluorescence is indicated. Black arrows in the graph depict the time point where the Ca\(^{2+}\)/ionomycin (2 mM/10 µM) solution was applied at t = 400 sec. The ratio of α-CaMKII-ECFP-WT fluorescence intensity to Cx36-EYFP-WT fluorescence intensity is 1.11 (± 0.034) at t = 400. Around mid-term the ratio is 1.07 (± 0.039) and approaches a maximum 1.47 around t = 1750 sec. At the end of the recording (at t = 1800 sec) the ratio of α-CaMKII-ECFP-WT fluorescence intensity to Cx36-EYFP-WT fluorescence intensity is 1.41 (± 0.051).

From a functional point of view, it is important to note that after the stimulation solution is applied the accumulation of α-CaMKII-ECFP-WT in GJ plaques shows a lag of about 600 sec before the translocation becomes visible. Obviously, the initial Ca\(^{2+}\)/ionomycin stimulus triggers a time consuming activation mechanism that needs to be initiated before translocation of α-CaMKII to the Cx36 target-sites happens.

The ratio of fluorescence intensities fluctuates during t = 400-500 sec and t = 1100-1200 sec intervals but overall, the increase after stimulation is significantly higher than the values obtained during baseline recording throughout the first 400 sec. (Experiment was performed 5 times in triplicate, n=15).

To further understand the importance of the Cx36 binding sites for the translocation of α-CaMKII-ECFP-WT, the above binding site mutants of Cx36 were exploited.
Figure 19: Display of different graphs, which show the ratio of $\alpha$-CaMKII-ECFP-WT to Cx36-EYFP in N2A cells co-transfected with $\alpha$-CaMKII-ECFP-WT and different Cx36-EYFP constructs.

The black arrow in all graphs indicates $t = 400$, the time point when $\text{Ca}^{2+}$/ionomycin (2 mM/10 µM) is added. The red boxes depict the time points $t = 900$ and $t = 1800$, respectively. (A) Time course of the ratio of $\alpha$-CaMKII-ECFP-WT to Cx36-EYFP-WT. (B) Time course of the ratio of $\alpha$-CaMKII-ECFP-WT to Cx36-EYFP-∆CLB with persistence of CTB. There is a minimal but not significant increase of the ratio. (C) Time course of the ratio of $\alpha$-CaMKII-ECFP-WT to Cx36-EYFP-∆CLB-∆CTB, with almost complete loss of translocation. (D) Time course of the ratio of $\alpha$-CaMKII-ECFP-WT to Cx36-EYFP-∆CTB. Partial translocation is still preserved.

As illustrated in Figure 20B, when N2A cells are co-transfected with $\alpha$-CaMKII-ECFP-WT and the Cx36-EYFP-∆CLB mutant, the ECFP fluorescence reveals a drastic reduction as compared to the WT probe (Figure 20A) with only a minimal increase after application of $\text{Ca}^{2+}$/ionomycin, indicating that CLB binding site of Cx36 is also important for the accumulation of $\alpha$-CaMKII protein in the GJ plaques (experiment was performed 4 times in triplicate, n=12).

The effect of $\text{Ca}^{2+}$/ionomycin treatment on $\alpha$-CaMKII-ECFP translocation approaches almost zero levels when both binding sites are deleted (Figure 20C). Deletion of CTB alone (Figure 20D) shows residual translocation (experiment was performed 4 times in triplicate, n=12). Like in the previous experiments, $\text{Ca}^{2+}$/ionomycin was applied at $t = 400$ sec and then the ratio of $\alpha$-CaMKII-ECFP-WT intensity to Cx36-EYFP-∆CTB was recorded during over time. Analysis shows that the ratio of $\alpha$-CaMKII-ECFP-WT intensity to Cx36-EYFP-∆CTB intensity is $1.042 \pm 0.047$ at $t = 400$ sec, where it increases to $1.11 \pm 0.050$ at $t = 900$ sec and reaches $1.248 \pm 0.042$ at $t = 1800$ sec (Figure 20A). These values demonstrate that the ratio of $\alpha$-CaMKII-ECFP-WT intensity to Cx36-EYFP-∆CTB intensity increased by 1.2 folds in a time frame of 1800 sec, which is consistent with the FRET data that showed persistent fluorescence transfer after deletion of the CTB binding site.

Due to the results of the FRET experiments, it was reasonable to assume that the deletion of both CLB and CTB sites would prevent the increase of the $\alpha$-CaMKII fluorescence in the GJ expressing plaques. To test this hypothesis, N2A cells were co-transfected with $\alpha$-CaMKII-ECFP-WT and Cx36-EYFP-∆CLB-
∆CTB plasmids. The ratio of α-CaMKII-ECFP-WT intensity to Cx36-EYFP-ΔCLB-ΔCTB intensity is 0.967 ± 0.009 at t = 400 sec, where this ratio increases to 1.013 ± 0.039 at t = 900 sec and reaches 1.040 ± 0.051 at t = 1800 sec (Figure 20C) (experiment was performed 5 times in duplicate, n=15).

In line with the FRET data, results of the live cell imaging experiments demonstrate that CLB and CTB sites of Cx36 are targets for α-CaMKII binding, which help the kinase to accumulate in plasmalemmal GJ domains after Ca\(^{2+}\)/ionomycin (2 mM/10 µM) stimulation. Deletion of both binding sites leads to a complete loss of translocation. Similarly, deletion of CLB, with persistence of CTB, also provokes an almost significant reduction of kinase mobility. Instead, CTB deletion is less effective.

Results in this chapter indicate that there is a possible regulation and activity in the Cx36 expressing GJ plaques, such as translocation of α-CaMKII to Cx36 expressing sites after Ca\(^{2+}\)/ionomycin treatment. It is shown that α-CaMKII translocates to Cx36 expressing sites in living N2A cells and that the absence of binding sides can effect the accumulation of α-CaMKII in the GJ regions. Combined with the FRET data these results strongly suggest that Cx36 is a substrate for CaMKII in living N2A cells and this can play a role in tuning the electrical synapse activity.

4.2.1 Is autophosphorylation of α-CaMKII a key event for the translocation of the kinase to Cx36 expressing GJ domains?

Autophosphorylation of α-CaMKII produces a form of the enzyme not requiring Ca\(^{2+}\)/calmodulin for sustained activity. In the present study, experimental support was provided to understand the relation between the autophosphorylated form of α-CaMKII-ECFP-WT and Cx36-EYFP-WT containing GJ plaques in living N2A cells.

Again, N2A cells were subjected to live cell imaging 48 h after co-transfection with the autophosphorylated α-CaMKII mutant (α-CaMKII-ECFP-T286D) and Cx36-EYFP-WT constructs.

In each experiment an initial 400 sec of baseline recordings were followed by injection of Ca\(^{2+}\)/ionomycin. A subsequent 1350 sec recording was performed to evaluate the influence of the applied Ca\(^{2+}\)/ionomycin. The stimulation solution was applied 400 sec after starting the experiment. As indicated in the previous chapter, during the imaging process the regions of interest were the Cx36-EYFP expressing plasma membrane domains. During the recording, the change in the distribution of α-CaMKII-ECFP-T286D was calculated.

As it is demonstrated in Figure 21A, the line graph shows the ratio of α-CaMKII-ECFP-T286D fluorescence to Cx36-EYFP-WT fluorescence in membrane associated GJ domains during the time. The black arrow in the graph indicates the application of Ca\(^{2+}\)/ionomycin (2 mM/10 µM) at t = 400 sec to α-CaMKII-ECFP-T286D and Cx36-EYFP-WT co-transfected N2A cells. Although the autonomous
activity of α-CaMKII-ECFP-T286D is known to be Ca\(^{2+}\)-independent, addition of stimulation solution initiates the translocation of the kinase. The ratio of α-CaMKII-ECFP-T286D intensity to Cx36-Cx36-EYFP-WT intensity is 0.949 (± 0.089) at t = 400 sec, while this ratio increases to 1.076 (± 0.087) at t = 900 sec and reaches 1.237 (± 0.097) at t = 1800 sec, resulting in a 1.3 fold increase in a time interval of 1800 sec. Taken together, these results demonstrate that in living N2A cells T286 autophosphorylation has a strong positive-regulatory effect on Ca\(^{2+}\)/ionomycin induced CaMKII translocation to Cx36 expressing GJ plaques (experiment was performed 5 times in triplicate, n=15).

In the next step, the aim was to show whether CaMKII activity would occur in the absence of T286 phosphorylation. For this α-CaMKII-ECFP-T286A and Cx36-EYFP-WT constructs were used to transfect the N2A cells. The α-CaMKII-ECFP-T286A mutant is unable to become phosphorylated due to the alanine residue in its structure. In Figure 21B the ratio of the intensity of α-CaMKII-ECFP-T286A to Cx36-EYFP-WT is illustrated. The ratio of intensity is 1.090 (± 0.046) at t = 400 sec, while it increases to 1.102 (± 0.076) at t = 900 sec and then reaches to 1.168 (± 0.084) at t = 1800 sec. (experiment was performed 7 times in triplicate, n=21).

Figure 20: Display of different graphs, which show the ratio of α-CaMKII-ECFP-WT to Cx36-EYFP in N2A cells that co-transfected with different α-CaMKII-ECFP-WT plasmids and Cx36-EYFP-WT.

The black arrow in all graphs indicates t = 400, the time point when Ca\(^{2+}\)/ionomycin (2 mM/10 µM) is added. The red boxes depict the time points t = 900 and t = 1800, respectively. (A) Time course of the ratio of α-CaMKII-ECFP-T286D to Cx36-EYFP-WT in α-CaMKII-ECFP-T286D and Cx36-EYFP-WT transfected N2A cells. (B) Time course of the ratio of α-CaMKII-ECFP-T286A to Cx36-EYFP-WT in α-CaMKII-ECFP-T286A and Cx36-EYFP-WT transfected N2A cells.

4.2.2 Accumulation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT expressing plaques can be blocked by CaM-KIIN and KN-93

Protein–protein interaction requires physical association of the interacting partners and this physical association can be stopped by the addition of blockers.

KN-93 is a membrane-permanent inhibitor of CaMKII and blocks the Ca\(^{2+}\) dependent activity of the enzyme by interfering with calmodulin binding sites. CaM-KIIN is a mimic peptide that binds to the α-CaMKII T-site and interferes with CaMKII binding to the NMDA-type glutamate receptor subunit.
NR2B, which is highly similar to a consensus sequence in the Cx36-CL binding site (Bayer et al., 2001; Bayer et al., 2006).

Figure 22A shows the recording of α-CaMKII-ECFP-WT and Cx36-EYFP-WT transfected N2A cells, which were incubated with 1 µM CaM-KIIN peptide for 10 min prior to imaging. Black arrows indicate the application of Ca²⁺/ionomycin (2 mM/10 µM) to the cells. The ratio remains constant over the entire recording, indicating that effective blocking of the CaM-KIIN exerts a robust blocking of translocation/accumulation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT positive GJ domains.

![Figure 22A](image)

Figure 21: Display of different graphs, which show the ratio of α-CaMKII-ECFP-WT to Cx36-EYFP under different drug applications in N2A cells that co-transfected with α-CaMKII-ECFP-WT and Cx36-EYFP-WT.

The black arrow in all graphs indicates t = 400, the time point when Ca²⁺/ionomycin (2 mM/10 µM) is added. The red boxes depict the time points t = 900 and t = 1800, respectively. (A) Time course of the ratio of α-CaMKII-ECFP-WT to Cx36-EYFP-WT under CaM-KIIN peptide application in α-CaMKII-ECFP-WT and Cx36-EYFP-WT expressing N2A cells. (B) Time course of the ratio of α-CaMKII-ECFP-WT to Cx36-EYFP-WT under KN-93 application in α-CaMKII-ECFP-WT and Cx36-EYFP-WT expressing N2A cells.

In Figure 22B, the graph, belongs to recordings from α-CaMKII-ECFP-WT and Cx36-EYFP-WT transfected N2A cells, which were treated with 10 µM KN-93 for 10 min prior to imaging. The ratio of α-CaMKII-ECFP-WT intensity to Cx36-EYFP-WT intensity is 0.974 (± 0.04) at t = 400 sec. This ratio is equal to 0.987 (± 0.03) at t = 900 sec. At the end of the recording, at t = 1800 sec, the ratio of α-CaMKII-ECFP-WT intensity to Cx36-EYFP-WT intensity is equal to 0.956 (± 0.03). The stable ratio of α-CaMKII-ECFP-WT intensity to Cx36-EYFP-WT intensity during time course indicates that 10 µM KN-93 application affected the translocation/accumulation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT expressing sites. The ratio of α-CaMKII-ECFP-WT intensity to Cx36-EYFP-WT intensity almost stayed the same in a time interval of 1800 sec. The following conclusions can be drawn from the above data:

CaMK-IIIN peptide binds to the CaMKII T-site and it interferes with accumulation of α-CaMKII-ECFP-WT in the Cx36-EYFP-WT expressing GJ plaques after Ca²⁺/ionomycin stimulation. Not only CaMK-IIIN but also KN-93, which is a less specific CaMKII inhibitor blocks the translocation of α-CaMKII.

[Both experiments were performed 3 times in triplicate, n=9].
4.3 Translocation of Cx36-EYFP-WT to α-CaMKII-ECFP-WT expressing sites in neurons

There are remarkable mechanistic parallels between the long-term potentiation of mammalian glutamatergic synapses and those operating in electrical synapses of the goldfish (Pereda et. al, 1995).

In chemical synapses redistribution of α-CaMKII appears to be a dynamic process that has been visualized in cultured hippocampal neurons and brain slices stimulated with glutamate/glycine (glu/gly) or electrical stimulation (Shen and Meyer et al., 1999; Dosemeci et al., 2001; Shen et al., 2000, Thalhammer et al., 2006). Subsequent studies indicated that redistribution of α-CaMKII in post synaptic densities effect the LTP and hippocampal-dependent learning (Elgersma et al., 2002).

So far, no data are available in living mammalian neurons, which show α-CaMKII distribution at sites of electrical synapses. In this chapter, the aim was to assess the translocational behavior of α-CaMKII at sites of Cx36-ECFP transfected primary neurons. Double transfected primary hippocampal neurons were taken to live cell imaging and cells were subjected to different stimulation paradigms under real time imaging conditions.

4.3.1 Translocation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT expressing sites in co-transfected neurons after chemical stimulation

Primary hippocampal neurons were obtained from P0-P1 Spraque Dawley rats and transfected with Cx36-EYFP-WT and α-CaMKII-ECFP-WT plasmids on DIV 11. α-CaMKII-ECFP-WT (Figure 23A and Figure 24A) and Cx36-EYFP-WT (Figure 23B and Figure 24B) expressing primary hippocampal neurons from wild-type animals developed normal morphologies with no evidence of differences in shape as compared to untreated neurons. The most significant difference in respect to N2A cells is that co-transfected hippocampal neurons do not form typical GJ plaques. They exhibit mostly vesicular structures of different sizes instead, which sometimes revealed close membrane association.

For time-lapse imaging cultures were taken to confocal microscopy and images were acquired every 30-45 sec before and after glu/gly (100 µM/10 µM) stimulation for 20-30 min (Figure 23 and Figure 24).

Images were obtained before (Figure 23D) and after (Figure 23E) glu/gly (100 µM/10 µM) application. Figure 23C shows a double transfected neuron that co-expresses α-CaMKII-ECFP-WT (Figure 23A) and Cx36-EYFP-WT proteins (Figure 23B) and the white box in Figure 23C depicts the region of
interest. Figure 23D and Figure 23E show a detailed view of the identical neuronal process before and after stimulation.

This experiment shows that in co-transfected primary hippocampal neurons, α-CaMKII-ECFP-WT protein translocates to Cx36-EYFP-WT expressing sites shortly after glu/gly (100 µM/10 µM) application at t = 100 sec (Figure 23E). White arrows in Figure 23E depict pearl-like formation of α-CaMKII-ECFP-WT in processes of neurons after glu/gly (100 µM/10 µM) application (t = 100 sec). Although a definite redistribution and accumulation of α-CaMKII-ECFP to Cx36-EYFP expression sites is evident after exposure of the neurons for 2 min to the glu/gly solution, we can not exclude that toxic effects are causal for the α-CaMKII-ECFP clustering. Such blebbing of neurons after glu/gly treatment has been frequently described (Froissard et al., 1994) and acclaimed to be a sign of excitotoxicity. Lowering of the glu/gly concentration to 50 µM did not have any effect on the distribution of the α-CaMKII-ECFP (Figure 24D and Figure 24E) (experiment was performed 4 times in duplicates, n=8).

Figure 22: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-WT expressing primary hippocampal neuron at DIV 13 before and after glu/gly (100 µM/10 µM) stimulation.

In neurons, α-CaMKII-ECFP-WT protein is distributed in the cytoplasm of the soma, dendrites and axons (A) while the Cx36-EYFP-WT protein disperses in form of vesicular structures (B). (C) shows the merged green and red channels; α-CaMKII-ECFP-WT and Cx36-EYFP-WT proteins and the white box in (C) indicates the region of interest. (D) presents the region of interest at higher magnification before the glu/gly (100 µM/10 µM) stimulation. Here, arrows show how α-CaMKII-ECFP-WT is distributed evenly in the process of the neuron. (E) presents the region of interest at higher magnification 2 minutes after glu/gly (100 µM/10 µM) application. Arrows in (E) depict how glu/gly application changes the distribution of α-CaMKII-ECFP-WT and causes pearl-like structures in the processes of the neurons. (Scale bar - 15 µm)
Although the glu/gly stimulation paradigm was identical to that previously described in several studies (Bayer et al., 2001, Shen et al., 1999), we render this phenomenon as critical. One reasonable explanation is that transfection and overexpression of both transgenes make the neurons more susceptible to glu/gly treatment, when a certain threshold is achieved.

Figure 23: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-WT expressing primary hippocampal neuron at DIV 13 before and after low glu/gly (50 µM/10 µM) stimulation.

4.3.2 Translocation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT expressing sites in co-transfected neurons after electrical stimulation

Electrical stimulation has also been used to elicit activity-dependent changes in transfected hippocampal neurons (Pettit, D. L. et al., 1994, Lledo, P. M. et al. 1995, Meyer et al., 2002). Meyer and coworkers (2002) reported that stimulation of hippocampal neurons with a series of electrical field pulses (10–100 Hz for one second) triggers transient translocations of GFP-CaMKII from the cytosol to
postsynaptic sites. However, the stimulus needed for maximal translocation can be variable with individual neurons, but typically requires a sequence between 20 and 50 field pulses.

Therefore a similar protocol was applied (see Chapter 3.8 under Material and Methods) on hippocampal neurons co-transfected with Cx36-EYFP-WT and α-CaMKII-ECFP-WT constructs. On DIV 13, primary hippocampal neuron cultures were transferred to confocal microscopy for time-lapse imaging and cells were visualized before and after electrical stimulation. Electrical stimuli were generated with a pulse generator and trains of 0.5 ms square pulses were delivered between 90 Hz using constant current mode (60 µA currents) through glass micropipettes that were placed near the imaged neuron.

Figure 25 displays Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-expressing primary hippocampal neurons at DIV13 before (Figure 25A and Figure 25D) and after electrical stimulation (Figure 25E). The electrical stimulus also caused α-CaMKII-ECFP-WT to form bulky structures in neuronal processes, which looked identical to those discovered after the glu/gly treatment.

In the next step, alternative electrical stimulation paradigms were used to avoid this effect. Electrical stimuli were generated with a pulse generator and trains of 0.5 ms square pulses were delivered between 10-100 Hz, using 10-70 µA currents. However, translocation of α-CaMKII-ECFP-WT was not successful under low current conditions and blebbing of neurons appeared in currents higher than 60 µA. Apparently, the neurons responded to electrical stimulation in a similar way as it was indicated in the glu/gly treatment. In all likelihood, we are facing a similar phenomenon and must render the double transfection with CaMKII-ECFP and Cx36-EYFP inappropriate for this kind of study. One rational for this phenomenon is the fact that double transfection may change the type of synaptic assemblies in a way that provokes increased susceptibility. As it will be shown in Chapter 4.4, mixed synapses are predominantly formed under the double transfection conditions and could be causal for the increased excitotoxicity after forced depolarization.
4 Results

Figure 24: Display of Cx36-EYFP-WT and α-CaMKII-ECFP-WT expressing primary hippocampal neuron at DIV 13 before and after electrical stimulation.

(A) α-CaMKII-ECFP-WT protein is distributed in the cytoplasm in the soma, and processes of the neurons, while the Cx36-EYFP-WT protein disperses in form of spots (B). (C) shows the merged green and red channels; α-CaMKII-ECFP-WT and Cx36-EYFP-WT proteins and the white box in (C) indicates the region of interest. (D) presents the region of interest at higher magnification before the electrical stimulation. Here, arrows show how α-CaMKII-ECFP-WT is distributed evenly in the process of the neuron. However, (E) presents the region of interest at higher magnification after electrical stimulation. The accumulation of α-CaMKII-ECFP-WT in the processes in (E) was interpreted as excitotoxicity. (Scale bar = 15 µm)

4.4 Assessment of the subcellular localization of Cx36-EYFP-WT

4.4.1 Cx36-EYFP-WT reveals close apposition to synaptophysin

Previous imaging studies from several groups agreed that α-CaMKII is associated with postsynaptic densities (PSDs) in primary hippocampal neurons and is enriched in PSD fractions after glutamate or electrical stimulation (Shen et al., 1999, Thalhammer et al., 2006). Therefore, the results described in the previous chapter raised the question concerning the exact localization of Cx36-EYFP-WT. In particular, the predominant occurrence of Cx36-EYFP-WT in form of vesicular structure might be indicative for a lack of intramembrane non-synaptic localization of the transcript.
To check whether Cx36-EYFP-WT localizes at sites of chemical synapses forming mixed synapses or occurs in form of pure electrical synapses, Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected primary hippocampal neurons were taken to immunofluorescence staining using anti-synaptophysin immunostaining as a presynaptic marker.

Quantification of synaptophysin positive sites showed that many synapses were present in the Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected neurons. Since synaptophysin positive sites represent presynaptic compartments, chemical and mixed synapses were determined by quantifying immunostaining of presynaptic synaptophysin-positive boutons and their association with or without Cx36-EYFP-WT labeling. Co-localization of synaptophysin and Cx36-EYFP-WT provided information on the number of the mixed synaptic type, which includes components of chemical and electrical synapses. Furthermore, only Cx36-EYFP-WT expressing sites indicated pure electrical synapses, and exclusive synaptophysin staining accounted for pure chemical synapses. Quantification did, of course, only enclose those neurons, which revealed adequate double transfection and appropriate synaptophysin staining.

The following distribution pattern of the different synaptic types was achieved:

i) 45 ± 3.4 % of the synapses revealed co-localization with fluorescence signals from Cx36-EYFP-WT and the presynaptic marker synaptophysin (Figure 27 indicating that Cx36-EYFP-WT protein mostly resides at mixed synapses (experiment was performed 3 times in triplicate, n=9).

ii) Apart from the co-localization of the two proteins, in 25 ± 4.4 % of the synapses, Cx36-EYFP-WT and synaptophysin proteins stand side by side where the synaptophysin indicates the presynaptic site and Cx36 in a pre-and postsynaptic position (experiment was performed 3 times in triplicate, n=9) (Figure 26). This side-by-side effect is presumably related to stereological reasons offering en face views of the mixed synapses assemblies. This kind of arrangement again indicates that a high number of Cx36-EYFP-WT is a component of mixed synapses.

iii) In 15 ± 6 % of the synapses Cx36-EYFP-WT protein did not co-localize with synaptophysin, suggesting that Cx36-EYFP-WT protein is part of pure electrical synapses (Figure 27).

iv) Only synaptophysin positive synapses occupied 10 ± 1.8 % of the total amount of stained synapses.

v) In addition, the double transfection and synaptophysin staining also allowed quantifying the number of Cx36-EYFP-WT positive with apposed α-CaMKII-ECFP-WT labeling.
particular, in the more peripheral parts of the dendrites, where the expression of α-CaMKII-ECFP-WT was low enough revealing no diffuse distribution along the entire dendritic stem, almost each Cx36-EYFP-WT plaque was associated with α-CaMKII-ECFP-WT label (≥ 90) from, which 72.4% revealed a symmetric staining on both sides of the Cx36-EYFP-WT plaques, and 17.2% only single-sided stain. 10.3% of the Cx36-EYFP-WT label did not reveal α-CaMKII-ECFP-WT association (Figure 27).

Figure 26 shows a Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-expressing primary hippocampal neuron, which was fixed and incubated with anti-synaptophysin antibodies.

Cx36-EYFP-WT is shown in red in Figure 26B, and it disperses in spot-like structures throughout the processes of the neuron. In Figure 26C, synaptophysin protein is depicted in the blue channel and marks the presynaptic site of neurons. Figure 26D shows the merged of Cx36-EYFP-WT, α-CaMKII-ECFP-WT and synaptophysin fluorescence. White arrows in Figure 26D point the regions of interest where Cx36-EYFP-WT and synaptophysin are localized in a side by side pattern. Figure 26E and Figure 26F are the zoomed views of Figure 26D. In both figures (Figure 26E and Figure 26F), arrows (1-2) the side-by-side localization of synaptophysin and Cx36-EYFP-WT is shown.
Figure 25: Display of synaptophysin staining in Cx36-EYFP-WT and α-CaMKII-ECFP-WT expressing primary hippocampal neurons at DIV 13.

Fluorescence images are illustrating the distribution of synaptophysin in Cx36-EYFP-WT and α-CaMKII-ECFP-WT in co-transfected hippocampal neurons. α-CaMKII-ECFP-WT protein is distributed all around the neuron (A). On the other hand Cx36-EYFP-WT protein is dispersed in a spot-like fashion (B). (C) displays the synaptophysin at presynaptic sites of the neurons. (D) An overlay of signals from Cx36-EYFP-WT, α-CaMKII-ECFP-WT and synaptophysin. The arrow 1 in (E) depicts side by side localization of Cx36 and synaptophysin indicating that Cx36 resides on the postsynaptic site. The arrow in (F) depicts side by side localization of Cx36 and synaptophysin indicating that Cx36 is localized on the postsynaptic site. (Scale bar = 15 µm)

In some of the neurons, instead of site-by-site association, synaptophysin staining and Cx36-EYFP-WT proteins co-localize with each other. Figure 27 demonstrates this phenomenon very well. In Figure 27A, two boxes B and C focus on the two different regions of the neuron. Figure 27 (B1-B2-B3) shows the three different channels of Box B separately (α-CaMKII, Cx36-EYFP-WT and synaptophysin staining) in higher magnification. Figure 27 (C1-C2-C3) show the three different channels of Box C separately (α-CaMKII, Cx36-EYFP-WT and synaptophysin staining) in higher magnification.

As it is shown in Figure 27 (B2 and C2) Cx36-EYFP-WT disperses in form of spot-like structures throughout the peripheral parts of the processes. In Figure 27 (B3 and C3) synaptophysin protein is depicted in blue and marks the presynaptic sites of the processes. Figure 27 (B and C) shows the overlay of Cx36-EYFP-WT, α-CaMKII-ECFP-WT and synaptophysin immunoreactivity.
Figure 26: Display of synaptophysin staining in Cx36-EYFP-WT and α-CaMKII-ECFP-WT expressing primary hippocampal neurons at DIV 13.

Fluorescence images are illustrating the distribution of synaptophysin in Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected hippocampal neurons (A). In (A) two regions of interests are shown in whites boxes: box B and box C. (B) and (C) are the magnified versions of box B and box C. B1, B2 and B3 show the each channel of picture (B) separately; in other words α-CaMKII-ECFP-WT, Cx36-EYFP-WT and synaptophysin. C1, C2 and C3 show the each channel of picture (C) separately. The arrows 1 and 6 depict the only synaptophysin positive boutons, while the arrows 2, 3 and 7 show the Cx36-EYFP-WT protein. The arrows 4 and 5 show the co-localization of Cx36-EYFP-WT and synaptophysin proteins. (Scale bar - 15 µm)

The arrow 1 in Figure 27B and Figure 27(B3) depicts the only synaptophysin immuno-reactive site. The arrows 2 and 3 in Figure 27B and Figure 27(B2) points the only Cx36 proteins. The arrow 4 in Figure 27B and Figure 27(B3) show the purple dot where Cx36-EYFP-WT (red channel) and synaptophysin (blue channel) co-localize and constitute the mixed synapse. Figure 27C is the magnified version of box C, which is shown in Figure 27A. The arrow 5 shows the co-localization of synaptophysin and Cx36-EYFP-WT protein, while arrow 7 shows only the Cx36-EYFP-WT protein. In the same region, the arrow 6 indicates the synaptophysin immunoreactive site.
Taken together, the above data confirms the synaptic localization of Cx36-EYFP-WT in primary hippocampal neurons after transfection. The high amount of close apposition of Cx36-EYFP-WT and synaptophysin strongly suggests that both components occur in the same synaptic compartment (Figure 26, Figure 27) and thus represent mixed synapses rather than pure electrical synapses. Definite clarification, however, of the synaptic type can only be achieved when systematic electron microscopic studies are performed.

4.4.2 Co-localization of Cx36-EYFP-WT with FM4-64

To corroborate the synaptophysin data, we used the dye FM4-64 that is able to embed into the membranes of synaptic vesicles when endocytosed during re-uptake in active synapses. The rational behind this additional approach was to identify the presynaptic sites associated with Cx36-EYFP-WT as active synaptic structures. In Figure 28, presynaptic vesicles of neurons are loaded with FM4-64 by stimulating Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected primary hippocampal neuron culture with incubation buffer containing 50 mM KCl and 15 µM FM4-64. After a washing step the remaining FM4-64 was found to be concentrated within the vesicles (Figure 28C). The results showed that 40 % of FM4-64 stained boutons co-localize with Cx36-EYFP-WT. indicative for co-localization of Cx36-EYFP-WT fluorescence with active presynaptic sites in co-transfected primary hippocampal neurons.
Figure 27: Display of FM4-64 staining in Cx36-EYFP-WT and α-CaMKII-ECFP-WT expressing primary hippocampal neurons at DIV 13.

Fluorescence images illustrating the distribution of FM4-64 stained active presynaptic sites in Cx36-EYFP-WT and α-CaMKII-ECFP-WT co-transfected hippocampal neurons. (A) α-CaMKII-ECFP-WT protein is distributed in the cytoplasm and processes of the neuron. (B) Cx36-EYFP-WT protein (red) is focused in a spot-like pattern. (C) displays the FM4-64 stained (blue) presynaptic boutons. (D) shows an overlay of signals from Cx36-EYFP-WT, α-CaMKII-ECFP-WT and FM4-64 stained structures. The arrow 1 (E, enlarged part of (D)) depicts Cx36-EYFP-WT that does not co-localize with FM4-64 stained presynaptic vesicles. The arrows 2 and 3 in (E, purple) depicts co-localization of Cx36 and FM4-64 stained sites indicating that Cx36 co-localizes with the activated presynaptic site. (Scale bar = 15 µm)
5 Discussion

5.1 Interaction of Cx36 with α-CaMKII: General Overview

For decays, one of the fundamental topics that came to foremost attention of neuroscientists was the issue of how neuronal connections shape brain functions. The key term “synaptic plasticity” was created to refer to changes in strength and in response to either use or disuse of transmission in synaptic pathways.

During the last 60 years scientists showed multidisciplinary effort in search for the cellular and molecular basis of “synaptic plasticity”, which as a main connotation includes the phenomenon of learning and memory. Most of the experimental efforts were related to the concept inaugurated by the Canadian psychologist J. Hebb in 1949. He was one of the first neuroscientists who suggested that synaptic plasticity serves a basis for learning. He quoted the following fundamental argument;

"When an axon in cell A is near enough to excite cell B and repeatedly and persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficacy in firing B is increased"

(Hebb, 1949)

Here, Hebb proposed that a synapse is prone to strengthening its output when its pre- and postsynaptic elements become activated over a certain time.

Today, it is widely accepted that formation and consolidation of memory traces depend on activity-dependent plastic changes that happen after the induction of sustained increase of electrical activity (long-term potentiation [LTP]) or sustained decrease of electrical activity (long-term depression [LTD]). (for reviews see; Cammarota et al., 2002, Daoudal et al. 2003, Lisman et al, 2011, Lisman et al., 2012)

Lately, the molecular mechanisms of LTP generation gained increasing interest. A keystone of these efforts was the discovery that α-CaMKII plays a fundamental in LTP activation in chemical synapses. Today the accepted model is that after generation of LTPs with high frequency stimulation, glutamate is released, which activates the ionotropic NMDAR and allows Ca\(^{2+}\) to enter the postsynaptic compartments. Influx of Ca\(^{2+}\) activates α-CaMKII and the kinase translocates to the PSD to phosphorylate a downstream synaptic targets like AMPAR, nitric oxide synthase (NOS), and SynGAP (a negative regulator of the MAP kinase pathway), to mention a few. These subsequent processes are found to be critical for synaptic plasticity and seem to constitute fundamental principles of memory and learning processes.

Although at the end of the last century the concept of "modifiability of synapses" (Bear et al., 1994,
Bear et al., 1995) was first described in chemically mediated excitatory synapses, scientist found an important model to study the basic mechanisms of electrical and chemical transmission in vertebrates. This model utilized the lateral dendrite of the goldfish Mauthner (M) cell, which is known as large myelinated club endings (club endings) (reviewed in Pereda et al., 2004). Because of its large size, characteristic myelination and dendritic localization and characteristic electrophysiological properties, the club endings are the most recognizable synaptic input to the Mauthner cells. In addition, what makes these cells so special is the dual input of the presynaptic activation with an excitatory (glutamatergic) component at club endings, and an electrically mediated transmission via GJ through connexin 35 (Cx35, the fish homolog of the mammalian Cx36) (Pereda et al., 2003, O’Brien et al., 1998; Condorelli et al., 1998). Remarkably, both types of synapses at these terminals are highly modifiable and can undergo activity-dependent potentiation of both the electrical and chemical components of their postsynaptic response (Yang et al., 1990; Pereda and Faber, 1996; Smith and Pereda, 2003). The potentiation of the club endings can be triggered by brief bursts of afferent activity and they require the activation of NMDAR and as well as α-CaMKII (Pereda et al., 1998).

The above investigations focusing on the club endings revealed that α-CaMKII is not only involved in the regulation of the chemical synapses but also contributes to the efficacy of GJ communication. From this observation it was concluded that the likely targets of α-CaMKII are not only the non-NMDA glutamate receptors of chemical synapses but also the GJ proteins (Pereda et al., 1998).

Although the exact effect of α-CaMKII on all GJ channel proteins is unclear, there are multiple studies, which provide stringent evidence for the association of α-CaMKII and GJ channel proteins. It is assumed that α-CaMKII effects on the Cx channel conductance helps to promote the insertion of Cx channels into the membranes and increases the open probability of the Cx channels. (Pereda et al., 2004)

To illuminate how α-CaMKII mediates the effects mentioned above, we performed a variety of studies to unravel the underlying mechanism. One of the essential discoveries was the identification of multiple α-CaMKII phosphorylation sites of the Cx36 and Cx35 proteins. This was an important finding to understand the mechanistic aspects underpinning the molecular interaction of α-CaMKII and the electrical synaptic protein Cx36 and Cx35 (Alev et al., 2008).

Alev and coworkers (2008) reported that Cx36 contains two juxtamembrane sequences that serve as potential binding sites for CaMKII and the amino acid residues S110 and T111 within the cytoplasmic loop and S315 at the carboxyl-terminal domain as potential phosphorylation residues. Binding and extent of phosphorylation of both cytoplasmic domains vary according to the autophosphorylated state of α-CaMKII and thus resemble behavior of fragments of the NR2B subunits of the NMDAR.

Furthermore, sequence alignment of the Cx36 CTB and CLB sites (Alev et al., 2008) showed that these areas are highly conserved in Cx35, with 86 and 92 % similarity for the CT and CL regions.
Discussion

respectively. O’Brien et al., (2005) reported similar results for Cx34.7, which is the perch Cx36 homolog. Cx34.7 showed 76.2 and 84 % similarity for the CT and CL regions, respectively.

Interestingly, homology studies showed that Cx36, Cx35, and Cx34.7 share identical phosphorylation sites: S315, S298, S300 respectively. An interesting aspect is that all these phosphorylation sites for α-CaMKII are not shared with any other kinases. The general conclusion drawn from this observation is that α-CaMKII association and presumably the regulatory mechanisms are highly conserved, among the phyla and may execute an important impact in the regulation of the activity of neuronal connexins.

All previous studies support the fact that activity-dependent coupling and uncoupling of neurons with GJ channels can be related to Cx36 interaction and phosphorylation by α-CaMKII. However, a striking caveat of this model was the lack of data in proof of a physical interaction of these proteins in vivo or in living cells. To fill this gap, the first aim of this study was to show such an interaction in a heterologous expression system, as a prerequisite for a better understanding of dynamics and plasticity at sites of electrical synapses. Further on, dynamic spatial properties of α-CaMKII in form of translocation events were taken into consideration since the distribution of the kinase was considered to be effective during the activation process of the electrical synapse.

5.1.1 Cx36 and α-CaMKII interact in response to forced Ca\(^{2+}\) influx

To assess the interaction between Cx36 and α-CaMKII the biophysical technique of fluorescence resonance energy transfer (FRET) was utilized. The FRET studies in this thesis embody the physical interaction of α-CaMKII and Cx36 in and outside of the GJ plaques, in the presence or absence of a cytoplasmic Ca\(^{2+}\) load. Ca\(^{2+}\) is known to mediate a variety of cellular processes and its regulation of α-CaMKII activity by the Ca\(^{2+}\)/calmodulin complex has been the subject of numerous studies (for review see: Lisman et al., 2002)

FRET was carried out in α-CaMKII-ECFP-WT and Cx36-EYFP-WT transfected N2A cells, which showed that α-CaMKII-ECFP-WT constitutes an interaction partner for Cx36-EYFP-WT exclusively at sites of GJ domains in the presence of an acute rise in internal Ca\(^{2+}\). No FRET signals could be measured between both proteins in reference points, i.e. outside of the GJ domain.

Similarly, when cells were exposed to control solution lacking Ca\(^{2+}\), no FRET is detected in GJ domains. From these findings, it can be concluded that α-CaMKII-ECFP-WT and Cx36-EYFP-WT interaction at GJ sites depends on the forced rise in internal Ca\(^{2+}\) after external treatment with a Ca\(^{2+}\)/ionomycin solution. These findings are consistent with data described by in vitro analyses, which revealed robust Ca\(^{2+}\) effects on the binding of α-CaMKII to GST-Cx36CL and GST-Cx36CT regions of Cx36 (Alev et al., 2008).
5.1.2 The C-binding region is crucial for the interaction of Cx36-EYFP-WT with α-CaMKII-ECFP-WT

One of the important issues raised within the previous chapter is the understanding of the binding mechanisms and whether the reported binding sites are essential for the interaction of the two proteins. To understand the contribution of potential binding in the interaction of the two proteins, three different Cx36 mutants were generated. These mutants were either lacking both binding sites (∆CLB-∆CTB) or exhibited only single site mutants (∆CTB or ∆CLB).

The results showed that when both binding sites of Cx36 protein are deleted FRET signals were completely abolished, regardless of the presence or absence of Ca\(^{2+}\)/ionomycin treatment. These observations indicate that physical interaction of Cx36-EYFP-WT and α-CaMKII-ECFP-WT proteins requires at least a bulk elevation of free Ca\(^{2+}\). Similarly, the ∆CLB mutant of Cx36 protein, led to a decrease of FRET signals. Therefore, when the CLB site of Cx36 is deleted, the remaining CTB site is not effective in terms of α-CaMKII binding.

The data are in accordance with the recent model proposed by previous findings in our laboratory (Alev et al., 2008). Here, in analogy with the current model of α-CaMKII interaction with the NR2B subunit of the NMDAr it was proposed (Alev et al., 2008) that after initial binding of Ca\(^{2+}\)/CaM to the kinase, which releases the enzyme from autoinhibition, the CLB domain interacts with the pseudotarget site in an un- or low-phosphorylated state. Subsequent, autophosphorylation of α-CaMKII at T286 enables the kinase to further facilitate the binding of the CTB domains of Cx36 to its corresponding substrate recognition motif at the catalytic segment of the kinase.

According to this model, the CLB domain constitutes the initial target, where the kinase docks to the Cx36 protein. This explains why FRET is absent when the CLB site is deleted. In accordance with the dominance of the CLB site for the initial interaction is the observation that deleting of the CTB site (Cx36-EYFP-∆CTB) does not inhibit FRET.

Interestingly, the previous in vitro experiments (Alev et al., 2008) also indicate that binding of CLB to CaMKII is less dependent on autophosphorylation of the kinase in contrast to binding to the CTB site. So far our in vivo data substantiate the model designed from the in vitro data, and acclaim the following sequence of events:

- Binding of Ca\(^{2+}\)/CaM to the kinase
- Opening of the kinase
- Binding of Cx36 at the CLB site
- Activation and autophosphorylation of the enzyme
- Binding of the CTB site at the substrate site of the kinase
Subsequent phosphorylation of Cx36

These observations do not only support the previously proposed model, but are also in agreement with the concept that interaction of α-CaMKII with the neuronal Cx36 follows principles previously described for interaction of CaMKII with the NR2B subunit of the NMDAR. Given the fact that chemical and electrical synapses frequently form mixed synapses, the repertoire of the postsynaptic molecular set up is shared by both synaptic domains. Whether this local association is also responsible for some kind of functional interactivity of the chemical and electrotonic elements of mixed synapses, provides an interesting matter for further exploration.

5.1.3 α-CaMKII mutants directed to the threonine 286 residue do not show increase of FRET after bulk influx of calcium

α-CaMKII undergoes autophosphorylation at threonine 286 (T286), which entails three important functional consequences: Firstly, autophosphorylation at T286 disables the autoinhibitory state of the kinase so that the enzyme becomes autonomous or independent from the Ca²⁺/calmodulin cue.

Second, T286 phosphorylation increases the affinity of the enzyme for Ca²⁺/CaM, almost 1000 times, a mechanism that is called CaM trapping.

Finally, autophosphorylation of T286 exposes a site on the enzyme that allows it’s binding to anchoring proteins, i.e. the NMDARs (Strack and Colbran et al., 1998 and Bayer et al., 2001).

To uncover possible similar mechanisms for CaMKII and Cx36 interaction two α-CaMKII mutants were exploited: (i) the autophosphorylation deficient mutant (α-CaMKII-ECFP-T286A, T286\textsuperscript{A286}) and (i) the constitutively active mutant (α-CaMKII-ECFP-T286D, T286\textsuperscript{D286}).

FRET analysis of double transfected N2A cells showed that FRET efficacy between constitutively active α-CaMKII-ECFP-T286D and Cx36-EYFP does not change after ionoporetic stimulation with Ca²⁺ indicating a lack of interaction.

Our model of a sequential event of α-CaMKII interaction with the Cx36 binding site offers reasonable explanations for this phenomenon. First, since initial binding of α CaMKII to the CLB binding site prefers the non-phosphorylated form of the enzyme, effective binding of Cx36 is not acquired in the highly activated (phosphorylated) form of the kinase. This in turn is a prerequisite for subsequent CTB binding. A further explanation of the lack of interaction of the T286D form of α-CaMKII is that residue T286 is found in a critical position at the so-called pseudotarget site of the kinase, responsible for target binding, i.e. subunits of the NMDAR. Since the pseudotarget site of α-CaMKII contains the binding motif of the CLB site, mutations at this domain may well interfere with binding efficiency of
the kinase to Cx36. Finally, the discovery that phosphatases (PP1 and PP2A, Kohlmann et al., 2009) are involved in terminating kinase activities at Cx36 sites, the constitutive active form of α-CaMKII may interfere with binding efficiencies in α-CaMKII/phosphatase conjugates.

Not surprisingly, the silent form of α-CaMKII (T286A) also lacks FRET, which is to be expected, particularly when the interplay of the CLB and CTB sites in respect to the autophosphorylation state of the kinase is considered.

5.2 Translocation of α-CaMKII to Cx36 expressing sites: a general overview

α-CaMKII is a key player in the plasticity of chemical synapses because of its holoenzyme structure, autoregulatory and mobility properties. (Hudmon and Schulman 2002)

In chemically mediated synapses, LTP is generated by activation of α-CaMKII, with the entry of Ca^{2+} through NMDARs. When autophosphorylation property of α-CaMKII is blocked by a point mutation at T286 LTP is not generated (Lisman et al., 2002). During LTP formation the main targets of α-CaMKII are AMPAR and NMDARs. In case of absence of α-CaMKII (as in GABAergic interneurons lacking CaMKII) (Sik et al., 1998) plasticity of the synapses depend on β-CaMKII (Lamsa et al., 2007).

One of the most obvious features of CaMKII activation through Ca^{2+}/CaM is translocation of the kinase to its target site. Stimulus type and the amount of extracellular Ca^{2+} levels can effect the translocation of the α-CaMKII to the synapses reversely or stably (Bayer et al., 2006; Shen et al., 2000).

To sum up, all these studies in chemical synapses have unraveled the critical role of α-CaMKII translocation and concentration at the sites of chemical synapses emphasizing the principle role of the translocation of α-CaMKII to glutamatergic PSDs for synaptic plasticity.

Data provided in the first part of the thesis indicated that α-CaMKII is one of the interaction partners of electrical synapse protein Cx36. Translocation events happening at the GJ sites would therefore provide additional evidence for co-evolved mechanisms guiding chemical and electrical synaptic transmission as well.

In order to find out and extend this idea, one of the further aims of our experiments was to provide evidence for translocation of α-CaMKII to Cx36 expressing sites after α-CaMKII stimulation. For this purpose, imaging of translocation events of the kinase to Cx36 containing GJ’s was initiated.

To achieve this goal, live cell imaging experiments were performed using confocal microscopy in double-transfected (α-CaMKII and Cx36 constructs) N2A cells and neurons.
5 Discussion

5.2.1 α-CaMKII translocates to Cx36-WT expressing sites in N2A cells

The elaborated data indicate (Chapter 4.6) reliable evidence that N2A cells reveal α-CaMKII-ECFP-WT translocation and accumulation to Cx36-EYFP-WT containing GJ plaques after Ca\(^{2+}\)/ionomycin stimulation and α-CaMKII-ECFP-WT amount increase significantly over time.

It is of particular interest that accumulation of α-CaMKII-ECFP-WT is significantly higher in the Cx36-EYFP-WT containing GJ plaques, as compared to GJ plaques consisting of Cx36 binding-site mutants. The differences in translocation efficiency between the different mutants are obvious. While the double site mutant (ΔCLB-ΔCTB) lacks any increase of α-CaMKII-ECFP the ΔCLB is more efficient than ΔCTB in terms of the translocation effect. These findings support our concept that CL binding site of Cx36 dominates the interaction of the two proteins, and CL binding is crucial for the kinase to dock itself to GJ plaques.

5.2.2 Enzyme activity of α-CaMKII is crucial for the translocation of the kinase to the GJ plaques

Pharmacological inhibitors that specifically target α-CaMKII represent a powerful tool for CaMKII-related research. Specific antagonists can acutely block α-CaMKII activity and help to reveal CaMKII-mediated effects within the cells. We here report that KN93 (water-soluble and cell membrane permeable α-CaMKII inhibitor) and CaM-KIIN (a natural α-CaMKII inhibitor protein, [Vest et al., 2007]) block the translocation of the kinase to the GJ expressing sites.

It is widely acknowledged that KN93 inhibits α-CaMKII by competing with CaM binding to the regulatory domain and prevents the association of CaM with α-CaMKII. This makes the KN93 a valuable inhibitor for the α-CaMKII activity (Sumi et al. 1991), although its specificity has been questioned. To substantiate the KN93 data a second antagonist was used in this study: CaM-KIIN which provides a promising alternative, because it only inhibits α-CaMKII, but not CaMKI, CaMKIV, PKA, or PKC [Chang et al., 1998, 2001].

Results obtained were essentially the same indicating that by inhibiting the CaMKII activity translocation of α-CaMKII-ECFP-WT to Cx36-EYFP-WT at GJ sites is abolished.

These findings are consistent with previous studies, which were reported in the goldfish. It was shown that intradendritic injection of CaM-KIIN and KN93 block the gap junctional and glutamatergic transmission at club endings [Pereda et al., 1998]. Therefore, the results reported in this thesis work explain why the potentiation of both components at the club endings is inhibited.

To sum up, the results described above indicate that for the translocation process both “naïve”
enzyme activity as well as substrate binding sites are crucial. The translocation of the kinase to the GJ protein correlates with the functional and structural integrity of this kinase.

5.3 Identification of expression sites of Cx36-EYFP in neurons

The subcellular distribution pattern of Cx36-EYFP-WT in transfected N2A cells occurred primarily in form of GJ plaques.

As mentioned Chapter 4.2.1., α-CaMKII translocation studies were also performed in double transfected primary hippocampal cultures. In vivo imaging showed that translocation of CaMKII to Cx36-EYFP-WT expressing sites is not a consistent event under low glu/gly stimulation. A major handicap for a quantitative analysis of translocation effects in neurons is the fact that transfection with Cx36-EYFP does not automatically induce formation of electrical synapses. We found abundant Cx36-EYFP fluorescence in vesicular structures, which occurred either in form of secretory vesicles or large cargo vesicles, which certainly do not contribute to synaptic transmission. Our double immunolabeling and additional triple labeling with the FM4-64 tracer indicated that we face at least three different synaptic complexes in the transfected neurons. (i) synaptophysin and/or FM4-64 positive sites, which consists of only a chemical synaptic component. (ii) Mixed synapses, which besides the presynaptic markers for chemical synapses, exhibit Cx36-EYFP in their close vicinity, and (iii) sites were exclusively Cx36-EYFP occurs in a membrane-bound form, although the latter is hard to separate from vesicle-bound Cx36-reactivity. We estimated that only in Cx36-EYFP GJ plaques visible CaMKII-ECFP expression was associated, since a co-localization of both in transport vesicles is hardly expectable. In addition, transfection efficiency in neurons was low and only a rare number of neurons (about 5% on a single slide) revealed visible double labeling and immunostaining with anti-synaptophysin. Also, transfection levels of α-CaMKII-ECFP needed to show locally defined expression. This was only achieved in fine processes, while close-to-soma expression and expression in the dendritic stems showed always diffuse overexpression without the chance to assess changes in α-CaMKII-ECFP concentrations. Definitely, we cannot exclude the possibility of translocation in neurons as found aside GJ plaques in N2A cells. The chance of monitoring this phenomenon under the described conditions is low, due to the high susceptibility of transfected neurons to glutamate/glycine treatment and the consequence of toxic effects. In addition we face the problem of rare double transfection in neurons. Interesting enough is the fact of a high number of mixed synapses found under synaptophysin-staining conditions and double transfection. A portion > 50% revealed co-localization, which at first glance is reasonably high. Recent data from FRIL labeling (Rash et al., 2000) and hippocampal immunohistochemistry (Nagy et al., in press) are consistent with this finding. Most remarkable is the fact that the building up of an chemical synapse with its complex complement of pre-and postsynaptic molecular constituents can be accompanied by a parallel processing of electrical synapse, i.e. GJ construct. If this mixed type of synapse is in fact functional remains to be further elaborated by combined electrical physiological and imaging approaches.
A first attempt was undertaken by our FM4-64 staining. Since FM4-64 labels active presynaptic sites by re-entrance of endocytosed vesicles loaded with this fluorescent-tracer, a co-localization of Cx36-EYFP-WT with FM4-64 under depolarizing conditions would be highly suggestive of a close side-by-side association of both synaptic components. As this was the case in a high number comparable to the synaptophysin/ Cx36-EYFP-WT co-localization, we are convinced that a major fraction of synaptic contacts occur in form of the mixed synapse type in transfected neurons.

5.4 Future directions

Results discussed in the previous chapters may serve as a basis for future studies. To fully understand the interaction of the two proteins the following questions should be addressed.

1. Immunochemical studies done in rats showed that Cx36 has a strong expression in the inferior olive, olfactory bulb, CA3/CA4 hippocampal subfields and several brain-stem nuclei (Condorelli et al., 2000). In this thesis, it is apparent that interaction of Cx36 and α-CaMKII may play distinct and important roles. Therefore, it may be of interest, to show the interaction of the two proteins in different regions of the nervous system in combination with electrophysiological studies focusing on the co-localization of these two proteins in acute slice preparations.

2. In N2A cells, FRET and translocation experiments with the wild type and mutant proteins showed that α-CaMKII-ECFP-WT translocates to GJ plaques and interacts with Cx36-EYFP-WT after the increase of [Ca^{2+}]. However, these observations provoke the question, how the dynamic pathway of α-CaMKII in the domains of GJ plaques can be regulated and which molecular components contribute to this process.

3. From previous studies on chemical synapses, α-CaMKII has a well-established role in modulating the structure and electrophysiological properties of chemical synapses (Malinow, R et al., 1988). Can the possible mechanism of action of α-CaMKII on GJ channels be the same as the well-known mechanism at chemical synapse sites?

4. Moreover previous electrophysiology studies in the Mauthner cell of goldfish suggest that α-CaMKII may be involved in changes of channel conductance as indicated by an increase in the open probability of the GJ channels or/and increase in the number of channels in the membrane (Pereda et al., 1995). Combining electrophysiology and FRET microscopy in one experimental setup, one can provide a complementary approach to track α-CaMKII translocation, the possible changes in the Cx36 trafficking and in the conductance of the GJ channels.

5. To obtain more evidence on the mobility of the α-CaMKII to Cx36 expressing GJ plaques the advantage of FRAP (fluorescence recovery after photobleaching) method can be applied. FRAP experiments in transfected cells may help to evaluate, recovery time of binding of α-CaMKII to F-actin in the cytosol and binding of α-CaMKII to Cx36 in GJ plaques.
plaques under different stimulation protocols. Also the recovery time of α-CaMKII at GJ plaques could provide information on the binding efficiency of α-CaMKII to Cx36.

5.5 Open Questions

One of the surprising outcomes of this work is presented in Chapter 4.3 α-CaMKII and Cx36, co-expressing neurons were exposed to chemical and electrical stimulation, in order to understand if binding of α-CaMKII to Cx36 is subject of activity dependent mechanisms such as translocation after stimulation. However, live cell confocal imaging showed that α-CaMKII does not translocate to Cx36 expressing sites in low concentrations of chemical stimulation and instead, application of higher concentrations of chemical stimulation causes toxicity. This conclusion needs additional confirmations, however we estimate the followings:

1. FRET experiments, which were performed in the GJ plaques of N2A cells, showed that Cx36-EYFP-WT and α-CaMKII proteins are interacting with each other. To support this result and show the translocation of the kinase to the Cx36 protein, living neurons were used and no translocation is observed. The main reason of this can be that neurons have a more complex structure compare to N2A cells. In N2A cells, constitute GJ plaques by connexin proteins. However as indicated in Chapter 4.4, Cx36 proteins are part of mixed synapses and couple/co-localize with synaptophysin immunoreactive synaptic boutons. Therefore, in neurons we do not only deal with GJ plaques but mixed synapses.

2. Failure of the translocation can be related with the coupling and the channel properties of the Cx36-EYFP-WT protein as well. Here, it is shown that Cx36-EYFP-WT can localize in mostly presynapses (Figure 28 and also chapter 4.4). In line with this result, Shakiryanova and his group, reported in their recent work that activation of presynaptic CaMKII by electrical stimulation is surprisingly slow and frequency dependent in the synaptic boutons. (Shakiryanova et al. 2011) It is an interesting estimation that maybe presynaptic CaMKII does not translocate to Cx36 sites when Cx36 protein co-localizes with synaptophysin on the presynaptic sides.

3. Consistent with the results presented here, it is also reported that in the club endings of Mauthner cells, Cx35 and CaMKII may interact, but this association might not be obligatory, because variable labeling was found even between plaques within the same terminal. (Flores et al., 2010)
6 Summary

In this thesis, the interaction between Cx36 and α-CaMKII proteins was explored. The research took place in three different steps.

First, the physical interaction between the two proteins was investigated by the FRET method. So far, there are some studies, which have touched on possible interaction of these two proteins. Moreover, the binding sites of two proteins had been identified previously using in silico analysis and pull down strategies (Alev et al., 2008). However, the physical interaction of Cx36 and α-CaMKII proteins was not confirmed. In this thesis, interaction of two proteins was successfully verified by FRET in a cell line expression system. In addition, further analyses using the Cx36 mutants revealed that the two proteins are likely to interact at two binding sites and the CL binding site of Cx36 is crucial for this interaction.

In the second part, stimulus induced translocation of α-CaMKII to Cx36 expressing GJ plaques was investigated in the cell line expression system. Using confocal imaging, it was shown that α-CaMKII is subject of activity dependent mechanisms in GJ plaques such as translocation after stimulation with Ca\(^{2+}\)/ionomycin in N2A cells. The translocation of the kinase is also dependent on the enzyme activity of the kinase. The physiological relevance of the translocation of the kinase was also investigated in the hippocampal neuronal cells. In contrast to the results obtained in N2A cells, α-CaMKII did not translocate Cx36 expressing sites in the hippocampal neurons after glu/gly stimulation.

In the final step, outcome from the translocation experiments in hippocampal neurons raised the question whether the Cx36-EYFP-WT protein localizes in the synapses. Therefore, localization of the Cx36-EYFP-WT protein was determined by using immunofluorescence techniques in Cx36 and α-CaMKII co-transfected neurons. Results showed that Cx36-EYFP-WT localizes in the presynapses and it is concluded that this can be the reason why α-CaMKII does not translocate to Cx36-EYFP-WT expressing synapses. However, the possible reasons, why translocation of the kinase did not take place in double neurons can be the focus of future research efforts.

Overall, this thesis significantly contributes to a better understanding of the interaction of Cx36 with α-CaMKII in living cells and provides first ever evidence for an interaction of both proteins in living system. α-CaMKII is not only a major component of glutamatergic chemical synapses but it is also the interaction partner of the Cx36 protein of electrical synapses. This shows that its modulatory role is not restricted to a specific type of synaptic transmission. Although the mechanism of action of α-CaMKII on GJ channels is not clear the work described here will open new avenues to further dissect the molecular and cellular mechanisms contributing to neuronal plasticity at sites of electrical synapses. In this context, the data reported in this thesis may foster novel strategies of how plasticity of electrical synapses.
7 References


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**Turkish Neuroscience Association**
- Apr 2006: Undergraduate travel award for 5th National Congress of Neuroscience in Zonguldak, Turkey

Publications

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