universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Untersuchung der Protein Expression verschiedener Mutanten des L-Typ Kalziumkanals Cav1.4“

Verfasserin

Sakine Korkmaz

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 441
Studienrichtung lt. Studienblatt:	Diplomstudium Genetik - Mikrobiologie (Stzw) UniStG
Betreuer:	Ao. Univ. Prof. Dr. Pavel Kovarik

Acknowledgements

My thanks go to Prof. Dr. Pavel Kovarik for accepting me as a diploma student. I would like to thank my supervisor Prof. Dr. Alexandra Koschak for her friendly guidance, assistance and advice with this diploma thesis. I also thank Verena Burtscher and Dagmar Knoflach for their help and time they provided me with my questions in the laboratory.

Furthermore, I thank my friends for their love and emotional support.

Last but not least, I would like to thank my dearest family for their love, understanding and support during my studies, in particular my niece Dila who always stood beside me.

Inhalt

Abstract:

The defects in L-type calcium channel (LTCC) can cause several human diseases (channelopathies). L-type calcium channels (LTCCs) comprise four different isoforms; $Ca_v1.1$ (α_{1S}), $Ca_v1.2$ (α_{1C}), $Ca_v1.3$ (α_{1D}) and $Ca_v1.4$ (α_{1F}).

$Ca_v1.4$ LTCCs are expressed mainly in retinal cells. The CACNA1F gene encodes the pore-forming α1 subunit. In the CACNA1F gene several mutations have been published. These mutations cause X-linked congenital stationary night blindness type 2 (CSNB2) (Bech-Hansen, Naylor et al. 1998, Boycott, Pearce et al. 1998, Strom, Nyakatura et al. 1998, Boycott, Pearce et al. 2000, Boycott, Maybaum et al. 2001), X-linked cone rod dystrophy (CORDX3) (Jalkanen, Mantyjarvi et al. 2006), Åland Island eye disease (AIED) (Jalkanen, Bech-Hansen et al. 2007), an X-linked retinal disorder (XRD) (Hope, Sharp et al. 2005), and retinal and optic disc atrophy. The symptoms of CSNB2 patients are varied levels of night blindness, low visual nystagmus, acuity, as well as myopia (Boycott, Pearce et al. 2000).

The purpose of this investigation was to clone various mutants of $Ca_v1.4$ α1 and characterize them biophysically. Therefore, two $Ca_v1.4$ α1 (G1339V and L216R) were constructed via site-directed mutagenesis. They were analysed by western blotting in order to analyse their expression on the protein level. The mutants (R1816X, L849P, G255E, and G359R) were previously generated by the group Alexandra Koschak and I tested them by western blot analysis. I have observed that the wild type (Newton, Bingham et al.) $Ca_v1.4$ α1 and all the mutants expressed protein, but the L849P mutant appeared to express less protein compared to WT. The position of the mutation at suggest that insertion of a Proline might affect plasma membrane trafficking.

Zusammenfassung

Der Defekt in L-Type-Kalzium-Kanal (LTCC) können zu unterschiedlichen menschlichen Krankheiten führen (Kanalopathien). LTCCs kommen in vier verschiedene Isoformen vor. Diese sind; die $Ca_v1.1$ (α 1S), $Ca_v1.2$ (α 1C), $Ca_v1.3$ (α1D) und $Ca_v1.4$ (α1F).

Cav1.4. Kanäle werden hauptsächlich in retinalen Zellen exprimiert. Im CACNA1F, welches für Cav1.4 Kanäle kodiert wurden mehrere Mutationen veröffentlicht. Diese Mutationen verursachen X-chromosomale kongenitale stationäre Nachtblindheit (CSNB2) (Bech-Hansen, Naylor et al. 1998, Boycott, Pearce et al. 1998, Strom, Nyakatura et al. 1998, Boycott, Pearce et al. 2000, Boycott, Maybaum et al. 2001), X-chromosomale-Zapfen-Stäbchen-Dystrophie (CORDX3) (Jalkanen, Mantyjarvi et al. 2006), Åland Island Augenkrankheit; AIED (Jalkanen, Bech-Hansen et al. 2007), eine X-chromosomale Netzhauterkrankung (XRD) (Hope, Sharp et al. 2005) und der Netzhaut- und Papillenatrophie. Die Symptome in CSNB2 Patienten sind unterschiedliche Grade der der Nachtblindheit, visueller Nystagmus, reduzierte Sehschärfe und Myopie (Boycott, Pearce et al. 2000).

Der Zweck dieser Untersuchung war zunächst verschiedene Mutanten von $Ca_v1.4$ α1 zu klonen und sie auf Proteinebene zu charakterisieren. Ich habe zwei $Ca_v1.4$ α1-Mutanten (G1339V und L216R) mittels Ortsspezifische-Mutagenese hergestellt und mittels Western Blotting analysiert, um die Expression der Proteine zu testen. Die Mutanten R1816X, L849P, G255E und G359R die zuvor von der Gruppe von Alexandra Koschak generiert wurden, habe ich ebenfalls durch Western Blotting getestet. Wildtyp (Newton, Bingham et al.) $Ca_v1.4$ α1 und alle Mutanten Kanäle haben Expression gezeigt, allerdings exprimierte die L849P Mutante im Vergleich zum WT weniger Protein. Eine Insertion der Aminosäure Prolin an der gegebenen Position dürfte daher der Transport des Cav1.4 Kanals an die Zellmembran verhindern.

1. INTRODUCTION

1.1 Voltage-gated Calcium Channels

Voltage-gated calcium channels (VGCCs) are transmembrane proteins, through which calcium flows along the electrochemical gradient into the cell. These channels are important for the calcium influx in response to membrane depolarisation and govern intracellular processes such as gene expression, secretion, contraction and neurotransmission (Catterall, Perez-Reyes et al. 2005).

VGCCs are high molecular complexes and constructing hetero-oligomeric complex of α1-subunit, β-, α2δ- and in some cases γ-subunit. The main properties of the calcium channels are determined by the ion-conducting α1 subunit of the channel (Bezanilla 2008).

The α1 subunit of voltage-gated calcium channels consists of four homologous domains (I - IV) each encloses six transmembrane segments (S1–S6) and a membrane-associated loop between transmembrane segments S5 and S6. The S4 segment works as the voltage sensor and the pore loop between transmembrane segments S5 and S6 in each domain is responsible for providing ion conductance and selectivity (Catterall 2000).

β subunits are cytosolic proteins and bind with high affinity to the α1 subunit of the channel (I-II loop) (Van Petegem and Minor 2006). The current amplitude is increased by coexpression of α1-with the ß-subunit (Singer, Biel et al. 1991). It is assumed that the beta-subunit performs a chaperone effect and so influences the density of expression of functional channels on the plasma membrane (Yamaguchi, Hara et al. 1998), facilitating the opening of pores (Neely, Wei et al. 1993, Josephson and Varadi 1996, Kamp, Perez-Garcia et al. 1996).

The extracellular α2-subunits of α2δ are bound to the δ-subunit through disulphide bonds (Cole, Lechner et al. 2005). It has been found that α2δ has four isoforms, which are α2δ-1, α2δ-2, α2δ-3, and α2δ-4 (De Jongh, Warner et al. 1990, Qin, Yagel et al. 2002). α2δ-1, α2δ-2 and α2δ-3 are expressed in CNS and the peripheral

nervous system. α2δ-1 exists in a lot of neuronal cell types (Cole, Lechner et al. 2005). This also includes dorsal root ganglion neurons (Newton, Bingham et al. 2001, Bauer, Nieto-Rostro et al. 2009).

The β and α2δ subunits increase channel trafficking, have influence on the channels biophysical properties and augment the expression of functional calcium channels at the plasma membrane (Dolphin 2012).

The γ subunit occurs in skeletal muscle calcium channels. Subunits that are related are expressed in heart and brain. The γ-subunit of the L-type channels possesses a modulating effect on the current amplitude as well as the activation and inactivation kinetics (Klugbauer, Dai et al. 2000).

The two groups in which the Ca_v channels are divided dependent on their α1-subunit are low-voltage activated (LVA) (Huguenard 1996), and high-voltage activated (HVA) channels (Hofmann, Biel et al. 1994, Dunlap, Luebke et al. 1995, Wheeler, Randall et al. 1995). The LVA channels are activated through low depolarization of the membrane potential whereas the HVA channels need higher membrane potentials.

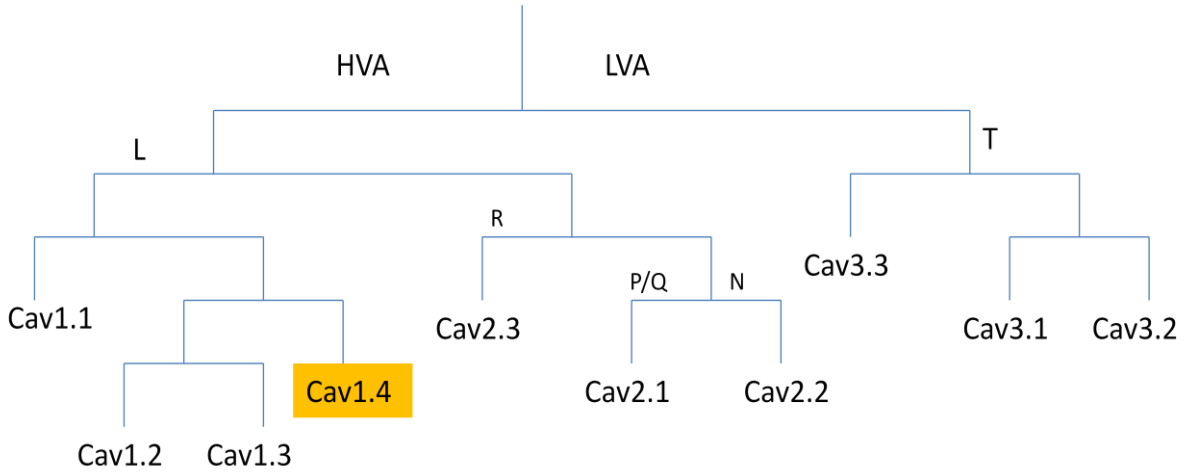

Figure 1. Phylogenetic tree of the voltage-gated calcium channels according to its α1- subunit. L-type channels: $Ca_v1.1$ -$Ca_v1.4$, P/Q-type channel: $Ca_v2.1$, N-type-channel: $Ca_v2.2$, R-type-channel: $Ca_v2.3$, T-type-channels: $Ca_v3.1$ -$Ca_v3.3$. Figure from Perez-Reyes, with slightly changes (Perez-Reyes 2003).

1.2 L-Type Calcium Channel

L-type calcium channels (LTCCs) is divided into four different α1-subunits: $Ca_v1.1$ (α_{1S}), $Ca_v1.2$ (α_{1C}), $Ca_v1.3$ (α_{1D}) and $Ca_v1.4$ (α_{1F}).

LTCCs show specific pharmacological properties that discriminate them from other VGCC. There are high sensitivity towards organic Ca^{2+} channel blockers and activators. Dihydropyridines (DHPs) are particularly important due to their high selectivity for LTCC and very high binding affinity. DHPs include both Ca^{2+} channel activators (e.g. BayK8644) and Ca^{2+} channel blockers (e.g. nifedipine,isradipine) (Striessnig, Grabner et al. 1998, Striessnig 1999).

$Ca_v1.1$ (α_{1S}):

The Cav1.1 channel is expressed almost exclusively in skeletal muscle cells and therefore has a restricted expression pattern. $Ca_v1.1$ channels transmit conformational changes of their voltage-sensing domains via mechanical linkage to the ryanodine receptors which release Ca^{2+} from the sarcolpasmic reticulum (Catterall, Perez-Reyes et al. 2005). Moreover, mutations in the CACNA1S gene, encoding $Ca_v1.1$ channels, can lead to Hypokalemic periodic paralysis and malignant hyperthermia sensitivity in humans (Striessnig, Bolz et al. 2010).

$Ca_v1.2$ (α_{1C}):

$Ca_v1.2$ is the predominantly expressed in the cardiovascular system, and together with $Ca_v1.3$ in several tissues such as including sinoatrial nodes (Platzer, Engel et al. 2000), heart atria, neurons (Olson, Tkatch et al. 2005), chromaffin cells (Marcantoni, Vandael et al. 2010). The CACNA1C gene encodes for the $Ca_v1.2$ channel. Mutations in this gene can cause Brugada Syndrome (Antzelevitch, Pollevick et al. 2007), sometimes also shorter as normal QT intervals (Burashnikov, Pfeiffer et al. 2010), and Early Repolarization Syndrome (Burashnikov, Pfeiffer et al. 2010), Timothy Syndrome (Splawski, Timothy et al. 2004, Splawski, Timothy et al. 2005).

Cav1.3 (α_{1D}):

$Ca_v1.3$ channels are prominently expressed in the inner ear and the sinoatrial node of the heart both in mice and humans (Platzer, Engel et al. 2000, Baig, Koschak et al.

2011). Mutations in the CACNA1D gene (encodes for the $Ca_v1.3$ channel) in humans cause a channelopathy with bradycardia and congenital deafness. Only human mutation is known so far (Baig, Koschak et al. 2011). As a reason mutations might either cause embryonic lethality in the homozygous state or dysfunction of the phenotypes is mild that they pass unnoticed in the heterozygous state (Platzer, Engel et al. 2000).

$Ca_v1.4$ (α_{1F}):

$Ca_v1.4$ α1 subunits primarily appears in retinal neurons but is also found in dorsal root ganglia (Murakami, Nakagawasai et al. 2001), lymphatic organs (McRory, Hamid et al. 2004), and immune cells (Kotturi and Jefferies 2005).

Mutations in the CACNA1F gene, which encode the $Ca_v1.4$ channels, lead to aberrations or the loss of the $Ca_v1.4$ protein. Mutation of CACNA1F can cause several diseases of which one is called Aland Iceland Eye Disease (AIED, Forsius-Eriksson syndrome) (Jalkanen, Bech-Hansen et al. 2007). Another one is the X chromosome-linked rod-cone dystrophy type 3 (CORDX3) with progressive progress (Jalkanen, Mantyjarvi et al. 2006) and the CSNB2 (congenital stationary night blindness type 2), an X-linked, congenital and non-progressive form the night blindness (Bech-Hansen, Naylor et al. 1998, Boycott, Pearce et al. 1998, Strom, Nyakatura et al. 1998, Boycott, Pearce et al. 2000, Boycott, Maybaum et al. 2001). CSNB2 symptoms range from various levels of night blindness to myopia, hyperopia, nystagmus, as well as vision loss and dystrophy of the photoreceptors can occur (Boycott, Pearce et al. 2000).

The biophysical and pharmacological characteristics of LTCCs in photoreceptors (Wilkinson and Barnes 1996, Taylor and Morgans 1998, Kourennyi and Barnes 2000, Stella, Bryson et al. 2002) and bipolar cells (von Gersdorff and Matthews 1996, Protti and Llano 1998, Berntson, Taylor et al. 2003) are the reason for distinguishing them from other LTCCs (I.g. Cardiac or smooth muscle LTCCs). Some of these characteristics are the fast activation and slow inactivation kinetics thought to be important to mediate neurotransmitter release at the photoreceptor synapses. Another characteristic is that they less sensitive to DHPs. LTCCs are not only found in the synaptic layers of the retina but also in the cell soma of photoreceptors and in

somatodendritic localization in bipolar and ganglion cells (Morgans 2001, Xu, Zhao et al. 2002, Berntson, Taylor et al. 2003).

1.3 Aim of the diploma thesis

$Ca_v1.4$ α1 subunits are predominantly expressed in the retina (Bech-Hansen, Naylor et al. 1998, Strom, Nyakatura et al. 1998). Several mutations in the CACNA1F gene which encodes the pore forming subunit of the $Ca_v.1.4$ channel have been described to cause the X-linked incomplete form of congenital stationary night blindness type 2 (CSNB2) (Bech-Hansen, Naylor et al. 1998, Strom, Nyakatura et al. 1998, Boycott, Maybaum et al. 2001, Wutz, Sauer et al. 2002). Symptoms of CSNB2 are low visual acuity, myopia, nystagmus and variable levels of night blindness (Boycott, Pearce et al. 2000).

In my diploma thesis I have biochemically analysed several CSNB2 mutants and I constructed two new mutants of $Ca_v1.4$ (G1339V and L216R) using the site- directed-mutagenesis technique. I was focusing on the following two questions.

1- Do $Ca_v1.4$- G1339V and L216R mutants show comparable protein expression as wild type $Ca_v1.4$ channels?

2- Why are the $Ca_v1.4$-L849P mutant-channels expressed at a lower level?

2. MATERIAL AND METHODS

2.1 Plasmid

The sequence of $Ca_v1.4$ wild type (Newton, Bingham et al.) α1 subunits were reported previously by Strom et al., 1998 (Gen Bank Accession Number: AJ224874). The $Ca_v1.4$ wild type α1 subunit cloned into the pCIneo-vector and can be checked by cutting with the enzymes EcoRI and AgeI comprising single restriction sites (Koschak, Reimer et al. 2003).

cDNA construct	**Reference**
α1-Wild Type	Strom *et al.,* 1998; Koschak et al., 2003
α1-L216R	unpublished
α1-G1339V	unpublished
α1-G255E	unpublished
α1-L849P	Strom *et al.,* 1998; Wutz *et al.*, 2002
α1-G359R	Simonz *et al.,* 2009
α1-R1816X	Wutz *et al.,* 2002
α2δ-1	Ellis *et al.,* 1988
β3	Castellano *et al.,* 1993

Figure 2. $Ca_v1.4$ subunit of WT and mutants as well as accessory subunits.

2.2 Site-Directed Mutagenesis

The method is based on the amplification of the whole plasmid using primers that include the desired changes. The primers were designed using the QuikChange primer design software and supplies from Agilent Technologies. With these, designed primers can produce single point mutation, multiple mutation, insertion or deletion.

For introducing the G1339V mutation Glycine was exchanged with Valin at nucleotide position 4016. As there couldn’t be introduced a restriction enzyme site for checking the clone directly at the mutated site an elimination mutation at nucleotide 4002 (C>T) was produced, so that the restriction enzyme SphI could not cut the cDNA carrying the mutation G1339V anymore.

Step1:	**Control:** Silent mutation to eliminate SphI-site at 4002C>T
Antisense strand	Rv 4002t: 5'-atctatgccgtcattggtatgcagatgttcggcaa-3'
Sense strand	Fv 4002t: 5'-ttgccgaacatctgcataccaatgacggcatagat-3'
Step2:	**Mutation:** G1339V (4016G>T):
Antisense strand	Rv 4016t: 5'-ggcatgcagatgttcgtcaaggtggctcttcag-3'
Sense strand	Fv 4016t: 5'-ctgaagagccaccttgacgaacatctgcatgcc-3'

Figure 3. Primers for PCR-mutagenesis of mutant G1339V. Red letters, silent mutation; blue letters, desired mutation.

For introducing the L216R mutation Leucin was exchanged with Arginin. Therefore, a mutation with the same primers was prepared at the position 648 where thymine changed to guanine. Another eliminated mutation was produced at the position 658 where guanine changed to adenine, for control purposes. This mutation eliminated a restriction site for the enzyme SanDI (KfII).

	Control: Silent mutation to eliminate SanDI (KfII)-site at 658G>A
	Mutation: L216R (648T>G)
Sense strand	5'-ccactgaggcgggtgtctggagtcccgagcc-3'
Antisense strand	5'-ggctcgggactccagacaccgcctcagtgg-3'

Figure 4. Forward and reverse primers for PCR-mutagenesis of mutant L216R. Silent mutation (red letters), desired mutation (blue letters)

Procedure of the PCR-Mutagenesis using PCR-device C1000 thermal cycler (Bio-rad)

1-Initial denaturation: The reaction is heated to a temperature of 94–96 °C. At first, different to step 2 (denaturation of tDNA-template), there is only one cycle of the initial denaturing process being performed for 2 minutes at 95°C. With this, a better denaturing of the DNA is provided to make sure that the template is being used effectively during the first amplification cycle.

2-Denaturation of tDNA-template: The reaction temperature in this first step is 94–98 °C for 10 minutes. It causes denaturing of the DNA-template by disrupting the hydrogen bonds between complementary bases, which gives single-stranded DNA molecules.

3-Annealing: The reaction temperature is lowered to 50–65 °C and allowing annealing of the primers to the single-stranded DNA template. The annealing temperature is chosen about 10°C below the melting temperature (Burashnikov, Pfeiffer et al.) of the primers used. Mutagenic primers contain the desired mutation. The chosen annealing temperature for Primers-G1339V was 56°C and for Primers-L216R 61°C.

4-Extension (Extension of primers with PfuUltra hotstart DNA polymerase)

The PfuUltra hotstart DNA polymerase has its optimum activity at a temperature of 68 - 72 °C, in this case 68°C.

5-Final Extension:

After the last PCR cycle at a temperature of 70-74 °C it was provided that any remaining single-stranded DNA is fully extended.

PCR Conditions		
Step	Temperature (°C)	Time
1	95°C	2 min
2	95°C	10 min
3	56*[1] / 61*[2] °C	30 sec
4	68°C	23 min 20 sec
	2 ∞ 5X	
5	95°C	10 sec
6	56*[1] / 61*[2] °C	30 sec
7	68°C	27 min
	6∞ 10X	
8	10°C	forever

Figure 5. PCR protocol used to introduce mutations G1339V and L216R: *[1] for Primers G1339V and *[2] for Primers L216R

Mix	1x
DNA Template (Cav1.4-pCIneo;50ng/µl)	2 µl
Fw Primer	1 µl
Rv Primer	1 µl
dNTPs (10mM)	1 µl
2% V/N DMSO	1 µl
10X PfuUltra	5 µl
PfuUltra hotstart	1 µl
dH_2O	38 µl
Total	50 µl

Figure 6. PCR reaction mixture

***DpnI* Digestion:**

Subsequently, the WT DNA was removed after the amplification of the reaction mixture with DpnI digestion. DpnI (Agilent Technologies, #500402) cleaves accurately at methylated and hemi-methylated sites, so it degrades the template plasmid but not the new PCR product.

The amount of 1 µL DpnI (which is equal to one enzyme unit) was added directly to the 50 µL PCR reaction mix and incubates at 37°C for 1 h.

After DpnI-Digest, plasmid DNAs which contain the desired mutations were transformed into E. coli XL1 blue cells.

2. 3 Amplifications and Purification of Plasmids

2. 3.1 Transformation

Transformation is one of three ways to transfer genetic information into bacteria. The other ways are transduction and conjugation. Under laboratory conditions free plasmid DNA can be added to bacteria that can take up the DNA with a suitable treatment.

For the transformation competent XL-Blue cells (Agilent Technologies), which are always stored -80 °C, were used. After taking 50 µl aliquots from the freezer, the cells are immediately put on ice. 100 ng pure plasmids DNA were transformed to the E. coli XL1-Blue competent cells. As a negative control purified water was used.

Procedure of Transformation

100 ng (approximately 5 µl) of the plasmid DNA and the same volume of purified water for the negative control were added to 50µl E. coli XL1-Blue competent cells in 1.5 ml Eppis and were incubated half an hour on ice. The bacterias were heat-shocked 90 seconds in 42 °C, so that the bacteria membrane became permeable and the plasmid DNA could be taken up. After that 1ml of LB medium was added to the bacteria (Steril! Beside the Bunsen burner). At 37 °C, 350-400 rpm, the bacteria were shaken for one hour to express the proteins. After one hour 200 µl transformation mix with sterile Drigalski-spatel were spread on antibiotic-containing (50mg/ml ampicillin) LB plates (beside the Bunsen burner). They were placed overnight, with the lid down (upside down), into the incubator. On the following day the negative control and single colonies of the positive control were checked.

2. 3.2 Mini-preparation (Small-Scale Plasmid DNA Purification)

The mini-prep is a practical method to isolate small amounts of plasmid DNA (~ 1 µg of DNA per millilitre of bacterial culture) from the transformed host. Small-scale DNA purification allows fast and easy control of transformants with restriction digestion (Becker, Caldwell et al. 1996).

Procedure of mini-preparation

Generally, the plasmid-DNA was isolated from 6 ml of an overnight shaking culture. For this isolation 10ml of LB medium was added to the plastic tube and 10µl Kanamycin (diluted 1:1000; 50mg/µl) was added too. A single colony was taken with a sterile tip of the agar plate and inserted into the medium (Steril! Beside the Bunsen burner). The samples were incubated overnight at 37 °C, 230 rpm. The next day, a Miniprep Kit "Isolation of High-copy plasmid DNA from E.coli" was used for the isolation of the DNA according to the manufacturer's protocol. 6ml of bacterial over night culture were centrifuged 12 000 rpm 4 minutes. Supernatant was removed and 250 µl cell resuspension Solution (vortex gently), 250 µl cell lysis solution (inverted 4-5 times, incubated for 5 minutes at RT.) and 350 µl neutralisation solution (inverted 4-5 times) were added one after another.

The Eppis were centrifuged for 1 minute at 12 000 rpm. Supernatant was transferred to a Spin Column and centrifuged again for 1 minute at 12 000 rpm. Flow-through was removed and 750 µl column wash solution were added to the column, then centrifuge at 12 000 rpm for 1 min and flow-through was removed. Finally 40 µl elution buffer were added und centrifuged again for 1 minute at 12000 rpm, so it was eluted into 1.5 ml Eppendorf tube.

2. 3.3 Midi-preparation (Large -Scale Plasmid DNA Purification)

After showing that the transformation has been successful, the DNA of the transformed bacteria was extracted in a larger scale. To 200 ml of LB medium 200 µl Ampicillin (50mg/µl) were added, and afterwards inoculated with a preculture which was incubated for 6 hours at 250rpm and 37°C. It was incubated overnight at 37 °C, 230 rpm.

For the isolation of DNA from maxi-prep a kit "NucleoBond Xtra Midi-Plus-EF Nucleic Acid and Protein Purification"was used according to the manufacturer's protocol.

2. 4 Control of Plasmids

2. 4.1 Restriction Enzym Digestion

Fermentas supplied restriction enzymes that were utilized according to the producer's suggestion. The control of compatibility and application of enzymes was used on "Fermentas DoubleDigest Tool" homepage.

Restriction enzymes and the expected fragments for WT and mutant $Ca_v1.4$ α1-plasmids and their accessory subunits are given in figure 7. All digestion solutions were mixed as presented in figure 8 and all prepared mixtures were incubated for one hour at 37°C.

Plasmid	**Vector**	**Restriction enzyme**	**Fragments (bp)**
α1-Wild type	pCIneo	SphI	5.565 , 3.118 , 2.013 , 779 , 72
α1-G1339V	pCIneo	SphI	5.565 , 5.131 , 779 , 72
α1-Wild type	pCIneo	SanDI (KfII)	11.027 , 520
α1-L216R	pCIneo	SanDI (KfII)	11.547 ,

Figure 7. Enzymes for restriction enzyme digestion and expected fragments after gel electrophoreses for control of $Ca_v1.4$ α1-subunits of Wild Type or mutants.

DNA	1 μg
Specific 10X buffer for Restriction enzyme	2 μl
Restriction enzyme	0.5-1 μl (5-10 U)
dH_2O (distilled water)	16-16.5 μl
Total	20 μl

Figure 8. Restriction enzyme digestion solution as stated by Fermentas.

2. 4.2 Gel electrophoresis

For the separation of DNA fragments gel electrophoresis was performed. The purpose of gel electrophoresis is to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualised in the gel by addition of ethidium bromide that binds strongly to DNA by intercalating between the bases and is fluorescent.

Procedure of gel electropheresis

The desired percentage of agarose (mostly 0.6-1%) in 1x TBE was dissolved in the microwave and poured after adding ethidium bromide (6μl EtBr/50ml) free of air bubbles into the gel chamber, where it got solid. The samples were mixed with 6x DNA loading dye and they were loaded. The first slot was loaded with marker on the gel. The gel was run in 1x TAE buffer at 4V/cm between 1h-4h, depending on the fragment's length as the amount of agarose.

2. 5 Cloning

Cloning is a copying process that enables copies of DNA-fragments. The vector contains special features. One of these features is the ability to insert or remove DNA-fragment in vector. To ensure the required homogeneity of the desired fragment, a selective amplification has to take place.

In this case, the cloning method was used for the generation of cDNA encoding for mutant Cav1.4 channels.

Procedure

The cloning process was carried out in several steps. At the beginning of the process, vector and Insert DNA were linearized with the enzymes (for L216R AgeI&EcoNI and for G1339V AgeI&EcoRI) by restriction enzyme digesting. After that the DNA fragment was separated via gel electrophoresis and then DNA was purified from agarose gel. The purified vector and insert DNA were ligated together and transformed into competent E. coli XL1 blue cells. The ligated plasmid DNA was amplified and purified. The plasmids were controlled by restriction enzyme digestion with previously predicted enzyme. The mutation cassette that appears as positive clones is sequenced by Biotech.

2. 5.1 DNA extraction from agarose gel

The DNA-fragments from insert- and vector-DNA were separated by electrophoresis and then desired DNA was extracted.

Procedure the DNA extraction from an agarose gel

The desired bands were cut as close to the DNA as possible to minimize the gel volume and the weight of the gel slice was recorded. To avoid damage, the time of gel-cutting under UV light exposure should be minimized. The bands were excised by a scalpel. The “GeneJet Gel Extraction Kit” was used to extract plasmid-DNA from the agarose gel. The excised gel was dissolved in a 1:1 volume (100 µl of Binding Buffer for every 100 mg of agarose gel) for 10 min at 50-60 °C. It was transferred to the “GeneJetTM purification column” and centrifuged for one minute. The flow-

through was discarded. 700 µl Wash Buffer was added to the GeneJetTM purification column and the process of centrifuging and discarding the flow-through was repeated. The empty GeneJetTM purification column was centrifuged again for one minute to completely remove residual Washing Buffer. As the last step 40µl Elution Buffer was added to the GeneJetTM purification column and centrifuged again for one minute. The flow-through involving DNA should be stored at -20°C.

For the ligation, the concentration from plasmid-DNA was calculated from the known concentration of the DNA ladder (20ng/5µl, each band). Integrated density value (IDVs) of DNA ladder was compared with IDVs of DNA fragment. For that 1-2µl of the purified DNA and 4µl of "FastRuler Middle Range DNA Ladder" were loaded on an agarose gel that was run at V/cm for 30 min or one hour.

2.5.2 Ligation of DNA

After linearization and purification from insert and vector DNA, these were ligated in a different ratio of linearised vectors to linearised inserts (1:5 or 1:10) using the "Rapid DNA Ligation Kit".

Procedure of Ligation

The ligation procedure was carried out as described in the figure 9 above and incubated for 5 minutes at 22°C. 5µl of this mixture was transformed as previously described into competent E. coli XL1-blue cells.

Mix	**1x**
Linearized Vector	10-100ng
Linearised Insert	variable; 1:5 or 1:10 ratio between linearised vectors and linearised inserts
5x Ligation Buffer	4 µl
T4 DNA Ligase (5U/µl)	1µl
dH_2O	Total 20µl

Figure 9. Ligation mixture components as being stated by Fermentas

2.5.3 Plasmids Control

The purpose of a plasmid control is to check whether the desired plasmids were acquired. Primarily, restriction enzyme digestion was performed and plasmid DNA was controlled by gel electrophoresis. Secondly, Multiple Sequence Alignment by Clustalw, an alignment software, was used to align the sequences derived from sequencing service "LGC Genomics" to WT-sequence. (http://www.genome.jp/tools/clustalw/). For sequencing, the primers were designed using athe program "Serial Cloner" with a length of 24 nucleotides and contained a CG-content of 50-60%.

Primer	**Sequences 5'-3'**
11467 fw	TCC ACA GGT GTC CAC TCC CAG TTC
1131 rv	TTG GAG AAC TCC CCA CTC AGG ACG
479 fw	CGC AAT GGC TGG AAC CTA CTC GAC
1568 rv	CCC AGT AGC AGG CAT TGG ACT TCA

Figure 10. Sequencing primer for the $Ca_v1.4$ mutation of L216R

Primer	**Sequences 5'-3'**
1325 fw	TGG TTC AGT CAT TCT ACT CGC TCC
2121 rv	TAC ATG ACC ACG TTC CAG TCC TCA
2098 fw	TGA GGA CTG GAA CGT GGT CAT GTA
2890 rv	CAG TAC TCG GAG TAC TCG CAG AAT
2867 fw	ATT CTG CGA GTA CTC CGA GTA CTG
3681 rv	ACC ATC TCA ATA GTG AAG AGG CCA
3658 fw	TGG CCT CTT CAC TAT TGA GAT GGT
4420 rv	GGC AAC CAC ATC CAA GTG TTT GAT

Figure 11. Sequencing primer for the $Ca_v1.4$ mutation of G1339V

2.6 Cell culture and transfection of mammalian tsA-201 cells

2.6.1 Splitting of tsA-201 cells:

TsA201 is a transformed human kidney (293, Sigma catalogue no. 85120602) cell line stably expressing an SV40 temperature-sensitive T antigen.

Cells were grown in 10cm culture dishes in high glucose Dulbecco`s Modified Eagle`s Medium (DMEM). A stabilized form of 2mM L-glutamine that prevents degradation and ammonia buildup in both adherent and 10% Fetal Bovine Serum (FBS, 2mM), a serum-supplement for better growth factors was added to the DMEM medium. The tsA-201 cells were incubated at 37°C in a 10% CO2 humidified air and splitted when the density of the cell was reached approximately 80%.

Procedure of passaging of tsA-201 cells

Primarily, medium was aspirated from 10cm culture dishes containing tsA-201 cells and 2ml of PBS-EDTA were added to the cells to detach cells. Cells were then centrifuged for 4 min at 631rpm (80G), room temperature. Supernatant was aspirated and the pellet was resuspended in 200µl DMEM medium. Desired dilution was prepared generally 1:10 or 1:20 for passaging or transfection. Cells were incubated at 37°C, 10% CO2.

2.6.2 Transient transfection into tsA-201 cells by using calcium phosphate precipitation

Wild Type $Ca_v1.4$α1-subunit and the mutants $Ca_v1.4$ α1 subunit of L216R, G1339V, R1816X, L849P, G255E, G359R were transfected, together with α2δ1 and β3 auxiliary subunits, pUC vector and GFP into the human embryonic kidney tsA-201 cells. GFP was also used for visualization of the transfection.

Transfection of tsA-201 cells was carried out using the calcium phosphate precipitation method to estimate the expression of α1 protein through Western blots. After transfection cells were incubated 48 hours at 37°C, 10% CO_2 humidified air. As a negative control, mock transfection was performed such that all subunits without α1 subunits were transfected into tsA201 cells. Mock transfection is a transfection of the

needed as a negative control to determine if there are any effects that are caused by the accessory subunits and the transfection reagent or process in general. Therefore α1-subunit plasmid DNA is omitted.

TsA-201 cells were transfected with 3 µg of α1 (Cav1.4-pCIneo-WT and Mutants) with 2 µg of β3, and with 2.5 µg of α2δ1 subunit cDNA, and with 2.5 µg of pUC carrier DNA. This was carried out as described in 10 cm culture dishes.

Procedure of Calcium Phosphate Preciptiation transfection

TsA-201 cells have to be grown optimally at 40-60% confluency. One day before transfection the medium has to be changed. The necessary amount of DNAs was calculated (3 µg of α1, 2 µg of β3, 2.5 µg, α2δ1-subunit and 2.5 µg pUC).

$CaCl_2$ and HEBS were thawed at 37°C in a water bath and let cool down to room temperature. Room temperature is important for precipitate formation. $CaCl_2$ was put in Eppendorf tubes and the previously given amount of DNA was added. Total volume of DNA and $CaCl_2$ should be 500µl. DNA/CaCl2 -Mix was added drop by drop to 500µl HEBS and the tube was gently tapped with a finger (final ratio: CaCl2: HEBS=1:1). DNA-$CaCl_2$–HEBS mixture was incubated exactly for 20 minutes at room temperature. After incubation, the DNA-CaCl2-HEBS mixture was distributed drop by drop on tsA201 cells while swaying the dish gently. The cells were incubated for 6 hours at 37°C, 10% CO2 humidified air and then the medium was changed. After two days the expressed cells were lysed so that obtained proteins could be detected by Western blot analysis.

2.7 Membrane preparation (Cell lysis):

The cells should be lysed to release the desired protein to the prepare samples for Western blotting. The tsA-201 cells were lysed with the below described methods. This enables the isolation of the expressed proteins.

Procedure of cell lysis

The growth medium was removed from cell dishes by vacuum suction. The cells were detached with 3ml PBS and the suspension was transferred into a 15ml tube. From now on, it is important that the cell suspension is stored on ice because the cells are sensitive and could be destroyed. Then the cells were centrifuged at 1,500rpm at 4°C for 5 min and the supernatant was removed through vacuum suction. The pellet was resuspended in 2 ml lysis buffer and incubated on ice for 15 minutes. It was pipetted 50x through a yellow pipette tip and the suspension was pressed 6x through a cannula (d=0.40mm) using 1ml Syringe thus the cells could break up. The cell suspension was centrifuged at 20,000rpm at 4°C for 20 min and the supernatant was collected avoiding the pellet into an ultracentrifuge tube. The cell suspension was centrifuged for 20 minutes at 30,900 rpm at 4°C (Beckman XL-90 Ultracentrifuge, 45 Ti Rotor). The supernatant was discarded and 200 µL of lysis buffer was added. The mix was incubated on ice for 30 minutes and was pressed again 6x through a cannula (d=0.40mm) using 1ml Syringe. The solution was aliquoted and shocked with liquid N_2 and stored at –80°C.

Lysis buffer (12.5ml)	**Stock solution**
125µl 1M Tris/HCl pH 7.4	24.23g Tris base in MQ water
12,5µl Approtinin	1mg/mL
12,5µl Trypsin inhibitor	100mg/mL
12,5µl Pepstatin A	1mM in EtOH
62,5µl Benzamidin	0.1M
62,5µl PMSF	40mM in EtOH
250µl Iodacetamid	0.1M
12,5µl Leupeptin	1mg/mL
total 12.5 mL with MQ water	

Figure 12. Lysis buffer mixture

2. 8 Lowry Protein Assay

There are different assays for protein quantification e.g Bradford, Lowry and UV spectroscopic protein assays. For my experiments I used the Lowry protein assay which Oliver H. Lowry has introduced and developed this colorimetric total protein assay method in 1951 (Lowry, Rosebrough et al. 1951).

2.8.1 Chemistry of the Modified Lowry Protein Assay

The assay is performed in two steps that are based on the Biuret and Folin-Ciocalteau reaction (figure 13). The first step is the Biuret reaction where the peptide bonds of proteins react with copper (Murakami, Nakagawasai et al.) ions under alkaline conditions to reduced Cu+. The second step is the Folin-Ciocalteau reaction, where the copper (Murakami, Nakagawasai et al.) is reduced by the oxidation of aromatic amino acids to copper (I) (Olson and Markwell 2007).

In this reaction, a strong blue color is the result due to the tyrosine and tryptophan content and less of cysteines or other amino acids. The copper (I) reduces the yellow Folin-Ciocalteau reagent (molybdenum(VI) - and Wolfram (VI)-heteropolyacids) to molybdenum blue. The initial unstable blue complex is rearranged which causes the stable blue complex with higher absorbance (Lowry, Rosebrough et al. 1951, Legler, Muller-Platz et al. 1985). The final blue colour is measured with a spectrometer at 750nm (Olson and Markwell 2007).

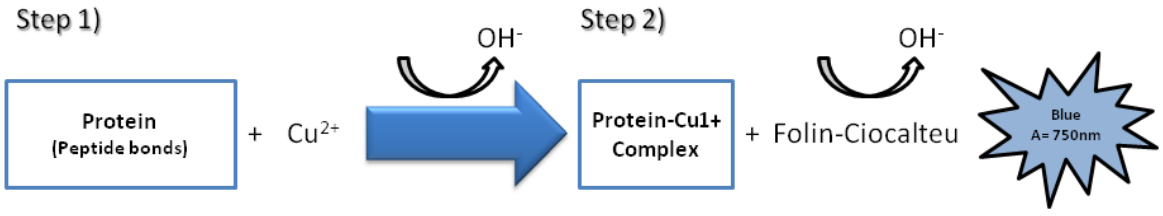

Figure 13. Two step procedure of the Lowry assay (Taken from: http://www.labome.com/method/Protein-Quantitation.html [27.08.2013])

The amount of protein in the samples was estimated using a standard curve of a preferred standard protein solution. In this case bovine serum albumin was used as standard protein (Thermo Scientific, #23209).

2.8.2 Lowry Factor Determination

Primarily, the Lowry factor was determined so that the protein concentration of each unknown sample can be calculated with help of the factor.

The standard curve was constituted by plotting the average blank corrected 750 nm value for each BSA standard. The standard concentration was plotted on the x-axis and the absorbance of the standards on the y-axis. The absorption is measured by photometry in µg/ml.

Procedure

Initially, increasing dilutions were prepared from the stock solution (BSA), the concentrations of which are known. The duplicate BSA dilution series was prepared with 0-20 µg BSA per 100 µl of stock solution containing 1mg/ml.

In each case 2x BSA in µg/100µl	dH_2O (µl)	Stock Solution BSA 1mg/ml in µl
1	495	5
2	490	10
3	485	15
4	480	20
5	475	25
6	470	30
7	465	35
8	460	40
9	455	45
10	450	50
11	445	55
12	440	60
13	435	65
14	430	70
15	425	75
16	420	80
17	415	85
18	410	90
19	405	95
20	400	100

Figure 14. Pipetting protocol for BSA dilutions in 1.5 mL Eppendorf tubes.

Afterwards 100µl of each dilution were transferred to new tubes and 100µl of MQ water to three tubes. MQ water was used as a blank which is subtracted from

obtained absorptions as a correction. At 15 second intervals, 100µl of 0.1% SDS was added to each tube and briefly vortexed. 1mL of Folin 1 was added again at 15 second intervals to each tube and was vortexed briefly and incubated for 10 minutes at room temperature. After 10 minutes at 15 second intervals, 100µL of Folin 2 was added to each tube in dark. Again the tubes were briefly vortexed and put into a rack which was wrapped in aluminium foil and were incubated in darkness for 30 minutes. After 30 minutes the absorbance of all samples was measured at 750nm.

A table with the obtained absorbance values was created. The obtained values were used for the reference protein by applying a calibration curve. The measured absorbance values were represented on the y-axis and the quantity of protein per reaction tube on the x-axis.

A

Conc. µg/100µl	Abs 1	Abs 2	MW	MW-BP
BP1	0,12	0,12	0,12	
BP2	0,119	0,119	0,119	
BP3	0,1195	0,1195	0,1195	0,120
1	0,143	0,143	0,143	0,024
2	0,168	0,168	0,168	0,049
3	0,183	0,183	0,183	0,064
4	0,2045	0,2045	0,205	0,085
5	0,214	0,214	0,214	0,095
6	0,283	0,283	0,283	0,164
7	0,227	0,227	0,227	0,108
8	0,24	0,24	0,240	0,121
9	0,33	0,33	0,330	0,211
10	0,265	0,265	0,265	0,146
11	0,315	0,315	0,315	0,196
12	0,397	0,397	0,397	0,278
13	0,296	0,296	0,296	0,177
14	0,3435	0,3435	0,344	0,224
15	0,369	0,369	0,369	0,250
16	0,3615	0,3615	0,362	0,242
17	0,329	0,329	0,329	0,210
18	0,375	0,375	0,375	0,256
19	0,3905	0,3905	0,391	0,271
20	0,401	0,401	0,401	0,282

B

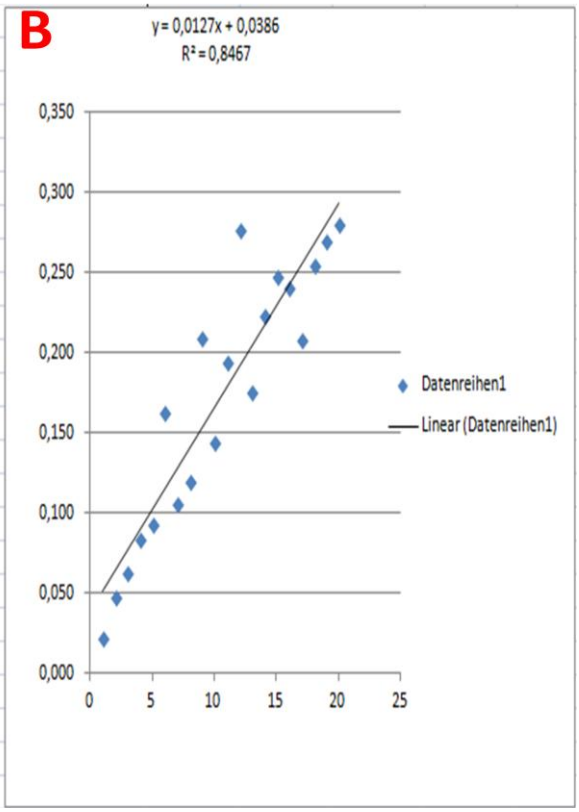

Figure 15. The dilutions of the samples and their measured absorption. In the above table the standard is showed as an example. BSA is 1mg/ml; **A,** the absorption corrected with average blank 750nm value for each BSA standard; Abs: absorption, MW: average of absorption MW-BP: average of absorption minus average of blank. **B,** Standard curve constituted by plotting the average blank corrected 750 nm values for each BSA standard.

The regression line occurs by the intersection point (x, y) and is determined by two parameters:

Each linear is mapped to a function y = bx + a. If the x-value is increased by one unit, then the y-value is changed by a factor of b. This factor is called the regression coefficient or effect coefficient. Factor "b" determines the upgrade of the straight line and constant "a" marks the point of intersection with the y-axis.

The standard curve was calculated by linear regression analysis using the equation. In this case:

y = 0, 0127x + 0, 0386

The regression coefficient is R^2 = 0, 8467

The factor was calculated: Factor = 1/slope; 1/0, 0127 = 78.7

2.8.3 Protein Quantification

The proteins concentrations were determined using linear standard curve with resulting factor.

Procedure

Two dilutions of each sample (1:20, 1:40, and 1:80) were prepared as described below.

Protein in µL	**MQ water in µL**	**receive dilution**
25 µl	475µL	1:20
210 µl of 1:20 dilution	475µL	1:40
210 µl of 1:40 dilution	475µL	1:80

Figure 16. Pipetting protocol for protein dilutions in 1.5 mL Eppendorf tubes.

After preparing the dilutions 100 µl of these dilution samples were transferred to new tubes. 100 µl of MQ water was pipetted into another three tubes. MQ water was used again as a blank and was subtracted from the absorptions obtained as a correction.

Next processes, 0.1% SDS, Folin I and Folin II were added and absorption was measured at 750nm. This corresponds to the amount of protein, which was added to the assay (100µl). With help of this equation the protein concentration was extrapolated.

Protein concentration of the sample = Abs x factor

blank 1	blank 2	blank 3	MW blank
0,11	0,114	0,173	0,1325
0,11	0,114	0,174	

Factor
78,7

WT						
Dilution	ABS 1	ABS 2	MW ABS	MW korr.	C [mg/ml]	c MW [mg/ml]
80	0,275	0,275	0,275	0,1425	8,9718	5,325366667
40	0,261	0,261	0,261	0,1285	4,04518	
20	0,321	0,32	0,3205	0,188	2,95912	

Total Volume [µl]: 30			5x Puffer [µl]: 6	
Sample	c MW [mg/ml]	33,40 µg [µl]	MQ water [µl]	5x Puffer [µl]
WT	5,325366667	6,271868604	17,7281314	6

Figure 17. The quantity of the proteins. In the above table WT is showed as an example. Abs: Absorption; MW: average of absorption; MW korr: MW blank-MW ABS; c [mg/ml]: MW korr*Factor*80/100; c MW [mg/ml]: average of c [mg/ml].

2.9 SDS-PAGE and Western blotting

After transfection and following cell lysis, the methods “SDS-Page and Western blotting” were used for detection of expression of $Ca_v1.4$ channel protein.

2. 9.1 SDS-PAGE

SDS-PAGE is a frequently used technique for the separation of proteins using gel electropheresis, in which the polyacrylamide gel enables separation of proteins in their molecular weights. For gel-electropheresis resolving and stacking gel was prepared. The concentration of the resolving gel is dependent on the size of the protein that is analzyed. In this case 8 % resolving gel and 4 % stacking gel were prepared. Initiation of polymerisation was applied to the solution of 10% APS and 10% TEMED to the mix. For observation of the weight of the proteins the marker “Precision Plus ProteinTM Kaleidoscope standards” and “Page Ruler Plus Pre-stained Protein Ladder (10-250 kDa)” were used. Around 24-30 µg of proteins was loaded to electrophoresis gel.

Procedure

All the parts of the gel casting device above all the glass plates and the comb were cleaned. The used glass plates had a thickness of 1.5mm. The gel casting device was built and resolving gel solution was carefully pipetted 1 cm below the comb between the plates. After that, it was overlaid with MQ water in order to avoid the gels to dry out. The gel was incubated until polymerization was finished (approximately 30 minutes). Stacking gel was pipetted and the comb was immediately inserted gently between the plates. Again, it was left for polymerization for 30 minutes. The gels were mounted into the electrophoresis device (Bio-Rad Mini Protean Tetra System + Power Pac 200 or 3000). It was filled up with 1x running buffer and the comb was removed.

The samples were prepared with MQ water in the desired quantity and 5x loading buffer that was previously heated to 57°C was added. The samples were incubated at 57°C for 15 minutes and loaded to the gel. The marker was loaded too by using a

long plastic pipette. Firstly, the electrophoresis was run for 20 minutes at 100 V and secondly for 1 hour at 180V.

Solution

Resolving Gel (total 10ml)	
	8%
dH_2O	5.28 mL
2M Tris/HCI pH 8.8	1.875 mL
20% (w/v) SDS	50 µl
30% / 0.8% (w/v) Acrylamide/Bisacrylamide	2.672 mL
10% APS	80 µl
TEMED	80µl

Stacking gel (total 10ml)	
	4%
dH_2O	6.7 mL
0.625M Tris/HCI pH 6.8	2 mL
20% (w/v) SDS	50 µL
30% / 0.8% (w/v) Acrylamide/Bisacrylamide	1.33 mL
10% APS	80 µl
TEMED	80 µl

Figure 18. Resolving gel and Stacking gel mixture

5X Sample Loading Buffer			
	25mL total	**concentration in 5X**	**final (in 1X)**
Tris (pH 6.8)	3.9ml of 2M stock	0.3125M	62.5mM
SDS	5g in 8mL MQ water	4% (w/v)	2% (w/v)
Glycerol	12.5mL	50% (w/v)	10% (w/v)
DTT	0.193mL	50mM	10mM
Bromophenole blue	Add a tiny crystal		
dH_2O	up to final 25ml		

8x Runnig Buffer		
	1l total	**8x**
Tris	24.28g	200mM
Glycine	114.86g	1.53 M
dH_2O	Up to final 1l	
1x Running Buffer		
	1L total	1X
200mM Tris	125mL of 8X stock	25mM
1.53 M Glycine		192mM
20% (w/v) SDS	5mL	0.1%
dH_2O	Up to final 1L	

Figure 19. Loading Buffer and Running Buffer mixture

2. 9.2 Western blotting (Immunoblotting)

Following the SDS-PAGE electrophoresis, the separated proteins were transferred from gel to a nitrocellulose (NC) membrane by wet-electroblotting. After the transfer, the non-specific sites were blocked and the NC membrane was incubated with a solution that involved specific primary and secondary antibodies. The primary antibody recognises and binds itself to the target protein and a secondary antibody.

The secondary antibody binds itself to the first antibody and links to a solution that involves an enzyme. The enzyme is catalysing a colorimetric reaction. By adding the ECL-solution the target protein gets visualised.

Procedure

The apparatus and the blot sandwich were prepared as previously described by the manufacturer (black side of holder, 2x sponges, filter paper, gel, NC membrane, filter paper, 2x sponges, and white side of holder). The apparatus was filled with transfer buffer and put in a box with ice. It was run constant for 90 minutes at 100V.

The gel with Coomassie staining was checked to see if the transfer was successful. The NC membrane was incubated in blocking buffer (1% Non-fat dry milk + 0.2% Gelatine powder) at room temperature. After 2 hours, the blocking buffer was removed and primary antibody (Rabbit anti-pan α1 voltage gated calcium channel antibody) was added by diluting it with blocking buffer (stock solution of antibody is 15µg/50µl; 1/1400 dilution). It was incubated overnight at 4°C while shaking on the table rocker. The primary antibody solution was removed and washed with washing buffer 5 times for 5 minutes each. The washing buffer was discarded and the secondary antibody (anti rabbit) was added diluting it in blocking buffer (1:20,000) that was incubated for 2 hours at room temperature shaking on the table rocker. The NC membrane was washed again with washing buffer 5 times for 5 minutes each. The ECL solution for detection was added and incubated for 5 minutes while gently shaking by hand. The membrane was picked with pincers and placed between two plastic foils in a film cassette. An x-ray film was cut in a dark room to an appropriate size and was put on the upper plastic foil enclosing the membrane, the film cassette was closed and exposed for various time frames (e.g. 5,10,15 minutes). The membrane was developed using the developing machine (AGFA).

Solution:

1x Transfer Buffer		
	1L total	**Final**
Tris & Glycine	125mL of 8x running buffer	25mM & 192mM
SDS	5mL 20% SDS	0.1%
Methanol	200mL	20%
dH_2O	Up to final 1L	

Washing Buffer		
	Total amount	**Final**
1M Tris/HCl pH 7.4	20mL	20mM
NaCl	9g	150mM
Triton X-100	10mL	0.5%
Tween 20	2mL	0.1%
dH_2O	Up to final 1L	

Blocking Buffer	
Non-fat dry milk	1.5g in 100mL washing buffer (RT)
Gelatine powder	0.3g in 50mL washing buffer (heat so the gelatine can dissolve better)

Figure 20. Solutions for Western blotting; 1x Transfer Buffer, Washing Buffer and Blocking Buffer

2. 9.3 Staining and Destaining of SDS-Polyacrylamide gels

Coomassie Blue staining is a practical and very easy method to see the location of a protein in gels. After protein transferring the total proteins on the membrane, the gel with Coomassie staining was checked to see if the transfer was successful.

Procedure

The gel was put into a plastic tray and stained gently with staining solution for two hours at room temperature shaking on the table rocker. After two hours the staining solution was removed and the gel was washed twice with de-staining solution. The first washing step of the gel took place for two hours at room temperature and the

second washing procedure was performed overnight at 4°C while shaking on the table rocker until the bands were visible on the gel.

Preparing of the dyer: 2g Brilliant Blue R (sigma Aldrich: B0149) was dissolved in 250 ml MilliQ H_2O and then 75 ml acetic acid and 500 ml Ethanol 96% were added. This mix was filled up with Milli Q H_2O to 1000 ml.

Preparation of decolorizer: 400 ml Ethanol 96% were mixed with 100 ml acetic acid and filled up to 1000 ml with Milli Q H_2O.

2. 10 Enzymatic de-glycosylation

The Intracellular trafficking of membrane proteins occurs through the secretory path. By this path are secretory proteins and membrane proteins translated, folded, modified and transported over the endoplasmic reticulum (ER), the ERGIC (ER-Golgi intermediate compartment) and Golgi apparatus to the plasma membrane.

Glycosylation is a form for post-translational modification of proteins (shown figure 21) which is important in the processing, folding and secretion of proteins from the Golgi apparatures and ER (Varki 1993).

The N-linked and O-linked glycosylation are both playing a part in maintaining protein conformation and activity and in protein intracellular trafficking and secretion. They also protect proteins from proteolytic degradation (Varki 1993). For N-linked glycosylation, glycans are attached to asparagine residues on the core protein, whereas for O-linked glycosylation glycans are attached to serine or threonine residues.

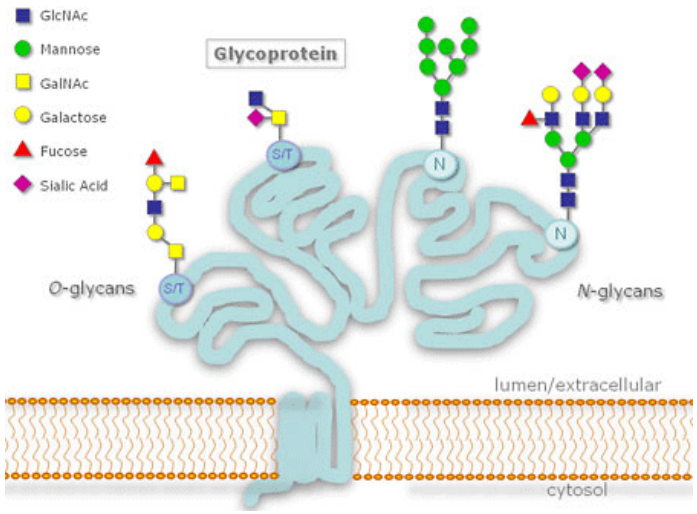

Figure 21. A glycoprotein modified with O-linked and N-linked glycosylation. (Taken from: https://www.neb.com/products/p6039-protein-deglycosylation-mix [22.08.2013])

N-linked glycolysation is the most common type of glycosidic bond. Observation of protein trafficking can be achieved by using PNGase F and Endo H which are specific endoglycosidases that characterize glycoproteins. The proteins in the endoplasmic reticulum react sensitive to Endo H digestion where N-glycosylation is

taking place whereas proteins in the Golgi body don't react in the same way thus being resistant because of the additional modifications that happen to the glycan.

Such approach should help us to clarify whether CSNB2 mutations that show reduced functional activity are indeed less expressed at the plasma membrane.

However, as a first step I set out to test whether $Ca_v1.4$ α1 subunits are glycosylated at all. Therefore, a kit from the company "New England Biolabs" was used for deglycosylation. This kit contains an enzyme mixture among other things, consisting of PNGase F Glycerol Free, Neuraminidase, O-Glycosidase, β1-4 Galactosidase and β-N acetylglucosaminidase. PNGaseF is an efficient enzyme for removal of every N-linked oligosaccharide of glycoproteins (Tarentino and Plummer 1994).

2. 10.1 Enzymatic Deglycosylation of Bovine Fetuin

Bovine Fetuin was used as a positive control for our assay because it is an N- and O-glycoprotein and highly glycosylated.

Procedure

There was a reaction procedure performed by the company New England Bio Labs (P6042) for Bovine Fetuin. The molecular weight of Fetuin is 64 kDa.

20 µg of Fetuin, 1 µl of 10x Glycoprotein and 5 µl of H_2O were mixed. The mixture was incubated at 95°C for 10 minutes. 1 µl of 10x G7 Reaction Buffer, 1 µl of 10% NP-40 and afterwards 1 µl of endoglycosidase were added to the sample. The mixture was then incubated at 37°C for 1 hour. Reaction products were visualized on SDS-PAGE.

2. 10.2 Enzymatic Deglycosylation of $Ca_v1.4$

To determine the glycosylation state of $Ca_v1.4$ α1 subunits transfected tsA-201 cells lysates were treated with deglycosylation enzyme mixture so that N-linked and O-linked carbohydrates of glycoproteins were removed. Then, samples were analyzed by SDS-Page and Western blotting. $Ca_v1.4$ protein was detected with the antibody directed against voltage gated calcium channels, Pan α1 showing a molecular mass of 219 kDa (figure 28).

An internal control was performed because there was no deglycosylation observed with the $Ca_v1.4$. TsA-201 cells were always con-transfected with the accessory subunits β3 and $\alpha 2\delta{-}1$. The latter is highly glycolsylated. Using an antibody directed against α2δ I found that α2δ underwent enzymatic deglcosylation with a weight loss from 175 kDa to 120 kDa (figure 29).

Procedure:

A reaction procedure by the company New England Bio Labs (P6039) was performed for the Protein Deglycosylation Mix.

Denaturing Reaction Conditions:

100 µg of glycoprotein were added to 18µl H_2O and then 2µl of 10x Glycoprotein Denaturing Buffer was added to make a 20µl total reaction volume. The glycoprotein was denatured by the heating reaction at 100°C for 10 minutes and after that it was chilled on ice and centrifuged for 10 seconds. To the denatured glycoprotein 5µl 10x G7 Reaction Buffer, 5µl 10% NP40 and 15µl H_2O were added and finally 5µl Deglycosylation Enzyme Cocktail. One sample was incubated at 37°C for 4 hours and another one overnight.

Non-denaturing Reaction Conditions:

Non-denaturing reaction was performed as a positive control for the fully deglycosylated protein. The non-denatured reaction can be compared to the denatured reaction to determine the extent of reaction completion.

100 µg of glycoprotein was dissolved in 40µl H_2O and 5µl 10x G7 Reaction Buffer was added and finally a 5µl Deglycosylation Enzyme Cocktail was added. One sample was incubated at 37°C for 4 hours and another one overnight. The longer the incubation time the more enzymes may be required.

3. RESULT

3.1 Identification of Cav1.4 G1339V and L216R-mutants by gel electrophoresis

Cav1.4-G1339V:

Cav1.4 -G1339V- pCIneo mutants were identified by enzyme digestion and gel electrophoresis. The plasmids (Ca_v1.4-G1339V) were digested with enzyme SphI and analyzed by agarose gel electrophoresis. To control the mutation of Ca_v1.4α1-G1339V mutant was inserted on the position 4002C>T so that one of the SphI-sites got eliminated. The expected fragments for Ca_v1.4α1-G1339V are 5.565, 5.131, 779, 72 bp. and for Wild Type Ca_v1.4α1 are 5.565, 3.118, 2.013, 779, 72 bp. A desired mutation on the position 4016G>T of Ca_v1.4 α1 was inserted and was controlled.

The position of the sequencing primers and the mutation sites is shown in plasmid maps (figure 32 in appendix).

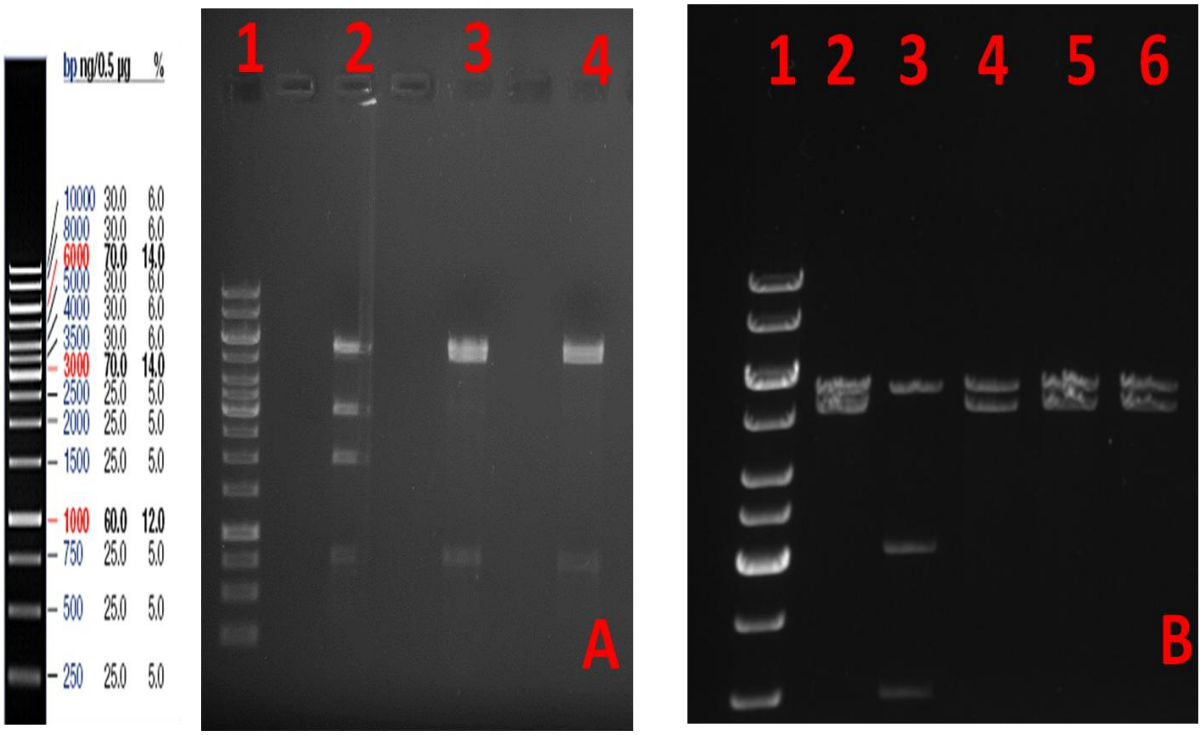

Figure 23: Agarose gel electrophoresis analysis of $Ca_v1.4$-G1339V after restriction digest with sphI Lane 1 is representing GenerulerTM 1Kb ladder of fermentas. A, Lane 2 is representing Wild Type of $Ca_v1.4$. Lane 3 and 4 are representing various samples of G1339V $Ca_v1.4$α1 mutant. 3% agarose gel, 4V/cm for 4 hours. B, Lane 3 is representing Wild Type of $Ca_v1.4$ α1. Lane 2, 4, 5 and 6 are representing various samples of G1339V $Ca_v1.4$ α1 mutant. 0.6% agarose gel, 4V/cm for 1 hours. Size of expected fragments are 5.565, 5.131, 779 , 72 bp

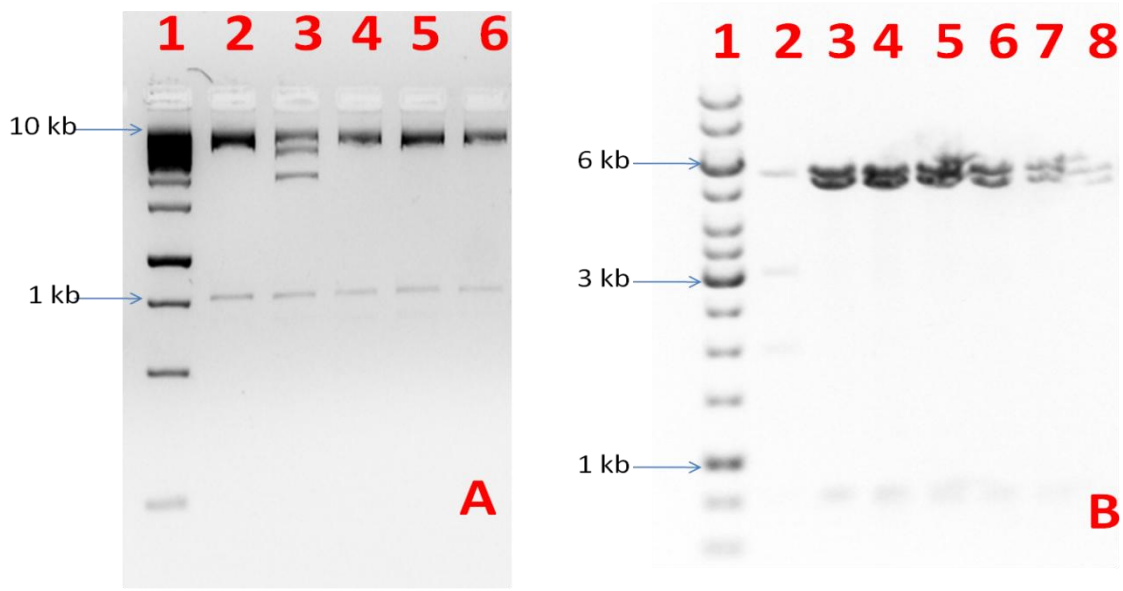

Figure 24: Agarose gel electrophoresis analysis of Ca_v1.4-G1339V after ligation. Plasmids were digested with enzyme SphI. The expected fragments for Ca_v1.4 α1-G1339V are 5.565, 5.131, 779, 72 bp. and for Wild Type Ca_v1.4 α1 are 5.565, 3.118, 2.013, 779, and 72 bp. Lane 1 is representing Generuler™ 1Kb ladder of Fermentas. **A,** Lane 3 is representing Wild Type of Ca_v1.4 α1. Lane 2, 4, 5 and 6 are representing various samples of Ca_v1.4 α1-G1339V mutant. 3% agarose gel, 4V/cm for 4 hours. **B,** Lane 2 is wild type of Ca_v1.4-G1339V. Lanes 3, 4, 5, 6, 7 and 8 are representing various samples of G1339V Ca_v1.4 α1 mutant. 0.6% agarose gel, 4V/cm for 1 hours.

$Ca_v1.4$ α1 L216R:

Mutation $Ca_v1.4$-L216R was inserted on the Position 648T>G of $Ca_v1.4$ α1 and for control an elimination mutation on the position G658>A so that it eliminates one of SanDI-sites. Plasmids were digested with enzyme SanDI (KfII) for 1 hour at 37°C. The expected fragment for $Ca_v1.4$ α1-L216R-pCIneo is 11.547 bp. The expected fragments for Wild Type are 11.027 and 529 bp.

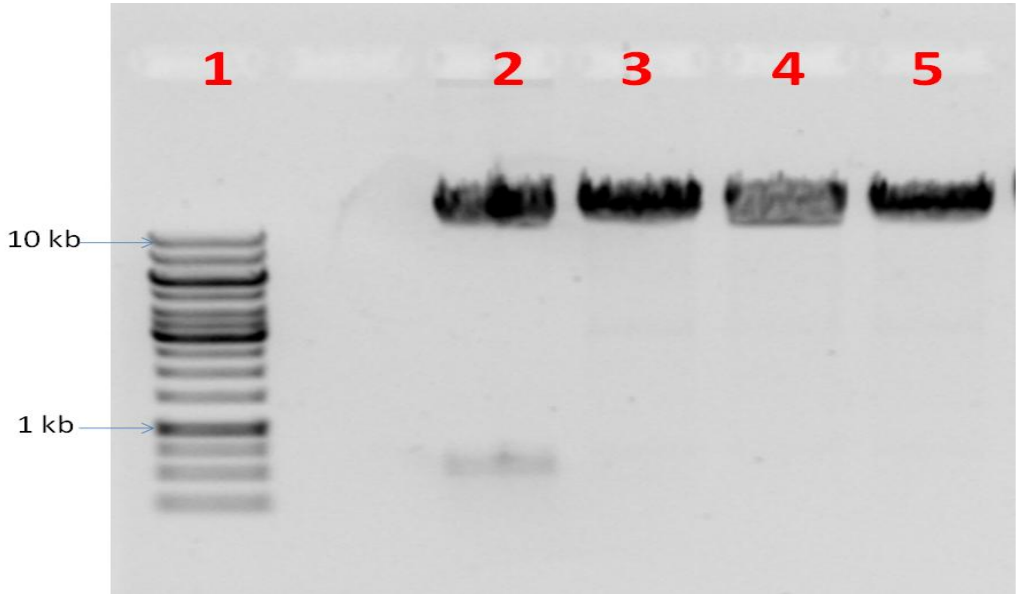

Figure 25: Agarose gel electrophoresis analysis of $Ca_v1.4$-L216R after restriction digest with SanDI (KfII). Lane 1: GenerulerTM 1Kb ladder of fermentas. Lane 2 is representing wild type of $Ca_v1.4$. Lanes 3, 4 and 5 are various samples of $Ca_v1.4$ α1-L216R mutant. 0.7% agarose gel, 4V/cm for 1 hour.

3.2 Isolation of Plasmid-DNA from agarose gel

The mutated Plasmid-DNA (Ca_v1.4 α1-G1339V and Cav1.4 α1-L216R) and Vector (pCIneo) were linearised with the enzymes of EcoRI/AgeI before the gel electrophoresis. Via gel electrophoresis the identified fragment (vector and plasmid) was removed with a scalpel from the gel and isolated.

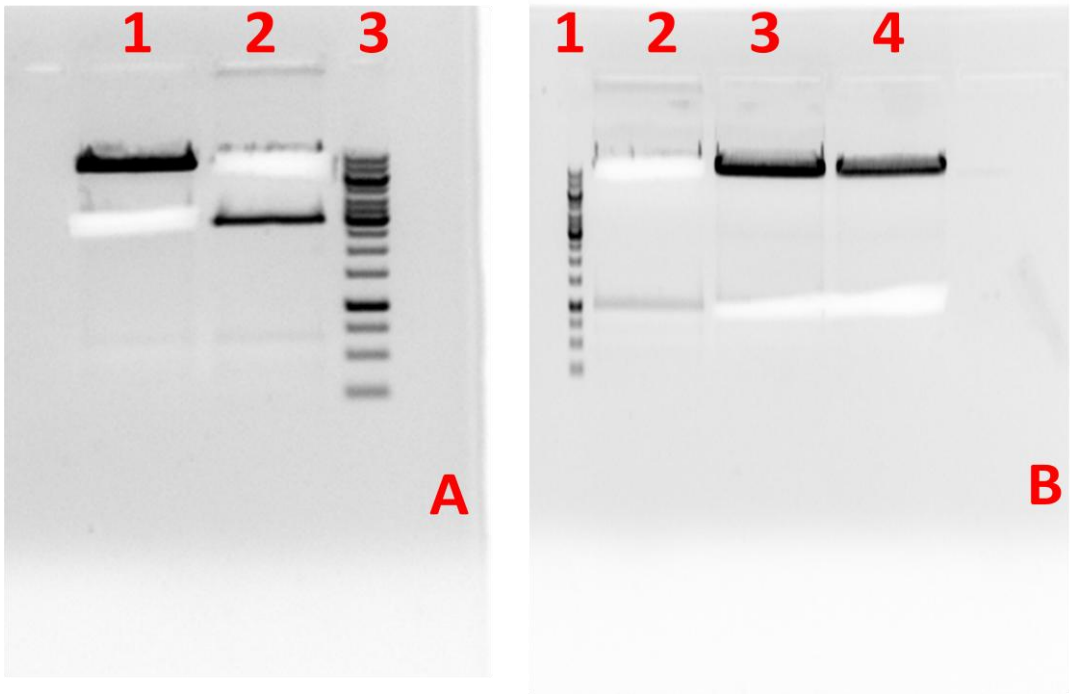

Figure 26: Purified plasmid-DNA and vector from an agarose. The holes on the gel are indicating the removed vector and plasmid**. A,** Ca_v1.4 α1-G1339V; Lane 1: Vector (pCIneo); Lane 2: Plasmid-DNA (Ca_v1.4 α1-G1339V), Lane 3: GenrulerTM 1Kb ladder (Fermentas); 0.8% agarose gel, 4V/cm for 1 hour. **B,** Ca_v1.4 α1-L216R; Lane 1: GenrulerTM 1Kb ladder (Fermentas); Lane 2: Vector (pCIneo); Lanes 3 and 4: The same Plasmid-DNA (Ca_v1.4 α1-L216R) as various samples; 0.8% agarose gel, 4V/cm for 1 hour.

3.3 Detection the protein expression of the WT ($Ca_v1.4$ α1) and various mutants of $Ca_v1.4$ α1

Human embryonic kidney tsA cells were transfected with $Ca_v1.4$ WT and mutants L216R, G1339V, G255E, G359R, R1819X, L849P and Mock, α1-subunits together with β3, α2δ1 subunit and also pUC and GFP. The protein expression by Western blotting after SDS-Page was quantified. $Ca_v1.4$ WT and mutants migrated with the expected molecular weight of 219 kDa.

The experiments tables e.g. the features of Western blot and transfection are shown figure 33 in appendix.

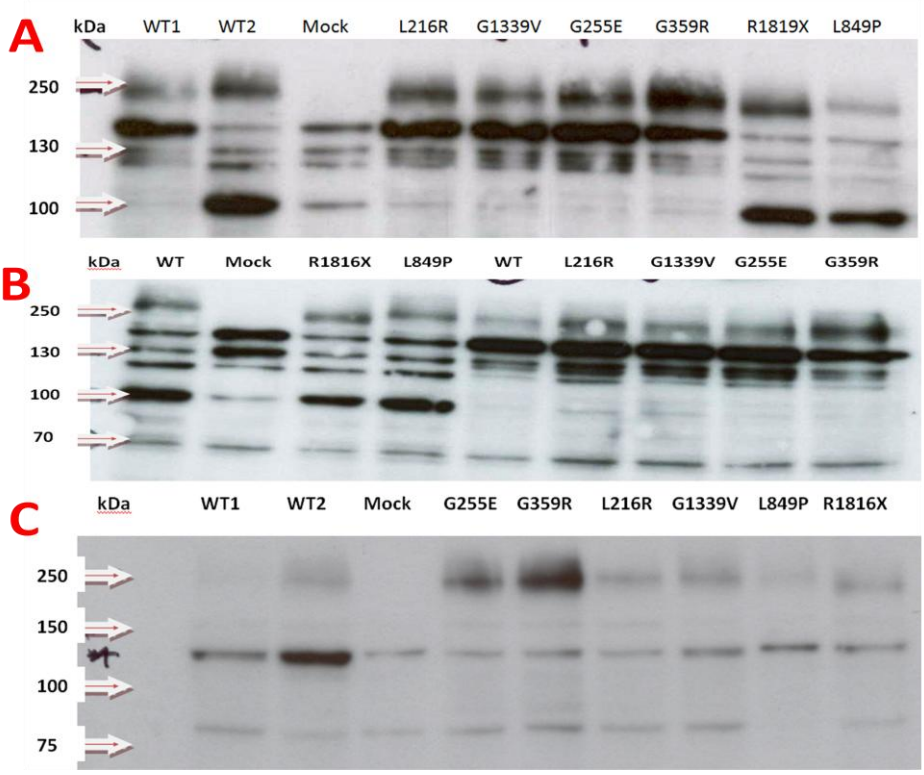

Figure 27: Heterologous expression of $Ca_v1.4$ WT and mutants α1-subunits in tsA-201 cells. 30 µg of each protein were blotted. For detection 1st Antibody Anti-Rabbit Pan α1 dilution for picture A, B 1:140 and for C 1:1500 were used and followed by 2nd Antibody Anti-Rabbit (Dilution 1:20000). A, B The used marker is PageRuler Plus Prostained Protein Ladder C, The used marker is the Kaleidoscope. The samples were run on 8% SDS-PAGE.

3.4 Enzymatic protein deglycosylation of $Ca_v1.4$ α1

$Ca_v1.4$ α1 WT was deglycosylated with deglycosylated methods that previously described in material and methods. The enzyme mixture is containing different enzymes; PNGase F, O-Glycoosidase, Neuraminidase, β1-4 Galactosidase, β-N-Acetylglucosaminidase (Biolabs New England Protocol P6039). Samples were incubated at 37°C for 4 hours. After that, the samples were loaded on the 8% SDS-PAGE (100V, 20 minutes then 180V, 1.5 hours) by electrophoresis. This was followed by transferring the Plasmid-DNA to the membrane. For detection 1st Antibody RbX Pan Alpha (Dilution 1:140) and 2nd Antibody anti Rabbit (Dilution 1:20000) were used one after another. 30 µg of $Ca_v1.4$ α1 WT and 50 µg of deglycosylation samples were loaded.

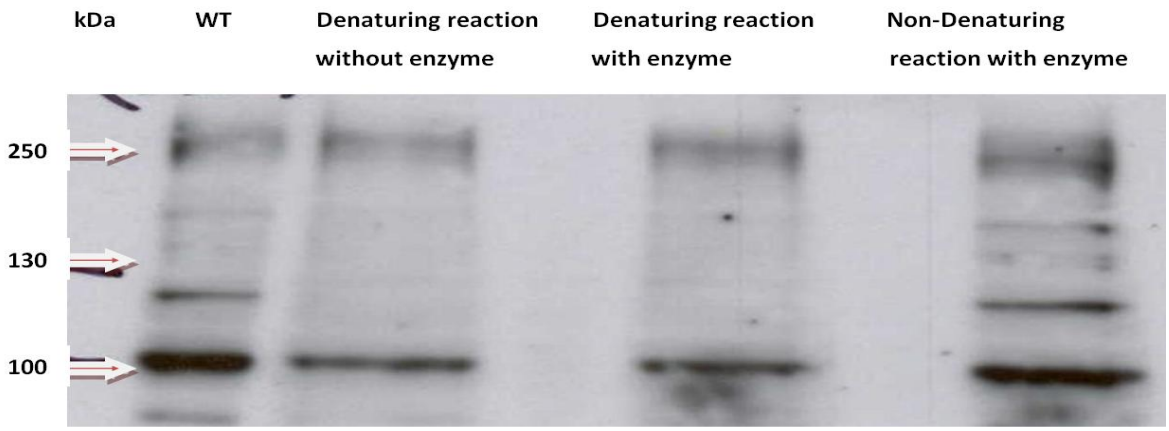

Figure 28: Enzymatic Protein Deglycosylation of the $Ca_v1.4$ α1 subunits Lane 1: 30 µg WT; Lane 2: 50 µg Cav1.4 α1 ; Lane 3: 50 µg Cav1.4 α1; Lane 4: 50 µg $Ca_v1.4$ α1. The used marker is PageRuler Plus Prostained Protein Ladder. On 8% SDS-PAGE, 1st Antibody Rabbit Anti-Pan α1 (Dilution 1:140) and 2nd Antibody Anti-Rabbit (Dilution 1:20000) were used one after another.

3.5 Enzymatic protein deglycosylation for internal control with α2δ Antibody

$Ca_v1.4$ α1 WT was also deglycosylated with deglycosylated methods that previously described in material and methods. The same enzyme mixture was used. For detection 1st antibody α2δ (Dilution 1:2000) and 2nd antibody anti mouse (Dilution 1:5000) were used one after another. 30 µg of $Ca_v1.4$ α1 WT and 50 µg of deglycosylation samples were loaded.

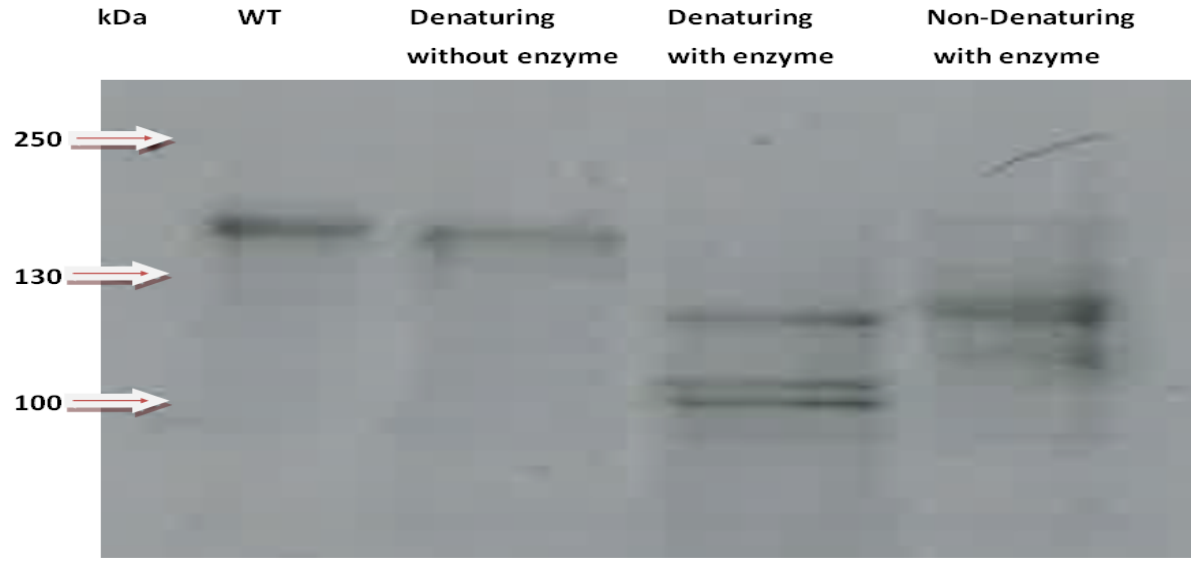

Figure 29: Detection of enzymatic protein deglycosylation of the $Ca_v1.4$ subunits α1 via 1st Antibody α2δ. Lane 1: 30 µg WT; Lane 2: 50 µg $Ca_v1.4$ α1; Lane 3: 50 µg $Ca_v1.4$ α1; Lane 4: 50 µg $Ca_v1.4$ α1.Ladder is PageRuler plus Prostained Protein Ladder; 8% SDS-PAGE; for detection 1st antibody α2δ (Dilution 1:2000) and 2nd Antibody Anti Mouse (Dilution 1:5000) were used.

3.6. Enzymatic protein deglycosylation of Bovine Fetuin for external control

Bovine Fetuin is provided as a deglycosylation substrate control (Biolabs New England Protocol P6039). Bovine Fetuin was deglycosylated with deglycosylation mixture too. For detection, the gel was stained with Coomassie-Blue-staining. If the Fetuin is deglycosylated the molecular weight is being reduced to approximately 50 kDa.

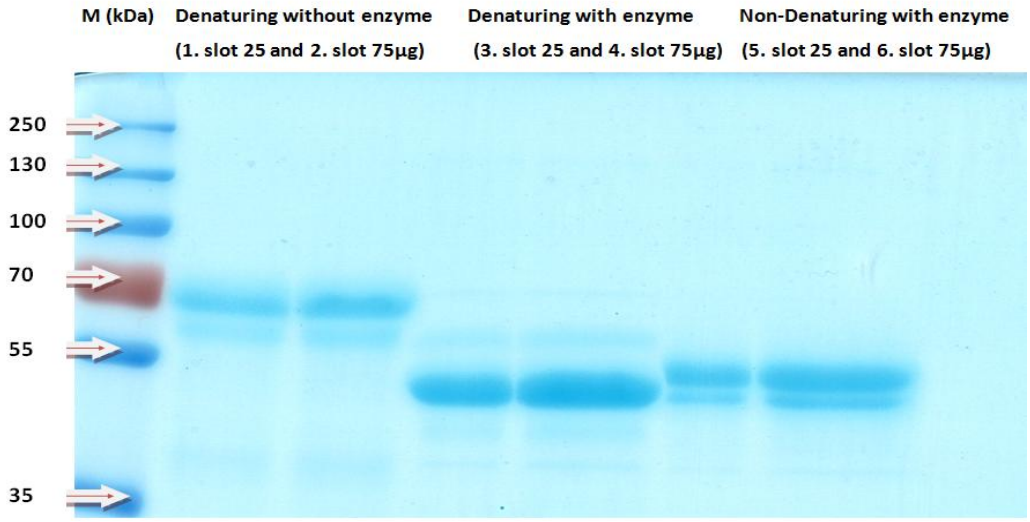

Figure 30: Enzymatic Protein Deglycosylation of the Bovine Fetuin. 25 µg and 75 µg Bovine Fetuin were deglycosylated, firstly by denaturing without enzymes, secondly by denaturing with enzymes, thirdly by non-denaturing with enzymes. 25 µg and 75 µg of the reactions were loaded on the 8% SDS-PAGE gel. Lane 1: PageRuler Plus Prostained Protein Ladder; for detection, the gel was stained with Coomassie-Blue-staining.

4. DISCUSSION

There are two types of CSNB which are mapped to varying loci of the X-chromosome. These are complete CSNB on Xp.11.4 and incomplete CSNB on Xp.11.23 (Bergen, ten Brink et al. 1996, Hardcastle, David-Gray et al. 1997).

Several mutations of the gene mapped to Xp11.23 in CACNA1F are the reason for CSNB2 (Bech-Hansen, Naylor et al. 1998, Strom, Nyakatura et al. 1998). This kind of night blindness is found primarily in males. This is due to the channel dysfunction of $Ca_v1.4$ being X-linked. If X-inactivation occurs in the CACNA1F gene it could be that heterozygote females are being affected too (Hemara-Wahanui, Berjukow et al. 2005). This is appearing by cellular mosaicism for healthy or mutant $Ca_v1.4$ channels.

$Ca_v1.4$ mutations are differentiated into two important groups based on their functional effects. One of these groups is loss-of-function where the Ca^{2+} influx is decreased or absent. The second group consists of gain-of-function mutations that increase Ca^{2+} influx, not always implying a better signaling.

In figure 31, the red boxes indicate the position of $Ca_v1.4$ α1-mutants of which I characterized my own constructed mutants (L216R, G1339V) with the mutants that were generated by the group Alexandra Koschak on their protein level via Western blotting.

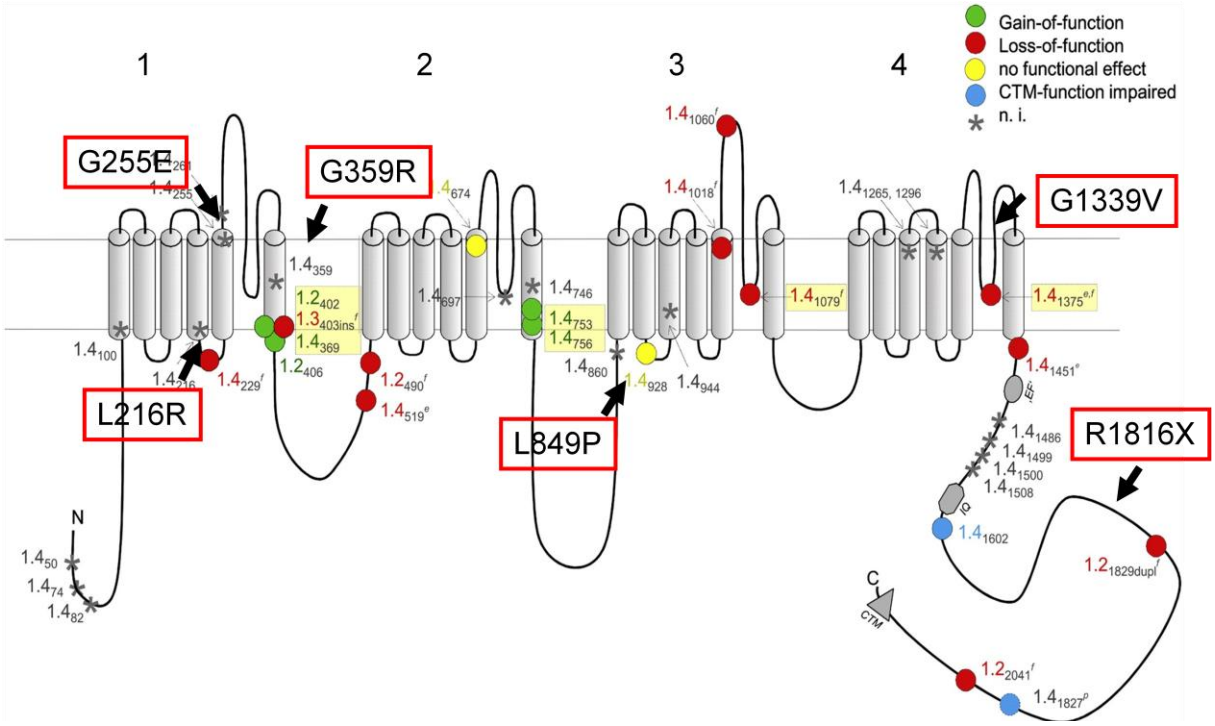

Figure 31: Shematic representation of the Ca^{2+} Channels $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ α1 subunits. The numbers refer to the position of the mutations in the corresponding LTCC (1.2, $Ca_v1.2$; 1.3, $Ca_v1.3$; 1.4, $Ca_v1.4$). The red boxes represent the $Ca_v1.4$ α1-mutants (L216R, G255E, G359R, L849P, G1339V and R1816) which are characterized on the protein level. $Ca_v1.4$ is composed of 4 domains with 6 transmembrane segments (S1-S6). S4, voltage sensor containing transmembrane segment; S5, inner pore forming transmembrane segment; S6, outer pore forming transmembrane segment. Figure from Stockner, T. and Koschak (2013), with slightly changes (Stockner and Koschak 2013).

Comparing the amount of the expressed proteins of the mutants to the expressed proteins of WT one could see that they were similar except for the LP mutants. It was observed in half of the Western blot experiments of LP mutants that they were less expressed compared to WT. In accordance with this finding Verena Burtscher and Klaus Schicker in the laboratory of Alexandra Koschak observed that the current density of the LP mutant was less than the WT. The current densities measured in pA/pF were −16.3 ± 1.6 (n = 38) and −2.5 ± 0.3 (n = 12) for WT and L849P, respectively ($p < 0.0001$; Ca^{2+}). The reduced current density might be explained by a reduced number of channels that are expressed at the plasma membrane due to a defect in channel trafficking from the endoplasmatic reticulum (ER) to the plasma membrane.

In the ER, glycosylation is used to monitor the status of protein folding. N-glycosylation often occurs co-translationally, in that the glycan is attached to the

nascent protein as it is being translated and transported into the ER. Therefore glycosylation acts as a quality control mechanism to ensure that only properly folded proteins are trafficked to the Golgi.

Glycosylation can be removed by using certain enzymes in this case a glycosylation mix containing PNGase F that specifically cleaves all N-linked oligosaccharides from glycoprotein. This reaction can be seen by SDS-PAGE with the electrophoretic mobility of the protein. Using this technique, one can suggest whether the protein is localized in the ER or at the plasma membrane. If the enzyme-mix induced cleavage, the mobility of the protein is expected to increase, showing a band lower than before, implying the protein to be in ER.

No reports on the (potential) glycosylation of $Ca_v1.4$ channels were found in the literature. To demonstrate biochemically, whether there are indeed fewer channels on the cell membrane or in the endoplasmic reticulum, I had to determine if the WT $Ca_v1.4$ is glycosylated in general or not. The glycosylation of the WT $Ca_v1.4$ α1 was not visible on the Nitrocellulose membranes (PVDF) (as shown in Figure 28).There may be two possibilities; either really there was no glycosylation of the WT or it was because of the kit, i.e. the enzyme mix could be deformed.

Therefore, I firstly carried out an external control with Bovine Fetuin which is an O- and N-glycoprotein and highly glycosylated (as shown in Figure 30). One can see that the Bovine Fetuin's weight decreased from 65 kDa to 50 kDa. Secondly, an internal control namely the accessory subunits α2δ-1, which was always contransfected with Cav1.4 and which is known to be glycosylated, was tested. As shown in Figure 29 the α2δ-1 has been successfully declycosylated.

Thus, we can conclude from these experiments that the enzyme-mix treatment works (both the external and internal positive controls showed successful deglycolsylation) but our protein of interest, the $Ca_v1.4$ channels are NOT glycosylated. Therefore we were not able to further use this assay to answer the question whether the functional phenotype of LP mutants results from poorly trafficked Cav1.4 to the cell membrane.

In the future therefore other methods to characterize plasma membrane proteins and their posttranslational modification should to be applied to answer this question. Biotinylation or mass spectrometric analyses are for instance two possible methods.

With biotinylation, membrane proteins on the plasma membrane are being labeled biochemically and separated from the ones in the intracellular compartments. When biotin and avidin communicate it can be useful for nonradioactive methods of purification, detection, immobilization, labelling, viral vector- targeting and drug targeting systems (Barat and Wu 2007). The biotinylating method is growing more and more popular and is one of the reasons for relevant advances in quantitative proteomics. As stated, biotinylation is useful for the analysis and characterization of how membrane proteins interact with other membrane proteins or intracellular adaptor and effector proteins combined with Western blotting and co-immunoprecipitation.

Another technique to characterize posttranslational modifications is mass spectrometry. Here, proteins are cut into peptides by using a specific protease such as Trypsin and loaded into a mass spectrometer that separates the sample into ions. The ions then run through a strong electric or magnetic field with speeds according to their mass-to-charge ratio, and so arrive at different time points at the detector. Many mass spectra arise, resulting in a typical fragmentation pattern which then can be searched for known masses in a database, resulting in a list of "hits", that is, proteins ranked by their likelihood to be the candidate. By choosing to look for N- or O-glycosylation in database search, glycosylated proteins can be detected in MS results, allowing distinguishing the surface and ER forms.

5. ABBREVIATIONS:

LTCC.....L-type Calcium Channel

VGCCs.....Voltage-gated calcium channels

CSNB..... congenital stationary night blindness

CSNB2……X-linked congenital stationary night blindness type 2

CORDX3…….X-linked cone rod dystrophy

AIED……Åland Island eye disease

XRD……X-linked retinal disorder

PCR.....Polymerase chain reaction

WT……...Wild type

HEK.....Human embryonic kidney

HVA.....High-voltage-activated

LVA.....Low-voltage-activated

BSA.....Bovine serum albumin

SDS-PAGE.....SDS-polyacrylamide gel electropheresis

IDV.....Integrated density value

ER.....Endoplasmic reticulum

ERGIC.....ER-Golgi intermediate compartment

nt.....Nucleotide

dH2O.....Distilled water

DHPs.....Dihydropyridines

Fw…….Forward

Rv…….Revers

RT.....Room temperature

dNTPs.....Desoxyribonukleosidtriphosphate

DMSO.....Dimethylsulfoxid

TBE.....Tris-borate-EDTA

TAE.....Tris-acetate-EDTA

EtBr.....Ethidiumbromid

DMEM.....Dulbecco`s Modified Eagle`s Medium

FBS.....Fetal Bovine Serum

PBS-EDTA.....Phosphate buffered saline-EDTA

GFP.....Green fluorescent protein

HEBS.....HEPES-buffered saline

APS.....Ammonium persulfate

TEMED.....N,N,N´,N´-tetramethylethylenediamine

DTT.....Dithiothreitol

NC.....Nitrocellulose

6. APPENDIX

6.1 Plasmid maps

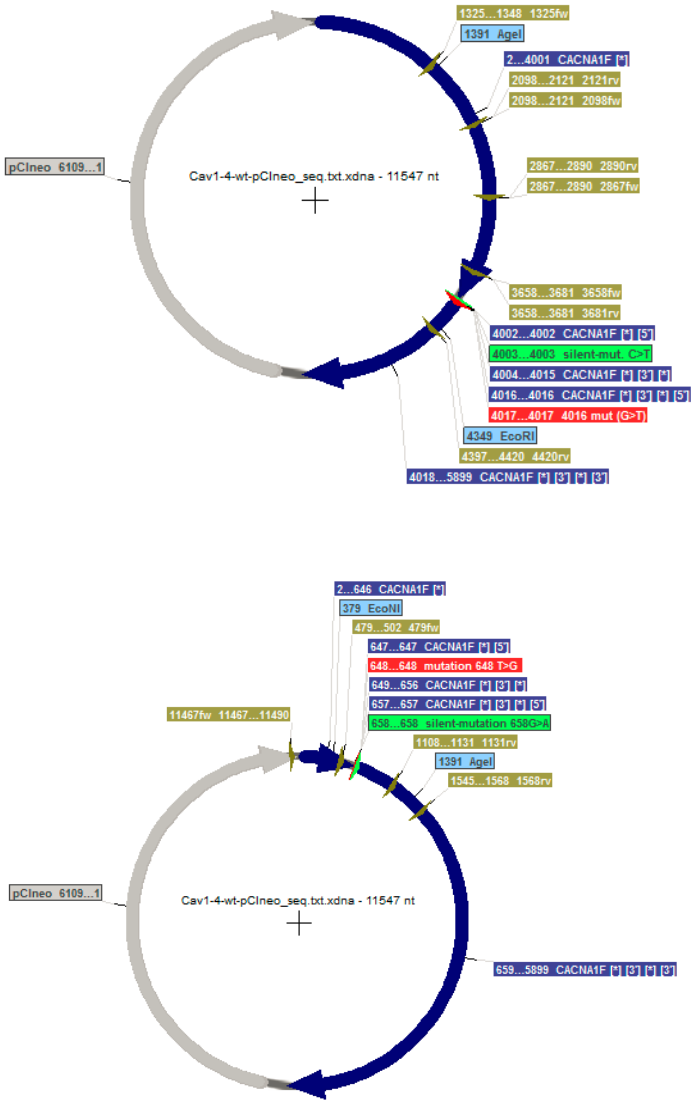

Figure 32: Plasmid maps (pCIneo) of $Ca_v1.4$ mutants G1339V and L216R. Established by Serial cloner DNA; Construct size: 11547 nucleotide (nt); grey: vector (pCIneo); dark blue: CaCNA1F; unique restriction sites are labeled light blue; nt position of silent mutants are labeled green; nt position of mutant are labeled red; nt position of forward (fw) and reverse (rv) sequencing primers are labeled brown. **A,** $Ca_v1.4$-G1339V plasmid: exchange of nt position from 4002 C to T for silent mutation and from 4016 G to T for desired mutation. A control plasmid was digested with AgeI and EcoRI, as manifested in materials and methods. **B,** $Ca_v1.4$-L216R plasmid: exchange of nt position from 658 G to A for silent mutation and from 648 T to G for desired mutation. Again, a control plasmid was digested, this time with AgeI and EcoNI.

6.2 Experiment Tables

Samples	Date of transfection	Date of Western Blot	Factor to Lowry-method	Analysis of Western Blot	Other annotations
Wt/retina, Wt/tsa, R1816X, L849P	06.09.2012	05.10.2012	51.27 (11.09.2012)	not observed	
Wt/2 ,Mock2, R1816X, L849P	18.09.2012	05.10.2012	51.27 (11.09.2012)	good	OK
Wt,Mock,L216R ,G1339V,G255E,G359R	27.09.2012	05.10.2012	51.27 (11.09.2012)	good	more expressed
Wt/1,L849P,R1816X	18.09.2012	08.11.2012	51.27 (11.09.2012)	good	OK
Wt,Mock,L216R ,G1339V,G255E,G359R	27.09.2012	08.11.2012	58.89 (03.10.2012)	good	only wt is not expressed.The other mutants are OK
Wt,G359R, G1339V	23.10.2012	16.11.2012	68.0	good	OK
R1816X, L849P	18.09.2012	16.11.2012	68.0	good	OK
Wt,Mock,L216R ,G1339V,G255E,G359R	29.10.2012	16.11.2012	68.0	good	G359R less expressed than wt and other mutants
WT1	16.11.2012	27.11.2012		good	
Wt2, Mock,L216R,G1339V, G255E,G359R, R1816X,L849P	22.11.2012	28.11.2012	68.0	good	LP less expressed
WT2 and Mock	16.11.2012	29.11.2012	68.0	good	more concentrated
WT1,L216R,G1339V, G255E,G359R, R1816X,L849P	22.11.2012	29.11.2012	68.0	good	
WT transfected 9 times, 1 was not ok					
Mock transfected 5 times					
R1816X transfected 4 times, thereof 3 good expressed.					
L849P transfected 4 times, thereof 2 good expressed.					
L216R transfected 4 times					
G1339V transfected 5 times					
G255E transfected 4 times					
G359R transfected 5 times					

Deglycosylation:					
Samples	Date of transfected	Date of Western Blot	Factor to lowry-method	Analysis of Western Blot	Other annotations
WT	23.10.2012	27.11.2012	68.0	good	No deglycosylation observed
Wt1,WT2	16.11.2012	29.11.2012	68.0	not good	No deglycosylation observed
WT 3	04.12.2012	05.12.2012	68.0	more concentrated	No deglycosylation observed
WT 4	08.12.2012	12.12.2012	68.0	Detected with α2δ:good	Deglycosylation observed
WT 4	08.12.2012	14.12.2012	68.0	Detected with α2δ: good, detected with anti pan not observed	

Figure 33: Names, dates and other annotations of held experiments

Curriculum Vitae

Sakine Korkmaz

sakinekorkmaz@hotmail.com

Education

1992 - 1998 High School in Istanbul/Turkey

Language of instruction Turkish and English

-Higher School Certificate

-Examination for University Entrance

1999 - 2002 Erciyes University in Kayseri/Turkey

2002 -2003 German Courses**;** Universitätslehrgang Vorstudienlehrgang UniStG from 01.10.2002 to 30.09.2003

2003-2006 University of Vienna; Diploma Study from 01.10.2003 to 11.07.2006

2006 University of Vienna Diploma Study; Genetics and Microbiology from 12.07.2006

Work and Research experience

September 2008

University of Vienna - Department of Physical Chemistry

Group: Dr. Georg Schmetterer: Molecularbiology of Cyanobacteria

Methods: Molecular Cloning, Electroporation, RT-PCR, Plasmid preparation, Agarose gel electrophoresis, Conjugation analysis, Mutagenesis analysis, Oxygraph measurement, Absorption of 14C-Glucose

Mai 2008

Max F. Perutz Laboratories, Department of Microbiology, Immunology and Genetics

Group: Dr. Alisher Touraev

Method: 1st part: Plant morphogenesis and embryogenesis in vitro.

2nd part: Techniques of plant transformation: Direct gene transfer, floral dip transformation and particle bombardment mediated gene transfer; Transient expression of screenable marker genes.

Mai – August 2011

Department of Pharmacology Medical University of Vienna

Group: Mg.Dr. Oliver Kudlacek

Method:

Basic molecular biology methods: DNA isolation and amplification (PCR), DNA sequencing, Molecular cloning, Electroporation,

Biochemical methods: SDS-Page

Microbiology methods: LAB culture, Aseptic technique

Mai –June 2012

Department of Pharmacology Medical University of Vienna

Group Univ.-Prof.Dr. Alexandra Koschak

Method: Molecular methods (Cloning, PCR etc.) and Cell biological methods (Introduction to cell culture, Transfection of eukaryotic cells)

SPECIAL SKILLS

Foreign Languages English: Fluently Spoken and Written

German: Fluently Spoken and Written

Turkish: Mother Tongue

Computer Skills MS Office

INTERESTS

Theatre, Painting, Travelling, Reading

7. BIBLIOGRAPHY

Antzelevitch, C., G. D. Pollevick, J. M. Cordeiro, O. Casis, M. C. Sanguinetti, Y. Aizawa, A. Guerchicoff, R. Pfeiffer, A. Oliva, B. Wollnik, P. Gelber, E. P. Bonaros, Jr., E. Burashnikov, Y. Wu, J. D. Sargent, S. Schickel, R. Oberheiden, A. Bhatia, L. F. Hsu, M. Haissaguerre, R. Schimpf, M. Borggrefe and C. Wolpert (2007). "Loss-of-function mutations in the cardiac calcium channel underlie a new clinical entity characterized by ST-segment elevation, short QT intervals, and sudden cardiac death." Circulation **115**(4): 442-449.

Baig, S. M., A. Koschak, A. Lieb, M. Gebhart, C. Dafinger, G. Nurnberg, A. Ali, I. Ahmad, M. J. Sinnegger-Brauns, N. Brandt, J. Engel, M. E. Mangoni, M. Farooq, H. U. Khan, P. Nurnberg, J. Striessnig and H. J. Bolz (2011). "Loss of Ca(v)1.3 (CACNA1D) function in a human channelopathy with bradycardia and congenital deafness." Nat Neurosci **14**(1): 77-84.

Barat, B. and A. M. Wu (2007). "Metabolic biotinylation of recombinant antibody by biotin ligase retained in the endoplasmic reticulum." Biomol Eng **24**(3): 283-291.

Bauer, C. S., M. Nieto-Rostro, W. Rahman, A. Tran-Van-Minh, L. Ferron, L. Douglas, I. Kadurin, Y. Sri Ranjan, L. Fernandez-Alacid, N. S. Millar, A. H. Dickenson, R. Lujan and A. C. Dolphin (2009). "The increased trafficking of the calcium channel subunit alpha2delta-1 to presynaptic terminals in neuropathic pain is inhibited by the alpha2delta ligand pregabalin." J Neurosci **29**(13): 4076-4088.

Bech-Hansen, N. T., M. J. Naylor, T. A. Maybaum, W. G. Pearce, B. Koop, G. A. Fishman, M. Mets, M. A. Musarella and K. M. Boycott (1998). "Loss-of-function mutations in a calcium-channel alpha1-subunit gene in Xp11.23 cause incomplete X-linked congenital stationary night blindness." Nat Genet **19**(3): 264-267.

Becker, J. M., G. A. Caldwell and E. A. Zachgo (1996). Biotechnology: A Laboratory Course, Elsevier Science.

Bergen, A. A., J. B. ten Brink, F. Riemslag, E. J. Schuurman, F. Meire, N. Tijmes and P. T. de Jong (1996). "Conclusive evidence for a distinct congenital stationary night blindness locus in Xp21.1." J Med Genet **33**(10): 869-872.

Berntson, A., W. R. Taylor and C. W. Morgans (2003). "Molecular identity, synaptic localization, and physiology of calcium channels in retinal bipolar cells." J Neurosci Res **71**(1): 146-151.

Bezanilla, F. (2008). "How membrane proteins sense voltage." Nat Rev Mol Cell Biol **9**(4): 323-332.

Boycott, K. M., T. A. Maybaum, M. J. Naylor, R. G. Weleber, J. Robitaille, Y. Miyake, A. A. Bergen, M. E. Pierpont, W. G. Pearce and N. T. Bech-Hansen (2001). "A summary of 20 CACNA1F mutations identified in 36 families with incomplete X-linked congenital stationary night blindness, and characterization of splice variants." Hum Genet **108**(2): 91-97.

Boycott, K. M., W. G. Pearce and N. T. Bech-Hansen (2000). "Clinical variability among patients with incomplete X-linked congenital stationary night blindness and a founder mutation in CACNA1F." Can J Ophthalmol **35**(4): 204-213.

Boycott, K. M., W. G. Pearce, M. A. Musarella, R. G. Weleber, T. A. Maybaum, D. G. Birch, Y. Miyake, R. S. Young and N. T. Bech-Hansen (1998). "Evidence for genetic heterogeneity in X-linked congenital stationary night blindness." Am J Hum Genet **62**(4): 865-875.

Burashnikov, E., R. Pfeiffer, H. Barajas-Martinez, E. Delpon, D. Hu, M. Desai, M. Borggrefe, M. Haissaguerre, R. Kanter, G. D. Pollevick, A. Guerchicoff, R. Laino, M. Marieb, K. Nademanee, G. B. Nam, R. Robles, R. Schimpf, D. D. Stapleton, S. Viskin, S. Winters, C. Wolpert, S. Zimmern, C. Veltmann and C. Antzelevitch (2010). "Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death." Heart Rhythm **7**(12): 1872-1882.

Catterall, W. A. (2000). "Structure and regulation of voltage-gated Ca2+ channels." Annu Rev Cell Dev Biol **16**: 521-555.

Catterall, W. A., E. Perez-Reyes, T. P. Snutch and J. Striessnig (2005). "International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels." Pharmacol Rev **57**(4): 411-425.

Cole, R. L., S. M. Lechner, M. E. Williams, P. Prodanovich, L. Bleicher, M. A. Varney and G. Gu (2005). "Differential distribution of voltage-gated calcium channel alpha-2 delta (alpha2delta) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia." J Comp Neurol **491**(3): 246-269.

De Jongh, K. S., C. Warner and W. A. Catterall (1990). "Subunits of purified calcium channels. Alpha 2 and delta are encoded by the same gene." J Biol Chem **265**(25): 14738-14741.

Dolphin, A. C. (2012). "Calcium channel auxiliary alpha2delta and beta subunits: trafficking and one step beyond." Nat Rev Neurosci **13**(8): 542-555.

Dunlap, K., J. I. Luebke and T. J. Turner (1995). "Exocytotic Ca2+ channels in mammalian central neurons." Trends Neurosci **18**(2): 89-98.

Hardcastle, A. J., Z. K. David-Gray, M. Jay, A. C. Bird and S. S. Bhattacharya (1997). "Localization of CSNBX (CSNB4) between the retinitis pigmentosa loci RP2 and RP3 on proximal Xp." Invest Ophthalmol Vis Sci **38**(13): 2750-2755.

Hemara-Wahanui, A., S. Berjukow, C. I. Hope, P. K. Dearden, S. B. Wu, J. Wilson-Wheeler, D. M. Sharp, P. Lundon-Treweek, G. M. Clover, J. C. Hoda, J. Striessnig, R. Marksteiner, S. Hering and M. A. Maw (2005). "A CACNA1F mutation identified in an X-linked retinal disorder shifts the voltage dependence of Cav1.4 channel activation." Proc Natl Acad Sci U S A **102**(21): 7553-7558.

Hofmann, F., M. Biel and V. Flockerzi (1994). "Molecular basis for Ca2+ channel diversity." Annu Rev Neurosci **17**: 399-418.

Hope, C. I., D. M. Sharp, A. Hemara-Wahanui, J. I. Sissingh, P. Lundon, E. A. Mitchell, M. A. Maw and G. M. Clover (2005). "Clinical manifestations of a unique X-linked retinal disorder in a large New Zealand family with a novel mutation in CACNA1F, the gene responsible for CSNB2." Clin Experiment Ophthalmol **33**(2): 129-136.

Huguenard, J. R. (1996). "Low-threshold calcium currents in central nervous system neurons." Annu Rev Physiol **58**: 329-348.

Jalkanen, R., N. T. Bech-Hansen, R. Tobias, E. M. Sankila, M. Mantyjarvi, H. Forsius, A. de la Chapelle and T. Alitalo (2007). "A novel CACNA1F gene mutation causes Aland Island eye disease." Invest Ophthalmol Vis Sci **48**(6): 2498-2502.

Jalkanen, R., M. Mantyjarvi, R. Tobias, J. Isosomppi, E. M. Sankila, T. Alitalo and N. T. Bech-Hansen (2006). "X linked cone-rod dystrophy, CORDX3, is caused by a mutation in the CACNA1F gene." J Med Genet **43**(8): 699-704.

Josephson, I. R. and G. Varadi (1996). "The beta subunit increases Ca2+ currents and gating charge movements of human cardiac L-type Ca2+ channels." Biophys J **70**(3): 1285-1293.

Kamp, T. J., M. T. Perez-Garcia and E. Marban (1996). "Enhancement of ionic current and charge movement by coexpression of calcium channel beta 1A subunit with alpha 1C subunit in a human embryonic kidney cell line." J Physiol **492 (Pt 1)**: 89-96.

Klugbauer, N., S. Dai, V. Specht, L. Lacinova, E. Marais, G. Bohn and F. Hofmann (2000). "A family of gamma-like calcium channel subunits." FEBS Lett **470**(2): 189-197.

Koschak, A., D. Reimer, D. Walter, J. C. Hoda, T. Heinzle, M. Grabner and J. Striessnig (2003). "Cav1.4alpha1 subunits can form slowly inactivating dihydropyridine-sensitive L-type Ca2+ channels lacking Ca2+-dependent inactivation." J Neurosci **23**(14): 6041-6049.

Kotturi, M. F. and W. A. Jefferies (2005). "Molecular characterization of L-type calcium channel splice variants expressed in human T lymphocytes." Mol Immunol **42**(12): 1461-1474.

Kourennyi, D. E. and S. Barnes (2000). "Depolarization-induced calcium channel facilitation in rod photoreceptors is independent of G proteins and phosphorylation." J Neurophysiol **84**(1): 133-138.

Legler, G., C. M. Muller-Platz, M. Mentges-Hettkamp, G. Pflieger and E. Julich (1985). "On the chemical basis of the Lowry protein determination." Anal Biochem **150**(2): 278-287.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). "Protein measurement with the Folin phenol reagent." J Biol Chem **193**(1): 265-275.

Marcantoni, A., D. H. Vandael, S. Mahapatra, V. Carabelli, M. J. Sinnegger-Brauns, J. Striessnig and E. Carbone (2010). "Loss of Cav1.3 channels reveals the critical role of L-type and BK channel coupling in pacemaking mouse adrenal chromaffin cells." J Neurosci **30**(2): 491-504.

McRory, J. E., J. Hamid, C. J. Doering, E. Garcia, R. Parker, K. Hamming, L. Chen, M. Hildebrand, A. M. Beedle, L. Feldcamp, G. W. Zamponi and T. P. Snutch (2004). "The CACNA1F gene encodes an L-type calcium channel with unique biophysical properties and tissue distribution." J Neurosci **24**(7): 1707-1718.

Morgans, C. W. (2001). "Localization of the alpha(1F) calcium channel subunit in the rat retina." Invest Ophthalmol Vis Sci **42**(10): 2414-2418.

Murakami, M., O. Nakagawasai, S. Fujii, K. Kameyama, S. Murakami, S. Hozumi, A. Esashi, R. Taniguchi, T. Yanagisawa, K. Tan-no, T. Tadano, K. Kitamura and K. Kisara (2001). "Antinociceptive action of amlodipine blocking N-type Ca2+ channels at the primary afferent neurons in mice." Eur J Pharmacol **419**(2-3): 175-181.

Neely, A., X. Wei, R. Olcese, L. Birnbaumer and E. Stefani (1993). "Potentiation by the beta subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel." Science **262**(5133): 575-578.

Newton, R. A., S. Bingham, P. C. Case, G. J. Sanger and S. N. Lawson (2001). "Dorsal root ganglion neurons show increased expression of the calcium channel alpha2delta-1 subunit following partial sciatic nerve injury." Brain Res Mol Brain Res **95**(1-2): 1-8.

Olson, B. J. and J. Markwell (2007). "Assays for determination of protein concentration." Curr Protoc Protein Sci **Chapter 3**: Unit 3 4.

Olson, P. A., T. Tkatch, S. Hernandez-Lopez, S. Ulrich, E. Ilijic, E. Mugnaini, H. Zhang, I. Bezprozvanny and D. J. Surmeier (2005). "G-protein-coupled receptor modulation of striatal CaV1.3 L-type Ca2+ channels is dependent on a Shank-binding domain." J Neurosci **25**(5): 1050-1062.

Perez-Reyes, E. (2003). "Molecular physiology of low-voltage-activated t-type calcium channels." Physiol Rev **83**(1): 117-161.

Platzer, J., J. Engel, A. Schrott-Fischer, K. Stephan, S. Bova, H. Chen, H. Zheng and J. Striessnig (2000). "Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca2+ channels." Cell **102**(1): 89-97.

Protti, D. A. and I. Llano (1998). "Calcium currents and calcium signaling in rod bipolar cells of rat retinal slices." J Neurosci **18**(10): 3715-3724.

Qin, N., S. Yagel, M. L. Momplaisir, E. E. Codd and M. R. D'Andrea (2002). "Molecular cloning and characterization of the human voltage-gated calcium channel alpha(2)delta-4 subunit." Mol Pharmacol **62**(3): 485-496.

Singer, D., M. Biel, I. Lotan, V. Flockerzi, F. Hofmann and N. Dascal (1991). "The roles of the subunits in the function of the calcium channel." Science **253**(5027): 1553-1557.

Splawski, I., K. W. Timothy, N. Decher, P. Kumar, F. B. Sachse, A. H. Beggs, M. C. Sanguinetti and M. T. Keating (2005). "Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations." Proc Natl Acad Sci U S A **102**(23): 8089-8096; discussion 8086-8088.

Splawski, I., K. W. Timothy, L. M. Sharpe, N. Decher, P. Kumar, R. Bloise, C. Napolitano, P. J. Schwartz, R. M. Joseph, K. Condouris, H. Tager-Flusberg, S. G. Priori, M. C. Sanguinetti and M. T. Keating (2004). "Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism." Cell **119**(1): 19-31.

Stella, S. L., Jr., E. J. Bryson and W. B. Thoreson (2002). "A2 adenosine receptors inhibit calcium influx through L-type calcium channels in rod photoreceptors of the salamander retina." J Neurophysiol **87**(1): 351-360.

Stockner, T. and A. Koschak (2013). "What can naturally occurring mutations tell us about Ca(v)1.x channel function?" Biochim Biophys Acta **1828**(7): 1598-1607.

Striessnig, J. (1999). "Pharmacology, structure and function of cardiac L-type Ca(2+) channels." Cell Physiol Biochem **9**(4-5): 242-269.

Striessnig, J., H. J. Bolz and A. Koschak (2010). "Channelopathies in Cav1.1, Cav1.3, and Cav1.4 voltage-gated L-type Ca2+ channels." Pflugers Arch **460**(2): 361-374.

Striessnig, J., M. Grabner, J. Mitterdorfer, S. Hering, M. J. Sinnegger and H. Glossmann (1998). "Structural basis of drug binding to L Ca2+ channels." Trends Pharmacol Sci **19**(3): 108-115.

Strom, T. M., G. Nyakatura, E. Apfelstedt-Sylla, H. Hellebrand, B. Lorenz, B. H. Weber, K. Wutz, N. Gutwillinger, K. Ruther, B. Drescher, C. Sauer, E. Zrenner, T. Meitinger, A. Rosenthal and A. Meindl (1998). "An L-type calcium-channel gene mutated in incomplete X-linked congenital stationary night blindness." Nat Genet **19**(3): 260-263.

Tarentino, A. L. and T. H. Plummer, Jr. (1994). "Enzymatic deglycosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from Flavobacterium meningosepticum." Methods Enzymol **230**: 44-57.

Taylor, W. R. and C. Morgans (1998). "Localization and properties of voltage-gated calcium channels in cone photoreceptors of Tupaia belangeri." Vis Neurosci **15**(3): 541-552.

Van Petegem, F. and D. L. Minor, Jr. (2006). "The structural biology of voltage-gated calcium channel function and regulation." Biochem Soc Trans **34**(Pt 5): 887-893.

Varki, A. (1993). "Biological roles of oligosaccharides: all of the theories are correct." Glycobiology **3**(2): 97-130.

von Gersdorff, H. and G. Matthews (1996). "Calcium-dependent inactivation of calcium current in synaptic terminals of retinal bipolar neurons." J Neurosci **16**(1): 115-122.

Wheeler, D. B., A. Randall, W. A. Sather and R. W. Tsien (1995). "Neuronal calcium channels encoded by the alpha 1A subunit and their contribution to excitatory synaptic transmission in the CNS." Prog Brain Res **105**: 65-78.

Wilkinson, M. F. and S. Barnes (1996). "The dihydropyridine-sensitive calcium channel subtype in cone photoreceptors." J Gen Physiol **107**(5): 621-630.

Wutz, K., C. Sauer, E. Zrenner, B. Lorenz, T. Alitalo, M. Broghammer, M. Hergersberg, A. de la Chapelle, B. H. Weber, B. Wissinger, A. Meindl and C. M. Pusch (2002). "Thirty distinct CACNA1F mutations in 33 families with incomplete type of XLCSNB and Cacna1f expression profiling in mouse retina." Eur J Hum Genet **10**(8): 449-456.

Xu, H. P., J. W. Zhao and X. L. Yang (2002). "Expression of voltage-dependent calcium channel subunits in the rat retina." Neurosci Lett **329**(3): 297-300.

Yamaguchi, H., M. Hara, M. Strobeck, K. Fukasawa, A. Schwartz and G. Varadi (1998). "Multiple modulation pathways of calcium channel activity by a beta subunit. Direct evidence of beta subunit participation in membrane trafficking of the alpha1C subunit." J Biol Chem **273**(30): 19348-19356.

Protein Quantitation. Taken from: http://www.labome.com/method/Protein-Quantitation.html (27.08.2013)

New England BioLabs. Taken from: https://www.neb.com/products/p6039-protein-deglycosylation-mix (22.08.2013)