Shear Force Activation of the Epithelial Sodium Channel (ENaC): Role of the β- and γ-Subunits

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Abstract

Mechanotransduction describes the ability of a living organism to sense mechanical forces that are caused through changes in the environment. It is a complex mechanism in which mechanical forces are converted into a cellular signal. Ion channels are important molecules for mechanotransduction. Epithelial sodium channel (ENaC) is one channel of the DEG/ENaC superfamily, mainly expressed in the lung, kidney and vasculature and is known to be important for blood pressure regulation. In the kidney and vasculature ENaC are continuously exposed to mechanical forces such as shear force (SF) caused by the urine or blood flow. Canonical ENaC is made up of three homologous subunits known as α, β, and γ which form a heterotrimeric channel. Till today, little is known about how single subunits and different combinations of these ENaC subunits respond to SF. Previous studies on the mechanosensitive channels of the nematode Caenorhabditis elegans (C. elegans) demonstrated that a linkage between the extracellular domain of the channel and the extracellular matrix (ECM) is important for sensing mechanical forces. The underlying molecular mechanism governing how ENaC senses SF remains unknown. To answer this question research in the Fronius lab derived a model, where N-linked glycans of glycosylated asparagines are tethered to the extracellular matrix to facilitate SF sensation and mechanotransduction. Therefore the aims of my PhD thesis were:

1) To investigate whether individual subunits of ENaC respond to SF. Electrophysiological experiments were performed by expressing (α, β, γ, or δ-subunit) as homotrimeric and co-expressing (αβ, αγ, βγ, δβ, or δγ-subunits) as heterotrimeric ENaC in Xenopus laevis oocytes followed by the determination of channel activity in response to SF.
2) To investigate whether N-linked glycans of glycosylated asparagines of the β- and γ-ENaC subunits are important for sensing SF. N-glycans of all ENaC subunits were enzymatically removed by PNGaseF and glycosylated asparagines removed in the β- and γ-subunits.

The main findings from aim 1 were that homotrimeric α-, β-, δ-ENaC expressed in oocytes can form functional channels that respond to SF, whereas γ-ENaC cannot. The co-expression of two ENaC subunits αβ-, αγ-, βγ-, δβ- and δγ-ENaC can form heterotrimeric functional channels that respond to SF. However, this finding was accompanied by decreased amiloride-sensitive currents compared to wild-type control channels (αβγ- or δβγ-ENaC), indicating an impaired trafficking/maturation of these channels.

The β-ENaC subunit was observed to consistently weaken the SF response when co-expressed with α-, δ- and γ-ENaC, identifying, for the first time, a modulatory role of β-ENaC subunit for SF activation.

The presence of the γ-ENaC subunit was observed to constantly increase the SF response when co-expressed with α- and δ-ENaC subunit. This increased response to SF was noted in the heterotrimeric αγ- and δγ-ENaC when compared to control αβγ- or δβγ-ENaC. These findings support the notion that γ-ENaC subunit enhances the ability of ENaC to sense SF. This was further confirmed by the elimination of the decreased SF response of homotrimeric β-ENaC, when the β-subunit was co-expressed with the γ-subunit.

In the second part of my thesis (aim 2) I observed that removal of N-glycans by enzymatic degradation resulted in a reduction in the measured SF response. This showed that N-glycans play an important role for SF activation of ENaC. In contrast to my hypothesis, removal of a single or multiple glycosylated asparagines
In conclusion, these results show that single subunits of ENaC expressed in oocytes as homotrimeric or heterotrimeric channels can form functional channels that respond to SF. This indicates that the β- and γ-ENaC subunit are important for the SF response. Changes in one of the subunits may affect the overall ability of ENaC to respond to SF. Furthermore, N-glycans are also important for this process. N-glycans of the β- and γ-ENaC subunit might have distinct functions that are involved in stabilisation, gating kinetics, trafficking and SF sensation of ENaC. This could be a new role for N-glycans in mechanotransduction and a new mechanism for how ENaC senses SF.
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<tr>
<td>aaseq</td>
<td>Amino acid sequence</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid-sensing ion channel</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeat</td>
</tr>
<tr>
<td>CulORi</td>
<td>Culture oocyte Ringer solution</td>
</tr>
<tr>
<td>DEG</td>
<td>Degenerin</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dubs</td>
<td>Deubiquitinating enzymes</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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PHA-1  Pseudohypoaldosteronism type 1
Po  Open probability
SEM  Standard error of mean
SF  Shear force
SNARE  Soluble N-ethylmaleimide -sensitive factor attachment protein receptor
SOC-medium  Super optimal broth with catabolite repression medium
t_c  Mean closed time
TEVC  Two electrode voltage-clamp
TMD  Transmembrane domain
t_o  Mean open time
TREK-1  Two pore domain potassium channel 1
TRPC1  Transient receptor potential channel 1
VSMC  Vascular smooth muscle cells
I_{M,ENaC}  ENaC-mediated amiloride sensitive current
min.  Minutes
h  Hour
Na+/K+ ATPase  Sodium potassium ATPase
1 Introduction
**1 Mechanotransduction**

The ability to sense mechanical forces that are caused through changes in the environment is an essential process in all living organisms. This ability to convert a mechanical stimulus into a cellular signal, is a highly conserved evolutionary feature that is called mechanotransduction (Martinac, 2004). Mechanotransduction is the conversion of mechanical forces into electrical and/or chemical signals which allows cells to perceive their mechanical environment and initiate an appropriate response (Hamill & Martinac, 2001; Martinac, 2004). In a physiological manner, mechanotransduction is important for a wide spectrum of physiological processes such as touch sensation (Lumpkin & Caterina, 2007), hearing (Howard & Hudspeth, 1988), proprioception (Woo et al., 2015) and blood pressure regulation (Shi & Tarbell, 2011). Hearing, for example, relies on mechanotransduction in the inner ear, where mechanical forces caused by the sound waves are transmitted through the fluid and sensed by highly specialised cells. In these cells mechanosensitive ion channels transduce the force into electrical signals (Howard & Hudspeth, 1988). At the cellular level studies have shown that mechanical forces are important to regulate cell growth, migration, differentiation, proliferation, electrolyte homeostasis and apoptosis (Vogel & Sheetz, 2006; Jaalouk & Lammerding, 2009; Weinbaum et al., 2010; Rennier & Ji, 2013). Abnormal mechanotransduction in cells, is associated with severe diseases such as hypertension (Drummond, 2012), atherosclerosis (Gimbrane et al., 2000) and cancer (Qazi et al., 2013). The sensation and transduction of mechanical forces due to mechanosensitive ion channels is suggested to be an important mechanism of cellular mechanotransduction (Martinac, 2004).
Mechanical forces are distinguished into two different types, mainly due to the direction from which they act on objects. In the human body, two major mechanical forces relevant for mechanotransduction are (1) pressure, and (2) shear force (SF) (Price et al., 2000; Bundey, 2007; Olsen et al., 2011; Shi et al., 2013).

(1) Pressure is a physical force that acts perpendicular to a surface area and leads to a deformation of tissues, cells and their membranes. This is apparent for instance in the vasculature, where blood present within the vessels by the pumping activity of the heart exerts pressure on the vessel walls. The pressure causes deformation (e.g. stretch) that can activate mechanosensitive ion channels which convert mechanical stimuli into intracellular signals (Bundey, 2007; Lumpkin & Caterina, 2007).

(2) SF is a physical force that is defined by molecules moving parallel, along a surface area and exerting force onto the surface of the cells. This is apparent again within the vasculature and other tubular structures (e.g. kidney tubules), where the flow of fluid over the surface of the endothelial and epithelial cells causes SF. SF mediates different cellular mechanisms and plays an important role in numerous physiological processes such as blood pressure regulation and alveolar fluid layer regulation (Satlin et al., 2001; Carattino et al., 2004; Althaus et al., 2007; Drummond, 2009; Althaus et al., 2011). This fluid movement over cells is tightly regulated via an interaction of a variety of ion channels, carriers and ion pumps, which facilitate the movement of ions along an electrochemical gradient. The movement of for example Na\(^+\), through the epithelial sodium channel (ENaC) and the sodium potassium ATPase (Na\(^+\)/K\(^+\)-ATPase), generates a transepithelial osmotic gradient that facilitates the movement of water across the epithelia (Koefoed-Johnsen & Ussing, 1958; Garty & Palmer, 1997). Proteins forming ENaC
belong to the Degenerin/Epithelial sodium channel (DEG/ENaC) protein superfamily. Members of this family are absent in plants, yeast, fungi but can be found in invertebrates such as *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster* and in vertebrates, including mammals (Kellenberger & Schild, 2002; Goodman & Schwarz, 2003). The next paragraph will focus on the function and structure of the DEG/ENaC protein superfamily.

### 1.1 The Degenerin/Epithelial sodium channel (DEG/ENaC) protein superfamily

The name of the DEG/ENaC family derived from the first identified degenerin (DEG) protein found in *C. elegans* and the mammalian epithelial sodium channel (ENaC) (Chalfie & Wolinsky, 1990; Driscoll & Chalfie, 1991; Canessa *et al.*, 1993; Lingueglia *et al.*, 1993; Canessa *et al.*, 1994b). DEG/ENaC proteins share similarities in their amino acid sequence and membrane topology. They have short intracellular N- and C-terminal domains, two membrane-spanning regions and a large extracellular loop containing cysteine rich domains (Canessa *et al.*, 1994a; Renard *et al.*, 1994; Snyder *et al.*, 1994; Benos *et al.*, 1995). The proteins have a length of approximately 550 to 950 amino acids and a molecular weight of approximately 80 to 95 kDa (Garty & Palmer, 1997; Tavernarakis & Driscoll, 2000, 2001). Channels formed by proteins of the DEG/ENaC superfamily are homo- and hetero-trimeric voltage-independent cation channels that can be blocked by amiloride, benzamil and phenamil (Cuthbert & Edwardson, 1979; Schild *et al.*, 1997; Benos & Stanton, 1999).

The activation profiles of the channels differ depending on their functionality in cells and species. Vertebrate ENaC in Na\(^+\) absorbing epithelia, for instance are
constitutively active (Palmer & Frindt, 1986; Canessa et al., 1994b) whilst DEG channels of *C. elegans*, called MEC (mechanosensitive channel related) are activated through membrane deformation by touch (Goodman & Schwarz, 2003; Eastwood & Goodman, 2012). Another sub-class of the DEG/ENaC superfamily is the Acid-sensing ion channels (ASICs), ubiquitously expressed in the nervous system of vertebrates and activated by ligands such as protons (Jasti et al., 2007). The close relationship of members of the DEG/ENaC superfamily is demonstrated by studies that show that sequence substitution of different family members can form functional channels (Hong & Driscoll, 1994). Hong & Driscoll (1994) demonstrated that substitution of the second transmembrane domain between MEC-4 and αENaC, can rescue a touch insensitive mutation that was identified in *C. elegans* MEC-4 (Hong & Driscoll, 1994; Waldmann et al., 1995b). This study indicated that mechanosensitivity might be an inherent functional property of the protein family.

Studies in *C. elegans* revealed that the channels are part of a mechanosensitive receptor complex and their activation involved the extracellular matrix (ECM). This complex consists of a connection between the mechanosensitive channel to the ECM and to the cytoskeleton (Ernstrom & Chalfie, 2002; Chalfie, 2009). The ECM is an organised network surrounding cells and tissues and consists of a variety of extracellular molecules (Theocharis et al., 2016). ECM macromolecules and their diverse composition differ between tissues (Alberts et al., 2008). ECMs can become rock-hard by calcification in teeth and bones, transparent in the cornea of the eye, mechanically resistant in tendons to withstand enormous tensile strength, or flexible as evident in the skin (Alberts et al., 2008).
1.2 Molecular structure of ECMs

To maintain a healthy tissue integrity and cell structure, cells are in direct contact with the ECMs. ECMs are formed by various molecules such as proteoglycans (PGs) and glycosaminoglycans (GAGs) and proteins such as collagen, elastin and fibronectin (Alberts et al., 2008; Jarvelainen et al., 2009; Frantz et al., 2010; Schaefer & Schaefer, 2010). Fibril-forming collagen types such as collagen I and collagen II, are one of the main components of ECMs with an estimated constitution of up to 30% of total ECM mass (Frantz et al., 2010). Elastin interacts with collagens and provides a recoil to tissues that are exposed repeatedly to stretch (Wise & Weiss, 2009). Fibronectin and laminin are adhesive glycoproteins which attach cells to the ECM. They are important in organising the interstitium, connecting cells with collagen fibres and function as an extracellular mechano-regulator (Smith et al., 2007). PGs form a highly hydrated, gel-like ground substance in which fibrous proteins are embedded. This large fibrillar scaffold in tissues ensures buffering, hydration, binding and force-resistance abilities (Frantz et al., 2010) as well as enabling the rapid diffusion of nutrients, metabolites and hormones between the blood and cell tissue (Alberts et al., 2008). An important GAG of ECMs is hyaluronic acid which provides cells with the ability to withstand physical forces by absorbing water, generating a counteracting turgor (Alberts et al., 2008).

1.3 Role of the ECM in mechanotransduction of DEG/ENaC channels

Due to the interactions between cells and their ECMs it is known that cells can sense and perceive their surroundings (Schwartz, 2010; Freedman et al., 2015;
Theocharis et al., 2016). It was previously shown that the glycocalyx, a specific form of ECM, is associated with mechanotransduction (Pahakis et al., 2007). It covers the luminal surface of endothelial cells and consists of glycolipids and glycoproteins (Li & Bonventre, 2016). Evidence suggested that the ECM is an important mechanotransducer but little is known about how mechanical forces such as SF are sensed and transduced into a cellular signal.

Over the last decade, growing evidence has shown that ECMs play an important role in mechanosensation. The first evidence for an involvement of extracellular tethers in mechanotransduction derives from studies done in C. elegans and vertebrate hair cells (Gillespie & Walker, 2001; Chalfie, 2009). Martin Chalfie and colleagues provided a hint that an interaction between ion channels and their ECM was important to sense mechanical forces (Du et al., 1996). They showed that specific genes (Mec-5 and Mec-9) of C. elegans encode putative extracellular proteins that might form an extracellular tether for mechanosensory channels (Du et al., 1996). Other evidence suggested that channels in vertebrate hair cells are tethered via their extracellular tip links to adjacent stereocilia and to the actin filament of the internal cytoskeleton (Pickles et al., 1984; Gillespie & Walker, 2001). Several years later another study of Chalfie and colleagues showed more evidence that a tethering of degenerin channels with surrounding ECM is necessary for mechanical sensation (Emtage et al., 2004). These studies underline that a tether between ion channels and their ECM might be an important mechanism to sense mechanical forces. However, the molecular structure of this tether remains unclear. A recent study from the Fronius lab provided evidence that glycosylated asparagines establish a connection to the ECM via their N-glycans (Knoepp et al., 2017). The potential role of N-glycans as molecules that facilitate interactions with their ECM is supported by studies showing that N-glycans facilitate cell-cell and
2 The Epithelial Sodium Channel

2.1 The stoichiometry of ENaC

Canonical ENaC consists of three homologous subunits in a combination of αβγ or δβγ forming a functional trimeric channel (Canessa et al., 1994b; Waldmann et al., 1995a; Fyfe & Canessa, 1998). Each ENaC subunit consists of 650 - 700 amino acids with a molecular weight of around 70 – 100 kDa (Canessa et al., 1993; Lingueglia et al., 1994; Garty & Palmer, 1997; Ji et al., 2012). The greatest sequence homology among the four subunits is between the α- and δ-ENaC subunit (34 % identity) and the β- and γ-ENaC subunit (34 % identity) and approximately 23 – 27 % between all subunits at amino acid level (Garty & Palmer, 1997; Hanukoglu & Hanukoglu, 2016).

The assumption that ENaC forms homotrimeric (ααα-, βββ-, γγγ- or δδδ-ENaC) and heterotrimeric (αββ/ααβ-, αγγ/ααγ-, ββγ/βγγ-, δββ/δδβ-, δγγ/δδγ-, αβγ-, or δβγ-ENaC) channels was underlined by biochemical, genetic, electrophysiological and crystallographic findings (Benson et al., 2002; Anantharam & Palmer, 2007; Jasti et al., 2007). Strong evidence that ENaC assemble to form trimers was shown by a publication by Jasti et al. (2007). By using crystallography the authors showed that the Acid-Sensing Ion Channel (ASIC), which are close relatives of ENaC, form trimeric channels (Jasti et al., 2007). This finding provided a novel insight into the molecular channel-structure of ENaC that different from previous studies, suggested that ENaC is formed by either four, or nine subunits (Firsov et al., 1998;
Kosari et al., 1998; Snyder et al., 1998; Dijkink et al., 2002; Anantharam & Palmer, 2007). Another study, using atomic force microscopy also indicated that ENaC assemble in the membrane as a trimer with a 1:1:1 ratio. (Staruschenko et al., 2005; Stewart et al., 2011). The previously assumed heterotrimeric ENaC channel structure was recently confirmed (after this thesis was submitted) by a study done by Noreng and colleagues (2018). They showed for the first time the crystal structure of ENaC and that the channel assembles with a 1:1:1 stoichiometry of α:β:γ subunits arranged in a counter clockwise manner (Noreng et al., 2018).

Despite the long confusion about the structure of ENaC, the functionality of ENaC is well studied and understood. In 1994, Canessa and colleagues showed that a robust expression of all three subunits (α, β, γ) in oocytes of Xenopus laevis is required to form a functional channel with highest activity. The expression of single subunits as a homotrimeric or co-expression of two subunits as a heterotrimeric ENaC, lead to minimal activity of the channel (Canessa et al., 1994b; Giraldez et al., 2007; Edelheit et al., 2011). Waldman and co-workers discovered a new ENaC subunit called the δ-ENaC subunit (Waldmann et al., 1995a). In contrast to the α-ENaC subunit predominantly expressed in Na⁺ absorbing epithelial tissues such as the lung or kidney (Rossier et al., 1994; Renard et al., 1995), the δ-ENaC subunit is mainly expressed in in the heart, liver, brain, pancreas, testis, skeletal muscle and blood leucocytes (Waldmann et al., 1995a; Su et al., 2004; Yamamura et al., 2004; Ji et al., 2012). Two splice variants of δ-ENaC (δ1) and (δ2) have been discovered in alveolar cells of the human lung (Zhao et al., 2012). Functional investigation revealed that homotrimeric δ-ENaC can form a functional channel with a small amiloride-sensitive whole cell current in oocytes of Xenopus laevis (Ji & Benos, 2004; Yamamura et al., 2004). The amiloride-
sensitive whole cell current of δ-ENaC co-expressed with β- and γ-ENaC is approximately 10-fold higher compared to αβγ-ENaC (Haerteis et al., 2009). Basic channel properties of δβγ-ENaC are changed compared to αβγ-ENaC. Differences are observed in cation selectivity (Ji et al., 2006), Na⁺ conductivity, open time of the channel and amiloride binding affinity (Ji et al., 2012).

2.2 Subunit topology of ENaC

The crystallised structure of ASIC1 revealed for the first time an insight into the structural organization of ENaC proteins (Jasti et al., 2007). The overall structure of ASIC was consistent with earlier studies on the membrane topology of α, β, and γ-subunits, proposing two transmembrane domains, short intracellular N- and C-terminal domains and a large extracellular-domain (70% of the protein) (Canessa et al., 1994a; Snyder et al., 1994; Kashlan & Kleyman, 2011). Jasti and colleagues introduced a high resolution protein structure, where each subunit resembles an up-right forearm, wrist and clenched hand (Jasti et al., 2007). They divided the channel into subdomains termed: transmembrane-, thumb-, finger-, palm-, knuckle-, β-ball-, wrist-domain (Jasti et al., 2007; Gonzales et al., 2009; Eastwood & Goodman, 2012) (Figure 1).
Figure 1: Putative model of an ENaC subunit

A) Simplified illustration of a β-ENaC subunit including all predicted subdomains. The different subdomains were coloured for better distinction: palm (yellow), finger (purple), thumb (green), knuckle (blue), β-ball (orange), wrist (black circle) and the transmembrane domains 1+2 (TM 1+2)(red). The wrist (black circle) is the junction between the TM 1+2 and the extracellular subdomains. The extracellular space is labelled as Out and the intracellular space as In. Modified from Eastwood et al., 2012. B) Predicted 3D model of a β-ENaC subunit rendered by SWISS-MODEL, based on the crystal structure of chicken ASIC1 (Jasti et al., 2007). The TM 1+2, palm- and knuckle-domain, consist of strong secondary protein structures (α-helix and β-sheets) that are considered to give the channel stability.

Every subunit of the channel is anchored through one transmembrane-domain (TMD1) into the lipid bilayer of the cell-membrane, while the second transmembrane-domain (TMD2) is lining the putative ion channel pore (Gonzales et al., 2009). The interaction of TMD1+2 of all three channel subunits is required to form the channel pore embedded in the cell-membrane (Jasti et al., 2007; Kashlan & Kleyman, 2011). The wrist-domain is a junction between the two transmembrane-domains and the extracellular-domains of each channel subunit (Jasti et al., 2007; Gonzales et al., 2009). The centre of the channel subunit is
defined by the palm-domain which is a β-strand rich scaffold that is directly connected to the two transmembrane-domains (TMD1 & TMD2) and reaches up to the knuckle-domain (Jasti et al., 2007). The thumb-domain is connected to the palm-domain through β-sheets, touches the above located finger-domain and they all together surround the β-Ball-domain. The highest sequence identity of 33 - 36 % is in the palm- and β-Ball-domains. The lowest sequence identity of 7 - 17 % is in the peripheral thumb-, knuckle-, and finger-domains of ENaC and ASIC that form the core of the assembled channel (Kashlan & Kleyman, 2011). The channel structure and integrity is guaranteed through a rigid network of disulphide bonds located in the thumb-, palm- and β-Ball-domains (Jasti et al., 2007; Eastwood & Goodman, 2012; Kellenberger & Schild, 2015). These rigid extracellular-domains might be required to transfer extracellular stimuli into conformational changes, initiating channel gating.

2.3 Physiological role of ENaC

The physiological role of ENaC is well characterised in epithelia, but little is known about the role of ENaC in non-epithelial cells such as endothelial cells of the vasculature where ENaC subunits have been detected (Drummond et al., 1998; Drummond et al., 2004; Jernigan & Drummond, 2005). In epithelial tissues the mechanism of how ENaC facilitate salt/water homeostasis was described by the so called “two membrane hypothesis” (Koefoed-Johnsen & Ussing, 1958). Here, Na⁺ enters the cell through apical ENaC down its electrochemical gradient and is extruded via the basolateral Na⁺/K⁺-ATPase (Koefoed-Johnsen & Ussing, 1958). The transport of Na⁺ across the epithelium generates a transepithelial osmotic gradient that facilitates the movement of water across the epithelia (Althaus et al.,
Given the role of ENaC in physiological and pathophysiological processes (Bhalla & Hallows, 2008) the following sections focus on its known function in the lung, kidney and vasculature. In accordance to its fundamental role for salt/water homeostasis, malfunctions and dysregulations of ENaC in epithelia is associated with a variety of different diseases such as pseudohypoaldosteronism type 1 (PHA-1), Liddle syndrome and hypertension (Shimkets et al., 1994; Chang et al., 1996; Hummler, 1999; Knight et al., 2006).

### 2.3.1 Role of ENaC in lung epithelium

The lung enables gas exchange in humans and vertebrates. Anatomically, the lungs of mammals can be divided into two main parts: the conducting airways and the respiratory part (Hollenhorst et al., 2011). The entire surface of the lung is lined with an epithelium that represents a barrier between the organism and the outside world. The alveolar epithelium in the respiratory units of the lung is covered with a fluid layer (alveolar fluid) ensuring an unproblematic gas exchange. ENaC is important for the maintenance of the alveolar fluid (Althaus et al., 2011; Hollenhorst et al., 2011). The importance of ENaC for this process was highlighted in a study of Hummler et al., (1996). The group showed that ENaC in epithelial cells of the lung is important for maintaining fluid reabsorption and fulfils a unique role in regulating foetal lung fluid clearance (Hummler et al., 1996). Transgenic animals lacking the α-ENaC subunit died within a few hours after birth because of an impaired reabsorption of fluid from the lung fluid. Likewise, there is evidence that a dysfunction of ENaC can lead to pathophysiological conditions such as lung oedema (Hummler & Vallon, 2005). Lung oedema is defined by fluid accumulation in the lungs that can be caused by a defective reabsorption of water through the
alveolar epithelium (Althaus et al., 2011; Fronius, 2013). Another disease that was recently linked to a mutation of ENaC is cystic fibrosis (CF) (Rauh et al., 2013). Commonly, CF is a genetic disorder where the function of the cystic fibrosis transmembrane conductance regulator (CFTR) channel is impaired, causing thickening of fluids and mucus in different organs including the lung (Yankaskas et al., 2004; Cutting, 2015). Rauh et al., showed that a mutation in the β-ENaC subunit caused a gain of function mutation in ENaC that may have increased Na\(^+\) reabsorption, resulting in reduced fluid level in the airways, similar to the situation with impaired Cl\(^-\) and bicarbonate secretion due to mutated CFTR (Rauh et al., 2013).

### 2.3.2 Role of ENaC in the kidney epithelium

The kidneys play a crucial role in regulating several physiological processes such as salt/water homeostasis (Opie & Rothbard, 1953), maintenance of acid-levels (Gilman & Brazeau, 1953) and regulation of blood pressure (Bianchi et al., 1974; Rossier et al., 1994). ENaC plays a central role in the late aldosterone-sensitive distal nephrons including the principal cells of the collecting duct, where they mediate Na\(^+\) reabsorption thus regulating water reabsorption (Almeida & Burg, 1982; Kellenberger & Schild, 2002), an important mechanism for blood pressure regulation (Bhalla & Hallows, 2008). Mutations of ENaC associated with impaired blood pressure regulation have been identified. For example, an ENaC loss of function mutation causes PHA-1 (Dillon et al., 1980) and a gain of function mutation results in a condition known as Liddle’s syndrome leading to hypertension (Liddle et al., 1963; Rossier et al., 2002; Knight et al., 2006). Liddle’s syndrome for instance, is an autosomal dominant inherited disease that is
characterized by hypertension due to increased renal Na\textsuperscript{+} absorption (Shimkets \textit{et al.}, 1994). These examples highlight the role of ENaC in the kidney as an important mediator of blood pressure regulation, where increased or decreased activity results in impaired blood pressure regulation (Rossier, 2002; Soundararajan \textit{et al.}, 2010).

2.3.3 Role of ENaC in the vasculature

The vascular system is made up of vessels that transport either lymph via lymphatic vessels or blood via arteries and veins through the body. Furthermore, the vascular system enables a supply of oxygen and nutrition for instance to the respiratory system, the digestive system, the kidney and urinary system. Recent studies have shown that ENaC is expressed in the endothelium of vessels and vascular smooth muscle cells (VSMCs) (Drummond \textit{et al.}, 2004; Jernigan & Drummond, 2005; Kusche-Vihrog \textit{et al.}, 2008). Interestingly, the number of ENaCs expressed on the apical membrane of epithelial cells compared to endothelial cells is 50\% higher, indicating a minor sodium influx in the vascular endothelium compared with the kidney epithelium (Kusche-Vihrog \textit{et al.}, 2010) despite the fact that the regulation of ENaC by aldosterone in the epithelium and endothelium is similar (Kusche-Vihrog \textit{et al.}, 2014). Studying the mechanical properties of endothelial cells \textit{in vitro} and \textit{ex vivo} using an atomic force microscopy (AFM), revealed that ENaC expression determines the mechanical stiffness of the endothelial cell cortex thereby affecting cell mechanical properties and vascular function (Jeggle \textit{et al.}, 2013), with increased ENaC expression associated with an increased endothelial cell stiffness. Accordingly, the knockdown of αENaC, decreased the mechanical stiffness of the endothelial cell cortex (Kusche-Vihrog \textit{et al.}, 2010; Warnock,
A similar outcome was observed with amiloride, where ENaC inhibition also decreased the endothelial cell stiffness (Oberleithner et al., 2006). It is suggested that these changes in cell stiffness mediate biophysical/biochemical signals from the blood to respective tissues (Pesen & Hoh, 2005).

ENaC of endothelial cells in blood vessels are continuously exposed to the haemodynamic traction forces of the blood stream – SF which are thought to be important for mediating vascular function (Davies et al., 1997; Davies et al., 2005). The blood flow is suggested to cause a deformation of endothelial cells, which stimulates the activity of the endothelial nitric oxide synthase (eNOS), the major enzyme for the production of the vasodilator mediator nitric oxide (NO) (Rubanyi et al., 1986; Fels et al., 2010). Thus, SF causes an increase of NO production, which results in an increased NO diffusion to the adjacent smooth muscle cells where it triggers a vasodilatation (Joannides et al., 1995). A study of Fels et al., (2010) showed that the NO production was reduced in stiff endothelial cells which coincided with increased ENaC expression (Fels et al., 2010). In this context, studies provided evidence that ENaC plays a role in mediating vasoconstriction in mesenteric arteries via an inhibitory effect on the production of NO (Perez et al., 2009) and also influences NO production in cultured endothelial cells (Guo et al., 2016). Besides a contribution of ENaC to vascular function via endothelial cells, recent studies have shown that ENaC are also expressed in VSMCs. A study performed in isolated rat cerebral arteries has shown that inhibition of ENaC in VSMCs prevents myogenic constriction (Drummond et al., 2004).

In addition to the role of ENaC in regulating the stiffness of the endothelial cortex, NO production and pressure-mediated vasoconstriction ENaC is also expressed in
the baroreceptor of rats (Drummond et al., 2001; Drummond et al., 2004; Jernigan & Drummond, 2005). Baroreceptors are sensory cells that are located in arteries and relay information of blood pressure changes to the brain. Taken together, there is sufficient evidence to highlight the importance of ENaC for blood pressure regulation that is independent of their epithelial function in the kidney.

2.4 Regulation of ENaC

Considering the importance of ENaC as a regulator of salt/water homeostasis and blood pressure regulation, its activity is regulated by various mechanisms that can be summarised in two main principles: 1) Regulating the number of channels in the membrane (N, via synthesis and/or trafficking to the membrane); or 2) regulating the Po of channels already present in the membrane. In altering the Po or open state of ENaC a fast change in Na\(^+\) conductance can be achieved (Benos et al., 1995; Ismailov et al., 1995), responsible for that is a change in gating of the channel. This for example can occur via proteolytic cleavage of the extracellular loops of the \(\alpha\)- or \(\gamma\)-ENaC subunits in the Golgi apparatus or at the cell surface (Vallet et al., 1997; Hughey et al., 2007; Butterworth, 2010). The proteolytic cleavage of ENaC increases its activity through changes in the Po (Hughey et al., 2007). Changing the number of channels in the membrane can also regulate Na\(^+\) conductance but this is slower. This form of regulation can be occur in response to hormones, such as aldosterone (Chen et al., 1999) and peptide hormones like vasopressin (Frindt & Burg, 1972), insulin (Benos et al., 1996) and angiotensin II (Ang II) (Beutler et al., 2003). The hormones primarily regulate the abundance of the channel in the membrane via changing the expression or via facilitating trafficking of already translated channel proteins to the membrane (Butterworth,
The next paragraphs will give a brief introduction about the regulation of ENaC via N-glycosylation, via trafficking and via affecting channel gating to influence Po.

2.4.1 N-linked glycosylation of ENaC

N-linked glycosylation is a common posttranslational modification occurring in concert with protein biosynthesis (Varki, 1993; Shental-Bechor & Levy, 2008). Besides phosphorylation, more than 50% of proteins are modified by attachment of sugar molecules at some point in their life cycle (Van den Steen et al., 1998). One of the main protein modifications for plasma membrane proteins is N-linked glycosylation. Here sugar molecules, also known as glycans, are attached to an asparagine residue in the consensus sequence Asn-X-Thr/Ser, where X is a random amino acid except for proline (Imperiali & Hendrickson, 1995; Imperiali & O’Connor, 1999). N-linked glycosylation is involved in several biological processes and is essential for the development of complex multicellular organisms (Mitra et al., 2006; Varki & Lowe, 2009). It affects a variety of cell processes including: signal properties of proteins (Wang et al., 2005), protein structure and folding (Mitra et al., 2006), protein quality control (Ou et al., 1993) and protein turnover (Shental-Bechor & Levy, 2008). The attachment of N-glycans to asparagines starts in the early stage of the protein maturation in the endoplasmic reticulum (ER) (Helenius, 1994; Fiedler & Simons, 1995). After this, N-glycans are further processed by a battery of enzymes that also facilitate the transport of the maturing protein to the Golgi-complex. This depends on specific recognition sites within the protein, and in the Golgi-complex the N-glycans can be further modified (Fiedler & Simons, 1995; Spiro, 2002).
Ion channels for instance, require N-linked glycosylation, to maintain functionality, stability and folding dynamics (Thayer et al., 2016; Lazniewska & Weiss, 2017; Kashlan et al., 2018). However, understanding the role of N-glycan attachments to ion-channels remains fragmented. During the last decades, ENaC has moved more and more into the spotlight to study the role of N-linked glycosylation of ion channels. The N-linked glycosylation of ENaC takes place in the ER, where the trimeric αβγ-channel complex assembles (Canessa et al., 1994a; Snyder et al., 1994; Adams et al., 1997). Early studies demonstrated that removing N-linked glycosylation of one single subunit of ENaC (α-ENaC) does not change the activity of the channel (Canessa et al., 1994a; Snyder et al., 1994). In contrast, another study by Kieber-Emmons and colleagues showed that degrading N-linked glycosylation decreases ENaC activity in Xenopus A6 cells (Kieber-Emmons et al., 1999). Another study by Kashlan and colleagues (2018) showed that the removal of all N-glycans in subunit specific experiments leads to changes in functional expression, surface expression, post-translational processing (maturation) and trafficking of ENaC (Kashlan et al., 2018). These studies showed that N-glycans are important for trafficking and maturation of ENaC. Furthermore, the absence of all N-glycans in the β-ENaC subunit changed the conformation of the α-ENaC subunit, leading to the assumption that N-glycans play an important role in subunit-subunit interactions (Kashlan et al., 2018).

2.4.2 Trafficking of ENaC

After mRNA synthesis of ENaC in the nucleus that can be initiated by the mineralocorticoid aldosterone (Escoubet et al., 1997; Epple et al., 2000), ENaC proteins are translated via ribosomes, inserted into the endoplasmic reticulum (ER)
and the proteins are either assembled to form heterotrimeric αβγ-ENaC (Adams et al., 1997; Butterworth, 2010) or single-subunits form homotrimers to be trafficked to the cell surface (Prince & Welsh, 1998). From there the trafficking of ENaC to the membrane starts through two main pathways (Hughey et al., 2004a; Hughey et al., 2004b). One is the transport of ENaC from the ER through the Golgi apparatus where the trafficking is mediated via so called COPII vesicles (Mueller et al., 2007). These complexes accumulate at the ER membrane and initiate budding of the protein-containing vesicle (Bonifacino & Glick, 2004). After the budding is finalised by the scission process, the mature vesicle including ENaC is transported via microtubules to the Golgi apparatus (Butterworth, 2010). After fusing of the vesicle in the Golgi membrane, ENaC become glycosylated, phosphorylated and either packed in lipid rafts or again packed in vesicles and trafficked to the apical cell surface. The vesicular trafficking of ENaC through the cytoplasm is facilitated by small GTPases and motor proteins that move membrane vesicles to their desired cell compartments or to the apical surface (Jing & Prekeris, 2009; Butterworth, 2010). At their destination, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins enable the docking and fusion with the membrane (Saxena et al., 2000; Condliffe et al., 2003; Bonifacino & Glick, 2004). How the vesicles fuse to incorporate ENaC in the cell membrane is a process that is not fully understood. The second pathway is the transport of immature ENaC (not glycosylated) from the ER directly to the cell membrane without passing the Golgi apparatus. Immature ENaC is stored in distinct pools beneath the cell membrane and can be activated by proteases in these post-Golgi compartments during transit from the ER to the plasma membrane (Hughey et al., 2004a; Hughey et al., 2004b; Butterworth, 2010).
2.4.3 Gating of ENaC

ENaC is a constitutively active channel since the channel switches in absence of a particular stimulus constantly between an open and closed state (Palmer & Frindt, 1986; Canessa et al., 1994b). How long a channel remains in the open and closed states is defined as the mean open or mean closed time. The open probability (Po) is a measure of the proportion of time that the ion channel spends in its open state (Rossier, 2003; Loffing & Schild, 2005). Canonical ENaC requires three different subunits (αβγ or δβγ) to form a fully functional channel with normal gating properties (Canessa et al., 1994b; Waldmann et al., 1995a).

The identification of subdomains, according to the crystallisation of ASIC1, revealed new aspects about the ENaC gating mechanisms (Figure 1). The thumb-domain for instance, is involved in transferring gating movements between extracellular-domains and transmembrane domains (Jasti et al., 2007; Li et al., 2009). The base of the thumb-domain of αENaC is involved in channel gating in response to external sodium (Shi et al., 2011). A study by Kashlan et al., (2010) showed that proteases influence channel gating of ENaC by removing specific residues in the thumb-domain (Kashlan et al., 2010). Insertion of multiple mutations in the thumb domain of ENaC affected gating by changing the Na⁺ self-inhibition of the channel (Maarouf et al., 2009). A study by Chen and colleagues (2015) showed that deleting the knuckle-domain of the α-ENaC subunit within the αβγ-ENaC trimer, activated the channel and simultaneously overcame the Na⁺ self-inhibition. The subsequent deletion of the knuckle-domain of the β- and γ-ENaC resulted in decreased activation of ENaC displayed by a reduced Na⁺ self-inhibition, surface expression and subunit maturation (Chen et al., 2015). Channels of the same DEG/ENaC superfamily showed an involvement of their extracellular subdomains in the gating process, supporting the assumption that all these channels
follow the same gating mechanism. For instance, adding mutations in the palm-domain of ASIC caused a hyperactivation of the channel (Eastwood & Goodman, 2012). The finger-domain shares the lowest sequence conservation between DEG/ENaC members and differs in cysteine rich insertions (Eastwood & Goodman, 2012). Previous studies showed that mutations in the finger-domain of DEG-channels causes channel-hyperactivation (García-Anoveros et al., 1995; Tavernarakis & Driscoll, 1997) and touch disruption (Hong et al., 2000). Furthermore, mutations in the palm-domain of DEG (Liu et al., 1996) and MEC-channels (Zhang et al., 2008) causes a hyperactivation (Eastwood & Goodman, 2012), or disruption in touch sensitivity (Tavernarakis & Driscoll, 1997; Hong et al., 2000). All these studies suggest that subdomain-subdomain interactions contribute to the channel gating machinery of ENaC.

2.5 ENaC is regulated by mechanical forces

The presence of ENaC in endothelial cells (Kusche-Vihrog et al., 2014) and VSMCs (Drummond et al., 2008) and the close exposure of ENaC to blood flow in the vasculature (Drummond, 2009), urine flow in the kidney (Morimoto et al., 2006) and the movement of alveolar fluid in the lung during breathing (Althaus et al., 2011), makes it likely that ENaC is influenced and regulated by mechanical forces. This is also supported by the fact that other members of the DEG/ENaC superfamily MEC, respond to SF (Shi et al., 2016). The close relationship between ENaC and the DEG (MEC) proteins of C. elegans supports the role of ENaC as a mechanosensor. The following section will provide a brief overview about how ENaC responds to mechanical forces.
2.5.1 ENaC is regulated by pressure

The first experimental evidence that ENaC responds to mechanical forces was published by Awayda et al., (1995). In this study bovine α-ENaC reconstituted in an artificial bilayer was activated by membrane stretch caused by negative hydrostatic pressure. The same group demonstrated an activation of ENaC in response to cell swelling by expressing bovine α-ENaC in Xenopus oocytes (Awayda et al., 1995). In agreement with these observations, Ismailov and colleagues (1997) published findings that showed that rat α-ENaC alone and rat αβγ-ENaC expressed in artificial lipid bilayers are activated through hydrostatic pressure (Ismailov et al., 1997). Contradictory findings, showed that αβγ-ENaC expressed in Xenopus oocytes, is inhibited by osmotic swelling (increased stretch) and activated by cell shrinkage (decreased membrane stretch) (Ji et al., 1998). Another study showed that hydrostatic pressure decreases amiloride-sensitive currents in response to stretch in lymphocytes (Achard et al., 1996). Contrary to previous reports, Awayda and Subramanyam indicated in 1998 that membrane stretch does not lead to a mechanical activation of rat ENaC in Xenopus oocytes (Awayda & Subramanyam, 1998). This finding was contradictory to previous findings (Awayda et al., 1995; Awayda et al., 1997), indicating that the mechano-sensitivity of bilayers reconstituted ENaC was not observed when the channel was expressed in cells (Fronius & Clauss, 2008). In this respect, Rossier and colleagues (1998) suggested it would be important to compare biophysical properties of a biochemical purified ENaC with biophysical properties of a native ENaC observed in the apical membrane of the kidney, to exclude contamination of non-ENaC proteins (Rossier, 1998). These controversial findings lead to the yet unsolved question as to whether pressure-induced membrane stretch can activate ENaC or not.
2.5.2 ENaC is regulated by shear force

ENaC is often exposed to SF such as the urine-flow in the kidney tubules (Morimoto et al., 2006) and blood flow in the vasculature (Drummond, 2009). Studies from the Kleyman group provided the first clear evidence that ENaC is activated by SF (Satlin et al., 2001; Carattino et al., 2004). An initial study from this group showed that SF in the cortical collecting duct of mice led to an activation of ENaC and increased Na\(^+\) absorption (Satlin et al., 2001). In addition, they confirmed their own finding by applying SF on ENaC expressing oocytes (Satlin et al., 2001). This was supported by another study showing that the amount of Na\(^+\)-reabsorption is in direct correlation with urine flow and therefore SF rates (Morimoto et al., 2006). Althaus et al., (2007), showed in single channel recordings that ENaC was activated by SF via increases in the Po of the channels (Althaus et al., 2007). Ongoing studies from Carattino and colleagues (2004, 2005) showed that the pore region plays an important role for channel gating in response to SF. Mutations in the pore region of the channel, resulted in a less responsive channel to SF compared with wild-type αβγENaC (Carattino et al., 2004, 2005). In addition, a study by Shi et al., (2013) showed that laminar SF led to a conformational change of the extracellular region of ENaC that is then transmitted to the channel gate, increasing Po of the (Shi et al., 2013).

Taking everything together, it is evident that ENaC is activated by SF and that different domains are involved in the SF-mediated activation of the channel. However little is known so far about the role of how single subunits of ENaC facilitate the response to SF.
2.5.3 Potential role of the β- and γ-ENaC subunit for mechanical activation

Previous studies identified that different ENaC subunits may play certain roles for mechanical activation. A study by Drummond and colleagues (1998) showed that only the γ-ENaC subunit is expressed in baroreceptors and might function as a mechanotransducer on its own to regulate blood pressure (Drummond et al., 1998). Further evidence that ENaC subunits may contribute differently to the response of mechanical forces was also published by the Drummond group, who demonstrated that the pressure-induced vasoconstriction in renal arteries depends on β- and γ-ENaC but not α-ENaC (Drummond, 2007, 2012). In addition, they demonstrated that β- and γ-ENaC subunits are expressed in mouse renal interlobar arteries and that ENaC inhibitors (amiloride and benzamil) abolished the pressure-induced vasoconstriction and increased cytosolic Ca$^{2+}$ and Na$^+$ concentration (Jernigan & Drummond, 2005). Interestingly, the group was not able to detect expression of the α-ENaC subunit. These results indicate that the β- and γ-ENaC subunits might play an important role for myogenic vasoconstriction in mouse renal interlobar arteries that is independent of the α-ENaC subunit (Drummond, 2012). In other studies, Jernigan & Drummond (2006) suggested that an interaction of the β- and/or γ-ENaC subunit with other members of the DEG/ENaC protein family such as the ASICs might form a functional channel. Here, in VSMCs, the expression of β- and γ-ENaC was detected together with ASIC1, 2, 3 and 4 (Jernigan & Drummond, 2006). These findings support the idea that the β- and γ-ENaC subunit without the α-subunit, can form a functional channel that might sense mechanical forces such as SF in the vasculature. Additionally, other studies indicate that β-ENaC is required for renal myogenic regulation in vitro and in vivo (Johnson et al., 2011; Chung et al., 2013). These findings underline the importance of the β-ENaC subunit as a mechanosensors in VSMCs.
Taken together, the expression of the β- and γ-ENaC subunit in endothelial cells and VSMCs supports the assumption that heterotrimeric βγ-ENaC can form functional channels that respond to SF without being co-expressed with the α-subunit. Thus, it is suggested that heterotrimeric βγ-ENaC might function as a mechanosensor in the vasculature. However, little is known about the underlying mechanism of how ENaC and in particular individual ENaC subunits sense SF.

### 2.6 Underlying mechanism of ENaC in shear force sensation

The underlying molecular mechanism of how ENaC sense SF is poorly understood so far. To answer this question, previous research proposed two different models that might explain mechanotransduction via ion channels (Kung, 2005; Christensen & Corey, 2007; Chalfie, 2009).

The first is the lipid bilayer model, where the only trigger is a mechanical force such as SF directed on the cell membrane. This deformation in the lipid bilayer (independent of other proteins) initiates a conformational change, inducing an activation of the channel (Martinac, 2004; Chalfie, 2009). Carattino and colleagues (2007) showed that changing the intrinsic properties in the plasma membrane did not affect activity of ENaC in response to SF (Carattino et al., 2007). This study provides evidence that the lipid bilayer model might not explain the activation of ENaC by SF.

The second model is the “tether model”, where intracellular and extracellular elements are tethered to the channel protein (Kung, 2005; Chalfie, 2009). The intracellular tether might be the connection between the N- or C-terminus with the cytoskeleton, and the extracellular tether might be the connection between the
extracellular domains of the channel with the ECM. These matrix-channel interactions sense and transmit mechanical stimuli followed by conformational changes of the channel (Gillespie & Walker, 2001; Sukharev & Corey, 2004; Christensen & Corey, 2007; Chalfie, 2009). Studies focusing on the intracellular tether, the cytoskeleton, reported that depletion of the C-terminus with its potential binding sites for F-actin (Mazzochi et al., 2006) affect the activation of ENaC in response to SF (Carattino et al., 2007). In this context, another study by Karpushev and colleagues (2010) demonstrated that disrupting the actin cytoskeleton did not alter SF induced activation of ENaC in CHO cells (Karpushev et al., 2010).

More recently it was predicted that the large extracellular domains of ENaC might participate in SF sensation (Fronius & Clauss, 2008; Kashlan et al., 2011; Shi et al., 2013). In this context, studies have shown that introducing mutations into the extracellular domain impaired the activation of ENaC in response to SF (Carattino et al., 2004, 2005). There have also been reports that mutations introduced in specific domains, such as the finger-, thumb- or wrist-domain can attenuate or augment ENaC activity in response to SF (Shi et al., 2011; Shi et al., 2012a; Shi et al., 2012b). Together, these studies underline the important role of the extracellular domains of ENaC in sensing SF, but the exact mechanisms remain unclear.

The close relationship between MEC-channel proteins in C. elegans and ENaC in vertebrates, led to the hypothesis that the sensing of mechanical forces might work in a similar fashion independent of the organism. This was shown in a study by Fronius and colleagues (2017), suggesting that SF sensation of ENaC depends on a connection to the ECM (Knoepp et al., 2017). This connection was likely to be facilitated by N-linked glycans of glycosylated asparagines, since removal of
glycosylated asparagines in α-ENaC reduced the SF response (Knoepp et al., 2017) (Figure 2). This indicated that glycosylated asparagines of the α-ENaC subunit and the ECM are essential to sense SF. This study and other studies support the assumption that the “tether model”, where the extracellular domain of the channel is tethered to the ECM, most probably explains how ENaC senses SF (Chalfie, 2009; Eastwood & Goodman, 2012; Knoepp et al., 2017).

Figure 2: Schematic drawing of putative αβγ-ENaC SF sensation

A) Showing a predicted heterotrimeric αβγ-ENaC that is tethered via N-glycans of glycosylated asparagines (Gly-ASN) to the extracellular matrix (ECM). B) It is believed that the application of SF acts on the ECM and gets transduced to the channel via glycosylated asparagines followed by a conformational change of the channel, leading to an increased Na\(^+\) influx. The extracellular space is labelled as Out and the intracellular space as In. Modified from Fronius Lab.

3 Hypothesis and objectives of the study

In summary, ENaC is activated by SF which is highly relevant for its function in the kidney and vasculature. Further evidence suggests that β- and γ-ENaC alone may be involved in the mechanotransduction processes. Still unknown is the role of individual ENaC subunits in these mechanotransduction processes and the
mechanism through which the β- and γ-ENaC subunits may contribute to SF sensation and activation by SF.

This project aims to characterize the role of β- and γ-ENaC for SF sensation. Additionally, as our lab has provided evidence that ENaC senses SF via glycosylated asparagines that are tethered to the extracellular matrix via N-glycans, this project aims further to investigate whether N-glycosylation sites of the β- and γ-ENaC subunit have a role or whether individual ENaC subunits might have a modulatory role in sensing SF.

My first hypothesis was:

The β- and γ-ENaC subunits have a modulatory role in sensing SF.

My second hypothesis was:

N-linked glycans of glycosylated asparagines of the β- and γ-ENaC subunits contribute to SF sensation via an interaction with the ECM.

Based on these two hypotheses the overall aim of my PhD project was to evaluate whether the β- and γ-ENaC subunits have a modulatory role and whether the extracellular domains of β- and γ-ENaC interact with the ECM via N-glycans of glycosylated asparagines and particularly if this is important for SF sensation. To address the hypothesis and to achieve the aim of my study the following objectives were defined:

Objective 1 Examination of whether or not the β- and γ-ENaC subunits respond to SF and have a modulatory role.

Objective 2 Identification of the role of N-linked glycans of glycosylated asparagines of the β- and γ-ENaC subunit for SF sensation.
2 Materials and Methods
1 Xenopus laevis

Adult females of the South African clawed frog *Xenopus laevis* were purchased through NASCO (Fort Atkinson, USA) and oocytes were surgically harvested. Frogs were kept in an aquatic housing system (XenoPlus, Tecniplast, Sydney, Australia) under the supervision of the University of Otago HTRU (Hercus Taieri resource unit) and the surgery procedures were approved by the Animal Ethics Committee (approval numbers: 114/13 and 83/16). Everything was conducted in accordance with the New Zealand Animal Welfare Act.

The next paragraphs will describe the housing of the animals, and the procedures for harvesting and using the oocytes for the electrophysiological and molecular biological experiments.

1.1 Housing of *Xenopus laevis*

*Xenopus laevis* were housed in an aquatic system of Tecniplast named XenoPlus. The system consists of 12, 26-litre tanks, with water temperature maintained between 20 – 21 degrees Celsius (°C), with each tank capable of holding up to six frogs. Here a maximum of four animals were kept per tank. The frogs were distinguished from each other by their unique skin pattern that was documented by photos (Figure 3). The system holds approximately 300 L water that circulates through the tanks to provide a constant water flow. Approximately 10-20% of the circulating water was exchanged by new tap water each day, to ensure a steady fresh water supply. The pH, temperature and conductivity of the water in the system was constantly monitored by probes and these parameters were accessible via touch panel. In order to ensure a clean water supply, a pre-filter (filter-wool),
mechanical filter (cartridge-filter), carbon-filter (activated carbon bag) and waste-filter (filter-bag) were included in the system. In addition, the system included a UV lamp for disinfection, to adjust and maintain a constant water quality.

Figure 3: Differences in the skin pattern of *Xenopus laevis*
Examples of three different *Xenopus laevis* frogs from the left to right (X17, X30, X32), to highlight the unique skin pattern of every individual frog.

1.2 Harvesting oocytes of *Xenopus laevis*

All *Xenopus laevis* frogs underwent up to 3 surgeries, to harvest oocytes for the experiments (*Section 3*). A fourth oocyte harvest was performed from euthanised animals. All surgeries were recorded in an animal register, to allow regular tracking of animals afterwards. Anaesthesia and euthanasia involved the use of MS222 (Tricaine methane sulfonate, Aldrich, Cat. No. E10521-50g) with a concentration of 1.3 g/L (buffered to a neutral pH; adjusting with HCl or NaOH), which is commonly used to sedate *Xenopus laevis* (Smith & Stump, 2000). The harvesting procedure of oocytes from *Xenopus laevis* was divided into two approaches: (1) surgery under anaesthesia and (2) surgery after euthanasia.
(1) Surgery under anaesthesia:

To minimise the stress level of every individual frog, surgeries on individual animals were done with an interval of approx. 1 year. For the harvesting procedure, animals were caught with a small fish net and transferred to a closable plastic box containing MS222. After the transfer into MS222, surgical instruments such as forceps, scissors, a compressor and a syringe with saline solution were disinfected using Trigene (0.5 % chlorhexidine (Sigma, Cat. No. 282227-5g) in 70 % ethanol (BDH, Cat. No. 10476)) and placed on a tray with a sterile tissue. After approx. 10-15 minutes in the anaesthetic, the frog was turned upside down to check the upright reflex, which is an indicator that the animal was anaesthetised. If the animal did not react, it was placed on a tray, one toe was pinched after pulling the leg carefully into a straight position to double check whether or not the frog was fully anaesthetised. Accordingly the frog was placed with its back on the tray to expose the abdomen and then covered with wet paper tissues (soaked with MS222) to prevent desiccation of the skin. To prevent an infection of the upcoming incision wound, the skin was carefully wiped with povidone-iodine prep pads (Dynarex, Cat. No. 1108).

An incision through the skin of approximately 1-2 cm was made with blunt pointed scissors on the abdomen above the groin, between the midline and the lateral aspect of the abdomen. Further on, a sharp pointed scissor was used to cut through the fascia and muscle layer, revealing oocytes, which are placed in sack shaped organs called ovaries. A part of the ovary was carefully picked with blunted forceps and pulled through the incision wound. It was then cut off and transferred into a 50 ml tube (Greiner, Cat. No. GRE227261) containing oocyte Ringers solution (CulORi) (Table 1). Both incisions (skin and muscle layer) were then sewed separately. For the muscle layer a needle (taper-point) with a self-dissolving thread was used (B.
BRAUN, Cat. No. G0024134) before the skin layer was stitched, using another type of needle (reverse cutting) with a self-dissolving thread (DemeTECH, Cat. No. PGA184019F4P). Following surgery, the frog was carefully placed in a separate box (recovery box) containing water that was taken from the housing system. During this process the nostrils of the frog were kept above the water surface to prevent drowning. Usually after 0.5 – 1 hour in the recovery box, the frog started to move and swim again, indicating recovery from the anaesthesia. Finally, the frog was placed in a separate tank of the XenoPlus system to make the recovery as stress-free as possible for five days.

During this period they were audited daily for abnormalities in behaviour and for signs of infection around the wound site. They were fed the day after surgery and placed back into their original tank on day five. Over a time period of three years no complications associated with surgery were observed indicating that the animals tolerated the procedure well.

(2) Surgery after euthanasia:

The animals were picked with a fishing net and placed in a closable box with MS222. For euthanasia, animals were placed for 30 min in MS222 that was followed by decapitation using a self-made punch, and subsequent destruction of the spinal cord by inserting a metal wire into the vertebral channel. Then, the frogs were placed with their back on a tray and the abdomen was opened by using blunt pointed scissors. Ovaries including oocytes were picked with blunted forceps and transferred into a 50 ml tube, filled with CulORi (Table 1). Thereafter, the carcass of the frog placed in a zip bag labelled with: number of the frog (X32), date of euthanasia, method of execution and then transferred into a chest freezer provided by the HRTU.
1.3 Separation and storage of oocytes

Immediately after surgery, the removed part of the ovary was washed 3-5 times with CulORi (see Table 1), poured into a petri dish (90 x 15 mm) (Technoplas, Cat. No. TECS9014UV20) containing CulORi and then carefully pulled apart by using two fine forceps. Oocytes were defollicated through enzymatic degradation using collagenase (1.5 mg dissolved in 1ml CulORi; Serva. Cat. No. 17465.02). Thereafter, oocytes were transferred into a Petri dish (90 x 15 mm) filled up with 20 ml of CulORi + 30 mg of collagenase and incubated for 90 minutes on a horizontal shaker by 90 rpm. Oocytes were immediately washed three times in Ca$^{2+}$-free ORi (Table 1) to remove cell debris and immature oocytes. Then the oocytes were incubated for 15 min in Ca$^{2+}$ ORi. Fully developed and healthy looking stage V – VI oocytes were collected (Dumont, 1972) and stored individually in a 96 well plate (Lab supply, Cat. No. GRE650201) containing CulORi.

Table 1: Composition of Ringer’s solutions used for oocyte separation and storage

<table>
<thead>
<tr>
<th>Components</th>
<th>CulORi [mM]</th>
<th>Ca$^{2+}$-free ORi [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Na$^{+}$-Pyruvate</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>EGTA</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50 μg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100 μg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>100 μg/ml</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>
2 Molecular biology

Molecular biology methods were used to generate cDNA and cRNA for heterologous expression of all ENaC subunits (α, β, γ, and δ) in oocytes of *Xenopus laevis* to either perform functional electrophysiology experiments (Section 3) or biotinylation experiments (Section 2.9). Therefore, the pTNT™ vector was used to amplify cDNA and synthesise cRNA which then was injected into oocytes, expressing various ENaC genes. The pTNT plasmids used for this thesis contained genes encoding ENaC subunits and were previously cloned from human lung tissue and provided from the Fronius Lab (Fronius *et al.*, 2010).

The pTNT™ vector (Promega) (Figure 4) is an expression vector that is commonly used to express cloned genes in vitro or in vivo. It contains either SP6- or T7 polymerase promoters that allow a highly efficient synthesis of RNA in vitro. Both the 5’β-globin leader sequence and the Poly (A) tail of 30 residues enhances the efficiency to translate cRNA into proteins (Falcone & Andrews, 1991; Wakiyama *et al.*, 1997). Ampicillin is included in the vector as a bacterial resistance to guarantee successful retention of the required DNA plasmid in the bacteria.
2.1 Site-directed mutagenesis

Site directed mutagenesis was performed to generate modified ENaC proteins. This included the replacement of glycosylated asparagines by alanines or the addition of an epitope tag to the N/C-terminus. The generated ENaC proteins were then used to perform functional electrophysiological experiments (Section 3) and biotinylation experiments (Section 2.9).

Before starting with the site directed mutagenesis, a sequence alignment was performed looking for a consensus sequence NXS and NXT that is specific for glycosylated asparagines. The β-ENaC subunit revealed eleven (Figure 5) and the γ-ENaC subunit five (Figure 6) glycosylated asparagines.
Figure 5: Sequence alignment of the human β-ENaC subunit

Based on the consensus sequence, NXS or NXT (N: asparagine; X: any amino acid except proline; S: serine; T: threonine), eleven glycosylated asparagines were identified in the human β-ENaC subunit. Every blue square with a number represents an individual glycosylated asparagine (N99, N135, N141, N146, N199, N207, N260, N364, N378, N449, N484). The two transmembrane domains are highlighted in grey. (NCBI Reference Sequence: NM_0002336.2)
The sequence alignment revealed five glycosylated asparagines following a consensus sequence NXS/T (N: asparagine; X: any amino acid except proline; S: serine; T: threonine). All in all five glycosylated asparagines were revealed (green numbers) in position N209, N248, N271, N291 and N497. The two transmembrane domains of γ-ENaC are highlighted in grey.

(SNCB Reference Sequence: NM_001039.3)

Self-made primers were designed to either add epitope tags or exchange glycosylated asparagines to alanines of the β- and γ-ENaC subunit. The primers were then generated according to the Primer Design Guidelines of the manufacturer’s protocol (QuickChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies, Cat. No. 210519-5) and then ordered from Life Technologies, Auckland, New Zealand. The β-ENaC subunit was tagged with a Flag-tag (aaseq: DYKDDDDK) and the γ-ENaC subunit with a His-tag (aaseq: HHHHHHH) (Table 2).
Table 2: Primers used for tagging the β- and γ-ENaC subunits

<table>
<thead>
<tr>
<th>Name</th>
<th>Tag</th>
<th>Orientation</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Flag</td>
<td>Human β-ENaC Flag-Tag</td>
<td>Forward</td>
<td>CCCTACCCCATGTGAGGACTACAAGAGCGATGAGCAGCACAGCCGCCCAAGCCTATGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTCATAGTTGGGGGGCGCTTGTCATGCCTTCTTGGATTCTCCTCAGGGTGGG</td>
</tr>
<tr>
<td>γ-His</td>
<td>Human γ-ENaC His-Tag</td>
<td>Forward</td>
<td>CCAATACACTACACCCACCACCACCACCTTITCCAAACCAGCTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GAGCTGTTGGAAAGTGGTGGTGGTGGGAATTGTATTTGGG</td>
</tr>
</tbody>
</table>

The primers that were used for generating asparagine deficient mutations of the β-ENaC (Table 3) and γ-ENaC (Table 4) are listed below:

Table 3: Primers used for asparagine deficient β-ENaC mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein</th>
<th>Orientation</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N99A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>GCCGTCAACCATCTGCCTGCAAGCCCTCTTCAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTTGACGGAGGCTAGCAGCGCAGATGTTGAGCGG</td>
</tr>
<tr>
<td>N135A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>GAGCTAAGCCATGCCGCTGCCACCAGGAACTGAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTTCAGTTCTCCATTGGCGAGCTGCTTAGCTTC</td>
</tr>
<tr>
<td>N141A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>ACCAGGAACCTGGGCTTCATCTCACCTTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CCAGATGGAGAAAGCCAGTTGCTCTGAGT</td>
</tr>
<tr>
<td>N146A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>CTGAACTTTCTCACCATCTGGGGCCACACACCCCTGGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GACCAAGGGGTGTTGGGCTGCAGATGGAGAAATTTGGTC</td>
</tr>
<tr>
<td>N199A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>CTATGTAGCCTGCAGCCAGGACCCAGGTACCACAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTGACACTGGTGCTCTGGCGAGGTACATAG</td>
</tr>
<tr>
<td>N207A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>CAGGTAGGCACTCTGTCGATGACCGCCAGGTAAGTACCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CGGTAATAGCCAGCTGCGATGACCGCCAGGTAAGTACCTG</td>
</tr>
<tr>
<td>N260A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>CCCTGCAATACCCGGGCTTCCTCCATCTTCTTCTACTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTAGAAGATGACGGAGGTGGAGCCGCGTGTTGAGG</td>
</tr>
<tr>
<td>N364A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>CCGTGACACCGTGCTGCTCTGAGGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GACCTCAGAAACCAGGACGGTCGCAAGG</td>
</tr>
<tr>
<td>N378A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>CAAAATCTTCTACAGCATCGACCCACCAGACTACTGCCCATCAGCAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGCCTGGATGAGTCTGATGCTGCTGCTACGTAGTCAGGATAGTTGG</td>
</tr>
<tr>
<td>N449A</td>
<td>Human β-ENaC</td>
<td>Forward</td>
<td>GATCTCCTGCGCTGACACCCAGTACAGAAGG</td>
</tr>
</tbody>
</table>

40
Table 4: Primers used for asparagine deficient γ-ENaC mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Tag</th>
<th>Orientation</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>N209A</td>
<td>Human γ-ENaC</td>
<td>Forward</td>
<td>GGATTCCAACGTGCAGCTGACACCTCCGACTGTGCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGCACAGTCGGAGGTTGAGCTGAGCACAGTGAGGAAGTCC</td>
</tr>
<tr>
<td>N248A</td>
<td>Human γ-ENaC</td>
<td>Forward</td>
<td>CCTCTGGAGAGAAGATCTGCAGTTCTCTGCTGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTCAGCAGAATAGCTCATGCGATTTCTCTCAGAGG</td>
</tr>
<tr>
<td>N271A</td>
<td>Human γ-ENaC</td>
<td>forward</td>
<td>GTGTCCCTGTGATGCCAGGGCTTTCACGCTTTCCACCAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>GTGGTGAAAGGCTGAAAGCCTGCCATACAGGAAC</td>
</tr>
<tr>
<td>N291A</td>
<td>Human γ-ENaC</td>
<td>forward</td>
<td>ACTTTCAACACAGAGAAGCTGAGACCATTCTAGACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>GGTCGTGAGAATTCCTGAGTTCTCTCTTTGTTGAAA GT</td>
</tr>
<tr>
<td>N497A</td>
<td>Human γ-ENaC</td>
<td>forward</td>
<td>GTAAACAAAAAGCTCGCAAGACAGACTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>CAAGTCTGTCTTGGCGAGCTTTTGTTFAC</td>
</tr>
</tbody>
</table>

Every site-directed mutagenesis sample was prepared following a site-directed mutagenesis protocol as shown in Table 5. A 200 µl PCR tube (Lab Supply. Cat. No. AXY321-01-102) was used to prepare the sample used for the site-directed mutagenesis PCR. Therefore, 5 µl of 10 x reaction buffer, 50 ng of DNA template (Fronius et al., 2010), 125 ng of oligonucleotide primer forward and reverse, 1 µl of dNTP mix, 1.5 µl of QuickSolution reagent and 38.5 µl Nuclease-free water up to a volume of 49 µl added. After adding the last reagent, 1 µl of QuickChange Lightning Enzyme, the sample was resuspended gently and thoroughly by using a
pipette. The final volume for every individual sample was set to 50 µl according to the manufacturer’s protocol (Table 5).

Table 5: Sample reactions for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Reaction buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>50 ng</td>
</tr>
<tr>
<td>Oligonucleotide Primer forward</td>
<td>125 ng</td>
</tr>
<tr>
<td>Oligonucleotide Primer reverse</td>
<td>125 ng</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>QuickSolution reagent</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>up to a final volume of 50 µl</td>
</tr>
<tr>
<td>QuickChange Lightning Enzyme</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The prepared samples were placed in a thermocycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, USA) and then the site-directed mutagenesis polymerase chain reaction (PCR) started (as described in Table 6).

Table 6: Protocol for site-directed mutagenesis PCR

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95°C</td>
<td>20 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>10 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>2.5 min</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68°C</td>
<td>5</td>
</tr>
</tbody>
</table>

After finishing the PCR protocol, a restriction enzyme Dpn I was added (provided by the kit). Dpn I is specific for methylated and hemimethylated DNA and is used
to digest the parental DNA template and to select for mutation-containing synthesized DNA (according to the manufacturer’s protocol). After resuspending the samples gently and thoroughly via pipetting, samples were placed in the thermocycler and incubated for another 15 min at 37 °C. Thereafter, the DNA was transformed into Ultracompetent cells (XL10-Gold) provided by the QuickChange Lightning Site-Directed mutagenesis Kit. After thawing the cells slowly on ice, 45 µl were transferred into a pre-chilled 15 ml tube. Afterwards 2 µl β-mercaptoethanol was added and samples were gently and thoroughly mixed via pipetting and incubated for 2 min on ice. Thereafter, 2 µl of mutated DNA was added, thoroughly mixed and incubated for 30 min on ice. Subsequently, the samples were heat-pulsed for 45 sec. in a pre-heated water bath at 42 °C and incubated on ice for 2 min. Then 0.5 ml pre-heated super optimal broth with catabolite repression (SOC-medium, Table 7), which was substituted for NZY+ broth, was added to the reaction and incubated on a shaker at 235 rpm for 1 hour.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>2 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>58.4 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>18.6 mg</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>95.2 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>360.3 mg</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>up to 100 ml</td>
</tr>
<tr>
<td>pH 7</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Reagents of the SOC-medium

Thereafter, the cell suspension (100µl) was plated on pre-heated agar plates (Table 8), including 75 µg/ml ampicillin and incubated at 37°C. After 24 hours bacterial
colonies were picked and transferred into 4ml of lysogeny broth (LB) medium 
(Table 9) including 75 μg/ml ampicillin and placed on a vigorous shaker at 235 
rpm overnight at 37°C.

Table 8: Reagents of the LB-medium for agar plates

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar for bacteria growth</td>
<td>7.5 g</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>up to 500 ml</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>750 μl</td>
</tr>
</tbody>
</table>

Table 9: Composition of LB-medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>up to 500 ml</td>
</tr>
</tbody>
</table>

The next morning, LB medium including mutated bacteria were harvested and used 
to produce a small amount of plasmid DNA.
2.2 Plasmid DNA purification

For small scale production of purified plasmid DNA, a minipreparation (Miniprep) kit (QuickClean II Plasmid Miniprep Kit, Cat. No. L00420-50, GenScript) was used.

LB media including bacteria as described before (Section 2.1), were taken and 1.5 ml of LB medium pelleted by centrifuging at 10,000 x g for 30 sec. and the supernatant was discarded. This step was repeated once to collect enough cells and the pellet was resuspended in 250 μl resuspension buffer until no clumps were visible. Then 250 μl of lysis buffer was added to the samples and gently inverted 4-6 times. 350 μl of neutralisation buffer was subsequently added and tubes were inverted for 4-6 times to arrest cell lysis. The sample was then centrifuged for 10 min at 13,000 x g until a compact white pellet was formed. The supernatant was then carefully transferred to a spin column (provided in the kit) and centrifuged at 6000 x g for 1 min. The DNA accumulates in the filter of the spin column and the flow through can be discarded. Afterwards, 650 μl washing buffer was added and the sample was centrifuged at 12,000 x g. This step was repeated once and another centrifuging step at 12,000xg for 1 min was added to remove/dry the filter of the spin column. The spin column was transferred to a new 1.5 ml Eppendorf tube and 50 μl of the elution buffer was pre-heated to 55°C. After pipetting the pre-heated elution solution onto the centre of the filter of the spin column, the sample was incubated for 1 min at room temperature and centrifuged at 12,000 x g for 1 min. The flow through of the elution buffer passing the filter of the spin column into the tube contained the plasmid DNA which was directly stored in a freezer at -20°C.
2.3 Sequencing of DNA

To verify if the mutant of interest was incorporated into the DNA, sequencing was performed. The sequencing was performed by the ABI 3730xl DNA Analyser (Thermo Fisher Scientific) in the Department of Anatomy, University of Otago, Dunedin, New Zealand. Sequencing was performed according to the instructions provided by the genetic analysis service, 200 ng of plasmid DNA and 3.2 pmol of sequencing primer were mixed to a total volume of 5 μl with MilliQ water.

2.4 Plasmid DNA linearisation

The DNA plasmids including mutated ENaC subunits had to be linearised before an *in vitro* transcription was performed. Therefore, 1 μg of DNA was mixed with 1 U of the restriction enzyme BamHI (Roche, Cat. No. 10567604001) and 10x reaction buffer (provided by the kit). The sample was then incubated for 90 min at 37°C and heat inactivated by an additional incubation of 15 min at 65°C.

2.5 In vitro transcription and RNA purification

An SP6 mMESSAGE mMACHINE Kit (Ambion, Cat. No. AM1340) was used to transcribe DNA into RNA. This was performed by following the instructions of the supplier’s protocol. The reaction of the *in-vitro* transcription was assembled in a 200 μl PCR tube. Therefore, 10 μl of 2 x NTP/CAP, 2 μl reaction buffer, 1μg of linear template DNA, 2 μl of enzyme mix and nuclease free water was added to a final volume of 20 μl (*Table 10*). The sample was resuspended thoroughly via pipetting and then placed in a thermocycler for 2 hr at 37 °C.
Table 10: in-vitro transcription protocol

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>up to 20 μl</td>
</tr>
<tr>
<td>2x NTP/CAP</td>
<td>10 μl</td>
</tr>
<tr>
<td>10x Reaction Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>linear template DNA</td>
<td>1 μg</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>2 μl</td>
</tr>
</tbody>
</table>

After this, 1μl Turbo DNase was added and further incubated in a thermocycler for 15 min at 37 °C.

The RNA mix from the in-vitro transcription reaction is full of unimportant nucleotides, short oligonucleotides, proteins and salts and has to be purified to obtain a high purity of RNA, necessary for an optimal translation of ENaC. Therefore, the RNA purification was performed using a MEGAclear™ Kit (Ambion, Cat. No. AM1908) according to the manufacturer’s manual. Therefore, the RNA sample was brought up to a volume of 100 μl with elution solution in a nuclease free 1.5 ml Eppendorf tube (Lab supply, Cat. No. AXY311-08-052). After mixing the sample gently, but thoroughly by pipetting, 350 μl of binding solution concentrate was added and also gently mixed by pipetting. 250 μl of 100 % ethanol (Merck, Cat. No. 64-17-5) was added, gently mixed by pipetting and then transferred into a spin column with a filter provided by the manufacturer. The sample was centrifuged for 1 min at 10,000 x g and the flow through discarded. Subsequently, 500 μl wash solution was added followed by another centrifugation for 30 sec at 10,000 x g. After discarding the flow through and repeating that step once, an additional centrifugation for 30 sec at 10,000xg was performed to remove remaining solutions. The filter of the spin column was then transferred to a new
1.5 ml tube (provided by the manufacturer) and immediately 50 μl pre heated (95 °C) elution solution was pipetted onto the centre of the filter and incubated for 1 min at room temperature. Columns were centrifuged at 10,000 x g for 1 min and the flow through passing the filter contains the cRNA that was immediately placed on ice and stored by -80 °C.

### 2.6 Quantification and qualification of cDNA and cRNA yield

To verify whether or not the mutated cDNA or cRNA had a good quantity and quality, spectrophotometry was employed using a NanoDrop 2000 (Thermo Scientific, Auckland, New Zealand) or microplate reader (Synergy 2, BioTek Instruments, USA). After confirming that the cDNA and cRNA was pure, cDNA was sequenced (Section 2.3) and cRNA injection into oocytes of *Xenopus laevis* was performed (Section 2.7 and 2.8).

### 2.7 Manual Microinjection of mRNA into oocytes of *Xenopus laevis*

To characterise ENaC activity in response to SF, cRNA encoding human ENaC subunits (α, β, γ, or δ) were injected into *Xenopus laevis* oocytes. Before starting the injection all instruments, and workbench, were treated with RNaseZap® (Ambion, Cat. No. AM9780) to prevent contamination of RNases that could degrade the RNA for injection. The injection capillaries (outer diameter 1.1 mm) (Drummond, Cat. No. 3-000-203-G/X) were pulled through a horizontal puller (P-87, Sutter Instruments, Novato, USA) and mounted onto a movable microinjector
piston. The tip of the capillary was opened by cutting off the tip using small scissors. After this, the capillary was back-filled air bubble-free with mineral oil (Sigma-Aldrich, Cat. No. 8042-47-5). The capillary tip was then submerged in a droplet of mineral oil placed in the cap of a 200 µl Eppendorf tube. The oocytes were placed in N-Methyl-D-glucamin (NMDG) CulORi (Table 12) on a custom-made plexiglass device, called “oocytes-sledge”, and lined up. Once the injection capillary was prepared the cRNA was taken from the –80 °C freezer and stored to allow the RNA to slowly thaw. Then a petri-dish (35 x 15 mm) (Thermo Fisher Scientific, Cat. No. 150460) was covered with parafilm (Lab supply, Cat. No. BEMPM996) and 1 µl of the unfrozen cRNA was pipetted on top of the parafilm forming a droplet. The injection capillary remaining attached to the injector was transferred from the mineral oil into the cRNA containing droplet. Then the droplet was sucked into the capillary. Each oocyte was impaled with the injection capillary and injected with 15 nl cRNA that corresponded to 24 ng of total cRNA. After injection the oocytes were placed into a 96-well plate (Lab supply, Cat .No. GRE650201) with NMDG-CulORi and stored at 17 °C degrees in an incubator for 24 hrs. The cRNA amount of individual ENaC subunits that was injected in each oocyte varied depending on the designated channel aimed to be investigated. Details are listed below:
Table 11: Amount of injected cRNA per oocyte

<table>
<thead>
<tr>
<th>Channel</th>
<th>cRNA amount (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβγ-ENaC</td>
<td>0.08 per subunit</td>
</tr>
<tr>
<td>α</td>
<td>0.24</td>
</tr>
<tr>
<td>β</td>
<td>0.24</td>
</tr>
<tr>
<td>γ</td>
<td>0.24</td>
</tr>
<tr>
<td>δ</td>
<td>0.24</td>
</tr>
<tr>
<td>αβ</td>
<td>0.12 ng per subunit</td>
</tr>
<tr>
<td>αγ</td>
<td>0.12 ng per subunit</td>
</tr>
<tr>
<td>βγ</td>
<td>0.12 ng per subunit</td>
</tr>
<tr>
<td>δβ</td>
<td>0.12 ng per subunit</td>
</tr>
<tr>
<td>δγ</td>
<td>0.12 ng per subunit</td>
</tr>
<tr>
<td>αβASN/ALAγ-ENaC</td>
<td>0.08 ng per subunit</td>
</tr>
<tr>
<td>αβγASN/ALA-ENaC</td>
<td>0.08 per subunit</td>
</tr>
<tr>
<td>H2O control</td>
<td>nuclease free water</td>
</tr>
</tbody>
</table>

NMDG-CulORi was used to replace Na\(^+\) on the ORi that was used for storage of ENaC RNA injected oocytes. It provides the same osmotic activity as sodium ions but is not permeable through Na\(^+\) channels, thus preventing a continuous influx of sodium ions into the cell.
Table 12: Composition of NMDG used for oocyte injection

<table>
<thead>
<tr>
<th>Components</th>
<th>NMDG-CuORi [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>10</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
</tr>
<tr>
<td>Na⁺-Pyruvat</td>
<td>2.5</td>
</tr>
<tr>
<td>NMDG</td>
<td>80</td>
</tr>
<tr>
<td>EGTA</td>
<td>-</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.06</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.02</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>Amikacin</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>pH 7.4</td>
<td></td>
</tr>
</tbody>
</table>

2.8 Automatic cRNA microinjection via Roboinject

Another way to inject cRNA into oocytes was performed through a fully automatic system called Roboinject (Multi Channel Systems MCS GmbH, Germany). Oocytes were placed into Nunc 96 well plates (Thermo Fisher Scientific, Cat. No. 249952) filled with NMDG-CuORi. These plates have a conical shape to centre individual oocytes. The 96 well plate was then placed on the well plate carrier and an alignment device, Nunc (WPN, Multi Channel Systems MCS GmbH), was plugged in well H12. The RNA samples were named and injection volumes (15 nl) defined before starting the injection process. The concentrations of all injected ENaC subunits are shown in Table 11. The microinjector capillary was prepared by backfilling (IN-25, Multi Channel Systems MCS GmbH) air bubble-free with mineral oil. After mounting the microinjection capillary on the steel plunger, cRNA was prepared. The well plate carrier can hold up to 11 0.5 μl reaction tubes (RT) (Multi Channel Systems MCS GmbH) in which the injection solution (e.g.
dissolved cRNA) will be held. From left: reaction tube servings 1-8 (red) are for cRNA, 9-10 (green) for nuclease-free water (minimum 200μl) and 11 (black) for oil removal (Figure 7).

![Figure 7: Nunc 96-Well plate carrier of the Roboinject](image)

Figure 7: Nunc 96-Well plate carrier of the Roboinject
Picture is showing an overview about the 96 well plate carrier of Roboinject. A Nunc 96 well plate is mounted and an alignment device placed in H12, for adjusting the microinjector capillary. The number 1 - 11 show reaction tube servings using a colour code: 1 – 8 (red) for cRNA, 9-10 (green) for nuclease free water and 11 (black) for oil removal.

The injection preparation was finished by moving the tip of the microinjection capillary into the centre of the alignment device using a portable microscope provided by Multi Channel Systems MCS GmbH. All manual or automatic oocyte injections were used for electrophysiological experiments (Section 3).

### 2.9 Biotinylation assay

A biotinylation assay was used to investigate whether or not β-ENaC as a homotrimeric ion channel alone assembles at the cell membrane of *Xenopus laevis* oocytes. Biotin is a water soluble vitamin that covalently binds to proteins, nucleic acid or other molecules and has a high affinity to streptavidin or avidin. In this
study a modified version of biotin, EZ-link® Sulfo-NHS-LC biotin (Thermo Fisher Scientific, Cat. No. 21335) was used, binding to specific streptavidin or avidin binding pockets (Weber et al., 1989; Livnah et al., 1993).

Therefore, oocytes were injected with β-ENaC cRNA (Section 2.7 and 2.8) and incubated for 24hrs or 48h at 17 °C. All biotinylation steps were performed on ice and in a 4 °C cold room. 2 x 50 oocytes were placed in a petri dish, filled with MBS buffer (Table 13) and then incubated for 30 min on ice in a cold room.

Table 13: Components of the MBS buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>88</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.4</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.8</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.4</td>
</tr>
<tr>
<td>Hepes</td>
<td>10</td>
</tr>
<tr>
<td>pH 7.2</td>
<td></td>
</tr>
</tbody>
</table>

After incubating oocytes for 30 min in the cold room, they were washed 3 times with MBS and after the last wash carefully removed. Subsequently, biotinylation buffer was added (10 mM triethanolamine, 150 mM NaCl, 2 mM CaCl₂, pH 9.5), whereas 50 oocytes were treated with biotin (+) (1 mg/ml) and 50 oocytes with no biotin (-) as a control. All oocytes were then incubated for 15 min by gentle agitation on a platform shaker running at 60-70 rpm in the cold room and the biotinylation reaction stopped by washing oocytes 2 times with quench buffer (192 mM glycine, 25 mM Tris-Cl, pH 7.5) After the last wash, oocytes were incubated in quench buffer on ice for 5 min in the cold room under gentle agitation on a
platform shaker 60-70 rpm. Oocytes were then washed for 2 times in MBS and homogenised in 500 μl lysis buffer (Table 14) by resuspension on ice. The homogenate was then vortexed for 30 sec and centrifuged at 5,000xg and 4 °C for 10 min. Three layers were visible after centrifugation: at the bottom the pellet containing cell debris, in the middle a liquid layer including proteins and at the top a layer with lipids.

Table 14: Components of the lysis buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Unit</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>mM</td>
<td>83</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>mM</td>
<td>1</td>
</tr>
<tr>
<td>Hepes</td>
<td>mM</td>
<td>10</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>%</td>
<td>1</td>
</tr>
<tr>
<td>Protease inhibitor tablets</td>
<td></td>
<td>1 tablet/10 ml</td>
</tr>
<tr>
<td>pH 7.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The middle layer was then collected by using a syringe (5 ml) with a cannula (1.10 x 38 mm) and transferred into a new 1.5 ml Eppendorf tube. This step was repeated 4-5 times to increase the purity of the samples. The received sample contains all protein and will be termed in the following as whole cell sample. A small amount of the whole cell sample (30 μl) of biotin (+) and control (-) were collected, mixed with 10 μl of 5x Laemmli sample buffer (Table 15) and denatured in a heat block at 100 °C for 10 min. Afterwards it was the sample was centrifuged at 13,000 x g for 30 sec, for collecting the condensate, and immediately frozen for the Western-blot performed the next day.
Table 15: Components of the 5 x Laemmli sample buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Unit</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>%</td>
<td>10</td>
</tr>
<tr>
<td>SDS</td>
<td>%</td>
<td>5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>%</td>
<td>25</td>
</tr>
<tr>
<td>bromphenol blue</td>
<td>%</td>
<td>0.8</td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>%</td>
<td>5</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The rest of the whole cell sample was used for continuing the biotinylation, where whole cell sample of biotin (+) and control (-) were treated with the enzyme PNGaseF (New England Biolabs, Cat. No. P0704S). PNGaseF was used to deglycosylate β-ENaC and performed following the protocol of the manufacturer. Therefore, both samples were treated with 3 μl of Glycoprotein Denaturing Buffer (10x) (provided by the kit), mixed with 27 μl of whole cell sample and denatured for 10 min at 100 °C. Afterwards, the samples were centrifuged at 13,000 x g for 1 min and then the PNGaseF reaction assembled. Therefore, 30 μl of denatured protein, 15 μl of MilliQ water, 6 μl 10 % NP-40, 6 μl GlycoBuffer and 3μl of PNGaseF was mixed in a 1.5 ml Eppendorf tube and were resuspended gently and thoroughly with a pipette (Table 16).

Table 16: Protein deglycosylation reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured Protein</td>
<td>30</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>15</td>
</tr>
<tr>
<td>10 % NP-40</td>
<td>6</td>
</tr>
<tr>
<td>GlycoBuffer</td>
<td>6</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>3</td>
</tr>
</tbody>
</table>
The PNGaseF reaction was then incubated for 1 hr at 37 °C in a heat block. In the meantime, 50 μl Neutravidin® Ultra Link® beads (Thermo Fisher Scientific, Cat. No. 53150) were aliquoted into a 1.5 ml Eppendorf tube and were transferred and centrifuged at 2,000 x g for 30 sec. Afterwards the supernatant was removed and washed with 250 μl lysis buffer. This step was repeated once to ensure a thorough wash of the beads. After incubating the samples with PNGaseF, 90 μl of lysis buffer (Table 14) was added to increase the volume of the samples and enable a better mixture of the sample with the beads. The samples, including the beads, were then placed in a cold room for an overnight shake at 4 °C.

On the next morning, samples, including the beads, were taken from the cold room and centrifuged at 2000 x g for 1 min. Subsequently, 30 μl of both samples were collected and transferred to a new 1.5 ml Eppendorf tube, depicting the cytoplasmic β-ENaC concentration of all oocytes. Then 10 μl of 5x Laemmli sample buffer was added and denatured for 10 min at 100°C. The beads were washed 5 times with 250 μl lysis buffer and centrifuged at 2,000 x g for 1 min after every wash step. Afterwards, 40 μl of 5x Laemmli sample buffer was added and denatured for 10 min at 100°C. All samples were centrifuged at 13,000 x g for 30 sec after denaturing to collect condensate.

2.10 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to separate the protein according to their molecular weights, following a standard protocol provided by Laemmli (Laemmli, 1970).

The gels consist of two different layers for resolving the protein sample, allowing an estimation of the molecular weight of the targeted protein. The first layer was
a separating gel (8%) and the second layer a stacking gel (4%) (Table 17). The gel was held together by a glass panel, 1.5 mm spacer and a ceramic plate, providing a loading capacity of 50 µl for each sample. For estimating the molecular weight of the targeted protein, two pre-stained protein markers were used (Kaleidoscope pre-stained marker, Bio-Rad Cat. No. 161-0324 and Precision plus, Dual colour, pre-stained marker, Bio-Rad Cat. No. 161-0374). The SDS-PAGE was run in a Hoefer Mini vertical system (Hoefer) according to the manufacturer’s manual, at 120 V for 2 hrs.

**Table 17: Components of the separating (8%) and stacking gel (4%)**

<table>
<thead>
<tr>
<th>Volume of solutions in ml</th>
<th>Separating gel 8%</th>
<th>Stacking gel 4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris pH 6.8</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide/Bis</td>
<td>3.34</td>
<td>0.665</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>10% ammonium persulphate (APS)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**2.11 Western blot**

After finishing the SDS-PAGE, proteins were transferred to a 0.2 µm polyvinylidene difluoride (PVDF) membrane (Roche, Cat. No. 3010040001). This PVDF membrane was activated in methanol for 10 sec. and placed in transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris-base). The transfer of the proteins from the gel to the PVDF membrane was performed using a Bio-Rad Turbo™ blotting system (Bio-Rad Cat. No. 1704155), in which a transfer sandwich was prepared, whereby the gel and the PVDF membrane were placed in the middle of
two stacks of blotting paper (Whatman, Cat. No. 3030917) soaked in transfer buffer. Everything was assembled in a Trans-Blot Turbo Cassette, mounted in the device and the Bio-Rad turbo transfer blot was performed at 25 V at a constant 1.3 A for 35 min.

Afterwards, the PVDF membrane was placed into blocking buffer (5% non-fat milk powder in TBS-T (Tris-buffered saline (50 mM Tris, 150 mM NaCl with 0.1% Tween)) and incubated on a horizontal shaker at room temperature for 1 h. Afterwards, the membrane was incubated with an Anti-Flag HRP antibody, diluted 1:2500 in TBS-T, to detect the targeted protein (Anti-Flag HRP antibody, Sigma). The membrane was then placed in a cold room at 4°C and incubated overnight. The next day, the antibody solution was removed and the membrane was washed 3 x 10 min with TBS-T and was then analysed by chemiluminescent detection.

Chemiluminescent detection was performed using enhanced chemiluminescent (ECL) (Amersham ECL Prime, Global Science Cat. No. GEHERPN2232) western blotting detecting reagent. The PVDF membrane was incubated for 5 min at room temperature, then placed in a photo-box and covered with a piece of X-ray film (Radiographic Supplies Cat. No. 16501454). The exposure time varied between a few sec. up to a maximum of 10 min. The X-ray film was then developed in developer solution (Radiographic Supplies Cat. No. 4037180), then washed in water and fixed with fixer solution (Radiographic Supplies Cat. No. 4037214). X-ray film was washed in water for another time to remove remaining fixer solution and then air dried by using a portable hair drier.
3 Electrophysiology

The oocytes of *Xenopus laevis* are a well-established heterologous expression model used to investigate ion-channel properties (Lane, 1983; Soreq, 1985; Weber, 1999). Therefore, frog oocytes were harvested and microinjected with mRNA encoding single or co-expressed as well as mutated ENaC subunits, and subsequently used for examining ion-transport processes via the TEVC method over a whole membrane (Section 3.1) or single channel measurements via the patch-clamp method (Section 3.5).

3.1 Two electrode voltage clamp (TEVC) measurement

TEVC was performed to record transmembrane currents of oocytes heterologously expressing ENaC. TEVC is a common electrophysiological method that is used to measure ion transport processes in *Xenopus* oocytes by manipulating the membrane potential of cells. The membrane potential is generated through an unequal distribution of ions at the intracellular and extracellular sides of living cells. With the voltage-clamp technique it is possible to manipulate the membrane potential to a specific value. This occurs via applying a current that is required to reach the clamped potential. This current can be measured and corresponds to the total ion current of a cell at the given command potential.

The potential and current electrode are both made of a chloride silver wire that is fixed to an electrode holder built of Plexiglas®. The glass capillaries that were mounted on the electrode holder of both electrodes were made in a vertical puller (P-87, Sutter Instruments, Novato, USA) by using a borosilicate glass with an outer diameter of 1.2 mm with filament (Hilgenberg, Cat. No. 1103237). The resistance
of the capillaries was between $2 - 9 \text{ M}\Omega$ and were used for impaling oocytes. The command potential was then set to -60 mV by using a TURBO TEC-05 TEVC amplifier (npi, Tamm, Germany). The TEVC experiments were recorded with a PowerLab 4/35 system (ADInstruments, Dunedin, New Zealand) connected to a PC.

### 3.2 Shear force application

For applying SF during TEVC measurements on ENaC expressing oocytes, a self-made perfusion chamber (Figure 8) combined with a self-made pressure controlled perfusion system (ALA Scientific Instruments, New York, USA) was used (Figure 9). Through this system it was possible to generate a specific liquid-flow to simulate SF in a relevant physiological manner.

The oocytes were placed in the centre of the self-made perfusion chamber in a small puncture, to position the oocyte. The chamber is round shaped with a channel in the middle to enable a constant application of SF onto the oocyte surface. The perfusion system allows a self-defined application of SF during the experiments. It also provides access to six reservoirs (50 ml syringes) to rapidly change solutions. Removals of passed solutions were sucked out into a waste flask via a vacuum pump (Figures 8 and 9).
Figure 8: Self-made perfusion chamber for SF experiments via the TEVC method
The oocyte was placed in a small puncture (black circle Oocyte) in the centre of the self-made perfusion chamber. The channel-like structure (of the chamber combined with a continuous in-flow (In) and out-flow (Out) of Oocyte ringer solution (Ori), guaranteed a permanent SF application onto the oocyte surface.

Figure 9: Overview of the two electrode voltage clamp setup
The oocyte was placed in a self-made perfusion chamber. The bath electrodes were placed in the chamber and then the oocyte was impaled with two microelectrodes. Afterwards the membrane potential was clamped to -60 mV via the voltage clamp amplifier TURBO TEC-05 npi. TEVC measurements were recorded via a PowerLab 4/35. Shear force 0.2 dyn*cm^{-2} was applied via a pressure regulator and a self-made perfusion system.
Oocyte Ringer solution (ORi) (Table 18) was used during all oocytes experiments to mimic the physiological environment of oocytes.

Table 18: Components of the oocyte Ringer solution (ORi)

<table>
<thead>
<tr>
<th>Components</th>
<th>ORi [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>90</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
</tr>
<tr>
<td>Na⁺-Pyruvate</td>
<td>2.5</td>
</tr>
<tr>
<td>pH 7.4</td>
<td></td>
</tr>
</tbody>
</table>

3.3 Shear force calculation

To calculate the approximate amount of SF that was applied onto the oocytes during the TEVC measurements, several equations were used. All equations are explained in the following paragraph. The flow velocity (ω) applied due to the perfusion system was calculated using the following equation:

\[ \omega = \frac{V}{A} \]

**Equation 1: calculation of the flow velocity**
- \( \omega \): flow velocity of the perfusion system [cm/s]
- \( V \): volumetric flow rate [cm³/s]
- \( A \): area of the channel (used chamber) [cm²]

In our experimental set up the velocity was 2.5 ml/min and an area of the channel of 2 mm the flow velocity of the perfusion system was 1.3 cm/sec.

To distinguish whether the flow dynamics are laminar and turbulent in the measurement chamber, Reynolds numbers (Re) were calculated. Therefore, the density of water (\( \theta \)) (0.9982 g/cm³ at 20°C), the flow velocity (\( \omega \)) (1.3 cm/sec), the diameter of the perfusion tube (D) (0.01 cm) and the dynamic viscosity of water (\( \lambda \)) (0.01002 g/cm³s) was used in the following equation:
\[ R_e = \frac{\theta \omega D}{\lambda} \]

**Equation 2: calculation of the Reynolds number**

- \( R_e \): Reynolds number
- \( \theta \): density of water at 20°C [g/cm³]
- \( \omega \): flow velocity of the perfusion system [cm/s]
- \( D \): diameter of the perfusion tube [cm]
- \( \lambda \): dynamic viscosity of water [g/cm·s]

The calculated Reynolds number was 25.9014. The \( R_e \) for laminar flow is between 14 and 80 (Hoger et al., 2002). The calculated Reynolds number of 25.9014 is in-between 14 and 80 showing that the flow of the solution is laminar. Drag coefficient (\( C_d \)) of a round objective (oocyte) is considered as 1 (Hoger et al., 2002). The next equation was used to calculate the effective drag force of the applied solution. Therefore, the density of water (\( \theta \)) (0.9982 g/cm³ at 20°C), the cross section area of an oocyte (\( A \)) (0.008 cm²), the flow velocity (\( \omega \)) (1.3 cm/sec) and the drag coefficient \( C_d \) (1) was used in the following equation:

\[ F_{\text{drag}} = 0.5\theta A \omega^2 C_d \]

**Equation 3: Calculation of the effective drag force**

- \( F_{\text{drag}} \): effective drag force [dyn]
- \( \theta \): density of water at 20°C [g/cm³]
- \( A \): cross section area of an oocyte [cm²] approx. 0.008 cm²
- \( \omega \): flow velocity [cm/s]
- \( C_d \): drag coefficient (1)

The calculated \( F_{\text{drag}} \) that is applied on an oocyte is 0.00676 dyn. The effective shear force (\( F_{\text{shear}} \)) value that was used for that project was fixed at 0.2 dyn/cm² and calculated using \( F_{\text{drag}} \) (0.00676) and the surface area of the oocyte (\( A_O \)) (0.03 cm²).

\[ F_{\text{shear}} = \frac{F_{\text{drag}}}{A_O} \]

**Equation 4: Calculation of effective shear force**

- \( F_{\text{shear}} \): effective shear force [dyn/cm²]
- \( F_{\text{drag}} \): effective drag force [dyn]
- \( A_O \): area of oocyte [cm²] approx. 0.03 cm²

The calculated SF value that act upon an oocyte in our experiments was 0.2 dyn·cm⁻².
3.4 Calculating the time constant (Tau)

In order to investigate the gating properties of homotrimeric or heterotrimeric ENaC in response to SF, tau was determined. Tau was defined as the time taken to reach a 63% increase in transmembrane current under SF exposure. For example, if the initial current is 1 μA and increases to 3 μA under SF, Tau is calculated using the time to reach a current of 2.26 μA (63% of the total increase of 2 μA). This procedure allows the time dependent activation properties of the investigated channels to be determined (e.g. homotrimeric, heterotrimeric and asparagine deficient mutants (αβASN/ALAγ or αβγASN/ASA).

3.5 Patch clamping

Another electrophysiological method that is commonly used for characterising ion channel properties is the patch clamp technique. Patch clamping allows the investigator a profound insight into a single ion channel located on a small cell membrane patch (cell-attached configuration).

For this project the patch clamp technique was used in a cell-attached configuration to investigate ion channel properties in ENaC expressing oocytes. The measurements were performed using a standard electrophysiology patch set-up connected to an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, USA). The amplifier signal was digitised through a Digidata 1440A (Molecular Devices, Sunnyvale, USA) and was filtered with 100 Hz via a low pass filter (900CT Tunable Active Filter, Frequency Devices, Ottawa, Illinois, USA). The amplifier was additionally connected to a Humbug 50 Hz noise eliminator (Quest Scientific, North Vancouver, Canada) to remove disruptive signals. Data were acquired (2
kHz) with an Axon clampex 10.4 and data analysis was performed by using the required software clampfit 10.4. The microelectrodes were chlorinated (using 205 bleach) and all solutions were filtered manually using a 0.22 μm sterile filter (Millex, Cat. No. SLGP033RS). The patch capillaries were pulled in a vertical puller (P-87, Sutter Instruments, Novato, USA) using borosilicate glass with a 1.5 mm outer diameter and fire polished to smoothen the edges of the capillary tip. The resistance of the patch capillaries were, in general, between 4 – 12 MΩ.

3.5.1 Cell attached patch clamp configuration

Cell attached configuration was used to further characterise ENaC properties, of ENaC mutations and ENaC subunit variants, expressed in oocytes. Oocytes are surrounded by a vitelline layer that prevents the accessibility of the microelectrode to the cell membrane. The ECM of oocytes consist of two compartments: (1) the vitelline layer and (2) the perivitelline space + glycocalyx (Larabell & Chandler, 1988; Hedrick & Nishihara, 1991); however, a previous PhD-thesis from our lab showed that the removal of the vitelline layer does not affect the response to SF of ENaC (Knoepp, 2014). For devitellinisation, oocytes were placed in 15 ml NMDG-CulORi including 1.5 g mannitol for approximately 20 min. (EMD Millipore Corp. Cat. No. 443907-100GM). Dissolving mannitol in solution changes the osmolality causing a shrinkage of the oocyte. Thereafter, oocytes were peeled with two fine tipped forceps and placed in a petri dish including intracellular patch solution (Table 19). A high K+ intracellular bath solution was used to hold the endogenous membrane potential close to 0 mV. The capillaries were filled with extracellular patch solution (Table 19) and mounted onto the electrode holder.
Table 19: Components of the intracellular/extracellular patch solution

<table>
<thead>
<tr>
<th>Components</th>
<th>Units</th>
<th>High K⁺ intracellular bath solution</th>
<th>Extracellular patch solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>mM</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td>KCl</td>
<td>mM</td>
<td>145</td>
<td>1</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>mM</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>mM</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>mM</td>
<td>5.5</td>
<td>-</td>
</tr>
<tr>
<td>HEPES</td>
<td>mM</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.2</td>
<td>7.4</td>
</tr>
</tbody>
</table>

An overpressure was applied on the capillary to prevent a blockage of the capillary tip and then moved to the oocytes. A change in resistance indicates an approach of the capillary to the cell membrane. A gigaseal between the membrane and the capillary was formed by applying negative pressure. The cell attached measurements were started by setting the membrane potential to 100mV.

3.5.2 Cell attached data analysis

Cell attached patch clamping recordings were analysed by using Excel™, GraphPad Prism7 and Clampfit 10.4. All single-channel properties shown in Table. 24 were calculated according the following equations. The open probability (P₀), the relative open probability (NP₀), the mean open time (T₀), the mean closed time (Tₐ) and conductance (g) of the examined channels were calculated in the following (Althaus et al., 2007). All missing variables used for the following equations were either obtained during experimental procedure using Clampfix 10.4 or via using the binominal distribution calculator (www.di-mgt.com.au/binomial-calculator.html) for Pn.
The open probability of more than one channel was calculated as shown in the following equation:

\[ P_o = \left( \sum_{n} nP_n \right) / N \]

**Equation 5: Calculation of channel open probability (P_o)**

- **P_o**: open probability
- **N**: number of active channels
- **n**: number of conducting levels at any given time
- **P_n**: probability that n out of N channels are open

The \( P_n \) of the membrane patch was compared to \( P_n \) generated by a theoretical binomial distribution, with the assumption that N channels all have the same \( P_o \). This was used to get some reliable information about how many channels were assembled in the membrane patch. The relative open probability (\( NP_o \)) was calculated as described below:

\[ NP_o = \sum \left( \frac{t_n n}{T} \right) \]

**Equation 6: Calculation of the relative open probability (NP_o)**

- **NP_o**: relative open probability
- **t**: open time of channel obtained at a distinct channel level (n) [s]
- **n**: distinct channel level
- **T**: total recording time [s]

The mean open time (\( t_o \)) and the mean closed time (\( t_c \)) were calculated as shown in the following equations:

\[ t_o = P_o NT / \sum E_n \]

**Equation 7: Calculation of mean open time**

- **t_o**: mean open time [s]
- **P_o**: open probability
- **N**: number of active channels
- **T**: total recording time [s]
- **E**: number of binned event obtained at a distinct channel level [n]
- **n**: distinct channel level
\[ t_c = (1 - P_0)NT / \sum E_n \]

**Equation 8: Calculation of mean open time**

- \( t_c \): mean open time [s]
- \( P_0 \): open probability
- \( N \): number of active channels
- \( T \): total recording time [s]
- \( E_n \): number of binned event obtained at a distinct channel level [n]
- \( n \): distinct channel level

The conductance of the patched channels was calculated using the I-V-curve (Figure 10) and the following equation:

\[ g_{v1} = \Delta I / \Delta V \]

**Equation 9: calculation the channel conductance**

- \( g_{v1} \): conductance in point V1 [pS]
- \( \Delta I \): \( \Delta \) current [pA]
- \( \Delta V \): \( \Delta \) voltage [mV]

![Figure 10: Showing an I-V-curve of a cell attached patch clamp experiment](image)

The I-V-curve shows which values were taken to calculate channel conductance. Conductance in point 1 (\( g_{v1} \)) in [pS], \( \Delta I = \Delta \) current in [pA], \( \Delta V = \Delta \) voltage in [mV].

4 Swiss model: fully automated protein structure homology-modelling

Swiss model is a software that makes it possible to render and predict protein structures (Xiang, 2006; Benkert *et al.*, 2011; Bordoli & Schwede, 2012). Since the protein structure of ENaC was revealed after submission of my thesis, Swiss
modelling was used to predict the protein structure of ENaC. Therefore, the nucleotide sequence β-ENaC (NM_0002336.2) and γ-ENaC (NM_001039.3) was copied into Swiss model and a protein model generated by using the crystal protein structure of Acid-sensing ion channels (ASICs) (Jasti et al., 2007) as a template (2qts.2.B).

5 Statistical analysis

The statistical data of all measurements are shown as standard error of the mean (SEM). All data were gathered in Microsoft Excel™ 2010 tables and allayed in GraphPad prism 7. The overall number of experiments was indicated by (n) and the overall number of different frogs indicated as (N). Comparison of dependent data that was recorded in the same measurement were analysed by using a two-tailed paired Student’s t test (comparing dependent values), whereas comparison of independent data sets of two different measurements were analysed by using the unpaired Student’s t test (comparing independent values), the comparison of 3 or more samples was done by a one-way ANOVA with multiple comparison. The statistical significances of the analysed experiments were indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The current traces of the measurements were done via LabChart, transferred to GraphPad Prism 7 and further adapted with the graphic program Inkscape (version 0.91). Data sets of experiments were visualised via Graphpad Prism7 as bar-graphs and also further adapted with Inkscape.
3 Results
1 Characterise whether the β- and γ-ENaC subunits have a modulatory role in shear force sensation
1.1 Introduction

The channel structure of a close family member of ENaC, which is the acid sensing ion-channel (ASIC), has a heterotrimeric channel composition of three subunits (Jasti et al., 2007). A study by Canessa et al., (1994) provided evidence that single ENaC subunits as well as co-expression of two ENaC subunits can form a functional channel that respond to SF (Canessa et al., 1994b). The composition of ENaC by co-expressing two subunits remains unclear so far. The co-expression of the α- and β-ENaC subunit would lead to a heterotrimeric channel composing of either, ααα-, ααβ-, αββ-, or βββ-ENaC. However, the exact composition of ENaC by co-expressing two subunits and how it impacts the ENaC response to shear force (SF) remains unclear. Therefore, I hypothesised that the expression of single ENaC subunits (homotrimeric) as well as co-expression of two ENaC subunits (heterotrimeric) form functional channels that respond to SF.

Therefore, I have investigated whether homotrimeric or heterotrimeric human ENaC respond to SF. Oocytes were injected with mRNA encoding single ENaC subunits aiming to express homotrimeric ENaC (α, β, γ, or δ) or two mRNAs encoding to express heterotrimeric ENaC (αβ, αγ, βγ, δβ, or δγ). These homotrimeric or heterotrimeric channels were then exposed to SF. The SF responses of these channels were compared with the SF responses of wild-type αβγ- or δβγ-ENaC. For a uniform comparison all experiments were performed 24 h after injection, except for β-, γ-, and βγ channels that were additionally incubated for 48, 72 or 96 h. An overview of all TEVC experiments performed for this first objective are indicated in the table below. Functional recordings were performed by the TEVC method (Materials and Methods; section 3.1) and the SF response was assessed by the application of 0.2 dynes*cm⁻² shear force.
Table 20: Overview of experiments performed to examine homotrimeric ENaC

<table>
<thead>
<tr>
<th>Homotrimeric ENaC</th>
<th>Oocytes number (n)</th>
<th>Batch (N)</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ENaC</td>
<td>18</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>25</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
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<td>4</td>
<td>72</td>
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<td></td>
<td>16</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>γ-ENaC</td>
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<td>24</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>13</td>
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<td>72</td>
</tr>
<tr>
<td>δ-ENaC</td>
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<td>4</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 21: Overview of experiments performed to examine heterotrimeric ENaC

<table>
<thead>
<tr>
<th>Heterotrimeric ENaC</th>
<th>Oocytes number (n)</th>
<th>Batch (N)</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
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<td>αβγ-ENaC</td>
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<tr>
<td>δβγ-ENaC</td>
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<tr>
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</tr>
<tr>
<td>βγ-ENaC</td>
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<tr>
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</tr>
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<td>δβ-ENaC</td>
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</tr>
<tr>
<td>δγ-ENaC</td>
<td>9</td>
<td>3</td>
<td>24</td>
</tr>
</tbody>
</table>

1.2 General procedure of TEVC measurements

All TEVC experiments were performed following the same protocol as described below:

To characterise ENaC activity in response of SF, all individual subunits (α, β, γ, δ), subunit combinations (αβ, αγ, βγ, δβ, δγ), as well as β- and γ-ENaC mutations
were injected into *Xenopus laevis* oocytes and measured via the TEVC technique. To begin, all oocytes were checked for morphological abnormalities under a microscope and removed if necessary. Afterwards, single oocytes were transferred into a self-made perfusion chamber, impaled and clamped at -60 mV after a resting period of 1-2 min. After plateauing of the transmembrane current (0 dyn*cm*²), SF (0.2 dyn*cm*²) was applied via a perfusion system. The increased transmembrane current under SF caused by a higher activation of ENaC was determined via the application of the amiloride (10μM; final concentration). The difference between the transmembrane current (Iₘ) and the remaining transmembrane current with amiloride (Iₐₐₘ) represents the ENaC mediated amiloride-sensitive current (I = Iₘ – Iₐₐₘ). In the following result chapters, all data is presented by using strictly the ENaC mediated amiloride-sensitive currents. The ENaC mediated amiloride-sensitive current without SF (0 dyn*cm*²) was termed as (I₀) and is composed of the baseline transmembrane current (Iₘ₀) and the remaining transmembrane current under amiloride (Iₐₐₘ) (Figure 11). The ENaC mediated amiloride-sensitive current with SF (0.2 dyn*cm*²) was termed as (I₀₂) and is composed of the baseline transmembrane current (Iₘ₀₂) and the remaining transmembrane current under amiloride (Iₐₐₘ). For comparison of two independent experiments SF induced current I₀₂ were normalised to I₀ (I₀₂/I₀).
Figure 11: General procedure of TEVC measurements
A representative TEVC current trace of an αβγ-ENaC expressing oocyte. The grey bar represents no SF (0 dyn*cm$^{-2}$), the orange bar the application of SF (0.2 dyn*cm$^{-2}$) and the black bar (a, 10 μM) the application of amiloride. The application of SF causes an increase of transmembrane current. The subsequent application of amiloride caused a rapid decrease in current. The ENaC mediated amiloride-sensitive current without SF (0. dyn*cm$^{-2}$) and with SF (0.2 dyn*cm$^{-2}$) is termed as $I_0$ and $I_{0.2}$, is composed of the transmembrane current ($I_{M0}$) or ($I_{M0.2}$) and the remaining transmembrane current under amiloride ($I_{ami}$), equation 1 and 2.

1.3 Human αβγ-ENaC is activated by SF

To clarify whether human αβγ-ENaC is activated by SF, oocytes were injected with αβγ-ENaC cRNA and current was measured via the TEVC method. Human αβγ-ENaC showed an increased amiloride-sensitive current in response to SF that is indicative for an activation of the channel due to an increased $P_o$ (Althaus et al., 2007) (Figure 12 A, B).
Figure 12: Human αβγ-ENaC is activated by SF

A) A representative TEVC current trace of an oocyte expressing αβγ-ENaC. The grey bar indicates no shear force application (0 dyn*cm$^{-2}$) and the orange bar the application of SF (0.2 dyn*cm$^{-2}$). The application of SF caused an increase in amiloride-sensitive current. The additional application of amiloride (a, 10 μM) led to a rapid current decrease. Amiloride was used as an ENaC blocker, to determine ENaC-mediated currents (I). B) Statistical analysis of the αβγ-ENaC-mediated currents (I) of all (n = 14) measured oocytes. The application of SF (0.2 dyn*cm$^{-2}$) caused a significant current increase compared to control (0 dyn*cm$^{-2}$) (**** $p < 0.0001$; n = 14; N = 3). C) Amiloride prevents SF responses of αβγ-ENaC. The wash-out of amiloride with 0.2 dynes SF increases the transmembrane current, revealing ENaC expression in these cells. D) Water injected oocytes did neither respond to SF nor amiloride. Two-tailed paired Student’s t-test.

The amiloride-sensitive current of αβγ-ENaC plateaus at 2.32 ± 0.38 μA in the absence of SF ($I_0$; 0 dyn*cm$^{-2}$). Following the application of SF ($I_{0.2}$; 0.2 dyn*cm$^{-2}$) a rapid increase to 4.12 ± 0.45 μA was observed (**** $p < 0.0001$; n = 14; N = 3; Figure 12 B). The subsequent application of amiloride (10 μM) blocks the bulk of currents from 4.12 ± 0.45 to 0.43 ± 0.10 μA, confirming that the measured
currents were due to the activity of ENaC. These experiments validate that αβγ-ENaC is activated by SF (Figure 12 A, B).

Oocytes expressing αβγ-ENaC were also exposed to SF in the presence of amiloride to ensure that the SF response is due to an activation of ENaC and not endogenous channels. Here no changes in membrane current in response to SF were observed (Figure 12 C) until amiloride was removed. Further experiments were performed using water-injected oocytes to reveal whether or not endogenous channels are blocked by amiloride. The water-injected oocytes did not show changes in membrane current due to an application of SF (0.2 dyn*cm⁻²) or amiloride (10 μA) (Figure 12 D). Both experiments support the observation that the SF response is indeed caused by ENaC and that endogenous channels of oocytes do not respond to SF.

Together, these results confirm that αβγ-ENaC forms a mechanosensitive ion channels that respond to SF. It also shows that the measured transmembrane current of αβγ-ENaC expressing oocytes is mediated by ENaC and not due to endogenous channels expressed in oocytes.

1.4 Homotrimeric α-ENaC is activated by SF

To test whether homotrimeric α-ENaC is activated by SF, oocytes were injected with α-ENaC and current was measured via the TEVC method. After equilibration of the amiloride-sensitive current and in the absence of SF, a current value of 31 ± 5 nA was measured ($I_0$; 0 dyn*cm⁻²). The application of SF caused a current
increase to 66 ± 7 nA ($I_{0.2}$; 0.2 dyn*cm$^{-2}$) (***, $p > 0.0001$; n = 18; N = 4; Figure 13).

**Figure 13:** Human α-ENaC responds to SF
A) Representative current trace of an oocyte expressing α-ENaC. The ENaC mediated current (I) plateaued after a short equilibration phase (0; grey bar). The subsequent application of SF (0.2; orange bar) caused an increased activation of α-ENaC. Amiloride (10 μM) was used to determine the ENaC mediated membrane current (a; black bar). B) Statistical analysis of the ENaC mediated currents (I) showed a significant increase under SF ($I_{0.2}$) exposure compared to $I_0$. (**** $p < 0.0001$; n = 18; N = 4). Two-tailed paired Student’s t-test.

This finding confirms that homotrimeric α-ENaC expressed in oocytes form functional channels that can be activated by SF.

### 1.5 Homotrimeric β-ENaC is activated by SF

Homotrimeric β-ENaC was expressed in oocytes and changes in response to SF recorded via the TEVC method. After around 2 min, the amiloride-sensitive current plateaued at 18 ± 10 nA and increased under SF application to 26 ± 11 nA. The comparison between the SF mediated currents ($I_{0.2}$) with the amiloride-sensitive current without SF ($I_0$) showed no significant difference (p = 0.104; n = 5; N = 6; Figure 14 A, C).
**Figure 14:** Homotrimeric β-ENaC incubated for 24 hours did not respond to SF

A) A representative current trace of an oocyte expressing homotrimeric β-ENaC. The application of SF leads to a slight increase in amiloride-sensitive current. B) Pie chart of β-ENaC showing in black: 3 of the oocytes responded to amiloride, in orange: 5 of the oocytes responded to amiloride + SF and in white 17 of the oocytes did not respond to amiloride + SF. C) The application of SF did not show a significant increase in ENaC mediated current (I) \((p = 0.074; n = 5; N = 6)\) Two-tailed paired Student’s \(t\)-test.

Previous observations of wild-type αβγ-ENaC (section 1.3) showed that all of the injected oocytes responded to SF. Conversely, just 5 out of 25 oocytes expressing homotrimeric β-ENaC displayed a response to SF following 24 h of incubation (Figure 14 B). After 24 h of incubation, 3 out of 25 of homotrimeric β-ENaC oocytes responded to amiloride, 5 showed an amiloride + SF response and the remaining 17 oocytes did not respond to amiloride + SF (Figure 14 B). Following 48 h of incubation, 5 out of 20 oocytes showed a response to amiloride, 8 responded to amiloride + SF and 7 did not respond to amiloride + SF. The ENaC mediated current plateaued at 7 ± 2 nA reaching a peak of 9 ± 2 nA under SF exposure \((** p < 0.01; n = 8; N = 5)\) (Figure 15 A and B). Following 72 h of incubation, 3 out of 15 oocytes responded to amiloride, 8 responded to amiloride + SF and 4 oocytes did not respond to either. After reaching a steady amiloride-sensitive current value at 7 ± 2 nA, SF caused a current increase of 10 ± 1 nA \((** p < 0.01; n = 8; N = 4)\); (Figure 15 C and D). The 96 h incubation, 7 out of 16 oocytes showed an amiloride response and 2 responded to amiloride + SF. The remaining 7 oocytes did not respond to amiloride + SF. The ENaC mediated current plateaued at a current value
of 3 ± 3 nA and increased to 5 ± 3 nA under SF exposure (n = 2; N = 4; Figure 15 E and F).

Figure 15: Increased incubation time of homotrimeric β-ENaC leads to a higher response to SF
A) Pie chart showing in black that 5 oocytes responded to amiloride, in orange 8 oocytes responded to amiloride + SF and in white 7 neither responded to amiloride nor SF. B) The statistical analysis of β-ENaC showed a significant increase of current under SF exposure following 48 h incubation (** p < 0.01; n = 8; N = 5). C) Pie chart showing that 3 oocytes out of 15 responded to amiloride, 8 responded to amiloride + SF and 4 did not respond to both. D) Incubation of β-ENaC for 72 h showed a current increase under SF application (** p < 0.01; n = 8; N = 4). E, F) From 16 oocytes, 7 responded to amiloride, 2 responded to amiloride + SF and 7 did not respond to amiloride + SF. Incubation of β-ENaC for 96 h led to the detection of 2 oocytes responding to SF (no statistical analysis was possible). Two-tailed paired Student’s t-test.

Taken together, figure 12 and 13 is showing that increasing the incubation time of homotrimeric β-ENaC to 48 or 72 h results in oocytes responding to SF. The
response to SF was lost after increasing the incubation time to 96 h (Figure 15). These results demonstrate that oocytes expressing homotrimeric β-ENaC respond to SF after increasing the incubation time to 48 h and 72 h, indicating a longer trafficking- or maturing-time of the channels is needed.

1.5.1 The insertion of the FLAG epitope tag in the β-ENaC subunit does not affect response to SF of αβγ-ENaC

To quantify homotrimeric β-ENaC expression in the oocyte membrane, biotinylation experiments were performed. Therefore, β-ENaC subunit was tagged with a FLAG epitope tag, a common method to label proteins, including ion-channels (Michlig et al., 2005; Brown et al., 2013; Fezai et al., 2015; Prechtel et al., 2018). The FLAG epitope tag was inserted into a β-ENaC plasmid (Fronius et al., 2010) via site directed mutagenesis.

The SF response of αβFlagγ-ENaC expressing oocytes were compared to αβγ-ENaC to confirm that the insertion of a Flag tag does not affect the activity of the channel in response to SF. Therefore, the amiloride-sensitive current under SF (I0.2) of αβγ-ENaC (control) was normalised to the amiloride-sensitive currents without SF (I0) and set to the value 1.0 ± SEM. Afterwards the normalised SF response of αβFlagγ-ENaC were compared to control. Oocytes expressing αβFlagγ-ENaC, showing a SF effect of 1.21 ± 0.17 (p = 0.3447; n = 16; N = 4; Figure 16 A) that did not change in comparison with αβγ-ENaC. In addition, to verify whether channel open kinetics were affected by the Flag epitope tag, Tau of αβγ-ENaC was set to the value 1.0 and normalised to Tau of αβFlagγ-ENaC. The comparison of the normalised time
constant Tau, between αβγ-ENaC (1.00 ± 0.12) and αβFlagγ-ENaC (1.16 ± 0.16) was unchanged \( (p = 0.4206; n \geq 16; N = 4; \text{Figure 16 B}) \).

**Figure 16**: Insertion of epitope tag (Flag) into β-ENaC co-expressed with α- and γ-ENaC does not change response to SF and tau compared to αβγ-ENaC

A) Statistical analysis of oocytes expressing αβγ- and αβFlagγ-ENaC. Channels were exposed to SF and the increased amiloride-sensitive current \( (I_{10.2}) \) was normalised to the amiloride-sensitive current \( (I_0) \). The SF response of αβγ-ENaC was set to the value 1.0 and normalised to the SF response of αβFlagγ-ENaC. The comparison between αβγ- and αβFlagγ-ENaC did not show a significant difference in response to SF \( (p = 0.3447; n \geq 16; N = 4) \). B) The statistical analysis of αβFlagγ-ENaC normalised to αβγ-ENaC did not show a significant change in Tau under SF exposure \( (p = 0.4206; n \geq 16; N = 4) \). Unpaired Student’s \( t \)-test.

This data demonstrates that the insertion of a Flag tag in the β-ENaC does not affect the SF activation of ENaC and the channel opening kinetics in response to SF.

### 1.5.2 Homotrimeric β-ENaC alone can form a functional channel that assembles on the membrane of oocytes

To quantify the expression of β-ENaC in the oocyte membrane the βFlag-ENaC subunit was expressed for 48 hours. Two groups of 50 oocytes expressing βFlag-ENaC were used for the biotinylation: one group was treated with biotin and one without biotin. Three samples were taken for each group: (1) βFlag-ENaC whole-cell after lysation, showing the whole amount of βFlag-ENaC of each group; (2)
β\textsubscript{Flag}-ENaC cytoplasm, showing the amount of cytoplasmic β\textsubscript{Flag}-ENaC of each group; and (3) β\textsubscript{Flag}-ENaC membrane, showing the amount of β\textsubscript{Flag}-ENaC that assembles at the membrane of each group.

Following western-blot analysis, results showed an unexpected band in column 3, indicating that the protein binds independently of the biotin to the beads (Figure 17).

A possible explanation for the band in lane 3 (Mb) could be the high number of 11 glycosylated asparagines, respectively the N-glycans attached to the asparagines.

This could make the protein very attractive to the beads, resulting in binding to the beads that is independent of the biotin. To investigate this possibility, further experiments were performed by using the enzyme PNGaseF. PNGaseF cleaves highly specific N-glycans attached to asparagines of proteins (Plummer & Tarentino, 1991; Tarentino & Plummer, 1994; Freeze & Kranz, 2010). Identical experiments were performed and whole-cell samples of both groups were incubated with PNGaseF to remove all N-glycans of β\textsubscript{Flag}-ENaC.
Figure 18: Biotinylation assay using a βFlag-ENaC expressing oocyte incubated for 48 h treated with PNGaseF

An identical experiment procedure was used as described in Figure 16. Representative western-blot of a βFlag-ENaC expressing oocyte (48 h) treated with PNGaseF. The molecular weight of βFlag-ENaC with (+) N-glycans is 96 kDa and the molecular weight of βFlag-ENaC after PNGaseF treatment without (-) N-glycans is 74 kDa. The two black bars with 100 kDa and 75 kDa indicates the protein-marker. The βFlag-ENaC protein was detected in all sample lanes but not in lane number 5 showing that βFlag-ENaC without biotin treatment did not bind to the beads. The quantification revealed a significant smaller amount of protein in percentage on the membrane (Mb) compared to Wc and Cyto (p < 0.0001). The same was observed by comparing Wc with Cyto (p < 0.0001, N = 3). Exposure time 5 sec. for Wc, Cyto and 10 min for Mb.

The successful cleavage of all N-glycans in βFlag-ENaC was achieved as indicated by a smaller molecular weight of cytoplasmic and membrane samples (Figure 18). The prediction that oocytes expressing βFlag-ENaC might bind to the beads, in the absence of biotin, was supported since removal of all N-glycans prevents the binding to the beads (see lane 5 (Mb), Figure 18) that was previously observed. The quantification of western-blots performed after 48 h of incubation of βFlag-ENaC revealed that the amount of protein in the membrane (Mb) is significantly smaller compared to Wc and Cyto (p < 0.0001, N = 3) (Figure 18).
experiment was performed using lysates of cells expressing $\beta_{\text{Flag}}$-ENaC for 24 h. The western-blots of these samples did not show any bands in lane 5 and 6 indicating that no, or a not verifiable amount, of $\beta_{\text{Flag}}$-ENaC in the cell-membrane after 24 hours (Figure 19).

![Figure 19: Biotinylation of a $\beta_{\text{Flag}}$-ENaC expressing oocyte incubated for 24 h and treated with PNGase F](image)

Identical biotinylation experiments were performed as described before (Figure 16). Lane 5 and 6 did not display a band indicating that no or a not verifiable amount of $\beta_{\text{Flag}}$-ENaC was expressed on the membrane of oocytes ($N = 3$). Exposure time 5 sec. for Wc, Cyto and 10 min for Mb.

In summary, these data indicates that $\beta_{\text{Flag}}$-ENaC requires 48 h to be expressed at the membrane surface in a detectable protein amount. After incubating $\beta_{\text{Flag}}$-ENaC for 24 h, not sufficient amount of protein was detected by biotinylation. Contradictory, 8 of all oocytes incubated for 24 h showed an amiloride-sensitive response and an overall of just 5 from 25 oocytes, from 2 oocyte batches out of 6, responded to SF. One explanation could be a change in the post-translational modification machinery throughout different oocyte batches (Green, 2002). This could result in a faster maturation of homotrimeric $\beta$-ENaC in some oocyte batches compared with others ones.
1.6 Homotrimeric γ-ENaC channel does not respond to SF

To examine whether homotrimeric γ-ENaC responds to SF, oocytes were injected with γ-ENaC cRNA, incubated for 24 h (n = 13; N = 3), 48 h (n = 14; N = 3), 72 h (n = 13; N = 4) and current was recorded via using the TEVC method (Figure 20). None of the subsequently measured oocytes expressing γ-ENaC showed an amiloride-sensitive current with and without SF. An example of a representative current trace and the statistical analysis of an oocytes expressing γ-ENaC incubated for 24 h is shown in figure 20. This result suggests that the channel might not be assembled on the membrane surface or in contrast to α- and β-ENaC may not be able to respond to SF.

Figure 20: Homotrimeric γ-ENaC does not respond to SF
A) A representative current trace of an oocyte expressing homotrimeric γ-ENaC. The application of SF did not induce an amiloride-sensitive current increase. B) Bar graph showing that none of the oocytes responded to SF (n = 13; N = 3). Two-tailed paired Student t-test.

1.7 Human δβγ-ENaC is activated by SF

To investigate whether human δβγ-ENaC is activated by SF, TEVC recordings were performed, using δβγ-ENaC expressing oocytes. The δβγ-ENaC expressing oocytes showed an amiloride-sensitive current after applying SF. The ENaC
mediated current of δβγ-ENaC plateaued at 4.06 ± 0.49 μA in the absence of SF. The application of SF caused an increase to 5.25 ± 0.63 μA (**** p < 0.0001; n = 12; N = 4; **Figure 21**). Together, these results confirm that δβγ-ENaC is mechanosensitive ion channels that respond to SF when expressed in *Xenopus* oocytes.

![Figure 21: Human δβγ-ENaC is activated via SF](image)

A) Current trace of a δβγ-ENaC oocyte, measured by the TEVC method. The amiloride-sensitive current increased under SF exposure and was blocked by amiloride. B) The statistical analysis of the ENaC mediated current (I) showed a higher activation of channels under SF (0.2 dyn*cm*²) compared to amiloride-sensitive current without SF (0 dyn*cm*²). (**** p < 0.0001; n = 12; N = 3); Two-tailed paired Student’s t-test.

### 1.8 Human δ-ENaC responds to SF when expressed as a homotrimeric channel

Oocytes expressing homotrimeric δ-ENaC were exposed to SF and changes in response to SF recorded via the TEVC method. After equilibration of the amiloride-sensitive currents at 28 ± 8 nA, the application of SF led to a significant current increase to 75 ± 20 nA (**** p < 0.0001; n = 17; N = 4; **Figure 22**). This data shows that δ-ENaC expressed as a homotrimeric channel can form a functional channel that responds to SF.
1.9 Amiloride-sensitive current of homotrimeric ENaC subunits varies in comparison to αβγ- and δβγ-ENaC

Previous work demonstrated that amiloride-sensitive currents of homotrimeric ENaC expressed in oocytes are smaller compared to wild-type ENaC (αβγ-ENaC) (Canessa et al., 1994b). Based on this study, amiloride-sensitive currents of α-, β48h- or β72h-ENaC were compared to αβγ-ENaC (control), and δ-ENaC was compared to δβγ-ENaC (control) to confirm that homotrimeric ENaCs exhibit a smaller amiloride-sensitive current. The amiloride-sensitive current is a common measure that reflects the number of ENaCs expressed on the cell membrane of oocytes (Firsov et al., 1996; Firsov et al., 1998). All amiloride-sensitive currents (I₀) of homotrimeric ENaC were normalised to controls and set at 1.0 ± SEM. Homotrimeric α-ENaC showed a smaller amiloride-sensitive current of 0.026 ± 0.004 compared with αβγ-ENaC (control) (**** p < 0.0001; n = 18; N = 4; Figure 23). Followed by homotrimeric β-ENaC incubated for 48 h which also showed a smaller amiloride-sensitive current of 0.0035 ± 0.0009 compared with control...
Homotrimeric β-ENaC incubated for 72 h, had a lower amiloride-sensitive current of 0.004 ± 0.0009 when compared with control (1.00 ± 0.2) (**p < 0.001; n = 8; N = 3; Figure 23). Homotrimeric δ-ENaC displayed a smaller amiloride-sensitive current of 0.007 ± 0.003 compared with δβγ-ENaC (**p < 0.0001; n = 17; N = 4; Figure 23).

In summary, all homotrimeric ENaC showed a much lower amiloride-sensitive current, indicating a smaller number or smaller Po of the channels expressed on the membrane surface of oocytes. In addition it has to be considered that different populations of ENaC are expressed on the membrane (cleaved/non cleaved ENaC channels) or changes in conductance, might affect amiloride-sensitive currents. This suggests a disrupted trafficking process resulting in a lower expression of all homotrimeric ENaC on the cell surface of oocytes, compared to control (αβγ- or δβγ-ENaC).
1.10 Heterotrimeric αβ-ENaC responds to SF

To investigate whether heterotrimeric αβ-ENaC respond to SF, currents in oocytes expressing αβ-ENaC were measured via the TEVC method. Heterotrimeric αβ-ENaC showed an increase in amiloride-sensitive current after applying SF. The quantification showed a significant increase from $14 \pm 3 \text{nA} \ (0 \text{ dyn/cm}^2)$ to $30 \pm 4 \text{nA}$ under SF exposure ($0.2 \text{ dyn/cm}^2$) (** ** $p < 0.0001; n = 21; N = 6$; Figure 24). This result demonstrated that heterotrimeric αβ-ENaC can form a functional channel that responds to SF without the co-expression of γ-ENaC, indicating that the subunit composition of ENaC can be changed to a channel consisting of two subunits without losing the entire SF responsiveness.

![Figure 24: Heterotrimeric αβ-ENaC respond to SF](image)

A) Representative current trace of a αβ-ENaC expressing oocyte. The amiloride-sensitive current increased under SF (0.2 dyn/cm²) exposure. B) Histogram shows that the ENaC mediated current (I) increased after applying SF ($I_{0.2}$) (** ** $p < 0.0001; n = 21; N = 6$). Two-tailed paired Student $t$-test.

1.11 Heterotrimeric αγ-ENaC is activated via SF

Further TEVC experiments were performed to investigate whether the co-expression of the α- and γ-ENaC subunit changes the SF response. The analysis of the amiloride-sensitive current of αγ-ENaC showed a robust increase current from...
166 ± 40 to 658 ± 132 nA after applying SF (** p < 0.0001; n = 16; N = 4; Figure 25). This result demonstrates that heterotrimeric αγ-ENaC can form a functional channel that responds to SF. In addition, the observed SF effect of αγ-ENaC without the β-ENaC subunits supports to the assumption that individual subunits of ENaC might have a relevant modulatory role in sensing SF.

**Figure 25:** Heterotrimeric αγ-ENaC is activated via SF
A) A current trace of an oocyte expressing αγ-ENaC. The application of SF leads to an increase of amiloride-sensitive current. B) The analysis of the ENaC mediated current (I) showed a significant current increase in response to SF (I_{0.2}) (** p < 0.0001; n = 16; N = 4). Two-tailed paired Student t-test.

### 1.12 Heterotrimeric βγ-ENaC responds to SF

The next ENaC combination that was tested via the TEVC method for changes in activity, due to SF exposure, was the heterotrimeric βγ-ENaC. Interestingly, incubating heterotrimeric βγ-ENaC for 24 h results in an inconsistent number of oocytes responding to SF, similar as previous described for homotrimeric β-ENaC (section 1.5). Following 24 h of incubation, 5 out of 15 oocytes expressing heterotrimeric βγ-ENaC responded to amiloride, 2 responded to amiloride + SF and 8 did not respond to amiloride + SF. Data analysis was not performed due to a lack of sufficient number of βγ-ENaC oocytes responding to SF (n = 2). After increasing
the incubation time to 72 h, 4 out of 13 oocytes responded to amiloride and, 9 responded to amiloride + SF. The amiloride-sensitive current of $\beta_{72h}$-ENaC plateaued at a current value of $25 \pm 10$ nA and increased to $49 \pm 27$ nA under SF exposure (*** $p < 0.001$; $n = 9$; $N = 4$; Figure 26). Following 96 h of incubation, 6 out of 12 oocytes responded to amiloride, 5 responded to amiloride + SF and 1 did not respond to amiloride + SF The response to SF did not change following 96 h of incubation ($p = 0.0625$; $n = 5$; $N= 3$; Figure 26).

Together, these data showed that increasing the incubation time of heterotrimeric $\beta\gamma$-ENaC to 72 h leads to a growing number of oocytes responding to amiloride and SF. A longer incubation time to 96 h did not improve the activity of the channel in response to SF, suggesting a loss of RNA potency or a reduced function of the protein translation machinery of oocytes (Figure 26). This further supports the assumption that the $\alpha$-ENaC subunit is the main subunit that is important for regular trafficking of ENaC in Xenopus oocytes (Butterworth et al., 2008; Butterworth et al., 2009; Butterworth, 2010).
Figure 26: Heterotrimeric βγ-ENaC partly respond to SF in a time dependent manner

A) Pie chart shows that 5 out of 15 oocytes respond to amiloride (in black), 2 responded to amiloride + SF (in orange) and 8 did not respond to either amiloride or SF (in grey). B) No statistical analysis was possible using βγ-ENaC expressing oocytes incubated for 24 h through a lack oocytes responding to SF. C) Pie chart shows that 4 oocytes respond to amiloride, 9 responded to amiloride + SF. D) Increasing the incubation time of βγ-ENaC to 72 h leads to a higher activation of heterotrimeric βγ-ENaC under SF exposure (*** p < 0.01; n = 14; N = 4). E) Pie chart of βγ-ENaC incubated for 96 h showing that 6 responded to amiloride, 5 responded to amiloride + SF and 1 did not respond to amiloride + SF). F) Histogram shows no significant increase of amiloride-sensitive current under SF exposure of oocytes expressing βγ-ENaC, incubated for 96 h (p = 0.0625; n = 12; N = 3) Two-tailed paired Student t-test.
1.13 The co-expression of δβ-ENaC causes an increased activation during SF exposure

After finishing all subunit combinations possible with αβγ-ENaC, experiments were continued substituting α- with δ-ENaC. The next tested combination was the co-expression of δ- with β-ENaC as a heterotrimeric channel. The amiloride-sensitive current equilibrated at a value of 11 ± 1 nA increasing to 13 ± 1 nA under SF exposure (**p < 0.01; n = 12; N = 3; Figure 27). In summary, the exposure of heterotrimeric δβ-ENaC to SF leads to an increased activation of the channel. The SF response of δβ-ENaC compared to αβ-ENaC (Figures 30 and 31) showed similarities, suggesting that the β-ENaC subunit co-expressed with α- or δ-ENaC might have a similar modulatory role in sensing SF.

![Figure 27: Heterotrimeric δβ-ENaC responds with an increased activation during SF exposure](image)

A) Current trace of a δβ-ENaC expressing oocyte, showing an increased activation of the amiloride-sensitive current after the application of SF. B) Statistical analysis of the ENaC mediated current (I) showed a significant current increase under application of SF (**p < 0.001; n = 12; N =3). Two-tailed paired Student t-test.

1.14 Heterotrimeric δγ-ENaC responds to SF

The activity of heterotrimeric δγ-ENaC in response to SF was examined via the TEVC method. The amiloride-sensitive current plateaued at 31 ± 7 nA and SF
application showed a current increase to 51 ± 11 nA (** p < 0.01; n = 9; N = 3; Figure 28). This result shows that δγ-ENaC can form a functional channel that is activated by SF.

**Figure 28:** Heterotrimeric δγ-ENaC exhibits a robust increase of activity to SF exposure

A) Current trace showing the SF response of an oocyte expressing δγ-ENaC. The application of SF (0.2 dyn*cm⁻²) caused a current increase compared to amiloride-sensitive current without SF (0 dyn*cm⁻²)

B) The quantification of the ENaC mediated current (I) showed a significant increase under SF exposure (** p < 0.01; n = 9; N = 3). Two-tailed paired Student t-test.

1.15 Co-expression of two ENaC subunits expressed as a heterotrimeric channel has a low amiloride-sensitive current in comparison to αβγ- and δβγ-ENaC

To investigate whether co-expression of two subunits of ENaC expressed as a heterotrimeric channel change the amiloride-sensitive current (expression level), these channels were compared to αβγ- or δβγ-ENaC. The amiloride-sensitive currents of αβγ- or δβγ-ENaC was normalised to the value 1.0 ± SEM. Heterotrimeric αβ-ENaC showed a lower amiloride-sensitive current of 0.009 ± 0.002 compared to αβγ-ENaC (**** p < 0.0001; n = 22; N = 6; Figure 29). The comparison of heterotrimeric αγ-ENaC showed a smaller amiloride-sensitive current of 0.11 ±0.026 with αβγ-ENaC (*** p < 0.001; n = 17; N = 4; Figure 29).
Heterotrimeric βγ-ENaC incubated for 72 h plateaued at an amiloride-sensitive current of 0.005 ± 0.0009 and was smaller compared to αβγ-ENaC (**** p < 0.0001; n = 14; N = 4; **Figure 29**). In addition, the amiloride-sensitive current of δβ-ENaC settled at 0.0026 ± 0.0003 and was lower compared to δβγ-ENaC (**** p < 0.0001; n = 12; N = 3; **Figure 29**). A smaller amiloride-sensitive current of 0.0075 ± 0.0017 was also observed in comparison to δβγ-ENaC (1.00 ± 0.12) (**** p < 0.0001; n = 9; N = 3; **Figure 29**). These data reveals that combining two ENaC subunits as a heterotrimeric channel leads to a lower amiloride-sensitive current when compared with αβγ- or δβγ-ENaC suggesting an impaired trafficking resulting in a lower number or smaller Po of these channel expressed at the cell surface of oocytes. The change in Po could be confirmed by further investigating heterotrimeric ENaC channels via the patch clamp technique. Another aspect that has to be considered is the possibility that different populations of channels are expressed on the cell membrane that represent fully mature/cleaved, immature or different compositions of ENaC channels.

**Figure 29:** Expression level of all heterotrimeric ENaC compared to control
Heterotrimeric ENaC were normalised to either αβγ- or δβγ-ENaC controls (1.0; dotted line). The amiloride-sensitive current of either αβ-, αγ- or βγ72h-ENaC was decreased compared with control (αβ-, βγ72h-ENaC: **** p < 0.0001; n = 21; N ≥ 4; αγ-ENaC; *** p < 0.001; n = 16; N = 4). The comparison of δβ- and δγ-ENaC showed a decrease in amiloride-sensitive current compared with control (**** p < 0.0001; n ≥ 9; N = 3). Unpaired Student’s t-test.
1.16 The β- and γ-ENaC subunit have a modulatory role in αβγ-ENaC activation in response to SF

Homotrimeric (α-, β-ENaC) and heterotrimeric (αβ-, αγ-, βγ-ENaC) combinations were compared to wild-type αβγ-ENaC, to elucidate whether the β- and γ-ENaC subunit have a modulatory role in sensing SF. Still questionable remains the composition of heterotrimeric (αβ-, αγ-, βγ-ENaC), which will be further elucidated in the discussion (section 1.2) The amiloride-sensitive response under SF (I_{0.2}) was normalised to the amiloride-sensitive response without SF (I_0) to eliminate differences in channel activity and expression. The SF response of αβγ-ENaC was set at 1.0 ± SEM, and subsequently the SF response of α-, β-, αβ-, αγ-, or βγ-ENaC were normalised to the SF response of αβγ-ENaC. The comparison of homotrimeric α-ENaC revealed a SF effect (I_{0.2}/I_0) of 1.413 ± 0.45 that did not change compared to αβγ-ENaC (p = 0.9999; n = 18; N = 4; Figure 30). After increasing the incubation time to 48 h of β-ENaC, the channel responded with a decreased SF effect of 0.554 ± 0.038 in comparison to αβγ-ENaC (* p < 0.05; n = 8; N = 6; Figure 30). After incubating β-ENaC for 72 h, the SF effect of 0.611 ± 0.096 was significantly decreased when compared with αβγ-ENaC (* p < 0.05; n = 8; N = 4; Figure 30). Heterotrimeric αβ-ENaC show a SF effect of 1.217 ± 0.29 that did not change compared to αβγ-ENaC (p = 0.8129; n = 21; N = 6; Figure 30), whereas αγ-ENaC showed a robust activation of the channel showing a SF effect of 2.249 ± 0.39 compared with αβγ-ENaC (*** p < 0.001; n = 16; N = 4; Figure 30). Finally, βγ-ENaC incubated for 72 h, displayed a SF effect of 0.652 ± 0.09 compared with αβγ-ENaC (Figure 30).

In summary, this analysis showed that when homotrimeric β-ENaC is incubated for 48 h and 72 h, a decreased SF response was observed compared to control,
indicating that β-ENaC might have a modulatory role in sensing SF. It suggests that the β-ENaC subunit may balance the αβγ-ENaC response to SF by fine-tuning the activity of the channel. Contradictory to β-ENaC, co–expression of the α- and β-ENaC subunit did not show a change in response to SF compared to αβγ-ENaC. However, the examination of heterotrimeric αγ-ENaC showed a strong response to SF, supporting the assumption that the β-ENaC subunit might work as a brake and regulate the SF response of αβγ-ENaC. To ensure a proper function of the αβγ-ENaC to SF, β-ENaC subunit may attenuate the SF response while the γ-ENaC subunit may augment the SF response. These observations lead to the conclusion that both β- and γ-ENaC have a modulatory function in the regulation of sensing SF.

Figure 30: The β- and γ-ENaC subunit modulates activity of αβγ-ENaC in response to SF. Histogram shows comparison of homotrimeric and heterotrimeric ENaC which respond to SF. The SF response ($I_{0.2}/I_0$) of every examined ENaC was normalised with respect to αβγ-ENaC (dotted line; αβγ-ENaC). The comparison of α-ENaC with αβγ-ENaC did not show any changes in response to SF ($p = 0.9999; n = 18; N = 4$). Homotrimeric β-ENaC incubated for 48 h and 72 h showed a decreased response to SF compared to αβγ-ENaC (* $p < 0.05; n = 8; N ≥ 4$). In addition, αβ-ENaC did not show a change ($p = 0.8129; n = 21; N = 6$), whereas αγ-ENaC respond to the exposure of SF compared to αβγ-ENaC (*** $p < 0.001; n = 16; N = 4$). Finally, βγ-ENaC incubated for 72 h did not show a response to SF compared to αβγ-ENaC ($p = 0.0955; n = 9; N = 4$). Unpaired Student’s $t$-test.
1.17 The β- and γ-ENaC subunits modulate activity of δβγ-ENaC in response to SF

Homotrimeric δ-ENaC and heterotrimeric (δβ- and δγ-ENaC) combinations were compared to wild-type δβγ-ENaC, to investigate whether the single subunits change activity in response to SF. The SF response of wild-type δβγ-ENaC was set on the value 1.0 ± SEM and then the SF response of δ-, δβ-, or δγ-ENaC normalised to that SF response of δβγ-ENaC. The comparison of homotrimeric δ-ENaC showed a strong SF effect (I₀/2/I₀) of 2.133 ± 0.3 compared with δβγ-ENaC (*** p < 0.001; n = 17; N =4; Figure 31). Furthermore, heterotrimeric δβ- ENaC showed a SF effect of 0.964 ± 0.04 compared to δβγ-ENaC (p = 0.4933; n =12; N =3; Figure 31), whereas δγ-ENaC showed increased SF effect of 1.236 ± 0.05 (*** p < 0.001; n = 9; N = 3; Figure 31). These experiments support previous findings that the composition of the channel affects the SF sensing of ENaC (section 1.16). Both the heterotrimeric αγ- as well as the δγ-ENaC showed an increased response to SF without the β-ENaC subunit, indicating a modulatory role of the β- and γ-ENaC subunit. The augmented SF response of homotrimeric δ-ENaC could provide another mechanism of how ENaC senses and regulates the channel activation in response to SF.
**Figure 31:** The β- and γ-ENaC subunits modulate activity of δβγ-ENaC in response to SF. Histogram shows the normalised SF response (dotted line represents control value at 1). The comparison of δ-ENaC with δβγ-ENaC (control) showed a strong activation of the channel due to SF exposure (** p < 0.01; n = 17; N=4). Heterotrimeric δβ- did not respond with a change to SF (p = 0.4933; n = 12; N= 3), whereas δγ-ENaC showed an increased response to SF compared to control (*** p < 0.001; n =9; N=3). Unpaired Student’s t-test.

### 1.18 Summary

Taken together, these results show that homotrimeric α-, β-, δ-ENaC expressed in oocytes can form functional channels that respond to SF, whereas γ-ENaC cannot. The co-expression of two ENaC subunits can form heterotrimeric αβ-, αγ-, βγ-, δβ-, or δγ-ENaC that can form functional channels that respond to SF. Theamiloride-sensitive currents of homotrimeric and heterotrimeric ENaC showed a sturdy reduction, reflecting a smaller membrane expression or Po of these channels compared to control (αβγ- or δβγ-ENaC), indicating an impaired trafficking or maturation of the channels. However, this assumption must be further elucidated by investigating basic channel properties via the patch clamp technique. Further on, homotrimeric β-ENaC responded to SF after increasing the incubation time to 48 h and 72 h, indicating a longer trafficking time to form a functional channel that senses SF. This was supported by biotinylation experiments (section 1.5.2)
showing that β-ENaC assembles in a detectable amount on the membrane surface after 48 h but not after 24 h. An explanation for why some β-ENaC expressing oocytes showed a response to SF after 24 h could be due to a change in their protein-translation machinery of different oocyte batches. Another reason simply could be that a functional β-ENaC expressed in an oocyte need in general a longer maturing time until they respond to SF. A sufficient amount of β-ENaC protein was detected at the membrane after incubating β-ENaC for 48 h, suggesting that a slower trafficking or maturing process of homotrimeric β-ENaC may explain the lack of SF response following 24 h of incubation. The decreased SF response in homotrimeric β-ENaC and increased SF response in homotrimeric δ- and heterotrimeric αγ-ENaC compared to control, led to the assumption that the β- and γ-ENaC subunit might have a modulatory role in sensing SF. In addition, the longer trafficking time of β-ENaC could be one way of how tissues, such as vascular smooth muscle cells, where β- and γ-ENaC subunits are expressed without α-ENaC (Drummond et al., 2008) and respond to mechanical forces such as SF. This findings supports the assumption that heterotrimeric βγ-ENaC can form functional channels (mechanotransducer) that respond to SF in vascular smooth muscle cells.
2 Characterising of the underlying mechanism of how the β- and γ-ENaC subunits participate in shear force sensation
2.1 Introduction

The second objective aimed to investigate the underlying molecular mechanisms of how the β- and γ-ENaC subunits sense SF. Till today, not much is known about the underlying mechanism of how ENaC subunits respond to SF exposure. Previous studies on mechanosensitive channels of *C. elegans* – that belong to the same protein family as ENaC proteins – revealed that the extracellular matrix (ECM) is required for mechanical activation (Emtage *et al.*, 2004; Fronius & Clauss, 2008). Unpublished work from the Fronius lab established that an intact ECM is required for effective shear force (SF) mediated activation of ENaC. Furthermore, glycosylated asparagines within the α-ENaC subunit may provide an “anchor” point to the ECM since mutation of these asparagines reduced shear force responses (Knoepp *et al.*, 2017). However, although these experiments demonstrated that the α-ENaC subunit is important for SF sensation, an additional residual activation in response to SF was always observed suggesting that other subunits (β and/or γ) may also contribute to SF sensation. In addition, there is the possibility that the β and γ subunit facilitate their effect also through a connection to the ECM. This is supported by the fact that in some tissues mechanosensitive responses were observed in cells expressing just the β- and γ-ENaC subunits (Drummond, 2012). Therefore, I hypothesised that the tether of N-linked glycans of glycosylated asparagines of the β- and γ-ENaC subunits to the ECM are important for SF sensation. Experiments were designed by removing glycosylated asparagines (ASN) of the β- and γ-ENaC subunit through alanine substitution (ALA) (αβASN/ALAγ or αβγASN/ASA) (*Materials and Methods; section 2.1*). An overview of all performed experiments for objective 2 are shown in the tables below (*Tables 22 and 23*). Functional recordings were performed by using the TEVC and patch clamp method.
Table 22: Overview of β-ENaC mutations performed to objective 2

<table>
<thead>
<tr>
<th>β-ENaC single mutants</th>
<th>Oocytes number (n)</th>
<th>Batch (N)</th>
<th>Incubation time</th>
<th>Conserved in three species (human, rat, <em>Xenopus laevis</em>)</th>
</tr>
</thead>
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</tr>
<tr>
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<td>24 h.</td>
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</tr>
<tr>
<td>N207A</td>
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<td>24 h.</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>N364A + N378A</td>
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<td>3</td>
<td>24 h.</td>
<td>-</td>
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<td>3</td>
<td>24 h.</td>
<td>-</td>
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<td>3</td>
<td>24 h.</td>
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</tr>
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<td>3</td>
<td>24 h.</td>
<td>-</td>
</tr>
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</table>

Table 23: Overview of γ-ENaC mutations performed for objective 2

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<th>Batch (N)</th>
<th>Incubation time</th>
<th>Conserved in three species (human, rat, <em>Xenopus laevis</em>)</th>
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<td>N291A</td>
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<th>Batch (N)</th>
<th>Incubation time</th>
<th>Conserved in three species (human, rat, <em>Xenopus laevis</em>)</th>
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<td>24 h.</td>
<td>-</td>
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2.2 PNGaseF decreases the SF response of ENaC expressed in Xenopus oocytes

To elucidate whether the SF response of ENaC is due to N-glycans of glycosylated asparagines (which may provide a tether to the ECM), experiments with PNGaseF were performed via the TEVC method.

Two approaches were implemented to investigate whether N-glycans play an important role in sensing SF. (1) Oocytes expressing αβγ-ENaC were either extracellular treated with; (2) or injected with PNGaseF and then the SF response compared to αβγ-ENaC (control). The extracellular treatment or injection of PNGaseF might cleave N-glycans of glycosylated asparagines during trafficking to the cell membrane, followed by a detachment to the ECM (Figure 32).

(1) The extracellular PNGaseF treatment of αβγ-ENaC oocytes with PNGaseF (treatment 30 min – 4 h) did not show a change in response to SF compared to non-treated αβγ-ENaC oocytes (control) \( (p = 0.7430; n \geq 8; N = 1; \text{Figure 33}) \).
Figure 33: The Extracellular treatment of αβγ-ENaC oocytes with PNGaseF does not change response to SF compared with αβγ-ENaC

Statistical analysis of oocytes expressing αβγ-ENaC either extracellular treated with PNGaseF or not (control). Channels were exposed to SF and the increased amiloride-sensitive current (I_{0.2}) was normalised to the amiloride-sensitive current (I_0). The SF response of αβγ-ENaC (control) was set to the value 1.0 and normalised to the SF response of extracellular treated αβγ-ENaC oocytes. The comparison between αβγ- and extracellular treated αβγ-ENaC did not show a significant difference in response to SF ($p = 0.7430; n \geq 8; N = 1$). Mann-Whitney test.

(2) Oocytes were injected with RNA encoding the αβγ-ENaC subunits and also injected with PNGaseF ~ 24 h after injection, incubated for 30 min for up to 6 h and in parallel the response to SF recorded. The amiloride-sensitive current under SF (I_{0.2}) was normalised with respect to the amiloride-sensitive current before SF (I_0). For each time interval the SF effect ($I_{0.2}/I_0$) of non-PNGaseF treated and PNGaseF injected oocytes was determined. SF effect of PNGaseF treated oocytes was considered as 1.0 ± SEM and the responses of the PNGaseF treated was normalised to this response. After 30 min – 1 h the SF effect of 1.128 ± 0.16 was observed ($p = 0.5787; n = 10; N = 4$; Figure 34), after 1 – 2 h a SF effect of 1.191 ± 0.15 ($p = 0.4234; n = 9; N = 4$; Figure 34), and after 2 – 3 h a SF effect of 0.84 ± 0.11 ($p = 0.3867; n = 11; N = 4$; Figure 34). These SF effects between 30 min – 3 h did not change compared to non-PNGaseF treated αβγ-ENaC. After 3 – 4 h after PNGaseF injection the SF effect was reduced to 0.609 ± 0.05 (**$p < 0.01; n = 11; N = 4$; Figure 34) and after 4 - 5 h of injecting oocytes with PNGaseF, the SF effect was a further decreased to 0.407 ± 0.04 (**$p < 0.01; n = 12; N = 3$;
Figure 34) compared to non-PNGaseF treated αβγ-ENaC. This attenuation of SF responsiveness might be due to newly generated ENaC that were assembled in the membrane of oocytes occurring at 3 - 5 h because these channels may not have N-glycans attached. However, the SF response recurred after 5 - 6 h by reaching almost the same SF effect of 1.001 ± 0.18 as in the beginning of the experiment, indicating that PNGaseF might lose its potency after 5 - 6 h (p = 0.7984; n = 8; N = 2; Figure 34).

**Figure 34:** PNGaseF decreases the response to SF in αβγ-ENaC in a time dependent manner

A) Current traces showing a decreased SF response in a time dependent manner. The enzyme PNGaseF was injected into a αβγ-ENaC expressing oocyte and exposed to SF after the indicated incubation times. The measured amiloride-sensitive current was decreased during 3-5h and the SF response was-recurred completely after 5-6 h. B) Statistical analyses of αβγ-ENaC expressing oocytes treated with PNGaseF over 6 h. The SF response of αβγ-ENaC treated with PNGaseF were normalised to the untreated αβγ-ENaC. Therefore, amiloride-sensitive current values under SF (I0.2) were normalised to the amiloride-sensitive current values without SF (I0). Normalised amiloride-sensitive currents of αβγ-ENaC were set to 1.0 (dotted line) for better depiction of the data. SF values were taken between time-intervals of 30 min to 6h. SF response was significantly reduced after 3 – 5 h and were rescued between 5 – 6 h (** p < 0.01; n ≥ 8; N ≥ 2). Mann-Whitney test.

The amiloride-sensitive currents between PNGaseF-treated αβγ-ENaC and non-PNGaseF treated αβγ-ENaC in the absence of SF did not show a significant change during the whole experiment (p > 0.05; n ≥ 8; N ≥ 3; Figure 35).
Figure 35: PNGaseF does not affect amiloride-sensitive current of αβγ-ENaC
Histogram shows the amiloride-sensitive currents of αβγ-ENaC with PNGaseF. The amiloride-sensitive current of the non-PNGaseF treated αβγ-ENaC (control) were normalised to 1.0 (dotted line) and then compared with PNGaseF-treated oocytes. None of the PNGaseF-treated oocytes showed a change in SF response compared to control ($p > 0.05$; $n \geq 8$; $N \geq 3$). Mann-Whitney test.

The present data demonstrates that injecting PNGaseF into oocytes expressing αβγ-ENaC leads to a decreased SF response of that channel. The reduction of this response observed after 3 h might be due to an exchange of “old” ENaC (with N-glycans) with “new” ENaC (without N-glycans), suggesting that N-glycans were cleaved by PNGaseF while trafficking to the cell membrane of oocytes. The amiloride-sensitive baseline current did not change over the time period of 6h, meaning that injecting PNGaseF into oocytes might not impair or disrupt trafficking. These findings support the assumption that N-glycans of glycosylated asparagines are involved and important for sensing SF. Due to the presence of glycosylated asparagines in the β- and γ-ENaC subunit, it is suggested that they could and in particular their N-glycans might also be important for SF sensation. However, PNGaseF is not ENaC specific and it might be that other transmembrane or ECM proteins being acted on by PNGaseF.
2.3 Single glycosylated asparagines of the β-ENaC subunit in the β-ball, finger-, thumb-, and knuckle-domains are important for SF dependent activation of ENaC

To investigate glycosylated asparagines of the β-ENaC subunit in terms of changes in channel properties such as SF response, open channel kinetics (Tau) and amiloride-sensitive currents of ENaC, site directed mutagenesis was performed and functional recordings performed via the TEVC method. Therefore, asparagines were exchanged to alanines in the β-ENaC subunit and then co-expressed with wild-type α- and γ-ENaC. To exclude differences in expression and activity of ENaC, the amiloride-sensitive SF response (I₀.2) was normalised to the amiloride-sensitive current without SF (I₀). The recorded SF response of αβγ-ENaC (control) was set at 1.0 ± SEM and then the SF response of the αβMut,γ-ENaC mutation normalised to the control. The time constant Tau as well as the amiloride-sensitive currents without SF (I₀) of αβγ-ENaC were set to the value 1.0 ± SEM and then normalised to the mutated channel. For each mutation corresponding recordings of αβγ-ENaC were performed as individual controls. The predicted model of how β-ENaC co-expressed with the α- and γ-subunit sense SF is shown in Figure 36.
Figure 36: Predicted model of how β-ENaC co-expressed with the α- and γ-subunit sense SF

A) All ENaC subunits are tethered due to N-glycans of glycosylated asparagines (Gly-ASN; turquoise circle) to the extracellular matrix (ECM). The different subunits are coloured as: α-ENaC (red), β-ENaC (blue) and γ-ENaC (green). B) The application of SF causes a shift of the ECM leading to a conformational change of the channel, increasing the open probability (Po) of ENaC. C) Removal of individual or multiple glycosylated asparagines of β-ENaC (red circle) detaches the extracellular loop from the ECM, followed by a less active ENaC in response to SF.

Removal of the glycosylated asparagine in position N378 in β-ENaC located in the thumb-domain increases channel activity in response to SF. The statistical analysis revealed that nine out of eleven β-ENaC mutations did not show significant differences in response to SF when compared with αβγ-ENaC (Figure 37). The exchange of a glycosylated asparagine into an alanine in position N99 caused an elevated SF effect of $1.59 \pm 0.26 (** p < 0.01; n = 12; N = 3; \text{Figure 37}$) compared to αβγ-ENaC. In addition, the exchange of glycosylated asparagine in position N378 also showed an increased SF effect of $1.44 \pm 0.21 (* p < 0.05; n = 11; N = 3; \text{Figure 37}$) compared to αβγ-ENaC.
Figure 37: Glycosylated asparagine N378 plays an important role in SF sensation
A) Histogram shows data of oocytes expressing glycosylated asparagine deficient point
mutations in β-ENaC co-expressed with the α- and γ-subunit. The measured SF response \( (I_{0.2}) \) of
the wild-type αβγ-ENaC control was normalised to the amiloride-sensitive current without SF
\( (I_0) \) and set to 1.0 (dotted line). Then the SF responses of mutated β-ENaC channels were
compared with an individual control experiment. Two of the eleven β-ENaC mutations (N99 and
N378) showed an increased amiloride-sensitive current induced by SF compared to control (**)\( \text{p} < 0.01 \); *\( \text{p} < 0.05 \); n ≥ 11; N = 3) but no effect was observed in the remaining nine
mutations (\( p > 0.05 \); n ≥ 11; N ≥ 3). B) 3D model of a β-ENaC subunit illustrating all locations of the
eleven glycosylated asparagines within their channel domains. The different colours indicate
different domains such as: finger- (purple), knuckle- (turquoise), palm- (yellow), β-Ball-
(orange), thumb- (green) and 1+2 TMD (red) transmembrane-domains. One-way ANOVA
with multiple comparison.

The examination of nine asparagine deficient β-ENaC mutations did not display
changes in Tau; however, one in position N99 and one in position N364 showed an
increase in time taken to full activation of all channels (Figure 38). The αβN99γ-
ENaC mutation induced a slower response to SF from 1 ± 0.05 to 1.42 ± 0.20 (**\( p < 0.01 \); n = 12; N = 3), and αβN364γ-ENaC mutation also generated a slower
response from 1 ± 0.05 to 1.64 ± 0.27 (**\( * \text{p} < 0.001 \); n = 13; N = 3; Figure 38)
compared with controls. These results suggest that the exchange of glycosylated
asparagine in both positions N99 and N364 in the β-ENaC subunit affects channel
kinetics of αβγ-ENaC.
The Tau of the glycosylated asparagine deficient mutation N99 and N364 was slower compared to αβγ-ENaC. The Tau of the control experiment αβγ-ENaC was normalised to the value 1.0 and compared to all glycosylated asparagine deficient β-ENaC mutations. Two β-ENaC mutations, one in position N99 (** p < 0.01; n = 12; N = 3) and another in N364 (** p < 0.001; n = 13; N = 3) responded slower to the application of SF compared to αβγ-ENaC. One-way ANOVA with multiple comparison.

In addition, to examine whether the amiloride-sensitive current without SF (I₀) of asparagine deficient β-ENaC mutants is impaired, amiloride-sensitive currents were analysed and compared to αβγ-ENaC. Five out of the eleven β-ENaC mutants did not show changes in amiloride-sensitive baseline current compared with αβγ-ENaC, but six β-ENaC mutants exhibited a decreased amiloride-sensitive current. This was observed with replacement of N99 (** p < 0.01; n = 12; N = 3), N135 (* p < 0.05; n = 15; N = 3), N141 (**** p < 0.0001; n = 11; N = 3), N146 (** p < 0.01; n = 12; N = 3), N260 (** p < 0.01; n = 13; N = 3) and N484 (** p < 0.01; n = 12; N = 3), (Figure 39).

Figure 38: The Tau of the glycosylated asparagine deficient mutation N99 and N364 was slower compared to αβγ-ENaC. The Tau of the control experiment αβγ-ENaC was normalised to the value 1.0 and compared to all glycosylated asparagine deficient β-ENaC mutations. Two β-ENaC mutations, one in position N99 (** p < 0.01; n = 12; N = 3) and another in N364 (** p < 0.001; n = 13; N = 3) responded slower to the application of SF compared to αβγ-ENaC. One-way ANOVA with multiple comparison.
Figure 39: The amiloride-sensitive current of asparagine deficient β-ENaC mutations is decreased in positions N99, N135, N141, N146, N260 and N484. The number of αβγ-ENaC channels were normalised to 1.0 (dotted line) and then compared to all glycosylated asparagine deficient β-ENaC mutations. Six out of eleven β-ENaC mutations in position N99 (** \( p < 0.01; n = 12; N = 3 \)), N135 (* \( p < 0.05; n = 15; N = 3 \)), N141 (** ** \( p < 0.0001; n = 11; N = 3 \)), N146 (** \( p < 0.01; n = 12; N = 3 \)), N260 (** \( p < 0.01; n = 13; N = 3 \)) and N484 (** \( p < 0.01; n = 12; N = 3 \)) showed a decreased number of channels expressed at the membrane of oocytes compared to control. One-way ANOVA with multiple comparison.

The findings in sections 1.16 and 1.17 support a role of the β-ENaC subunit as a modulatory ENaC subunit for the SF response within αβγ-ENaC. The glycosylated asparagine in position N99 in the β-Ball domain and glycosylated asparagine in position N378 in the thumb-domain showed an increased response to SF. Contrary results were shown in previous studies from our lab showing that N-glycans of glycosylated asparagines in the α-ENaC subunit decrease response to SF (Knoepp et al., 2017). In addition, two β-ENaC mutations in position N99 and N364 presented a decreased response in Tau, indicating an impairment in the channel open kinetics of the channel. The changes in the amiloride-sensitive current of six β-ENaC mutations in position N99, N135, N141, N146, N260 and N484 leads to the assumption that the decreased number of channels expressed at the membrane of oocytes, reflecting a disrupted trafficking of the mutated channels. This and
previous findings in the α-ENaC could confirm that removing all N-glycans of glycosylated asparagines of ENaC (Section 2.2) and mutating single N-linked glycosylated asparagines in the β-ENaC subunit are important for sensing SF. In summary, the results confirm my hypothesis that glycosylated asparagines of β-ENaC are important for sensing SF and the accuracy of our model.

2.4 The increased SF response of αβN378Aγ-ENaC might be due to interaction with the α- and γ-ENaC subunit

The glycosylated asparagine in position N378, located in the thumb-domain showed an increased activity of the channel in response to SF. To investigate whether the increased SF response is due to changes in channel properties within the β-ENaC subunit or due to interaction with the α- and γ-ENaC subunit, experiments were performed expressing either homotrimeric βN378A, or heterotrimeric βN378Aγ- or αβN378A-ENaC.

SF responses of homotrimeric β- and βN378A-ENaC were measured 24 h, 48 h, 72 h and 96 h after injection and the SF response of βN378A normalised (I0.2/I0) to wild-type control (β-ENaC). None of the homotrimeric βN378A-ENaC expressing oocytes incubated for 24 h responded to SF, however 5 out of 16 oocytes showed an amiloride-sensitive response. The increased incubation time of βN378A-ENaC to 48 h showed that 8 out of 20 oocytes showed an amiloride-sensitive response and 5 responded to amiloride + SF. The comparison of the SF response of homotrimeric βN378A to wild-type β-ENaC showed no significance difference (p = 0.8444; n ≥ 4; N ≥ 3; Figure 40). After 72 h of incubation, 2 out of 13 oocytes
showed an amiloride response, 6 responded to amiloride + SF and 5 did not respond to amiloride + SF. After 96 h, 7 out of 16 oocytes responded to amiloride, 1 responded to amiloride + SF and 8 did not respond to amiloride + SF (Figure 40).

Figure 40: Homotrimeric βN378A shows no change in response to SF compared to control

A) Pie chart showing 8 out of 20 oocytes responding to amiloride (black), 4 responded to amiloride + SF (orange) and 8 did not respond to amiloride + SF (white). B) Homotrimeric βN378A was expressed in oocytes, incubated for 48 h and then SF response compared to β-ENaC (control). The SF response of control was normalised (I0.2/I0) and set to 1.0. The statistical analysis showed no change in SF response compared to control (p = 0.8444; n = 4; N = 5). C) Pie chart shows that 2 out of 13 oocytes responded to amiloride, 6 responded to amiloride + SF and 5 showed no response to amiloride + SF. D) The further incubation to 72 h of βN378A did not show any changes in response to SF compared to β-ENaC (p = 0.8518; n = 6; N = 3). E, F) Increasing the time to 96 h led to 7 out of 16 oocytes responded to amiloride, 1 responded to amiloride + SF and 8 did not respond to amiloride + SF. Unpaired Student’s t-test.
To further investigate whether the increased SF response of αβN378Aγ-ENaC is due to changes of the channel properties in the β-ENaC subunit itself or due to interactions with the α- or γ-ENaC subunit, SF responses of heterotrimeric βN378Aγ or αβN378A were determined. Identical experiments were performed by normalising the SF response of the mutated ENaC (βN378Aγ) to the wild-type control (βγ-ENaC). After 72 h of incubation, 1 out of 14 of oocytes responded to amiloride, 6 responded amiloride + SF and 7 did not respond to amiloride + SF. After 96 h, 2 out of 11 oocytes responded to amiloride, 5 responded to amiloride + SF and 4 did not respond to amiloride + SF. Both βN378A (72 h and 96 h) showed no difference in response to SF compared to control ($p > 0.05$; $n \geq 5$; $N \geq 3$; Figure 41).
Figure 41: The amiloride-sensitive current of βN378Aγ-ENaC shows no change in response to SF compared to βγ-ENaC

A) Out of a group of 14 oocytes, 1 responded to amiloride, 6 responded to both amiloride + SF and 7 did not respond to amiloride + SF. B) Histogram shows the normalised SF response of βN378Aγ-ENaC compared to the control βγ-ENaC (set to 1.0). The quantification of βN378Aγ-ENaC incubated for 72 h did not show any changes in response to SF (p = 0.7756; n = 6; N = 4). C) Pie chart showing 2 out of 11 oocytes responding to amiloride, 5 responded to amiloride + SF and 4 did not respond to amiloride + SF. D) The increased incubation time of βN378A-ENaC to 96 h did not change the response to SF in comparison to the control (p = 0.4206; n = 5; N = 3). Unpaired Student’s t-test.

Finally, heterotrimeric αβN378A-ENaC was examined and changes in SF response determined. Therefore, αβN378A was expressed in oocytes, incubated for 24 h, exposed to SF and compared to wild-type αβ-ENaC (control). The normalised (I_{0.2}/I_0) SF response showed no change in SF when compared to control (p = 0.9487; n = 12, N = 3; Figure 42).
Figure 42: Heterotrimeric αβN378A does not change response to SF compared to wild-type αβ-ENaC

Histogram showing normalised SF response ($I_{0.2}/I_0$) of wild-type αβ-ENaC (set to 1.0) in comparison to mutated αβN378A-ENaC. The statistical analysis showed no change in response to SF compared to control ($p = 0.9487$; $n=12$; $N=3$). Unpaired Student’s $t$-test.

The substitution of β-ENaC with mutated βN378A did not change response to SF in homotrimeric (βN378A) or heterotrimeric (βN378Aγ or αβN378A) ENaC. This data suggests that the glycosylated asparagine in position N378 did not change any channel properties within the β-ENaC subunit itself, indicating that the increased SF response of αβN378Aγ-ENaC might be due to an interaction between all three ($α$, $β$, $γ$) subunits. A disruption of this functional interaction of all three ENaC subunits could lead to a higher open probability ($P_0$) or higher conductivity ($G$) of the channel. To investigate this, cell attached patch clamp experiments were performed by using αβN378Aγ-ENaC and wild-type control αβγ-ENaC.

2.5 Single channel properties of αβN378Aγ-ENaC are not changed in comparison to control αβγ-ENaC

To determine whether the increased SF response of αβN378Aγ-ENaC were due to an enhanced SF sensation or due to mutant related changes causing a higher open
probability (Po) or conductivity (G), oocytes were injected with mRNA encoding αβN378Aγ-ENaC and αβγ-ENaC (control) and subsequently used for patch-clamp experiments. Cell-attached configuration experiments were performed to measure single-channel activity of αβN378Aγ-ENaC and αβγ-ENaC. The permeability (P) for Na⁺ was calculated by measuring different channel current amplitudes, analysing stages of membrane potential ranging from -20 mV to -100 mV (Figure 43).

![Figure 43: Single-channel recordings of αβγ-ENaC in a cell-attached configuration, clamped at different membrane potentials](image)

Patch-clamp current trace of αβγ-ENaC at different membrane potentials ranging from -20 mV to -100 mV. The dotted line represents the closed state of the channels (zero current).

Values at different membrane potentials were then plotted in a current-voltage curve (I-V-curve) (Figure 44). The data was fitted by using the Goldman-Hodgkin-Katz current equation. The calculation of the permeability at an extracellular Na⁺ concentration of 90 mM and an intracellular Na⁺ concentration of 11 mM (Sobczak et al., 2010) was performed by using the Goldmann-Hodgkin-Katz equation. The
αβγ-ENaC had a permeability of $1.43 \pm 0.026 \times 10^{-12} \text{ cm}^3/\text{s}$ and the αβN378Aγ-ENaC had a permeability of $1.47 \times 10^{-12} \pm 0.095 \times 10^{-12} \text{ cm}^3/\text{s}$. The comparison of both channels showed no difference in permeability (Table 24), indicating that the movement of Na$^+$ cations through the channel pore is not impaired.

![Membrane potential (mV)](image)

**Figure 44: Current-voltage curve (I-V-curve) of αβγ-ENaC and αβN378Aγ-ENaC mutation**

Current-voltage curve (I-V-curve) showing a comparison of αβγ-ENaC (black circle) with αβN378Aγ-ENaC (black squares). The conductance (G) was calculated by using data achieved by generating the I-V-curve. The data was fitted by using the Goldman-Hodgkin-Katz current equation.

The conductance (G) of αβγ-ENaC was $4.98 \pm 0.39 \text{ pS}$ and the conductance of αβN378Aγ-ENaC was $4.31 \pm 0.32 \text{ pS}$ and no difference was observed in both channels (Table 24). Furthermore, open probability (Pₒ) and relative open probability (NPₒ) did not show any difference by comparing αβγ-ENaC with αβN378Aγ-ENaC (Table 24). The data for the Pₒ was obtained from recordings at a membrane potential of -100 mV that lasted between 5-15 minutes. The comparison of the time where channels are closed (tₒ) and when the channels are
open (to) were also analysed and no difference was found in both channels (Table 24).

**Table 24: Single-channel properties of αβγ-ENaC and αβN378Aγ-ENaC mutant**

<table>
<thead>
<tr>
<th></th>
<th>P (cm²/s)</th>
<th>G(pS)</th>
<th>P₀</th>
<th>N₁₀₀₀₀</th>
<th>t₀ (s)</th>
<th>tₑ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (αβγ-ENaC)</td>
<td>1.43 ± 0.026</td>
<td>4.98 ± 0.39</td>
<td>0.17 ± 0.016</td>
<td>0.26 ± 0.06</td>
<td>2.22 ± 0.78</td>
<td>10.56 ± 3.5</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=4)</td>
<td>(n=4)</td>
<td>(N=4)</td>
<td>(n=4)</td>
<td>(n=4)</td>
</tr>
<tr>
<td>β-ENaC Mutation (N378A)</td>
<td>1.47 ± 0.095</td>
<td>4.31 ± 0.32</td>
<td>0.13 ± 0.083</td>
<td>0.24 ± 0.07</td>
<td>2.11 ± 0.41</td>
<td>14.3 ± 5.05</td>
</tr>
<tr>
<td>(n=7)</td>
<td>(n=7)</td>
<td>(n=4)</td>
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P: permeability; G: conductance; P₀: open probability; NP₀: relative open probability; t₀: mean open time; tₑ: mean close time. The values that are shown are means ± SEM; Unpaired Student’s t-test

The single channel properties of αβγ-ENaC and αβN378Aγ-ENaC showed no differences in comparison. Previous findings (Knoepp et al., 2017) and this data supports the assumption that N-glycans of glycosylated asparagines attached to the extracellular matrix (ECM) are essential for ENaC to sense SF. The removal of a glycosylated asparagine in position N378 in the thumb-domain, including N-glycans in the β-ENaC subunit leads to a detachment to the ECM. This data demonstrates that the glycosylated asparagine N378 in the β-ENaC subunit affect the SF sensation of the channel without affecting basic channel properties.
2.6 Domain-specific mutations of glycosylated asparagines of the β-ENaC subunit located in the palm-, knuckle- and β-ball-domains increase sensation to SF of ENaC

After mutating all single glycosylated asparagines of β-ENaC, experiments were continued by generating domain-specific mutations, to investigate how domain-specific N-linked glycosylated asparagines affect ENaC activation in response to SF. Therefore, glycosylated asparagines located within single domains were removed and the response to SF determined. The three dimensional protein structure model of β-ENaC revealed two glycosylated asparagines in the thumb-domain, two in the β-ball-domain, five in the finger-domain and one in the palm- and knuckle-domain (Figure 45 B). The SF response of wild-type ENaC (αβγ) was normalised (I0.2/I0) and set to the value 1 and then the mutated β-ENaC, co-expressed with the α- and γ-ENaC subunit, normalised to that SF response. The β-Ball mutation, including two glycosylated asparagines (N99 + N260) showed an increased SF effect of 2.45 ± 0.38 compared to αβγ-ENaC (**** p < 0.0001; n = 11; N = 3; Figure 45). The second β-ENaC mutation that was generated was a double mutation where glycosylated asparagines of the knuckle- (N484) and palm-domain (N449) were combined. The SF response increased to 1.44 ± 0.11 compared to αβγ-ENaC (* p < 0.05; n = 12; N = 3; Figure 45). The other domain-specific mutations: one mutation in the thumb-domain (N364 + N378), two mutations in the finger domain, one (1: N135 + N199) and the other (2: N135 + N199 + N207) showed no change in response to SF (p > 0.05; n = 12; N = 3; Figure 45).
Figure 45: Domain-specific β-ENaC mutations in the palm-, knuckle- and β-Ball-domains increase channel sensation to SF
A) Multiple mutations in specific-domains of β-ENaC were generated and expressed in oocytes. The SF response was normalised ($I_{0.2}/I_0$) and the value of control $\alpha\beta\gamma$-ENaC set to 1 (dotted line). The double mutation in the β-Ball domain (**** $p < 0.0001$; $n = 12$, $N = 3$) and the knuckle- and palm-domain (* $p < 0.01$; $n = 12$, $N = 3$) showed an increased response to SF compared to $\alpha\beta\gamma$-ENaC. The other mutations in the thumb- and finger-domain (1, 2) showed no response to SF ($p > 0.05$; $n = 12$, $N = 3$). B) 3D model of the β-ENaC subunit showing all domain in different colours: finger- (purple), thumb- (green), palm- (yellow), knuckle- (turquoise), β-Ball- (orange), 1+2 transmembrane-domain (red). The positions of all eleven glycosylated asparagines are shown as coloured circles to highlight in which domains they are located. One-way ANOVA with multiple comparison.

To further investigate whether open channel kinetics of domain-specific β-ENaC mutations change, tau was determined. One out of five domain-specific β-ENaC mutation in the β-Ball showed a slower response to SF from 1 ± 0.06 to 1.34 ± 0.17 (* $p < 0.05$; $n = 11$, $N = 3$; Figure 46). The other four domain-specific mutations of β-ENaC did not show a change in response to SF ($p > 0.05$; $n = 12$, $N = 3$; Figure 46) compared to $\alpha\beta\gamma$-ENaC.
Multiple domain-specific mutations of β-ENaC were generated and co-expressed with the α- and γ-ENaC subunits. The tau value of the control experiment (αβγ-ENaC) was normalised to the value 1.0 (dotted line) and compared to the mutated channels. One of the β-ENaC mutations showed a change in tau in comparison to the control (* p < 0.05; n = 11; N =3), whereas the other four did not (p > 0.05; n = 12; N =3). One-way ANOVA with multiple comparison.

Changes in amiloride-sensitive baseline currents were determined by comparing domain-specific β-ENaC mutation with control (αβγ-ENaC). The domain-specific β-ENaC mutation in the β-Ball- (**** p < 0.0001; n = 11; N = 3; Figure 47) and in the finger-domain (1+2) (*** p < 0.001; n = 12; N = 3; Figure 47) showed a decreased amiloride-sensitive current, reflecting a smaller expression of these channels. The other mutations in the thumb- and palm + knuckle-domain showed no change in amiloride-sensitive current compared to αβγ-ENaC, indicating no change in expression of these channels (p > 0.05; n = 12; N =3; Figure 47).
Figure 47: Amiloride-sensitive current of the domain-specific β-ENaC mutations in the β-Ball and in the finger domain (1+2) changes compared to αβγ-ENaC

The amiloride-sensitive current of control (αβγ-ENaC) was normalised to the value 1.0 and then individual domain-specific β-ENaC mutations normalised to that value. The domain-specific mutation in the β-Ball (**** p < 0.0001; n = 12; N = 3) and finger-domain (1+2) (*** p < 0.001; n = 12; N = 3) showed a decreased amiloride-sensitive current. Mutations in the thumb-, finger-(1) and palm + knuckle-domain did not affect amiloride-sensitive current compared to control (p > 0.05; n = 12; N = 3). One-way ANOVA with multiple comparison.

In summary, these results showed that generating domain-specific β-ENaC mutations in the β-Ball-, palm-, and knuckle-domain, increases activation of ENaC to SF, indicating that the glycosylation sites, including the N-glycans, are important for ENaC SF sensation. These domain-specific β-ENaC mutations might disrupt the internal interaction of all three (α, β, γ) subunits, followed by a functional impairment that might affect the rigidity of the channel during SF exposure.
2.7 Single glycosylated asparagines in the finger-domain (N248) of γ-ENaC play an important role in sensing SF

To investigate whether glycosylated asparagines of the γ-ENaC subunit are important for sensing SF, mutations lacking these asparagines were generated and examined via the TEVC method (Figure 48). Therefore, identical experiments were performed as described previously for investigating the β-ENaC mutations (section 2.3).

![Figure 48: Hypothetical model of how γ-ENaC co-expressed with α- and β-ENaC senses SF](image)

A) Showing a heterotrimeric wild-type αβγ-ENaC were all 3 subunits are labelled in different colours: α-ENaC (red), β-ENaC (blue) and γ-ENaC (green). The extracellular loops of every ENaC subunit is tethered via N-glycans of glycosylated asparagines (Gly-ASN; turquoise) to the extracellular matrix (ECM). B) SF application is causing a conformational change of the channel by shifting the ECM leading to a higher activity. C) Mutating glycosylated asparagines of γ-ENaC by exchanging them to alanines (Ala; red) breaks the linkage between extracellular loop and ECM leading to a less active ENaC in response to SF.

After completing analysing the β-ENaC subunit for changes in response to SF, identical experiments were performed for the γ-ENaC subunit. Therefore, channel properties of asparagine deficient γ-ENaC mutations were investigated by determining SF responses, channel open kinetics via Tau and amiloride-sensitive
currents. The SF response, Tau, as well amiloride-sensitive currents of αβγ-ENaC were determined and normalised to the value 1.0 ± SEM. Afterwards, recorded data of γ-ENaC mutations were normalised to the αβγ-ENaC control. Five glycosylated asparagine deficient mutations were generated and co-expressed with α- and β-ENaC. In short, the quantification of the normalised (I_{0.2}/I_0) SF response showed no change in all five γ-ENaC mutations compared to control (αβγ-ENaC) (p > 0.05; n ≥ 11 ; N ≥ 3 (Figure 49 A).

**Figure 49:** Removal of glycosylated asparagines of γ-ENaC causes no change in response to SF

A) Mutations were generated in five individual positions of γ-ENaC by performing point-mutations where one asparagine was exchanged by an alanine. The amiloride-sensitive current under SF exposure (I_{0.2}) is normalised to the amiloride-sensitive current without SF (I_0). The dotted line represents the SF response of the control (αβγ-ENaC) normalised to the value 1.0. None of the five γ-mutants showed a significant amiloride-sensitive current increase in SF compared to αβγ-ENaC (p > 0.05; n ≥ 12; N ≥3). B) 3D model of human γ-ENaC subunit including specific channel domains such as: finger- (purple), knuckle- (turquoise), Palm- (yellow), β-Ball- (orange), Thumb- (green) and 1+2 TMD (red) transmembrane-domains. The positions of all five glycosylated asparagines are shown as coloured circles to highlight in which domains they are located. One-way ANOVA with multiple comparison.

Furthermore, tau and the amiloride-sensitive currents were determined to show whether there is a change in channel expression or open channel kinetics of ENaC. The quantification of the recorded data revealed that one out five of the γ-ENaC
mutations in the finger-domain (N248) showed an increase in Tau from $1.42 \pm 0.17$, compared to control $\alpha\beta\gamma$-ENaC (* $p < 0.05$; n = 12; N = 3; Figure 50 A). The other four $\gamma$-ENaC mutations did not show a change in Tau overall ($p > 0.05$; n $\geq 11$; N $= 3$; Figure 50 A). The analysis of three out of five $\gamma$-ENaC mutations, one in the finger- (N248), one in the $\beta$-ball- (N271) and another one in knuckle-domain (N497), showed a decreased amiloride sensitive baseline current compared to the control $\alpha\beta\gamma$-ENaC, suggesting a decrease in membrane expression (* $p < 0.05$; ** $p < 0.01$; n $\geq 12$; N $\geq 3$; Figure 50 B). The other three $\gamma$-ENaC mutations showed no change in amiloride-sensitive current ($p > 0.05$; n $\geq 12$; N $\geq 3$; Figure 50 B).

In conclusion, this results show that none of the five glycosylated asparagine deficient $\gamma$-ENaC mutations responded differently to SF in comparison to control (wild-type $\alpha\beta\gamma$-ENaC), indicating that glycosylated asparagines in $\gamma$-ENaC are not important for sensing SF. However, the quantification of Tau showed a mutation in the finger-domain (N248) that increases the time taken until the channel is fully
activated by SF, suggesting an impairment in the open channel kinetics due to this mutation. Furthermore, mutations in the finger- (N248), β-Ball- (N271) and knuckle-domains (N497) showed a decrease in membranae expression, suggesting a disrupted trafficking of these channels.

2.8 Domain-specific mutations of glycosylated asparagines of the γ-ENaC subunit do not affect channel response to SF

To identify how domain-specific N-linked glycosylated asparagines affect ENaC activation in response to SF, γ-ENaC mutations were generated by removing glycosylated asparagines of entire domains (Figure 51 B). The mutated γ-ENaC subunits were co-expressed with α- and β-ENaC and compared to control via the TEVC method (wild-type αβγ-ENaC). The quantification of two double mutations, one in the finger- and the other one in the β-ball-domain did not change channel activity in response to SF (p > 0.05; n ≥ 12; N ≥ 3; Figure 51 A).
Double γ-ENaC mutations were generated removing all glycosylated asparagines in the finger- and β-ball-domain. The SF response of the control αβγ-ENaC were normalised (Io,2/Io) to the value 1.0 (dotted line) and then compared to the γ-ENaC mutations. The comparison showed no change in response to SF in comparison to αβγ-ENaC (p > 0.05; n ≥ 12; N ≥ 3).

B) 3D model of human γ-ENaC showing positions of all five glycosylated asparagines (coloured circles) and channel domains: finger- (purple), knuckle- (turquoise), Palm- (yellow), β-Ball- (orange), Thumb- (green) and 1+2 TMD (red) transmembrane-domains. Mann-Whitney test and Unpaired Student’s t-test.

To further investigate whether channel open kinetics or amiloride-sensitive currents are changed due to γ-ENaC mutations, tau, as well as amiloride-sensitive currents, were analysed. Tau of the two double mutations did not show any difference compared to wild-type control (αβγ-ENaC) (p > 0.05; n ≥ 12; N ≥ 3; Figure 52 A). In addition, the amiloride-sensitive current decreased in both γ-ENaC mutations compared to control (* p < 0.05; n ≥ 12; N ≥ 3; Figure 52 B).
Figure 52: Tau and amiloride-sensitive current of domain-specific γ-ENaC mutations
A) The time constant Tau of the control αβγ-ENaC was normalised to the value 1.0 (dotted line and compared to the two double γ-ENaC mutations. The statistical analysis showed no change in tau compared to the control (\( p > 0.05; n \geq 12; N \geq 3 \)).

B) Both double mutations showed a decrease in amiloride-sensitive current, reflecting a lower expression level of the channel (* \( p < 0.05; n \geq 12; N \geq 3 \)). Mann-Whitney test and Unpaired Student’s t-test.

In summary, the SF effect of γ-ENaC mutations in the finger- and β-ball-domains did not change in comparison to wild-type control (αβγ-ENaC). These experiments underline that mutating glycosylated asparagines of the γ-ENaC subunit and thereby removing N-glycans, did not play an important role in sensing SF of ENaC. However, the double mutations of γ-ENaC did affect the amiloride-sensitive current of the channels, indicating a change in trafficking, expression level due to the inserted mutations.

2.9 Summary

This section shows that N-glycans of glycosylated asparagines of the β-ENaC play an important role in sensing SF. The experiments performed with PNGaseF showed that a cleavage of N-glycans of all ENaC subunits decreases SF response. This interaction between glycosylated asparagines, via N-glycans, to the ECM might be
one mechanism of how ENaC senses SF. Due to an observed rest current after PNGaseF treatment and SF application, it is highly likely that our described mechanism is one part of a complex that is involved in SF sensation of ENaC.

Furthermore, by mutating single and multiple glycosylated asparagines of the β-ENaC, contradictory results were observed showing an enhanced SF response compared to wild-type αβγ-ENaC instead of an expected attenuation. One possible explanation might be that N-glycans of glycosylated asparagines of the β-ENaC subunit might provide a connection as either an intra-tether within the subunit or an inter-tether to an adjacent subunit rather than a direct linkage to the ECM. This detachment of N-glycans could then loosen the rigidity of the channel leading to a more active channel in response to SF. Another contradictory result was shown by analysing the double mutation (N364 + NN378) in the thumb-domain, including the glycosylated asparagine (N378), which previously showed an increased SF response (section 2.3). This double mutation in β-ENaC did not show a change in response to SF, indicating that the combined mutation of N364 + N378 might have a compensatory effect in ENaC SF sensation. Mutating single and multiple glycosylated asparagines of γ-ENaC showed no change in SF, indicating that N-glycans or glycosylation sites are not important in sensing SF. However, γ-ENaC mutation in position N271 showed an impaired Tau, indicating a change in channel open kinetics, reflecting an impaired gating of Na⁺ due to this mutation.
4 Discussion
1 The β- and the γ-ENaC subunit have a modulatory role for sensing SF

Previous studies have shown that ENaC is a mechano-sensitive ion channel which responds to SF in various expression systems as well as in native tissues such as kidney epithelium (Satlin et al., 2001; Carattino et al., 2004; Morimoto et al., 2006; Abi-Antoun et al., 2011) and arteries (Ashley et al., 2018). SF regulates ENaC activity by increasing the Po of the channel (Althaus et al., 2007). Evidence from the literature proposed a model for ENaC SF sensation, where SF initiates a conformational change within the large extracellular domains (Shi et al., 2013). Studies in C. elegans provided evidence for the involvement of an extracellular tether that connects the mechano-sensitive MEC channel to the ECM (Du et al., 1996; Emtage et al., 2004). However, the molecular structure of the extracellular tether remains unknown. The close relationship between MEC-channel proteins in C. elegans and ENaC in invertebrates, led to the assumption that the sensation of SF by ENaC might work in a similar way. A previous study from our lab provided evidence that ENaC might be connected to the ECM via N-linked glycans of glycosylated asparagines (Knoepp et al., 2017). The removal of domain specific glycosylated asparagines within N-glycans of the α-ENaC subunit caused a reduction in channel activity in response to SF (Knoepp et al., 2017). Further evidence suggested that degradation of the ECM also impaired SF sensing of ENaC. This indicated that N-glycans of α-ENaC and the ECM are important for SF sensing. Notably, different studies provided the evidence that in some tissues, either β- or γ-ENaC can be expressed without α-ENaC and may respond to mechanical forces such as SF (Drummond et al., 1998; Drummond, 2012). However, whether homotrimeric β- or γ-ENaC form a functional channel that
responds to SF remains unknown. Based on this lack of information the two main questions of my thesis were:

(1) Do the β- and γ-ENaC subunits have a modulatory role for sensing SF?

(2) Are N-linked glycans of glycosylated asparagines of β- and γ-ENaC potential tethers that facilitate SF sensation?

To identify the role of individual ENaC subunits for the ability to respond to SF, homotrimeric (α, β, γ or δ) or heterotrimeric (αβ, αγ, βγ, δβ, or δγ) ENaC were expressed in oocytes of *Xenopus laevis* and the response to SF was characterised.

### 1.1 The α-ENaC subunit is activated by SF

A study by Canessa *et al.*, (1994) provided the first evidence that homotrimeric α-ENaC, presumably assembled as ααα-ENaC channels, can form a functional channel on the membrane surface of *Xenopus* oocytes, indicated by a small amiloride-sensitive current (Canessa *et al.*, 1994b). The assessment of the amiloride-sensitive current is a common way to determine the number of ENaC expressed in the cell membrane of *Xenopus* oocytes. In this context a study by Firsov and colleagues (1996, 1998) showed that the amiloride-sensitive current of ENaC correlated exactly with the number of expressed channels expressed in the cell membrane of *Xenopus* oocytes (Firsov *et al.*, 1996; Firsov *et al.*, 1998). In other words, a low amiloride-sensitive current reflects a small number of channels expressed on the membrane of *Xenopus* oocytes. In my study, amiloride-sensitive currents of homotrimeric α-ENaC were determined and compared to αβγ-ENaC respectively, to evaluate differences in numbers of ENaC expressed in the
membrane. The comparison revealed a decreased amiloride-sensitive current that could reflect 1) a decreased Po or 2) a low number of expressed channels on the cell membrane (Figure 23). The low amiloride-sensitive current of homotrimeric α-ENaC, showing a low expression of channels on the membrane, makes it difficult to characterise homotrimeric α-ENaC at a single channel level. This might be a reason why evidence regarding this topic is very limited. However, Fyfe and Canessa (1998) did show that the subunit composition of ENaC might be a main determinant of the Po (Fyfe & Canessa, 1998). They demonstrated that the co-expression of α- and β-ENaC increases the Po and the co-expression of α- and γ-ENaC decreased the Po of the channel (Fyfe & Canessa, 1998). This finding provides evidence that the Po of homotrimeric α-ENaC might be changed. In short, either the low Po or low number of channels expressed on the membrane might explain the low amiloride-sensitive current observed in homotrimeric α-ENaC.

Theses amiloride-sensitive currents of expressed homotrimeric α-ENaC are well investigated in Xenopus oocytes, where several studies clearly show a functional channel with a low current (Canessa et al., 1993; Canessa et al., 1994b; Snyder et al., 1994; Fyfe & Canessa, 1998). However, the question that still remain is whether homotrimeric α-ENaC exists in native tissues and responds to SF or not.

The exposure of homotrimeric α-ENaC to SF caused an activation of the channel, showing clearly that α-ENaC alone can form a functional channel that responds to SF (Figure 13). To determine whether there are differences in the SF response between homotrimeric α-ENaC and αβγ-ENaC, the normalised SF response of both channels were compared. The comparison showed no changes in the normalised SF response, indicating no impairment of the SF sensation in homotrimeric α-ENaC (Figure 30). This finding demonstrates that the α-ENaC subunit (without β- and γ-subunit) responds in the same ratio to SF as αβγ-ENaC, indicating a major role of
the α-subunit for SF sensation. In agreement with this observation, Knoepp et al., (2017) provided evidence that the α-ENaC subunit plays an important key role for sensing SF (Knoepp et al., 2017), as did other groups (Carattino et al., 2004; Shi et al., 2013).

Taken together these results show that homotrimeric α-ENaC can form a functional channel that is expressed on the cell membrane and responds to SF. In addition, the SF responsiveness of homotrimeric α-ENaC did not change in comparison to αβγ-ENaC, indicating a key role of the α-subunit for SF sensation.

1.2 The β-ENaC has a modulatory role for SF sensation of ENaC

In my study the expression of heterotrimeric αβγ-ENaC in oocytes showed a consistent amiloride-sensitive current and response to SF. Homotrimeric β-ENaC showed an amiloride-sensitive current and these channels also responded to SF, indicating that homotrimeric β-ENaC can form a functional channel that responds to SF. Interestingly, the expression of homotrimeric β-ENaC, presumably forming βββ-ENaC channels, resulted in an inconsistent number of oocytes responding to amiloride and SF (Figure 14). This inconsistency was reduced by increasing the incubation time for expression. Incubation of ≥ 48 hours resulted in a higher number of oocytes responding to amiloride and SF (Figure 15). This finding was further supported by biotinylation experiments showing that homotrimeric β-ENaC assembles on the membrane in a detectable amount after 48 h but not after 24 h of incubation (Results; section 1.5.2). One explanation for why some oocytes incubated for 24 h showed a response to SF and others did not, could be due to instabilities in the protein-translation machinery of oocytes derived from different
oocyte batches (Green, 2002). This assumption was supported by other studies reporting quality differences in oocytes due to unexplained and unexpected seasonal variations in laboratory environments (Wu & Gerhart, 1991; Goldin, 1992; Delpire et al., 2011). Another explanation could be that functional homotrimeric β-ENaC expressed in oocytes needs a longer trafficking or maturation time to the membrane (further discussed in section 1.2.1). However, this is also consistent with other channels expressed in oocytes that require ≥48 h of incubation after mRNA injection. This is in agreement with observations in our lab showing that the expression of ASICs in Xenopus oocytes require a longer incubation time as well (Barth, 2017).

The co-expression of β- with α-, δ- or γ-ENaC aiming to form channels consisting of only two subunits in Xenopus oocytes showed a considerably low amiloride-sensitive current when compared with αβγ- or δβγ-ENaC. In agreement with my observations, studies showed that heterotrimeric αβ- and βγ-ENaC can form functional channels with a decreased amiloride-sensitive current. Again, this may be reasoned by either a lower Po or a decreased number of channels present on the membrane (previously discussed in section 1.1) (Canessa et al., 1994b; McNicholas & Canessa, 1997). The high intrinsic Po of heterotrimeric αβ-ENaC confirms that the reason for the low amiloride-sensitive current is indeed a decreased number of channels expressed on the cell membrane (Fyfe & Canessa, 1998). Another interesting observation was the longer incubation time of heterotrimeric βγ-ENaC, similar as observed for homotrimeric β-ENaC. Increasing the incubation time to 72 h led to formation of channels that responded to amiloride and SF (Figure 26). The reason for that could be either an impaired trafficking or prolonged maturation time. In agreement with my findings, Bonny et al., (1999) showed that heterotrimeric βγ-ENaC needed longer incubation times (6-7 days) to
assemble a functional channel that responded to amiloride compared to αβγ-ENaC when expressed in Xenopus oocytes (Bonny et al., 1999). Channels consisting of only βγ-ENaC subunits have significantly lower currents in Xenopus oocytes which may also be caused by impaired trafficking (Canessa et al., 1994b; Bonny et al., 1999). However, these results are contradictory to studies showing that βγ-ENaC subunits are localised on the membrane of VSMCs without any indications of impaired trafficking (Jernigan & Drummond, 2005, 2006).

To investigate whether the β-ENaC subunit has a modulatory role for SF sensation, the normalised SF response of homotrimeric β-ENaC was compared with αβγ-ENaC. The normalised response of homotrimeric β-ENaC, presumably forming βββ-ENaC, to SF was decreased in comparison with corresponding control (αβγ-ENaC) (Figure 30). This decreased SF response could be due to a missing connection to another adjacent subunit, indicating an impaired ability of homotrimeric β-ENaC to respond to SF. In agreement with my finding Berdiev and colleagues (2000) showed that an interaction between the α-ENaC subunit with the β- or γ-ENaC subunit played an important role in modulating gating of heterotrimeric ENaC (Berdiev et al., 2000). By taking this study into account it might be that the decreased SF response of homotrimeric β-ENaC is due to a lack of connection/interaction with the α- and/or the γ-ENaC subunit.

Furthermore, none of the β-ENaC heterotrimeric channels (co-expressed either with α-, γ- or δ-ENaC) revealed a change in the relative activation in response to SF in comparison with αβγ-ENaC or δβγ-ENaC (Figures 30 and 31). Based on the assumption that the β-ENaC subunit may have a modulatory role in sensing SF, this observation was unexpected. The decreased SF response of homotrimeric β-ENaC was gone, when the β- was combined with the α-ENaC subunit, supporting
the major role of the α-ENaC subunit for sensing SF. This also supports the idea that an interaction between the α- and β-ENaC subunit is important for normal channel function including the ability for SF sensation. A similar response was observed in heterotrimeric βγ-ENaC, also indicating a close interaction between the β- and γ-ENaC subunit for sensing SF. Interestingly, the increased SF response of homotrimeric δ-ENaC was completely eliminated in co-expression with the β-ENaC subunit, supporting that the β-ENaC subunit may have a modulatory role for SF sensation (Figure 31). The subsequent comparison of the γ- with either the α- or δ-ENaC subunit, revealed an increased response to SF compared with αβγ- or δβγ-ENaC (Figures 30 and 31). This finding demonstrates that the β-ENaC subunit might weaken the ability of ENaC to sense SF, highlighting again a modulatory role of β-ENaC for sensing SF.

What remains uncertain is the composition of ENaC when two subunits were co-expressed with each other. The co-expression of the α- with the β-ENaC subunit for instance will presumably form channels such as ααα-, ααβ-, αββ or βββ-ENaC. The most obvious explanation could be the combined assembly of all ααα, ααβ-, αββ or βββ-ENaC together on the membrane of Xenopus oocytes. However, the lack of literature available regarding ENaC composition makes it difficult to draw a final conclusion.

Taken together, homotrimeric β-ENaC can form a functional channel that shows a time-dependent activity when exposed to SF. The appearance of these functional channels correlated with the detection of β-ENaC protein in the membrane of the cells. The time-dependent activity indicates a slower maturation and/or trafficking time of these channels (further discussed section 1.2.1). The decreased SF response of homotrimeric β-ENaC was “rescued” when co-expressed with α- or γ-
ENaC subunit, indicating the minor role of the β-ENaC subunit for sensing SF compared to the α- or γ-ENaC subunit. Additionally, the increased SF response of homotrimeric δ-ENaC was completely eliminated in co-expression with the β-ENaC subunit, supporting the modulatory role of the β-ENaC subunit (Figure 31). The increased SF response observed in heterotrimeric αγ- and δγ-ENaC provides evidence suggesting that the modulatory role of the β-ENaC subunit might weaken the ability of ENaC to sense SF via balancing the SF response and holding ENaC in a physiological state.

1.2.1 Maturation and trafficking of homotrimeric β-ENaC is impaired

This paragraph will discuss the possibility of whether the time-dependent activity of homotrimeric β-ENaC might be due to a slower trafficking or maturation of these channels. Canonical αβγ-ENaC undergoes post-translational modification via proteolytic cleavage and glycosylation to mature to a fully functional channel (Hughey et al., 2003; Hughey et al., 2004a; Ergonul et al., 2006; Kashlan et al., 2018). Hughey and colleagues (2003) showed that the α- and γ-subunits pass through proteolytic cleavage during channel maturation (Hughey et al., 2003). These cleavage sites might speed up the maturation of wild-type αβγ-ENaC and the lack of proteolytic cleavage sites in the β-ENaC subunit could explain the observed longer maturation time of the homotrimeric channel. In addition, the absence of cleavage sites in the β-ENaC subunit (Hughey et al., 2003), might lead to a less active channel that cannot respond to SF in a sufficient way. In agreement with this assumption, Caldwell et al., (2004) provided evidence that the activity of a non-cleaved ENaC is very low, reflected in a low Po (Caldwell et al., 2004).
Since SF clearly increases the Po of ENaC the missing cleavage site in homotrimeric β-END might explain the decreased SF response (Figure 30).

Besides the maturation of homotrimeric β-END the intermittent number of oocytes responding to SF, could be due to an impaired and prolonged trafficking. In brief, ENaC mRNA is synthesised in the nucleus, followed by mRNA translation by ribosomes on the ER, and then incorporation into the ER, where they undergo N-linked glycosylation (Canessa et al., 1994a; Renard et al., 1994; Snyder et al., 1994). A potential explanation for prolonged trafficking could be due to a change in the number of glycosylated asparagines representing glycosylation sites where N-glycans are attached to the channel (Hughey et al., 2003; Kashlan et al., 2018). These N-glycans play an important role in trafficking of ENaC (Kashlan et al., 2018). They showed that subunit specific removal of N-glycans impairs trafficking of ENaC (Kashlan et al., 2018). The human ENaC subunits have various glycosylation sites: the α- and γ-subunit have 5- and the β-subunit has 11-glycosylated asparagines. The change from potentially 21 glycosylated asparagines normally present in heterotrimeric αβγ-END to 33 glycosylated asparagines of presumably homotrimeric βββ-END might interfere with trafficking of the protein. Interestingly, the attachment of every individual N-glycan core to a glycosylated asparagine increases the molecular weight by ~3 kDa (Kornfeld & Kornfeld, 1985; Alvarez de la Rosa et al., 2002). This increase in the number of N-glycans in homotrimeric β-END accompanied with an increased molecular weight might be the reason for a slower trafficking.

The assumption that the increased number of N-glycans of homotrimeric β-END might interfere with the trafficking was further supported by an unexpected observation during biotinylation experiments. Surprisingly, the β-END subunit
was bound to the beads independent of the biotin. One explanation, could be the high number of glycosylated asparagines, respectively the N-glycans attached to the asparagines, could make the protein “stick” to the beads, resulting in binding to the beads without biotin. To confirm that the N-glycans of β-ENaC were the reason for the unexpected binding, further experiments were performed using PNGaseF. PNGaseF is an enzyme that specifically cleaves all N-glycans attached to the glycosylated asparagines (Plummer & Tarentino, 1991; Tarentino & Plummer, 1994; Freeze & Kranz, 2010). The deglycosylation of homotrimeric β-ENaC with PNGaseF led to a successful biotinylation experiment, indicating the impairment was due to a high number of N-glycans (Figure 18). The increased molecular weight and the increased affinity to bind cellular compartments might slow down the trafficking of homotrimeric β-ENaC.

Thus, it may be concluded that the absence of β-ENaC on the membrane after 24 h of incubation and the decreased response to SF might be due to a different subunit composition (βββ-ENaC instead of αβγ-ENaC) which prolongs the maturation or trafficking time of the channel. However, the complexity of maturation and trafficking of ENaC subunits makes it likely that more than one mechanism is required to explain the observed changes.

1.3 The γ-ENaC has a modulatory role for SF sensation of ENaC

Although different strategies were trialled neither amiloride-sensitive currents nor SF responses were observed with homotrimeric γ-ENaC. This indicates either that γ-ENaC cannot form a functional channel in the membrane, or that the γ-ENaC subunit alone might not be trafficked to the membrane. Both processes may be
reasoned by the lack of post-translational modifications such as proteolytic cleavage. Hughey et al., (2004) showed that cleavage of γ-ENaC subunits is required for activation of ENaC channels (Hughey et al., 2004b). This led to the assumption that homotrimeric γ-ENaC might be silent on the membrane and requires activation via proteolytic cleavage to enable the channel to respond to SF. This notion is supported in a study by Caldwell et al., (2004) showing that non-cleaved ENaC remain almost silent with a really low Po until they become cleaved (Caldwell et al., 2004). This might explain the lack of SF sensation of homotrimeric γ-ENaC.

The co-expression of the γ-subunit with either the α- or δ-subunit of ENaC showed a lower amiloride-sensitive current when compared with corresponding controls (αβγ- or δβγ-ENaC) (Figures 30 and 31), indicating either a low Po or impaired number of channels expressed on the membrane. In agreement with my results, a previous study showed that heterotrimeric αγ-ENaC can form functional channels expressed in Xenopus oocytes with modest differences in amiloride-sensitive currents when compared with αβγ-ENaC (McNicholas & Canessa, 1997). The similarities in Po of heterotrimeric αβγ- and αγ-ENaC excluded the fact that the low amiloride-sensitive current of αγ-ENaC might be due to a decreased number of channels on the membrane of Xenopus oocytes (Garty & Palmer, 1997; Fyfe & Canessa, 1998).

To further investigate whether the γ-ENaC subunit has a modulatory role in sensing SF, normalised SF responses of heterotrimeric αγ- or δγ were compared with corresponding αβγ- or δβγ-ENaC SF responses. The subsequent comparison of αγ- or δγ-ENaC to corresponding controls, revealed an increase in channel activation when exposed to SF (Figures 30 and 31). These findings show that the γ-ENaC
subunit co-expressed with the α- or δ-ENaC can form a channel with enhanced SF sensation features, indicating a modulatory role of the γ-ENaC subunit. The modulatory role was further supported by the fact that the decreased SF response of homotrimeric β-ENaC was eliminated due to a co-expression with the γ-ENaC subunit. In addition, it seems that the γ-ENaC subunit plays a more important role for the ability of ENaC to sense SF than the β-ENaC subunit.

Taken together, these results clearly show that homotrimeric γ-ENaC cannot form a functional channel that responds to either amiloride or SF (Figure 20). In addition, the co-expression of the γ-ENaC subunit with either α- or δ-ENaC results in a functional channel that responds to amiloride and SF (Figures 30 and 31). The comparison of heterotrimeric αγ- and δγ-ENaC with corresponding control (αβγ- or δβγ-ENaC), revealed an increased response to SF, indicating a modulatory role of the γ-ENaC subunit. The modulatory role of the γ-ENaC subunit is further supported due to the fact that the decreased SF response of homotrimeric β-ENaC was eliminated when co-expressed with the γ-ENaC subunit. These findings showed for the first time that the γ-ENaC subunit might have a modulatory role for SF sensation.

1.4 The δ-ENaC subunit is activated by SF

The expression of homotrimeric δ-ENaC showed a decreased amiloride-sensitive current when compared with δβγ-ENaC. This is in agreement with all previous data describing homotrimeric and heterotrimeric ENaC (sections 1.1, 1.2 and 1.3). Yet, homotrimeric δ-ENaC showed an increased response to SF when compared with corresponding control (δβγ-ENaC) (Figure 31). This result was unexpected and it underlines the importance of the interactions between the β-subunit and the δ-
ENaC subunit. It further supports the modulatory role of the β-subunits for SF sensation when co-expressed with the δ-subunit. However, the increased response to SF was not observed in homotrimeric α-ENaC, indicating that the β- and γ-subunits might have different roles for sensing SF depending on the subunit they are co-expressed with. The most likely explanation could be that the β- and γ-ENaC subunits might interact differently with the respective subunits. This can result in an elevated Po of heterotrimeric channels versus homotrimeric δ-ENaC and a subsequent higher response to SF. In other words, the high intrinsic Po of homotrimeric α-ENaC might blunt the additional activation of the channel via SF.

In summary of this chapter, the findings of this thesis provide evidence about the role of the α- , β- , γ- and δ-ENaC subunits in the ability of ENaC to sense SF. 1) The α- and the δ-ENaC subunits have a major role for sensing SF. This is further supported by previous studies performed in our laboratory (Barth, 2017; Knoepp et al., 2017). 2) The modulatory role of the β-ENaC subunit might be to weaken the ability of ENaC to sense SF. 3) The modulatory role of the γ-ENaC subunit might be to enhance the ability of ENaC for SF sensation. Assumption 2) and 3) is further supported by comparing heterotrimeric βγ-ENaC with αβγ-ENaC (Figure 30). There was no difference observed, indicating that both subunits co-expressed will balance the SF response back to a normal physiological state of the channel. It seems that the β- and the γ-ENaC subunits have modulatory roles and that the role of the γ-subunit is to enhance and the β-subunit is to weaken the ability of ENaC for SF sensation.
1.5 Physiological relevance of homotrimeric and heterotrimeric ENaC

The modulatory role of β- and γ-ENaC might provide an insight to how epithelial cells in the kidney, endothelial cells and VSMCs in the vasculature might retain their physiological function during changes in blood pressure. ENaC as a mechanosensor might modulate enormous changes in SF rates from approximately 0.06 to 20 dyn*cm⁻² in the collecting duct of the kidney (Cai et al., 2000; Friedrich et al., 2006), between 20 – 40 dyn*cm⁻² in large arteries (Davies, 1995) and up to 100 dyn*cm⁻² in vascular beds (Nerem et al., 1993), due to adjusting ENaC subunit compositions. The mechanical stimulus becomes translated into cellular signals and might provide a new possible mechanism of how epithelial cells, endothelial cells or VSMCs respond to physiological changes in blood pressure. The new concept that β- and γ-ENaC modulate the activity of ENaC in response to SF, provides crucial evidence for a better understanding of normal physiological and pathophysiological processes within blood pressure regulation.

Furthermore, the possibility of changing the subunit composition of ENaC might be a common feature of cells to form channels with distinct functional characteristics to regulate the Na⁺ transport in the kidney, lung or vasculature. This assumption is supported by a study from Farman et al., (1997) providing evidence that in the epithelia of the rat lung a large prevalence of α- and γ-subunit mRNA expression was found with little or no β-ENaC (Farman et al., 1997). According to that expression pattern it could be possible that the number of presumably ααγ- or αγγ-ENaC would be much higher in respiratory epithelia than the number of αβγ-ENaC. This study provided evidence that heterotrimeric αγ-ENaC indeed has an important physiological role in rat lung epithelia. Another report by
Randrianarison et al., (2008) revealed an interesting finding, showing that low mRNA expression of β-ENaC in mice lung, results in to a compensatory up-regulation of α- and γ-ENaC and surprisingly, led only to a modest impairment in ENaC function (Randrianarison et al., 2008). This study supports again that co-expressing the α- and the γ-ENaC subunit might have an important physiological function. Further supporting this assumption are studies showing that β- and γ-ENaC deficient mice, unlike α-ENaC deficient mice, survive, supporting the assumption that heterotrimeric αβ- or αγ-ENaC have sufficient channel activity (Hummler et al., 1996; Barker et al., 1998; Pradervand et al., 1999). The constant exposure of ENaC to SF in the lung supports the assumption that at least in certain tissues only one or two ENaC subunits may function as a physiological mechanosensor.

2 Glycosylated asparagines of ENaC are important for sensing SF

Asparagines become N-linked glycosylated via an attachment of sugar molecules, also known as glycans, to a nitrogen atom (Imperiali & Hendrickson, 1995). The N-linked glycosylation follows a specific consensus sequence Asn-X-Thr/Ser, where X is a random amino acid except for proline (Imperiali & Hendrickson, 1995; Imperiali & O’Connor, 1999). Several studies provide evidence that the formation of N-glycans within ENaC might play an important role in trafficking and maturation of the channel (Hughey et al., 2003; Hughey et al., 2004b; Kashlan et al., 2018). In addition, another role for N-glycans derived from studies performed in mechanosensitive channels of C. elegans that belong to the same
protein family as ENaC proteins revealed that the ECM is required for mechanical activation (Emtage et al., 2004). Based on these studies it has been proposed that a tether between the channel and the ECM facilitates a physical connection (Du et al., 1996; Emtage et al., 2004; Chalfie, 2009). Since the literature revealed that glycoproteins such as integrins are connected to the ECM via attached sugar chains (Hynes, 1987; Frantz et al., 2010), it is possible that N-glycans provide a suitable tether structure. This concept was investigated by the removal of all N-glycans via mutating glycosylated asparagines within the α-ENaC subunit (Barth, 2017; Knoepp et al., 2017). The study showed that degradation of the ECM via enzymes (hyaluronidase) and mutations of glycosylated asparagines at position N312 and N511 in the α-ENaC subunit lead to a lower SF response (Knoepp et al., 2017). Although these experiments demonstrated that glycosylated asparagines of α-ENaC are important for SF sensation a residual current in response to SF was observed. This indicates that other subunits (β and/or γ) may also contribute to SF sensation through a connection to the ECM. This is supported by the fact that both subunits contain glycosylation sites in their extracellular domain. The attached N-glycans may also form tethers that contribute to SF sensation of ENaC.

Taken together with the previous findings, it was shown that glycosylated asparagines, including N-glycans of the α-ENaC subunit, are important (Knoepp et al., 2017), and the β- and γ-ENaC subunits have a modulatory role for sensing SF (see previous chapter). This raised the question of whether glycosylated asparagines of the β- and γ-ENaC subunits, in particular their N-glycans, are facilitating the modulatory role for SF activation. To address this question, two general approaches were implemented:
(1) To investigate whether N-glycans are important for sensing SF, N-glycans were removed enzymatically by treatment with PNGaseF.

(2) Site-directed mutagenesis was performed to generate glycosylated asparagine deficient β- and γ-ENaC mutations by replacing the asparagines within the glycosylation motif against alanines.

2.1 N-linked glycans are important for SF sensation of ENaC

To clarify whether N-glycans are involved in SF sensation, an enzymatic degradation was performed. Therefore, heterotrimeric αβγ-ENaC was extracellular treated (Figure 33) or injected with PNGaseF (Figures 34 and 35) and the subsequent SF response was determined. The extracellular treatment with PNGaseF of αβγ-ENaC oocytes did not show a change in response to SF (Figure 33). One explanation for this observation might be due to high costs and small volumes (30 μl) of available PNGaseF, which complicated the experimental procedure. However, the injected αβγ-ENaC oocytes showed a reduced SF response 3 – 5 h after injection of PNGaseF and a recovery of the SF response after 5 – 6 h (Figure 34). This finding underlines the importance of ENaC N-glycans for SF sensation. The reduced SF effect after 3-5 hours PNGaseF injection and its recovery is an interesting observation.

One explanation for this observation could be the exchange of “old” ENaC still with N-glycans present remaining in the membrane for up to 2 hours after PNGaseF injection. After two hours “new” ENaC subunits that were exposed to the PNGaseF and lack N-glycans are getting inserted into the membrane. In other words the delay of reduced SF effect and the recovery period may be caused by the turnover
of “old” ENaC (with N-glycans) with “new” ENaC (N-glycans removed). This assumption is supported by studies showing that the turnover of ENaC in oocytes from Xenopus laevis takes approximately 3 - 4 h (Valentijn et al., 1998). These studies support the idea that the decreased SF effect observed after 3-5 h might be due to ENaC turnover. The recovered SF response observed between 5 – 6 h might be due to a loss of enzyme activity of PNGaseF. Taking both observations together 1) the decreased SF response after 3 – 5 h and 2) the recurred SF response after 5 - 6 h, legitimates the assumption that the change in SF activation is due to an exchange of “old” ENaC into “new” ENaC.

To reveal whether the decreased SF activation was due to an impaired trafficking that reduces the number of αβγ-ENaC in the membrane, amiloride-sensitive currents were compared. The amiloride-sensitive current of αβγ-ENaC with and without PNGaseF showed no changes during the entire experiment. Even within the 3-5 h period the amiloride-sensitive current between non-treated and PNGaseF-treated oocytes was not changed. This indicates that the removal of N-glycans by PNGaseF does not change the population of channels expressed on the cell-membrane of Xenopus oocytes.

Still questionable is how PNGaseF can reach and cleave N-glycans during channel maturation and trafficking to the cell membrane. This derives from the fact that PNGaseF was injected into the cytoplasm of the oocytes and that normal channel maturation and trafficking would occur in the form of vesicles. Here the extracellular glycosylated asparagines would face towards the vesicle lumen and should not be exposed to the cytoplasm (and the PNGaseF). To address this question the trafficking of ENaC to the cell membrane will require more attention. By assuming that PNGaseF accumulates in the cytoplasm after injection, one
possible scenario could be the cleavage of N-glycans of ENaC while being trafficked from the ER to the Golgi apparatus or directly to the cell membrane in vesicles. This assumption remains absolute speculative due to a wide range of possibilities of how PNGaseF might interact with ENaC and other transmembrane or ECM proteins.

Taken together, the decreased response to SF of a αβγ-ENaC expressing oocyte, after injection of PNGaseF, indicates a cleavage of N-glycans attached to ENaC and that the N-glycans play an important role for the ability of ENaC to sense SF. The decreased response to SF after 3 h, might provide information about the turnover of ENaC in Xenopus oocytes. The question as to how PNGaseF interact with ENaC remains controversial and must be investigated further. This experiment shows that the removal of N-glycans decreases ENaC activity in response to SF without affecting the expression of channels in the membrane. It indicates that the N-glycans are important for SF activation of ENaC to support the role of the N-glycans as potential tethers for mechanotransduction.

2.2 Glycosylated asparagines of the β-ENaC subunit have a modulatory role in sensing SF

Previous in-house data revealed that glycosylated asparagines located in the palm- (N312) and knuckle- (N511) domains of α-ENaC are involved in SF sensation (Knoepp et al., 2017). Interestingly, two out of five glycosylated asparagines located in the palm- (N312) and knuckle (N511) domains of α-ENaC are involved in SF sensation, whereas the other three located in the finger- (N232), thumb- (N397) and β-ball (N293) domains are not involved (Knoepp et al., 2017). In agreement with this observation the substitution of corresponding glycosylated
asparagines in the δ-ENaC subunit, located in the finger- and thumb-domains did not affect SF sensation (Knoepp et al., 2017). Since, the β-ENaC subunit displays 11 glycosylated asparagines itself, these may have an important role in SF as well.

2.2.1 Glycosylated asparagines 99 and 378 of β-ENaC is involved in modulating the SF response

To investigate whether glycosylated asparagines, of the β-ENaC subunit have a modulatory role for SF sensation, glycosylated asparagines were removed and exchanged to alanines. This would eliminate the specific glycosylation site and remove the N-glycan as a potential tether to the ECM. The protein sequence analysis of the β-ENaC subunit revealed a total of 11 glycosylated asparagines in the extracellular domain, 5 in the finger, 2 in the β-Ball, 2 in the thumb-, 1 in the knuckle- and 1 in the palm-domain (Figure 53).
Figure 53: Homology model of the protein structure of the human β-ENaC subunit. 3D model illustrates the locations of the 11 glycosylated asparagines within the channel domains. 5 glycosylated asparagines were found in the finger (purple), 1 in the knuckle (turquoise), 1 in the palm (yellow), 2 in the β-Ball (orange), 2 in the thumb-domain (green).

After mutating all single glycosylated asparagines by exchanging these to alanines, each mutated subunit was co-expressed with α- and γ-ENaC and the resulting SF responses were compared to wild-type αβγ-ENaC. Two glycosylated asparagine one in the β-Ball and one in the thumb-domain (N378), showed a changed SF response. Interestingly, both β-ENaC mutations (αβN99Aγ-ENaC and αβN378Aγ-ENaC) revealed an increased response to SF compared to αβγ-ENaC. This is contradictory to results observed with the replacement of asparagines in the α-subunit where decreased response to SF was observed (Knoepp et al., 2017). These contrary observations might provide evidence for distinct roles of N-linked glycans besides forming a potential tether to the ECM (Knoepp et al., 2017). In addition to the tether to the ECM, two other scenarios could be possible where N-glycans might provide a tether within the subunit itself (intra-tether) or to another adjacent subunit (inter-tether) (Figure 54).
To further investigate whether the increased SF response of the αβN378Aγ-ENaC mutation is due either an intra- or an inter-tether was addressed by comparing the SF response of homotrimeric and heterotrimeric wild-type ENaC with corresponding βN378A mutations. To address the question whether the observed increased SF response of glycosylated asparagine N378 is due to disturbing the intra-tether N-glycan network, normalised SF responses of homotrimeric wild-type β-ENaC was compared with homotrimeric mutated βN378A-ENaC. The comparison revealed no change in SF response, indicating that the N-glycan of N378 might provide an inter-tether to the α- or γ-ENaC subunit rather than an intra-tether. To further investigate whether the increased SF response of glycosylated asparagine N378 is caused due to a N-glycan inter-tether interaction, the SF response of heterotrimeric wild-type αβ-, or βγ-ENaC was compared with mutated
αβN378Aγ-ENaC. The comparison revealed no change in response to normalised SF, indicating that the increased SF response of αβN378Aγ-ENaC might be due to a missing N-glycan inter-tether connection to both adjacent α- and γ-ENaC subunits rather than an individual subunit. However, another aspect that might explain why the SF response of wild-type and mutant channels (βN378A) did not change, could be explained by the orientation of the channel when exposed to SF. With presumably three mutated βN378A-ENaC subunits co-expressed together as one homotrimeric channel, might neutralise conformational changes due to a misalignment of individual subunits preventing channel opening.

The importance of N-glycans for the interaction between subunits is highlighted in a study of Kashlan et al., (2018) showing that the removal of all N-glycans of the β-ENaC subunit affects the conformation of co-expressed α-subunits. This suggests that the β-ENaC subunit without asparagines causes misfolding of the α-subunit (Kashlan et al., 2018). In addition, other studies showed that the removal of all N-glycans of the β-ENaC subunit affected the proteolytic cleavage of the α-subunit in the finger domain (Hughey et al., 2004a; Bruns et al., 2007; Kashlan et al., 2012), indicating that the channel might be folded in an alternate conformation. These studies, combined with results shown in my thesis, highlight that the increased SF response of the αβN378Aγ-ENaC might be due to a missing N-glycan connection to the adjacent α- and γ-ENaC subunits, supports the notion of distinct functions of N-glycans for the channel beside SF sensation such as folding or stability.

To further investigate whether or not removal of glycosylated asparagine N378 affects single-channel properties (conductance, permeability or Po), single channel measurements were performed, comparing αβN378Aγ-ENaC with the
corresponding control ($\alpha\beta\gamma$-ENaC). The subsequent comparison revealed there was no change in single-channel properties caused by $\beta$-ENaC mutation N378A (Table 24). In addition, the comparison of the amiloride-sensitive current of $\alpha\beta$N378A$\gamma$-ENaC compared with control ($\alpha\beta\gamma$-ENaC) revealed no change, indicating a similar number of channels are expressed on the membrane of Xenopus oocytes.

Further, the removal of glycosylated asparagine N99 in the $\beta$-Ball-domain revealed a change in Tau, indicating a change in channel open kinetics (Figure 38). Tau is a time constant that is commonly used to determine changes in channel open kinetics (Carattino et al., 2005; Karpushev et al., 2010; Chen et al., 2013). The increase in Tau demonstrates that glycosylated asparagine N99 slows down the Na$^+$ gating in response to SF. Hence, it could be that the N-glycan of glycosylated asparagine N99 provides an important intra- or inter-tether that is linked to channel gating. The comparison of $\alpha\beta$N99A$\gamma$-ENaC with corresponding control ($\alpha\beta\gamma$-ENaC) showed a decreased amiloride-sensitive current, reflecting again either a low Po or small number of channels expressed on the membrane (Figure 39). This finding indicates that N-glycan of glycosylated asparagine N99 might play an important role in trafficking/maturation and gating in response to SF. The same was observed by removing glycosylated asparagine N364 in the knuckle-domain (Figure 39). Interestingly, the similarities of N99 and N364 support the assumption that individual N-glycans might have distinct functions within ENaC that could affect trafficking/maturation or gating of the channel in response to SF. Another removal of glycosylated asparagine N135, N141, or N146 in the finger-, N260 within the $\beta$-Ball- and N484 in the knuckle-domain revealed a decreased amiloride-sensitive current, again indicating a lower Po or smaller number of channels on the membrane of Xenopus oocytes (Figure 39).
The previous discussion highlights the possibility that N-glycans attached to glycosylated asparagines of ENaC might have distinct functions. Questions remains about whether the distinct function of N-glycans can be explained by 1) insufficient glycosylation or 2) localisation/positioning of the glycosylated asparagine within the β-ENaC subunit.

1) One explanation could be insufficient glycosylation, independent of their glycosylation consensus sequence. There are three different stages of N-linked glycosylation that are divided into three major types such as high mannone, complex, and hybrid that differ in their outer branches (Kornfeld & Kornfeld, 1985). The high mannone type is composed of three branches each with two sugar residues. The complex types has two branches each with three sugar residues each and the hybrid type have features of both other types (Kornfeld & Kornfeld, 1985; Varki, 2017). These complex glycan structures might generate a complex network that interact with their surroundings and might provide an intra- or inter-tether to multiple structures. This complexity in structure supports the assumption that different types of N-glycans might have other functions than other N-glycans. In other words, it might be that a high mannone N-glycan has a role different to a complex or hybrid N-glycan within ENaC subunits.

2) Another potential explanation that might explain the distinct functions of N-glycans within the β-ENaC, could be the N-glycan location/positioning within the extracellular-domains. A 3D surface model (provided by collaboration with the Justus Liebig University Giessen) revealed that 10 out of 11 glycosylated asparagines of the β-ENaC subunit are located towards the outside of the protein (Figure 55).
Figure 55: Putative 3D surface structure-model of heterotrimeric αβγ-ENaC, highlighting glycosylated asparagines of the β-ENaC subunit
A) Shows the side-view of the channel trimer, B) Top-view and C) Bottom view of αβγ-ENaC, including all glycosylated asparagines of the β-ENaC subunit in colour and corresponding number. Glycosylated asparagines locations were coloured as: the finger- (purple), the knuckle- (turquoise), the palm- (yellow), the β-Ball- (orange), the thumb-domain (green). One glycosylated asparagine N99 in the β-ball domain did not face towards the outside of the β-ENaC subunit, whereas all the other did.

The only glycosylated asparagine that did not point outwards was N99 located in the β-Ball-domain of the β-ENaC subunit. The removal of glycosylated asparagine N99, including its N-glycan, might disrupt the stability/folding of the β-Ball domain of the β-ENaC subunit, causing increased activity of ENaC in response to SF (further discussed section 2.2.2). This might provide an example for a glycosylated asparagine where the N-glycan provides an intra-tether that has a stabilising/folding role within the channel rather than being a tether for inter-subunit interactions or a connection to the ECM. Dubious remains whether the N-
glycan structure or types of every individual glycosylated asparagines of ENaC. Following this up might reveal that specific types of N-glycans have distinct functions such as providing an intra-tether, an inter-tether or an ECM-tether (Figure 54) that might be involved in folding/stabilisation within the channel, affecting Na⁺ gating in response to SF or SF sensation of ENaC.

Taken together, previously described findings support the assumption that N-glycans of glycosylated asparagines of the β-ENaC subunit might have distinct functions that modulate the SF response due to an intra-tether within the subunit or an inter-tether to adjacent subunits. The third scenario where the N-glycan might provide an ECM-tether might be excluded for the β-ENaC subunit, supported by studies showing that removal of the ECM-tether decreases responses to SF in the α-ENaC subunit (Barth, 2017; Knoepp et al., 2017). In the case of the β-ENaC subunit it might be more likely that N-glycans have intra- or inter-tether functions that affect the stability/folding of the channel which then influences the gating and activity of ENaC in response to SF. In agreement with my assumption, studies demonstrated that the removal of single or multiple N-glycans affect either the activity or folding of the protein (Helenius, 1994; Mitra et al., 2003). This is further supported by a study showing that N-glycans might act like molecular glue, holding residues around the glycosylation sides to stabilise the protein structure (Imperiali & O'Connor, 1999; Lee et al., 2015). In this context, another study demonstrated that just two out of five N-glycans in the α-ENaC subunit decreased (Knoepp et al., 2017) and just two out of eleven N-glycan of the β-ENaC subunit increased the activity of ENaC in response to SF (Figure 38).
2.2.2 Domain specific glycosylated asparagines of the β-ENaC subunit are involved in modulating SF response

To further investigate whether domain specific glycosylated asparagines might be important for modulating the SF response, domain specific asparagine deficient mutations of β-ENaC were generated.

The removal of all glycosylated asparagines within the β-Ball (N99 + 260) revealed an increased activity of ENaC in response to SF when compared to corresponding control (αβγ-ENaC) (Figure 45). Interestingly, glycosylated asparagine N99 is the only asparagine of the β-ENaC that faces towards the subunit itself rather than to the outside (Figure 55). This glycosylated asparagine N99 might represent an example of an intra-tether N-glycan that affects the stability/folding of the subunit itself followed by an increased ENaC activity in response to SF. The double β-ENaC mutation (N99A + N260A) did show a change in Tau under SF exposure when compared with the corresponding control (αβγ-ENaC) (Figure 46), indicating a change in channel open kinetics in response to SF. In addition the comparison of the amiloride-sensitive current of double β-ENaC mutation (N99A + N260A) with corresponding control (αβγ-ENaC) revealed a decreased current, indicating either by a low Po or an impaired trafficking/maturation reflected by a smaller number of channels expressed on the membrane. Interestingly, the fact that glycosylated asparagine N260 showed a decreased amiloride-sensitive current in a single (N260A) and double mutation (N99A + N260A), supports the assumption that N-glycans have distinct functions within the channel. Another supportive aspect is the finding that removing glycosylated asparagine N99 alone did affect SF sensation, indicating that in the β-Ball-domain of the β-ENaC subunit an intra-tether between individual N-glycans might be important for SF sensation. The intra-tether function of N-glycan attached to glycosylated asparagine N99 is also
supported due to the fact that this asparagine faces towards the subunit itself. This result highlights again the complexity of intra- and inter-tether N-glycan interactions and the possibility that two N-glycans can interact with each other, affecting trafficking/maturation and SF sensation together.

Previous findings from our lab provided evidence that glycosylated asparagines in the palm- and knuckle-domain (double-mutation) of the α-ENaC subunit play an important role for SF sensation (Barth, 2017; Knoepp et al., 2017). For this very reason a double mutation was generated by removing all glycosylated asparagines within the palm- and knuckle (N449 + N484) of the β-ENaC subunit. The comparison of the double mutation (N449 + N484) with corresponding control (αβγ-ENaC) revealed an increased activity of ENaC under SF exposure (Figure 45). In contrast, mutating two glycosylated asparagines in the palm- and knuckle-domain (N312 + N511) of the α-ENaC subunit revealed a decreased SF response, indicating subunit dependant functions of N-glycans. Based on these results it could be that N-glycans of the palm- and knuckle-domain of the α-ENaC subunit provides an ECM-tether whereas corresponding N-glycans of the β-ENaC might provide an intra- or inter-tether rather than an ECM-tether. The double mutation within the palm- and knuckle (N449 + N484) of the β-ENaC subunit did not show any changes in Tau and amiloride-sensitive current, indicating that these N-glycans neither affect channel open kinetics (gating of Na+ in response to SF) in response to SF nor the trafficking or maturation of the channel (Figures 46 and 47).

The exchange of all glycosylated asparagines located in the thumb-domain (N364 + N378) showed no change in response to SF (Figure 45). This finding is controversial regarding the previous observation that removal of single glycosylated asparagine N378 showed an increased response to SF. By assuming
that the removal of the N-glycan of N378, could cause a structural change of the thumb-domain, a lack of another glycosylated asparagine in position N364 might compensate the SF effect by reversing the structural change back to the domain’s original conformation. The structural change in the thumb domain could occur due to disruption to the intra- or inter-tether N-glycan interactions within the β-subunit or the adjacent α- or γ-subunit. Another aspect that might explain the increased SF response of single glycosylated asparagine N378 and the missing response after removing N-glycans attached to glycosylated asparagines (N364 + N378), could be the strong variability between oocyte batches of *Xenopus laevis* during SF exposure. The double mutation (N364A + N378A) of the thumb domain did not show any changes in Tau and amiloride-sensitive currents when compared with the corresponding control (αβγ-ENaC) (Figures 46 and 47), indicating that N-glycans of the thumb-domain neither affect trafficking, maturation or channel open kinetics reflecting the gating of Na\(^+\) in response to SF.

The removal of two (N135 + N199) and three glycosylated asparagines (N135 + N199 + N207) together in the β-ENaC subunit from the finger-domain revealed no change in response to SF when compared with corresponding control (αβγ-ENaC) (Figure 45). None of the mutations of the finger-domain showed any changes in Tau when compared with control, indicating that these N-glycans did not impair the channel open kinetics, reflecting the gating of Na\(^+\) in response to SF (Figure 46). However, the amiloride-sensitive current of the double (N135A + N199A) and threefold mutation (N135A + N199A + N207A) decreased when compared with control (Figure 47). The decreased amiloride-sensitive current might be explained again by either a low Po or a smaller number of channels expressed on the membrane of *Xenopus* oocytes. This finding supports the assumption that the intra- or inter-tether interaction between multiple N-glycans might affect
trafficking/maturation or stability/folding that influence the gating of the channel in response to SF.

The potential importance of domain specific glycosylated asparagines might be explained by taking a closer look into the protein structure that was derived from the ASIC crystallised structure. The palm-domain provides a connection between the two TMDs, the thumb- and β-Ball-domains are dominated by secondary protein structures such as β-sheets and disulphide bonds which provide a rigid scaffold within the subunit (Jasti et al., 2007; Eastwood & Goodman, 2012). This rigid network/scaffold is fortified by N-glycans of glycosylated asparagines and this increase the stability of the protein (Imperiali & O’Connor, 1999). Removing, for instance, N-glycans via mutating glycosylated asparagines might disturb this rigid network/scaffold via breaking intra- or inter-tether interactions that could cause a conformational change followed by a loss of stability of the channel. The subsequent application of SF might change the conformation of the channel causing an increased gating that reflects a higher activation of the channel.

In summary, previous literature and findings of this thesis supports the importance of glycosylated asparagines of the β-ENaC subunit in modulating responses to SF of ENaC (Barth, 2017; Knoepp et al., 2017). In this section it was shown for the first time that removal of glycosylated asparagines in β-ENaC of the thumb-, β-Ball-, palm- and knuckle-domains increase the activity of ENaC in response to SF which contradicts previously described data from our lab showing a decreased SF response when asparagines in the α-ENaC subunit were removed (Barth, 2017; Knoepp et al., 2017). This observed difference may provide new insights into the putative roles of N-glycans of ENaC function and the ability of the channel to respond to SF. The observations of this thesis suggest that N-glycans attached to
glycosylated asparagines play a more complex role for SF sensing than just a connection to the ECM. It can also not be excluded that each N-glycan may have multiple “anchor points”. Particularly given that N-glycans have a diverse tree-like structure with multiple branches. It can be hypothesised that some branches may provide intra-tethers within the subunit, whereas other branches of the same N-glycan may provide inter-tethers that reach out to adjacent subunits. In addition, it could be hypothesised that different types of N-glycans can interact with different substrates and therefore have different roles in sensing SF. For example some N-glycan types may interact with protein components and other types may prefer carbohydrate molecules. The results of this section suggest that glycosylated asparagines including the N-glycans of the β-ENaC subunit of certain domains are more important for SF sensation than others, indicating different functions for N-glycans such as protein stabilisation and folding or trafficking and maturation of the channel. Finally these findings support the notion that glycosylated asparagines including their N-glycans of the β-ENaC subunit modulate the ability of ENaC to sense SF.

2.3 Glycosylated asparagines of the γ-ENaC subunit do not contribute to SF activation

The γ-ENaC subunit reveals 5 glycosylated asparagines (Figure 56). The co-expression of the 5 single glycosylated asparagines mutant deletions of γ-ENaC with the wild-type α- and β-ENaC subunit, revealed no change in response to SF when compared with the corresponding control (αβγ-ENaC). This indicates that the glycosylated asparagines (their N-glycans) of the γ-ENaC subunit do not play a role for the ability of ENaC to sense SF (Figure 49). However, replacement of
certain glycosylated asparagines did change channel function. For example, N248 of the finger-domain showed an increase in Tau when compared with control, indicating a change in the opening kinetics of the channel (Figure 50). As with the asparagines of β-ENaC, this might provide evidence that this specific N-glycan affects the integrity leading to an impaired gating of the channel. Furthermore, the removal of a single glycosylated asparagine N271 of the β-Ball-domain and N497 of the knuckle-domain showed a decreased amiloride-sensitive current (Figure 50), indicating that these N-glycans might impair the trafficking/maturation or the Po of the channel.

![Figure 56: Homology model of the protein structure of a γ-ENaC subunit](image)
3D model of the protein structure of a γ-ENaC subunit illustrates all locations of the 5 glycosylated asparagines within their channel domains. 2 glycosylated asparagines were found in the finger- (purple), 1 in the knuckle- (turquoise), 2 in the β-Ball- (orange). The palm- (yellow) and the thumb-domain (green) did not reveal a glycosylated asparagine.

To further investigate whether domain specific glycosylated asparagines of the γ-ENaC subunit has a role in modulating the SF response, double mutations of the finger- (N209A + N248A) and β-Ball-domain (N271A + N291A) were generated.
The application of SF did not change the response of either double mutations when compared to the corresponding control (αβγ-ENaC) (Figure 51). In addition, both double mutations did not show a change in Tau in comparison with control, indicating that these N-glycans did not impair channel open kinetics or the gating process (Figure 52). Interestingly, both double mutations revealed a decreased amiloride-sensitive current, indicating that N-glycans of the γ-ENaC subunit might have an important role in channel trafficking and maturation. The small amiloride-sensitive current observed in the single (N248A) and double mutation (N209A + N248A) of the γ-ENaC subunit, reveals a distinct N-glycan function of N248 in trafficking and maturation of the channel.

To further investigate whether the position of glycosylated asparagines is important for the distinct function of attached N-glycans, a 3D surface model of the γ-ENaC subunit was generated (provided by collaboration with the Justus Liebig University Giessen). All glycosylated asparagines faced towards the outside of the γ-ENaC subunit (Figure 57). The γ-ENaC subunit did not reveal glycosylated asparagines that faced towards the inside of the subunit as observed for glycosylated asparagine N99 of the β-ENaC subunit. By assuming that glycosylated asparagines, in particular their N-glycans, of the γ-ENaC subunit play an important role for trafficking or maturation of the channel, it would make sense that N-glycans face towards the outside to provide recognition sides to enable normal trafficking. In agreement with this assumption, a study by Kashlan et al., (2018) showed that the removal of all N-glycans of the γ-ENaC subunit impaired trafficking of the channel (Kashlan et al., 2018).
Figure 57: Putative 3D surface structure-model of heterotrimeric αβγ-ENaC highlighting all glycosylated asparagines of the γ-ENaC subunit. 

A) Presents the side-view, B) the top-view and C) the bottom-view of a heterotrimeric αβγ-ENaC protein. The glycosylated asparagines of γ-ENaC were highlighted in the same way as described for figure 3. All glycosylated asparagines face towards the outside of the γ-ENaC subunit.

Taken together, these findings in this section of the thesis showed for the first time that the removal of N-glycans attached to glycosylated asparagines in the γ-ENaC subunit did not change activity of ENaC in response to SF. However, one single mutation in the finger-domain showed an impairment of channel open kinetics or gating in response to SF. Furthermore, one double mutation in the finger- and one in the β-Ball-domain revealed a lower amiloride-sensitive current, indicating a more important role of attached N-glycans in trafficking and maturation. In this context, it is likely that N-glycans of the γ-ENaC subunit have a more important
role in gating and trafficking or maturation rather than a modulatory role for SF sensation.

2.4 Physiological relevance of glycosylated asparagines of the β- and γ-ENaC subunits

The removal of N-glycans of the β-ENaC subunit which results in a more active channel in response to SF, coincides with another well-known β-ENaC mutation called Liddle’s syndrome. This mutation is also characterised by an over activation of ENaC causing severe hypertension. The Liddle’s syndrome mutation is characterised by an increased number of ENaC on the membrane, whereas the β-ENaC mutation in this thesis is characterised by a normal number of channels on the membrane but an increased activation of ENaC in response to SF. However, the fact that both β-ENaC mutations show an increased activation of ENaC, supports the assumption that the β-ENaC subunit has a modulatory role by holding the channel in a normal physiological state when exposed to SF. Taken together, the observation that asparagine deficient mutations in the β-ENaC subunit increase ENaC activity in response to SF provides new information about a possible β-ENaC mutation that causes hypertension in the human body. A better understanding of the so called gain of function mutations of ENaC could help to treat patients with severe hypertension.

The assumption that N-glycans of the α-, β-, or γ-ENaC subunit provide intra-tether within the individual subunit, inter-tether to adjacent subunits and ECM-tether for sensing SF, could provide a new insight into understanding the molecular mechanism of how ENaC senses SF. The interactions within the subunit itself
(intra-tether), between adjacent subunits (inter-tether) or between the channel and
the ECM (ECM-tether) via N-glycans could provide fundamental information to a
better understanding of the function of ENaC as a mechanosensor.

3 Conclusion

The aim of this thesis was to address two hypotheses:

1) The β- and γ-ENaC subunits have a modulatory role in sensing SF.

2) N-linked glycans of glycosylated asparagines of the β- and γ-ENaC subunit contribute to SF sensation via an interaction with the ECM.

The first hypothesis was accepted in considering all performed experiments. The second hypothesis addressing the importance of N-linked glycans of the β- and γ-ENaC subunit for SF activation via an interaction with the ECM was rejected.

1) This thesis demonstrates that homotrimeric and heterotrimeric ENaC can form functional channels that respond to SF. In addition, it provides evidence that the β- and γ-ENaC subunits can have a modulatory role for SF activation in *Xenopus* oocytes. These findings support evidence that in tissues, where β- and γ-ENaC subunits were detected without α-ENaC subunit, these ENaC subunit combinations are capable of responding to SF on their own.

2) The second part of this thesis showed that N-glycans are involved in SF sensation but no evidence whether this occurs via interaction with the ECM was found. Furthermore, N-glycans of glycosylated asparagines of the β-ENaC subunit, may for instance, form a connection to an adjacent subunit or extracellular-domain
rather than a tether to the ECM. The deletion of N-glycans might disturb the protein structure resulting in a more active ENaC. The investigation of glycosylated asparagines of the γ-ENaC did not show a change in SF sensation, indicating that attached N-glycans do not play an important role. In addition, it highlights the possibility that N-glycans of individual glycosylated asparagines might have different functions beside an ECM-tether such as an intra-tether within the subunit, an inter-tether to adjacent subunits that might play an important role in stabilisation or folding within the protein. Since all experiments performed in this thesis were done in Xenopus oocytes the relevance of this data for SF sensation in vivo needs to be further elucidated.

4 Further direction

The fact that homotrimeric and heterotrimeric ENaC can form functional channels which respond to SF in Xenopus oocytes needs further confirmation by backing up the result using reliable mammalian cell models. Therefore, epithelial Fischer rat thyroid gland cells (FRT-cells) could be transfected with ENaC combinations, exposed to SF and followed by SF patch clamp experiments. In addition, HUVECs (endothelial cell model) could be grown, stimulated with aldosterone to boost ENaC expression, treated with siRNA to suppress different ENaC subunits and SF patch clamp experiments performed. Further experiments could be performed by using a mouse model to investigate the modulatory role of ENaC as well as the involvement of the ECM in SF sensation in vivo and ex vivo. Investigations in a mouse model with reduced levels of β-ENaC (Pradervand et al., 1999) could provide more information about the modulatory role of β-ENaC. Therefore, vessels
could be harvested, treated with enzymes to degrade, for instance, the ECM and then measured pressure myography or patch clamp experiments, to confirm the modulatory role of the β-ENaC subunit in a mammalian model. Furthermore, a mouse model could be generated by introducing specific asparagine deficient mutations (Knock in) through viral vectors. For instance, primary data from our collaborators showed that introducing a double asparagine deficient α-ENaC mutation, as reported previously (Knoepp et al., 2017), using an adenoviral vector (AdVs) prevents the development of hypertension in a mouse model (Knoepp et al., unpublished). Another possibility to investigate the importance of glycosylated asparagines in response to SF in a mammalian model, through gene editing technologies such as Transcription activator-like effector nuclease (TALENs) and CRISPR/Cas9. Furthermore, it would be really interesting to determine which N-glycan types are attached to individual glycosylated asparagines. It might be that N-glycans of glycosylated asparagines that are attached to the ECM are different ones than N-glycans that stabilise the protein. In other words, it could be that the decreased SF responses observed in α-ENaC is due to tethers to the ECM via a different type of N-glycans than the increased SF response observed in the β-ENaC subunit. Therefore, mass spectrometry could be used. A further investigation of the domain specific mutations of the β-ENaC subunit could provide more information about the observed increased SF response. These changes could be due to either an increased Po or conductance of these channels and could be addressed by single channel recordings via patch clamping. These experiments would help to understand whether individual N-glycans of glycosylated asparagines of ENaC have different tasks in trafficking, stabilisation or SF sensing.
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