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Involvement of SNARE components in the transfer of cholesterol from lipid droplets to mitochondria in rat adrenals and ovaries

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Abstract

Dysfunction in cholesterol storage and transport contributes to various different diseases such as Niemann Pick C Disease, congenital adrenal hyperplasia or foam cell formation in atherosclerosis. Cholesterol plays an important role in adrenals and ovaries where it serves as the basis for the synthesis of steroid hormones.

Intracellular cholesterol is stored in lipid droplets. However, it needs to be transported into the mitochondrial matrix to be converted into pregnenolone as the first step of steroidogenesis. While a protein complex consisting of the so called transduceosome and metabolon has been identified to mediate cholesterol transport across the outer and inner mitochondrial membrane respectively, the transportation mechanism of cholesterol from lipid droplets to the outer mitochondrial membrane is still elusive. As SNARE proteins (*s*oluble *N*-ethylmaleimide-sensitive-factor *a*ttachment *re*ceptor proteins) can mediate vesicular transport and have been found on lipid droplets, it was the aim of this study to investigate if cholesterol is transported from lipid droplets to mitochondria in a SNARE-mediated manner.

Mitochondria were isolated from rat ovaries and adrenals. In an *in-vitro* reconstitution bioassay, various combinations of purified recombinant His-tagged SNAREs (SNAP23, SNAP25, NSF, α-SNAP and Stx17) were added to mitochondria and cholesterol lipid droplets. It was analysed via ELISA how much pregnenolone was synthesised from the cholesterol added in the form of lipid droplets. The more cholesterol is transported into the mitochondrial matrix, the more pregnenolone can be produced. Furthermore, the effect of GTP (guanosine triphosphate) and cytosol on pregnenolone production was investigated.

Addition of SNARE proteins resulted in a 46fold increase in pregnenolone production ($p<0.0001$, Tukey's multiple comparison test), indicating their

involvement in cholesterol transport from lipid droplets to mitochondria. In particular, the combination of SNAP23, SNAP25, NSF and α-SNAP was found to mediate cholesterol transport to the outer mitochondrial membrane ($p<0.05$, Sidak's Multiple Comparison Test) while the detailed role of Stx17 remains unclear. Furthermore, it was observed that cytosolic components enhance SNARE-mediated cholesterol transport without need for GTP ($p<0.05$, Sidak's Multiple Comparison Test).

Overall, these results allow proposing a model of SNARE-mediated cholesterol transport from lipid droplets to the outer mitochondrial membrane and across the mitochondrial membranes into the matrix for steroidogenesis. Cholesterol might join the cholesterol pool in the outer mitochondrial membrane by hemifusion of lipid droplets with the outer mitochondrial membrane. StAR (steroidogenesis acute regulatory protein) could bind cholesterol from this cholesterol pool before it induces its transport across the mitochondrial membranes via the transduceosome/metabolon. Cholesterol is then converted into pregnenolone as the first step of steroidogenesis in the mitochondrial matrix.

This study sheds new light on transportation of cholesterol from lipid droplets to the outer mitochondrial membrane *in vitro*. Incorporating the results into previous findings, a model of SNARE mediated cholesterol transport from lipid droplets into the mitochondrial matrix for steroidogenesis is proposed. The exact molecular mechanism of SNARE mediated cholesterol transport and the detailed role of each SNARE within this process *in vivo* remain to be determined.

Content

1 Introduction

1.1 Sources of cholesterol for steroidogenesis

In the public but also in the medical community cholesterol is primarily perceived as a cardio-vascular risk factor. Although this is true cholesterol also plays an important role in many biological processes, for example as an essential structural component of mammalian cell membranes (Balodimos, Kealey, & Hurxthal, 1968; Cooper, 1978). Furthermore, it provides the basis for many steroid hormones such as estrogens, androgens, glucocorticoids or mineralocorticoids, which are produced in steroidogenic cells like adrenocortical cells or granulosa cells of ovaries (Brown, Kovanen, & Goldstein, 1979; Cooper, 1978; Elliott & Schally, 1955). Its essentialness is illustrated by various diseases - such as congenital adrenal hyperplasia, Niemann Pick Disease and foam cell formation in arteriosclerosis - deriving from cholesterol transportation and storage dysfunctions (Newman, Murad, & Geer, 1971; Ribeiro et al., 2001; Tee et al., 1995).

In steroidogenic cells, intracellular cholesterol originates from several different sources (Morris and Chaikoff 1959). The first supply is granted by endogenous synthesis from acetate at the endoplasmic reticulum (ER) (Srere, Chaikoff, & Dauben, 1948). Endogenous cholesterol synthesis is regulated by various mechanisms, i.a. sterol-induced degradation of the rate limiting enzyme 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGCoA-reductase) (Horton, Goldstein, & Brown, 2002; Sever, Yang, Brown, Goldstein, & DeBose-Boyd, 2003).

Second, Verschoor-Klootwyk, Verschoor et al. (1982) could show that normal adrenal steroidogenesis in rats depends upon cholesterol delivery from plasma and that adrenocorticotropic hormone (ACTH)-stimulated steroid production is increased remarkably in the presence of lipoproteins (Verschoor-Klootwyk, Verschoor, Azhar, & Reaven, 1982). Lipoprotein derived cholesteryl ester uptake can be distinguished into two pathways: receptor-mediated endocytosis and selective cellular uptake via scavenger

receptor class B, type 1 (SR-BI) (Acton et al., 1996; Hoekstra et al., 2009).

For receptor-mediated endocytosis, apolipoprotein B- or E containing lipoproteins – mainly low density lipoproteins (LDL) - are processed via the LDL-receptor (Brown & Goldstein, 1986). After LDL has bound to the LDL-receptor, the ligand-receptor complex moves within the plasma membrane by diffusion to a region called "coated pits", which contains predominantly the protein clathrin (Anderson, Brown, & Goldstein, 1977; Burridge, Feramisco, & Blose, 1980). These regions invaginate and form vesicles, which fuse with early endosomes, then with each other and finally with lysosomes (Goldstein, Brunschede, & Brown, 1975). While the LDL-receptor is recycled and moves back to the cell membrane, lysosomal acid lipase hydrolyses the delivered cholesteryl esters (Brown, Anderson et al. 1983). The unesterified cholesterol is transported through the lysosomal membrane by Niemann Pick C Protein type 1 and 2 (NPC1 and NPC2) for steroid synthesis or re-esterification by acyl CoA-cholesterol acyltransferase for storage in lipid droplets (LDs) (Goldstein, Dana, Faust, Beaudet, & Brown, 1975; Kwon et al., 2009; Xu, Benoff, Liou, Lobel, & Stock, 2007).

Nevertheless, in rodents very large amounts of lipoprotein derived cholesteryl esters are processed by selective cholesteryl ester uptake via SR-BI (Glass, Pittman, Weinstein, & Steinberg, 1983). SR-BI seems to be essential for optimal adrenal function as impairment of this receptor induces adrenal insufficiency (Azhar, Leers-Sucheta, & Reaven, 2003; Hoekstra et al., 2009). Especially ACTH (or LH (luteinizing hormone)) stimulated steroid hormone production relies heavily on this cholesterol source (Azhar et al., 2003; Gwynne & Hess, 1980; Kraemer et al., 2007). For selective uptake, cholesterol-rich lipoproteins bind to SR-BI and release cholesteryl esters regardless of their lipoprotein composition without uptake of the lipoprotein itself (Glass et al., 1983; Reaven, Tsai, & Azhar, 1995). The cholesteryl esters are stored in LDs or hydrolyzed by hormone sensitive lipase (HSL) for transferral of cholesterol to mitochondria (Kraemer et al., 2004).

The third source of cholesterol, which can be transported to mitochondria for steroid synthesis, is provided by mobilisation of cholesteryl esters stored in lipid droplets. HSL hydrolyzes these cholesteryl esters to cholesterol. Experiments with *HSL* knockout adrenal cells (from mice) suggest that cholesterol coming from these stores accounts for approximately half of the basal steroid production (Kraemer. 2007).

All three sources supply cholesterol for biosynthesis of steroid hormones, of which the first step is conversion of cholesterol to pregnenonlone by the rate limiting enzyme cholesterol side chain cleavage enzyme (P450scc), which is found in the inner mitochondrial membrane (Black, Harikrishna, Szklarz, & Miller, 1994). Pregnenonlone can be converted to progesterone by 3-hydroxysteroid dehydrogenase (3HSD) at the inner mitochondria membrane in association with the translocase of the inner mitochondria membrane 50 (Tim50) (Pawlak, Prasad, Thomas, Whittal, & Bose, 2011). Otherwise, pregnenolone exits the mitochondrion and serves as substrate for the steroid 17 alpha-hydroxylase/17,20 lyase (P450c17) in the ER. All further steps take place at the ER until the final step of glucocorticoid and mineralocortocoid synthesis, which involves the two isoenzymes steroid 11β-hydroxylase (P450c11β) and aldosterone synthase (P450c11AS) resulting in cortisol and aldosterone, respectively (Ishimura & Fujita, 1997; Takayama et al., 1996; White, Curnow, & Pascoe, 1994).

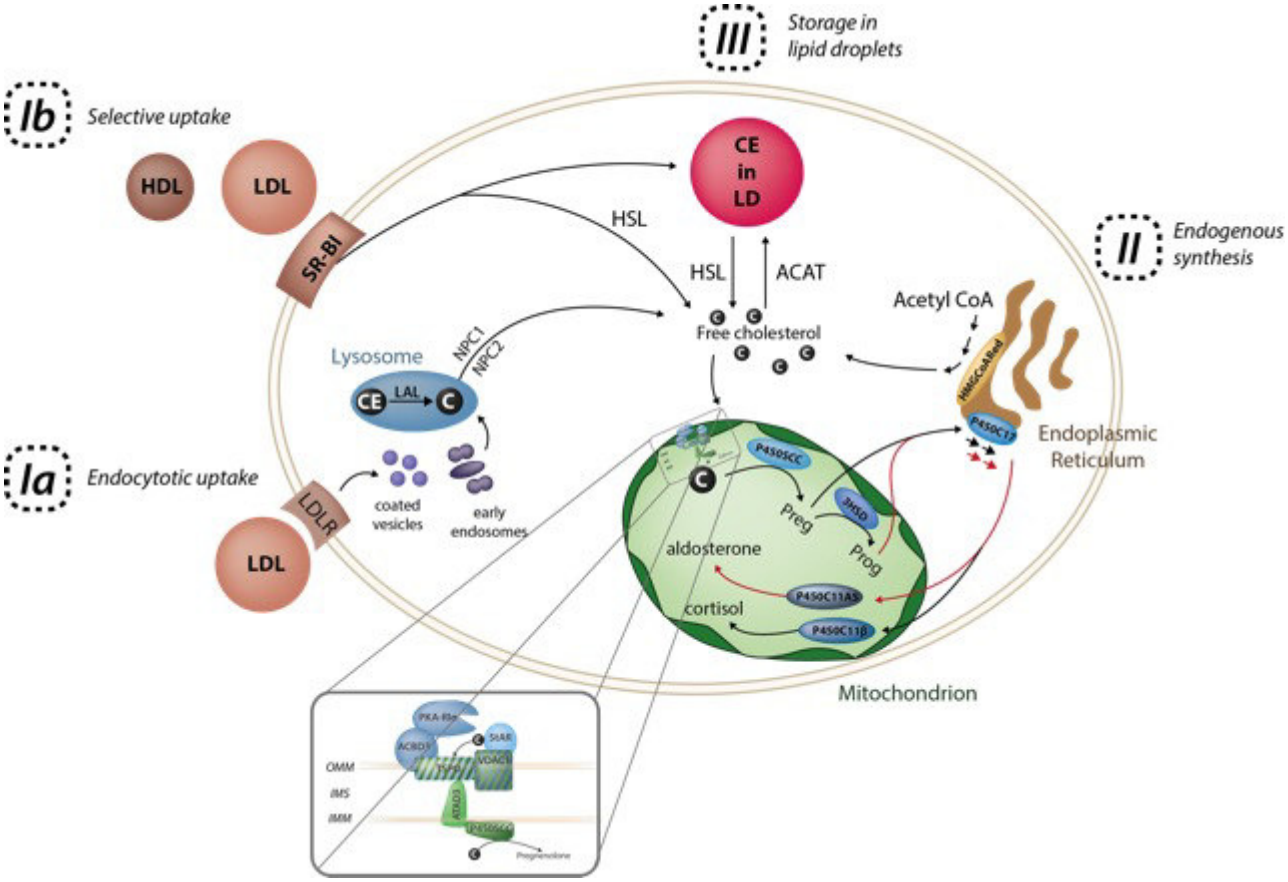

Fig. 1 Sources of cholesterol for steroidogenesis
Three main sources supply cholesterol for steroidogenesis:

I. a) Endocytotic uptake of cholesteryl esters (CE) from low density lipoproteins (LDL), uptake via the LDL receptor (LDLR). CEs are hydrolysed in lysosomes by the lysosomal acid lipase (LAL) and exported by the two proteins Niemann Pick C type 1 and 2 (NPC1 and NPC2).
b) Selective CE uptake from high density lipoproteins (HDL) and LDL via scavenger receptor B type 1 (SR-BI). CEs are hydrolysed by hormone sensitive lipase (HSL) or stored in lipid droplets (LDs).
II. Endogenous synthesis of cholesterol (C) from acetyl CoA at the Endoplasmic Reticulum (ER), rate limiting enzyme is Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMG-CoA-Red).
III. CEs stored in LDs are hydrolysed by HSL. Free cholesterol can be stored in LDs as CE after re-esterification by acyl CoA-cholesterol acyltransferase (ACAT).

Free cholesterol is imported into mitochondria via the transduceosome (see Figure 2). Steroidogenesis begins in mitochondria with conversion of C to Pregnenonlone (Preg.) by the cholesterol side chain cleavage enzyme (P450scc). Preg. is either exported to the ER or transformed into progesterone (Prog.) by the 3-hydroxysteroid dehydrogenase (3HSD). At the ER, the steroid 17 alpha-hydroxylase/17,20 lyase (P450c17) works on the substrates. After re-import into mitochondria, final transformations by the isoenzymes steroid 11β-hydroxylase and aldosterone synthase (P450c11β and P450c11AS) result in cortisol and aldosterone respectively.

Figure 1 was designed according to sources quoted on page 1 to 3

1.2 Transport of cholesterol to mitochondria – assumptions, hypotheses and speculations

It is currently unclear how cholesterol is transported from its various sources – endogenous synthesis at the ER, lipoprotein derived free cholesterol and cholesteryl esters stored in LDs - to the inner mitochondrial membrane for conversion to pregnenolone.

The best described part of this transportation process is the last step, in which cholesterol is transferred through the outer mitochondria membrane to the inner mitochondrial membrane by a protein complex named transduceosome (J. Liu, Rone, & Papadopoulos, 2006). Confocal microscopy and immunoblot studies could reveal the components: Acyl-CoA binding domain containing 3 (ACBD3), the RIα subunit of cAMP dependent Protein Kinase A (PKARIα), voltage dependent anion channel 1 (VDAC1), translocator protein 18kilodalton (kDa) (TSPO) in the outer mitochondrial membrane as well as the hormone-induced steroidogenesis acute regulatory protein (StAR) (J. Liu et al., 2006). Within this complex, TSPO is thought to constitute the channel for cholesterol import with its five α-helices serving as transmembrane segments through the outer mitochondrial membrane and the cholesterol-recognition amino acid consensus at the cytosolic carboxy-terminus (Korkhov, Sachse, Short, & Tate, 2010; H. Li, Yao, Degenhardt, Teper, & Papadopoulos, 2001). VDAC1 serves as anchor protein in the outer mitochondrial membrane and helps importing StAR (M. Bose, Whittal, Miller, & Bose, 2008). StAR binds and releases cholesterol dependent on phospholipid-induced conformational changes (Baker, Yaworsky, & Miller, 2005; H. S. Bose, Whittal, Baldwin, & Miller, 1999). Furthermore, its activation requires its localisation at the outer mitochondrial membrane so that it acts as the initiator of the transduceosome supplying cholesterol for ACTH induced steroidogenesis (H. S. Bose, Lingappa, & Miller, 2002). ACBD3 is a Golgi associated protein. Upon hormone stimulation it dissociates from the Golgi and brings protein kinase A (PKA) along to the outer mitochondrial membrane by binding to TSPO (H. Li, Degenhardt, et al., 2001). It is thought to function as an A-kinase anchoring protein (AKAP) bringing PKA

in close proximity with its substrates TSPO and StAR resulting in their phosphorylation (Papadopoulos, Liu, & Culty, 2007). Moreover, StAR activity was shown to be enhanced by phosphorylation (Dyson et al., 2008). In 2012, Rone et al. identified an 800kDa protein complex, which includes polymerised TSPO and VDAC1 as well as the ATPase family AAA domain-containing protein 3 (ATAD3) and P450scc (Rone et al., 2012). ATAD3 is a well conserved ATPase (S. Li & Rousseau, 2011). It is anchored in the inner mitochondrial membrane, enriched at mitochondrial contact sites and its knock-out was found to result in decreased cholesterol flow between the ER and mitochondria (Da Cruz et al., 2003; Gilquin et al., 2010; S. Li & Rousseau, 2012). Hence, Rone et al. propose this complex acting as a metabolon transferring cholesterol directly to P450scc where it is transformed into pregnenolone (Rone et al., 2012).

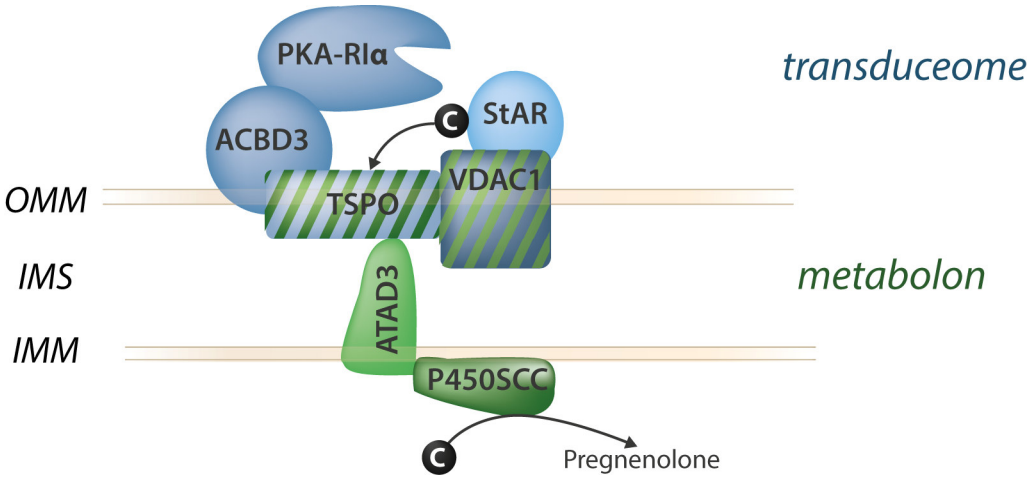

Figure 2 Import of cholesterol into mitochondria
The transduceosome: voltage dependent anion channel (VDAC1) and translocator protein, 18kDa (TSPO), recruit Acyl-CoA binding domain containing 3 (ACBD3), which binds the RIα subunit of cAMP dependent Protein Kinase A (PKA-RIα), and steroidogenic acute regulatory protein (StAR) in response the hormone stimulation. TSPO is proposed to serve as channel, while VDAC1 anchors the complex to the OMM and StAR acts as initiator supplying cholesterol (C). ACBD3 functions as A-kinase anchoring protein so that PKA can phosphorylate StAR enhancing its activity.
The metablonon: 800kD protein complex which contains the inner mitochondrial membrane proteins ATAD3 and P450scc in addition to TSPO and VDAC1. It is thought to bring cholesterol directly to P450scc for pregnenolone synthesis.

OMM = outer mitochondrial membrane, IMS= intermembranous space, IMM = inner mitochondrial membrane

Figure 2 was designed according to sources quoted on page 5 to 6

However, much less is known about the transportation of cholesterol from its various sources to the outer mitochondrial membrane before it is transferred to P450scc by the transcudeome/metabolon. As cholesterol is essentially insoluble in aqueous solution, it could be moved via protein-bound transport, vesicular transport or transport within larger organelles, e.g. lipid droplets (Haberland & Reynolds, 1973).

In steroidogenic cells, two members of the START domain super family – StAR-related lipid transfer (START) domain containing 4 (StARD4) and StAR-related lipid transfer (START) domain containing 5 (StARD5) – can bind cholesterol similarly to StAR and are discussed to be responsible for protein-bound transport of cholesterol to mitochondria (Soccio, Adams, Maxwell, & Breslow, 2005; Soccio & Breslow, 2003). These proteins are highly conserved and are lacking an amino-terminal sequence so that they are thought to be cytosolic proteins (Alpy & Tomasetto, 2005). Furthermore, StARD4 co-localises with acyl CoA-cholesterol acyltransferase and increases its activity in the ER (Rodriguez-Agudo et al., 2011). However, knock-out of StARD4 in mice appears to be compensated and only results in minor changes of weight and serum lipids (Riegelhaupt, Waase, Garbarino, Cruz, & Breslow, 2010).

The second assumption – that vesicles transport cholesterol to the outer mitochondrial membrane – is based on numerous microscopic observations showing ER vesicles in close proximity to mitochondria (Frey & Mannella, 2000). Furthermore, a high concentration of cholesterol within cell membranes leads to increased rigidity of the membrane and hence promotes formation of vesicles through hydrophobic mismatch (Garcia-Saez, Chiantia, & Schwille, 2007; Simons & Vaz, 2004). Thus, vesicles seem to play an important role in lipid homeostasis.

The third possible mode of cholesterol transport involves lipid droplets, which are also often found in close proximity to mitochondria and discovered to be in physical contact with mitochondria (Novikoff, Novikoff, Rosen, & Rubin, 1980; Pu et al., 2011). Once thought of as an inert fat deport, lipid droplets are now recognised to be dynamic organelles with the ability to move along the cytoskeleton, e.g. to mitochondria (Shen et

al., 2012; Weibel et al., 2012). LDs consist of a neutral lipid core – mainly cholesteryl ester or triacylglycerine depending on cell type - surrounded by a monolayer of phospholipids and a wide array of associated proteins. While the proteome of cholesteryl ester containing LDs it not well studied, it was found out that on triacylglycerine lipid droplets these proteins include structural proteins, enzymes involved in metabolism, vesicular transport machinery and several cytoskeletal proteins (Cermelli, Guo, Gross, & Welte, 2006) laying the basis for an active role in lipid metabolism with storage, metabolic and transport properties.

1.3 Lipid droplets (LDs) as potential deliverer of cholesterol to mitochondria

1.3.1 LDs as dynamic organelles in lipid metabolism

The major break-through in accessing the role of LDs as dynamic organelles was reached by proteomic analyses of purified LD fractions revealing at least parts of the protein machinery on their surface.

Some of the best investigated LD proteins are the perilipin family proteins (Plins), which not only have a structural function stabilising LDs but also control lipolysis by regulating access of HSL and adipose triglyceride lipase (ATGL) to LDs (Egan, Greenberg, Chang, & Londos, 1990; Greenberg et al., 1991). Only recently it was found out that the abundance of different Plin members differs between triacylglycerine and cholesteryl ester containing LDs. In steroidogenic cells, cholesteryl ester lipid droplets mainly contain Plin1c and Plin5 (Hsieh et al., 2012). The biological significance as it relates to steroidogenesis remains yet unknown (Kraemer, Khor, Shen, & Azhar, 2013).

Mass spectrometry experiments have revealed more proteins – many of them enzymes - on LD surfaces that manifest the active role of LDs in lipid metabolism, such as HSL itself, lanosterol synthase, nicotinamide adenine dinucleotide (phosphate) (NAD(P))-dependent steroid

dehydrogenase-like protein, acyl-CoA synthetase and long chain family member (ACSL) 1,(Brasaemle, Dolios, Shapiro, & Wang, 2004).

Furthermore, cytoskeleton proteins could be identified on LDs indicating their role as a dynamic organelle in lipid metabolism and explaining their broad distribution across the cell (Brasaemle et al., 2004; Krahmer et al., 2013; Targett-Adams et al., 2003). For triacylglycerine lipid droplets, intact microtubules were found out to be required for triacylglycerine independent growth by complex formation and kinesin-1 to mediate plus-end droplet motion (Bostrom et al., 2005; Shubeita et al., 2008). In adrenals, the intermediate filament vimentin, which is part of the cytoskeleton, was found to form a capsule around cholesteryl ester lipid droplets (Fuchs & Weber, 1994; Hall & Almahbobi, 1992, 1997). Vimentin not only interacts with proteins that show motor-like properties but also proteins with sterol binding properties as well as HSL (Chou et al., 2007; Shen et al., 2012; Wang, JeBailey, & Ridgway, 2002). Shen et al. discovered that the interaction with HSL affects lipolysis in addition to translocation of HSL to LDs (Shen, Patel, Eriksson, & Kraemer, 2010). In *vimentin* null mice, serum corticosterone concentrations, progesterone peaks in the oestrus cycle (in female mice) and stimulated serum progesterone concentrations were reduced suggesting a defect in adrenal and ovarian steroidogenesis (Shen et al., 2012).

In summary, lipid droplets seem to be active organelles in lipid metabolism transporting their cargo along the cytoskeleton. However, the exact mechanisms mediating cholesteryl ester/cholesterol delivery from LDs to the transduceosome in the outer mitochondrial membrane for steroidogenesis are still unknown.

1.3.2 Role of soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNAREs) on LDs

Interestingly, SNAREs were found to mediate fusion of triacylglycerine LDs (Bostrom et al., 2007). Originally, SNAREs were well known from membrane fusion of secretory pathways, e.g. synaptic exocytosis (Baumert, Maycox, Navone, De Camilli, & Jahn, 1989; Bennett, Calakos, &

Scheller, 1992; Trimble, Cowan, & Scheller, 1988). They can be differentiated into R- (arginine) and Q- (glutamine) SNAREs according to a key residue in the centre of their SNARE domain or into v- SNAREs (vesicular) and t-SNAREs (target-membrane) according to their position (Fasshauer, Sutton, Brunger, & Jahn, 1998). All SNAREs share a similar basic structure: At the carboxy-terminus, SNAREs are linked to a membrane, either by thioester linked acylgroups (e.g. synaptosomal-associated protein 25 (SNAP25)) or by transmembrane domains (e.g. syntaxin) (Han, Wang, Bai, Chapman, & Jackson, 2004; Ungermann & Langosch, 2005). In-between the carboxy-terminus and the divergent amino-terminus the SNARE motif is found (Sutton, Fasshauer, Jahn, & Brunger, 1998). To initiate membrane fusion, the SNARE motifs of opposing SNAREs and a soluble N-ethylmaleimide sensitive factor adaptor proteins (SNAP) assemble into parallel, twisted, coiled-coil, four helix bundles (Antonin, Fasshauer, Becker, Jahn, & Schneider, 2002; Sutton et al., 1998). This exothermic reaction starts at the amino-terminus of the SNAREs and ends at the carboxy-terminus according to the zip-model and provides the energy for membrane fusion (Fasshauer, Otto, Eliason, Jahn, & Brunger, 1997; Sorensen et al., 2006). The disassembly of this complex is driven by concerted action of N-ethylmaleimide sensitive factor (NSF) and α-SNAP (alpha-soluble NSF-attachment protein) (Hayashi, Yamasaki, Nauenburg, Binz, & Niemann, 1995). The amino-terminus of NSF binds to the SNAP-SNARE complex while one of its two ATP (adenosine triphosophate) binding domain shows ATPase activity needed for disassembly (Nagiec, Bernstein, & Whiteheart, 1995; Tagaya, Wilson, Brunner, Arango, & Rothman, 1993).

SNAREs interact with ras related in brain (rab) proteins, which are members of the Ras superfamily of GTPases, to mediate membrane fusion (Salminen & Novick, 1987; Schardt et al., 2009). Rab proteins cycle between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state (Schlichting et al., 1990; Zerial & Stenmark, 1993). In the cytoplasm, GDP-Rab are bound by GDP-dissociation inhibitors (GDI) and kept soluble (Alexandrov, Horiuchi,

Steele-Mortimer, Seabra, & Zerial, 1994; Ullrich et al., 1993). Removal of GDI by GDI-displacement factors and exchange of GDP for GTP by guanine nucleotide exchange factors leads to recruitment of Rabs to donor membranes and conversion into their active GTP-form (Andres et al., 1993; Dirac-Svejstrup, Sumizawa, & Pfeffer, 1997; Ullrich et al., 1993). This allows Rab proteins to coordinate their switch regions so that effectors, such as tethering factors, can bind to the Rab protein (Barr & Lambright, 2010; Simonsen et al., 1998). For inactivation of Rabs at the acceptor membrane after vesicle fusion, a GTPase activating protein is required to assist with GTP hydrolysis so that GDP-Rab can be bound by GDI for re-cycling (Araki, Kikuchi, Hata, Isomura, & Takai, 1990). Rab proteins were found on LDs and shown to regulate interaction of LDs with early endosomes (Bartz et al., 2007; P. Liu et al., 2007). Furthermore, it was observed in an *in-vitro* assay that GTP affects interaction of LDs with mitochondria indicating that Rab proteins might mediate LD-mitochondria interaction (Pu et al., 2011).

Beside Rab proteins SNAREs were found on LDs as well. In muscle and liver cells, LDs were found to be associated with NSF, α-SNAP and the SNAREs SNAP23 (synaptosomal-associated protein of 23 kDa), syntaxin-5, VAMP4 (vesicle-associated membrane protein 4) as well as SNAP25 (Bostrom et al., 2007). Furthermore, in NIH 3T3 fibroblast, triacylglycerine LDs were found to form a complex with mitochondria, whose formation is decreased by siRNA (small interfering ribonucleic acid) knockdown of SNAP23 (Jagerstrom et al., 2009). SiRNA knockdown of SNAP 23 also leads to decreased beta-oxidation which indicates that the SNAP23 mediated complex is of functional relevance (Jagerstrom et al., 2009). So SNARE components seem to play an important role for transport of triaclyglycerine to mitochondria. However, it is still unknown if SNARE components are also involved in transport of cholesterol via LDs to mitochondria for steroidogenesis.

2 Aim of this study and research strategy

2.1 Aim of this study

It is known that intracellular cholesterol is transported from its various sources to mitochondria for steroidogenesis. However, the exact transportation pathway and mechanisms involved are still unclear. LDs seem to play an important role in this process and may mediate the transport of cholesterol to mitochondria. The aim of this study is to determine if cholesterol is transported from lipid droplets to mitochondria in a SNARE-mediated manner. It will be investigated if the SNARE components SNAP23, SNAP25, NSF, syntaxin 17 and α-SNAP are involved in this process. Furthermore, the effect of cyotosolic components and GTP on cholesterol transport from LDs to mitochondria will be analysed.

2.2 Research strategy/ approach

Rat ovaries and adrenals will be used as a model. An *in-vitro* reconstitution bioassay will be used to model transportation of cholesterol via lipid droplets to mitochondria. Briefly, mitochondria of rat ovaries and adrenals, LDs and StAR will be added to various combinations of purified recombinant histidine (His)-tagged SNAREs. The medium will be analyzed for pregnenolone via a quantitative enzyme-linked immunosorbent assay (ELISA). The more cholesterol reaches mitochondria, the more pregnenolone will be produced. Cytosol and GTP will be added to investigate their effect on pregnenolone production and hence cholesterol transport from LDs to mitochondria.

In particular, the effect of SNAP23, SNAP25, NSF and α-SNAP will be investigated as they have been found on triacyl glycerine lipid droplets (Bostrom et al., 2007). The effect of syntaxin 17 (Stx17) will be examined as it is abundantly expressed in steroidogenic cell types and specifically

localizes to smooth membranes of the ER, which are highly involved in lipid metabolism (Steegmaier, Oorschot, Klumperman, & Scheller, 2000). Purified recombinant StAR will be added to the in-vitro reconstitution assay as well to insure sufficient cholesterol transport across the mitochondrial membranes. Cytosol and GTP will be added as Rab proteins were found on LDs and GTP was observed to affect interaction of LDs with mitochondria in an *in-vitro* assay (Bartz, Zehmer et al. 2007; Liu, Bartz et al. 2007; Pu et al., 2011).

3 Material and Methods

3.1 Cloning of recombinant SNARE proteins

Cloning was used in order to produce vectors carrying a recombinant protein, which was then transferred into *Escherichia coli (E. coli*) (*Bl21DE3*) for induced expression. The purified proteins were later on used for reconstitution assays.

3.1.1 Vector, deoxyribonucleic acid (DNA) sequence of SNARE proteins, primer design and restriction enzymes

Vector: pET15b purchased by Novagen

The polymerase chain reaction (PCR) amplification product was inserted into the vector behind the coding sequence of a 6xHis-tag in reading direction resulting in an amino-terminal His-tag. Hence, a fusion protein of the 6xHis-tag and the protein coded for by the amplified PCR was produced.

DNA sequence, primers and restriction enzymes

- Structure of the sense-primer
  Cytosine/Guanine rich base triplet for protection against nucleases – cutting sequence of the restriction enzyme – additional base to keep the insert within the reading frame if needed – Start codon and/or template sequence
- Structure of the anti-sense-primer
  Cytosine/Guanine rich base triplet for protection against nucleases – cutting sequence of the restriction enzyme – complementary base pairs to the leading strand of the template beginning at the 3'end

Restriction enzymes and their cutting sequence are marked in green, start codons (or the first codon of the coding DNA sequence for truncated proteins) in yellow, stop codons in red (see below).

The primers were produced by Elim Biopharm.

Homo sapiens synaptosomal-associated protein, 23kDa (SNAP23), transcript variant 1
NM_003825.3

```
  61 gtggcctcgc ccgcttgagt tttgattcat catggataat ctgtcatcag aagaaattca
 121 acagagagct caccagatta ctgatgagtc tctggaaagt acgaggagaa tcctgggttt
 181 agccattgag tctcaggatg caggaatcaa gaccatcact atgctggatg aacaaaagga
 241 acaactaaac cgcatagaag aaggcttgga ccaaataaat aaggacatga gagagacaga
 301 gaagacttta acagaactca acaaatgctg tggcctttgt gtctgcccat gtaatagaac
 361 aaagaacttt gagtctggca aggcttataa gacaacatgg ggagatggtg gagaaaactc
 421 accttgcaat gtagtatcta aacagccagg cccggtgaca aatggtcagc ttcagcaacc
 481 aacaacggga gcagccagtg gtggatacat taaacgcata actaatgatg ccagagaaga
 541 tgaaatggaa gagaacctga ctcaagtggg cagtatcctg ggaaatctaa aagacatggc
 601 cctgaacata ggcaatgaga ttgatgctca aaatccacaa ataaaacgaa tcacagacaa
 661 ggctgacacc aacagagatc gtattgatat tgccaatgcc agagcaaaga aactcattga
 721 cagctaaagc tactgctgtt cttctttatc atttattcac ttccgtagct cctccttgaa
 781 agttattacc ttttcagagt ttaagttttc ggttccacgc tcttctaatt gggagataat
```

Primer 1 5′ TCCGGATCCGATGGATAATCTG 3′ bp = 22 Tm = 66 GC = 50% BamHI
Primer 2 5′ TGCGGATCCTTAGCTGTCAATG 3′ bp = 22 Tm = 66 GC = 50% BamHI

Homo sapiens N-ethylmaleimide-sensitive factor attachment protein, alpha (NAPA), transcript variant 1
NM_0038727.3

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  61 ctgagtccct ttgtggccgc catggacaat tccgggaagg aagcggaggc gatggcgctg
 121 ttggccgagg cggagcgcaa agtgaagaac tcgcagtcct tcttctctgg cctctttgga
 181 ggctcatcca aaatagagga agcatgcgaa atctacgcca gagcagcaaa catgttcaaa
 241 atggccaaaa actggagtgc tgctggaaac gcgttctgcc aggctgcaca gctgcacctg
 301 cagctccaga gcaagcacga cgcagccacc tgctttgtgg acgctggcaa cgcattcaag
 361 aaagccgacc cccaagaggc cattaactgt ttgatgcgag caatcgagat ctacacagac
 421 atgggccgat tcacgattgc ggccaagcac cacatctcca ttgctgagat ctatgagaca
 481 gagttggtgg acatcgagaa ggccattgcc cactacgagc agtctgcaga ctactacaaa
 541 ggcgaggagt ccaacagctc agccaacaag tgtctgctga aggtggctgg ttacgctgcg
 601 ctgctggagc agtatcagaa ggccattgac atctacgaac aggtggggac caatgccatg
 661 gacagccccc tcctcaagta cagcgccaaa gactacttct tcaaggcggc cctctgccac
 721 ttctgcatcg acatgctcaa cgccaagctg gctgtccaaa agtatgagga gctgttccca
 781 gctttctctg attcccggga atgcaagttg atgaaaaaat tgctagaggc ccacgaggag
 841 cagaatgtgg acagctacac cgagtcggtg aaggaatacg actccatctc ccggctggac
 901 cagtggctca ccaccatgct gctgcgcatc aagaagacca tccagggcga tgaggaggac
 961 ctgcgctaag ccccacccag ccccccagtg cccgtcttcc tgtcccatct gctcagagag
```

Primer 1 5′ TCTGGATCCGATGGACAATTCC 3′ bp = 22 Tm = 66 GC = 50% BamHI
Primer 2 5′ TTAGGATCCTTAGCGCAGGTCC 3′ bp = 22 Tm = 68 GC = 55% BamHI

Rattus norvegicus syntaxin 17 (Stx17)
NM_145723.1

```
 121 ggctgcagga tgtccgaaga tgaggaaaag gtgaagctac gcaggcttga accagccatc
 181 cagaaattca ccaagatcgt gatcccaaca gacctggaga ggctaaagaa gcaccagata
 241 aacatcgaga agtatcaacg gtgcagaatc tgggacaagt tgcatgaaga gcacatcaat
 301 gccgggcgca ctgttcagca actgcgctcc aatatccgag aaatggagaa gctttgctta
 361 aaagtgcaca aagatgatct gatccttttg aagcgaatga tagatcctat gaaggaagca
 421 gcagcagccg ccacagccga gtttctccag ctccacttgg aatctgtgga agaactcaag
 481 aaacaagtca agaatgaaga agctttacta cagccttctc tgaccagatc cacaaccatt
 541 gatggagtcc acactggaga ggctgaagct gcttcccaga gtctgactca gatatatgcc
 601 ttgcctgaaa ttcctcgaga ccaaaatgct gctgagtcct gggaaacctt agaagcggac
 661 ttaattgagc ttagccatct ggtcaccgat atgtccctcc tagtaaattc tcagcaggag
 721 aagattgaca gcattgcaga ccatgtcaac agtgctgctg tgaatgttga agagggaacc
 781 aaaaacttgc agaaggctgc aaaatacaag ctggcagccc tgcctgtggc aggtgcagtc
 841 atcggaggag tggtgggggg tcccatcggc ctcctcgcag gcttcaaagt ggcaggaatt
 901 gcagctgcac ttggtggtgg ggtgttaggc ttcacaggtg gaaaattgat acaaagaagg
 961 aaacagaaaa tgatggagaa gctcacttcc agctgtccag atctccctag ccaaagtgac
1021 aaaaaatgca gttaaagccc agcttccagc tggtactgtg cacccgacct aaggagcaac
```

Primer 1 5′ TCTGGATCCGATGTCCGAAGAT 3′ bp = 22 Tm = 66 GC = 50% BamHI
Primer 2 5′ TGCGGATCCTAACTGACTTTTTTG 3′ bp = 24 Tm = 68 GC = 42% BamHI

Mus musculus steroidogenic acute regulator protein (Star), nuclear gene encoding mitochondrial protein
NM_0011485.4

```
                                                          atgt
tcctcgctac gttcaagctg tgtgctggaa gctcctatag acatatgcgg aatatgaaag
gattaaggca ccaagctgtg ctggccattg gccaagagct caactggaga gcactggggg
attccagtcc cgggtggatg ggtcaagttc gacgtcggag ctctctgctt ggttctcaac
tggaagcaac actctatagt gaccaggagc tgtcctacat ccagcaggga gaggtggcta
tgcagaaggc cttgggcata ctcaacaacc aggaaggctg gaagaaggaa agccagcagg
agaacgggga cgaagtgcta agtaagatgg tgccagatgt gggcaaggtg tttcgcttgg
aggtggtggt agaccagccc atggacagac tctatgaaga acttgtggac cgcatggagg
ccatgggaga gtggaaccca aatgtcaagg agatcaaggt cctgcagagg attggaaaag
acacggtcat cactcatgag ctggctgcgg cggcagcagg caacctggtg gggcctcgag
acttcgtgag cgtgcgctgt accaagcgca gaggttccac ctgtgtgctg gcaggcatgg
ccacacattt tggggagatg ccggagcaga gtggtgtcat cagagctgaa cacggcccca
cctgcatggt gcttcatcca ctggctggaa gtccctccaa gactaaactc acttggctgc
tcagtattga cctgaaggggg tggctgccga agacaatcat caaccaggtc ctatcgcaga
cccagataga gttcgccaac cacctgcgca agcgcctgga agccagccct gcctctgagg
cccagtgtta a
```

Primer 1 5′ TATGGATCCGGAAGCAACACTC 3′ bp = 22 Tm = 66 GC = 50% BamHI
Primer 2 5′ TTAGGATCCTTAACACTGGGC 3′ bp = 21 Tm = 62 GC = 48% BamHI

Homo sapiens N-ethylmaleimide-sensitive factor (NSF), transcript variant 1
NM_006178.3

```
  61 ccgagcgcag agctgcagcc gccgagccgg acgtgtccgc gaagatggcg ggccggagca
 121 tgcaagcggc aagatgtcct acagatgaat tatctttaac caattgtgca gttgtgaatg
 181 aaaaggattt ccagtctggc cagcatgtga ttgtgaggac ctctcccaat cacaggtaca
 241 catttacact gaagacacat ccatcggtgg ttccagggag cattgcattc agtttacctc
 301 agagaaaatg ggctgggctt tctattgggc aagaaataga agtctcctta tatacatttg
 361 acaaagccaa acagtgtatt ggcacaatga ccatcgagat tgatttcctg cagaaaaaaa
 421 gcattgactc caacccttat gacaccgaca agatggcagc agaatttatt cagcaattca
 481 acaaccaggc cttctcagtg ggacaacagc ttgtctttag cttcaatgaa aagctttttg
 541 gcttactggt gaaggacatt gaagccatgg atcctagcat cctgaaggga gagcctgcga
 601 cagggaaaag gcagaagatt gaagtaggac tggttgttgg aaacagtcaa gttgcatttg
 661 aaaaagcaga aaattcgtca cttaatctta ttggcaaagc taaaaccaag gaaaatcgcc
 721 aatcaattat caatcctgac tggaactttg aaaaaatggg aataggaggt ctagacaagg
 781 aattttcaga tattttccga cgagcatttg cttcccgagt atttcctcca gagattgtgg
 841 agcagatggg ttgtaaacat gttaaaggca tcctgttata tggacccccа ggttgtggta
 901 agactctctt ggctcgacag attggcaaga tgttgaatgc aagagagccc aaagtggtca
 961 atgggccaga aatccttaac aaatatgtgg gagaatcaga ggctaacatt cgcaaacttt
1021 ttgctgatgc tgaagaggag caaaggaggc ttggtgctaa cagtggtttg cacatcatca
1081 tctttgatga aattgatgcc atctgcaagc agagagggag catggctggt agcacgggag
1141 ttcatgacac tgttgtcaac cagttgctgt ccaaaattga tggcgtggag cagctaaaca
1201 acatcctagt cattggaatg accaatagac cagatctgat agatgaggct cttcttagac
1261 ctggaagact ggaagttaaa atggagatag gcttgccaga tgagaaaggc cgactacaga
1321 ttcttcacat ccacacagca agaatgagag ggcatcagtt actctctgct gatgtagaca
1381 ttaaagaact ggccgtggag accaagaatt tcagtggtgc tgaattggag ggtctggtgc
1441 gagcagccca gtccactgct atgaatagac acataaaggc cagtactaaa gtggaagtgg
1501 acatggagaa agcagaaagc ctgcaagtga cgagaggaga cttccttgct tctttggaga
1561 atgatatcaa accagccttt ggcacaaacc aagaagatta tgcaagttac attatgaacg
1621 gtatcatcaa atggggtgac ccagttactc gagttctaga tgatggggag ctgctggtgc
1681 agcagactaa gaacagtgac cgcacaccat tggtcagcgt gcttctggaa ggccctcctc
1741 acagtgggaa gactgcttta gctgcaaaaa ttgcagagga atccaacttc ccgttcatca
1801 agatctgttc tcctgataaa atgattggct tttctgaaac agccaaatgt caggccatga
1861 agaagatctt tgatgatgcg tacaaatccc agctcagttg tgtggttgtg gatgacattg
1921 agagattgct tgattacgtc cctattggcc ctcgattttc aaatcttgta ttacaggctc
1981 ttctcgtttt actgaaaaag gcacctcctc agggccgcaa gcttcttatc attgggacca
2041 ctagccgcaa agatgtcctt caggagatgg aaatgcttaa cgctttcagc accaccatcc
2101 acgtgcccaa cattgccaca ggagagcagc tgttggaagc tttggagctt ttgggcaact
2161 tcaaggataa ggaacgcacc acaattgcac agcaagtcaa agggaagaag gtctggatag
2221 gaatcaagaa gttactaatg ctgatcgaga tgtccctaca gatggatcct gaataccgtg
2281 tgagaaaatt cttggccctc ttaagagaag aaggagctag ccccccttgat tttgattgaa
```

```
Primer 1 5' TTTATTACATATGATGGCGGGCCGG 3' bp = 25 Tm = 48 GC = 50% NdeI
Primer 2 5' TGGCTCGCATATGTCAATCAAAATC 3' bp = 25 Tm = 40 GC = 50% NdeI
```

Homo sapiens synaptosomal-associated protein, 25kDa (SNAP25), transcript variant 1
NM_003081.3

```
 181 gcccaggcgc ccagccactc cccaccgcta ccatggccga agacgcagac atgcgcaatg
 241 agctggagga gatgcagcga agggctgacc agttggctga tgagtcgctg gaaagcaccc
 301 gtcgtatgct gcaactggtt gaagagagta aagatgctgg tatcaggact ttggttatgt
 361 tggatgaaca aggagaacaa ctcgatcgtg tcgaagaagg catgaaccat atcaaccaag
 421 acatgaagga ggctgagaaa aatttaaaag atttagggaa atgctgtggc cttttcatat
 481 gtccttgtaa caagcttaaa tcaagtgatg cttacaaaaa agcctggggc aataatcagg
 541 acggagtggt ggccagccag cctgctcgtg tagtggacga acgggagcag atggccatca
 601 gtggcggctt catccgcagg gtaacaaatg atgcccgaga aaatgaaatg gatgaaaacc
 661 tagagcaggt gagcggcatc atcgggaacc tccgtcacat ggccctggat atgggcaatg
 721 agatcgatac acagaatcgc cagatcgaca ggatcatgga gaaggctgat tccaacaaaa
 781 ccagaattga tgaggccaac caacgtgcaa caaagatgct gggaagtggt taagtgtgcc
```

```
Primer 1 5' TATGGATCCGATGGCCGAAGAC 3' bp = 22 Tm = 66 GC = 55% BamHI
Primer 2 5' TCCGGATCCTTAACCACTTCC 3' bp = 21 Tm = 64 GC = 52% BamHI
```

a= adenine, c = cytosine, g= guanine, t = tyrosine

3.1.2 DNA amplification by polymerase chain reaction (PCR)

The PCR for amplification of DNA was conducted as described previously by (Sambrook. J., 1989). Isolated DNA from mouse tissue was used as DNA template for the PCR if the PCR product was used for cloning. Whole cell extracts of *E. coli* were used as template for evaluation if the cloning was successful (check-PCR). Thereby, a single *E. coli* colony was resuspended in 15µl sterile water (H2O). After incubation at 30°C for 10 minutes (min), 1µl of the cell suspensions was used as template DNA.

Thermus aquaticus (*Taq*) DNA Polymerase (for check-PCRs) and Platinum *Taq* DNA Polymerase High Fidelity (*Taq*HIFI, Invitrogen) served as thermostable DNA polymerases whereupon *Taq*Hifi ensured the sufficiency of the PCR product for cloning due to its proof reading ability.

	Amount	**Volume**
DNA	20ng plasmid DNA or 200ng genomic DNA as templates	3µl
10xbuffer	1% Triton X-100, 500 mM KCl, 15mM MgCl2, 100mM Tris·HCl, pH 8.8	5µl
dNTPs	10mM each	4µl
Primer_s	20pmol/µl each	1µl
Primer_as	20pmol/µl each	1µl
Taq or *Taq* HIFI		1µl
H20 autoclaved, distilled (dist.)		35µl
Total		50µl

dNTP= deoxynucleoside triphosphate, Tris = tris-(hydroxymethyl)-aminomethane, primer_s= sense-primer, primer_as= anti-sense-primer

PCR-Program with 35 cycles

	Temperature	**Duration**
Initial denaturation	94°C	2min
Denaturation	94°C	30seconds (sec)
Annealing	60 °C, Δ 20°C	30sec
Elongation	68°C	500 basepairs/min for Taq HIFI, 100 basepairs/min for Taq
Final elongation	68°C	2x elongation time
Hold at	4°C	Hold

PCR machine: Mastercycler gradient – Eppendorf

Afterwards, the PCR products were analysed by agarose gel electrophoresis.

The PRC product was sequenced by ElimBio.

3.1.3 Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was used to separate DNA fragments according to their molecular weight.

1% weight per volume (w/v) agarose was dissolved in TAE buffer (40mM Tris-acetate, pH7.5, 20mM Na-acetate, 1mM EDTA) in a microwave. After cooling down to 60°C, ethidium-bromide (0.5µg/ml) for DNA visualization under UV light was added with a pipette and mixed with the pipette top before pouring the mixture into a cuboid mould with a comb for cooling down to room temperature (RT) and solidifying.

The PCR product was mixed in 4:1 ratio with 5x loading dye (30% volume per volume (v/v) glycerol, 0.25% (w/v) bromphenol-blue, 0.25% (w/v) xylencyanol) and loaded into the pockets on the agarose gel. Commercially available molecular weight markers were used. The gels

were run in TAE buffer at 50V or 100V depending on the size of the gel. The negatively charged DNA, hence, moved to the anode and was separated according to its molecular mass. The separated DNA fragments were visualized under UV light (366 nm).

3.1.4 Purification of PCR amplification products

To purify PCR amplification products the "QIAquick Gel Extractoin Kit" was used.

In brief, 3 volumes of buffer QG were added to 1 volume of gel (100mg ~100µl). The mixture was incubated at 50°C for 10min and vortexed in-between. After the gel had dissolved completely, 1 volume of isopropanol was added for DNA precipitation. After mixing, the sample was applied to a QIAquick spin column, which binds DNA, and centrifuged for 1min at 179000g. The supernatant (sup) was decanted and the column was washed with 0.75ml of PE buffer (centrifugation at 17000g for 1min). The centrifugation was repeated after decanting the sup to remove the residual washing buffer. The QIAquick column was placed into a 1.5ml microcentrifuge tube. For elution, 40µl of EB buffer (10mM TrisCl, pH 8.5) were added. The column was rested at RT for 3min and then centrifuged for 1min at 17900g. The concentration of DNA was measured as described below. The DNA was stored at 4°C (PCR product) or at -20°C (plasmids).

3.1.5 Measurement of DNA concentration

In order to measure the DNA concentration the NanoDrop 1000 Spectrophotometer by Thermo Scientific was used. 1µl sample was pipetted onto the end of a sampling arm containing a fibre optic cable. By closing the sampling arm a second fibre optic cable was then brought into contact with the sample causing the liquid to bridge between both fibre optic ends. A spectral measurement was made using a pulsed xenon flash lamp as light source. The instrument is controlled by PC software which saves the measured DNA concentration in a file.

3.1.6 Digestion of DNA with restriction endonucleases

Restriction endonucleases were used for digestion of PCR products and vectors according to the following pipetting scheme.

	For PCR products	**For vectors**
DNA	50µl (1 µg/µl)	20µl (1µg/µl)
Enzyme specific buffer	5µl	5µl
Enzyme	5U/1µg of DNA	5U/1µg of DANN
H2O dist.	Filling the tube up to 50µl	Filling the tube up to 50µl
Total	50µl	50µl

For 2h at 37°C

The buffers were used according to the recommendations of NewEngland BioLabs Inc. After digestion, the DNA fragments were analysed by agarose gel electrophoresis and purified (as described above).

If the plasmid was digested with only one restriction endonuclease, heat inactivated alkaline phosphatase (Invitrogen) was added to the digestion mixture for 20min at 37°C to prevent re-closing of the plasmid by removing 5’ phosphates prior to insert ligation.

3.1.7 Ligation of DNA fragments with ligation enzymes

	Amount
Vector	100-200ng
Insert	6x molar excess of the vector
Buffer (10 fold) (50mM Tris·HCl, 10mM MgCl2, 1mM DTT, 1mM ATP, 5% (w/v) PEG-8000, pH 7.6)	1.5µl
T4-ligase (from bacteriophage T4)	0.5µl
H2O dist.	To fill up to 15µl
Total	15µl

15h at 16°C

The buffer and the T4-ligase were purchased from NewEngland BioLabs Inc.

The ligation product was used for the transformation of competent *E. coli*.

3.1.8 Heat shock transformation

After ligation, the plasmid was transformed into *DH5a E. coli* for plasmid replication.
For protein induction and expression the correct plasmid containing the insert was transformed into *BL21-DE3 E. coli*. For both *E. coli* stems the same transformation procedure was used.

Competent *E. coli* cells were taken from the -80°C freezer and put on ice for 5min in an eppendorf tube (eppi). For testing and replicating plasmids, 100µl of *DH5a* were transformed using the ligation mix. For protein expression 50µl of *BL21-DE3* were used. The tubes were kept on ice and 50ng of DNA was added for incubation on ice for 30min. Afterwards, the tubes with *E. coli* and DNA were heat-shocked for 45sec at 42°C to allow the plasmid to enter the bacteria and then put on ice for 2min to reduce damage to the *E. coli* cells. 1ml of lysogeny broth (LB) was added followed

by incubation for 1.5h at 37°C at 220rounds per minute (rpm). 100µl of the culture were spread on a LB-ampicillin (LB-amp) plate which was inoculated over night. Single colonies were picked after 12-16h.

The constitution of the used media was as follows.

For liquid cultures

LB-medium: 0.5% (w/v) yeast extract, 1% (w/v) bacto-tryptone, 1% (w/v) NaCl.

LB-Amp medium: LB-medium supplemented with ampicillin (100µg/ml)

For solid media (plates)

LB-medium supplemented with 2% (w/v) bacto-agar, autoclavation at 120ºC for 20min.

After the plates had cooled down to at least 50°C, ampicillin was added resulting in an ampicillin concentration of 100µg/ml.

3.1.9 Miniprep for DNA Isolation from *Escherichis coli* (*E. coli*)

For isolation of plasmid DNA from *E. coli* the QIAprep Spin Miniprep Kit (250) was used.

Briefly, a single *E. coli* colony was incubated in 5ml LB-amp medium over night at 37°C at 200rpm. The cells were harvested by centrifugation (6800g, 5min, RT) and then resuspended in 250µl of Buffer1. To lyse the cells 250µl of Cell Lysis Solution (Buffer2) were added and the tubes were inverted 8 times and then incubated for 3min at RT. After neutralisation with 350µl of Neutralisation Solution (Buffer3), the tubes were inverted several times again, followed by centrifugation (17900g, 10min, RT). The supernatant was transferred into QIAprep spin column by pipetting. The column was spun for 1min at 13000rpm for the lysate to pass trough the clearing membrane and the DNA to bind to the binding membrane. The column was washed with 750µl of PE Buffer by centrifuging the column for 1min at 13000rpm. After decanting the buffer, the column was spun again

to dry the binding membrane. The QIAsprep spin column was placed into a clean eppi. 40µl of nuclease-free water or Elution Buffer were added to the DNA binding membrane to elute the DNA. The eppi was rested at RT for 2min and then centrifuged at 13000rpm for 1min. Afterwards, the filtrate was stored at -20°C.

3.1.10 Control of orientation of the insert

If an insert is digested with the same restriction endonuclease at both ends, orientation of the insert within the plasmid needs to be checked. Therefore, each plasmid containing the insert was digested with a restriction endonuclease that only cuts once in the insert and once in the vector (procedure see above). Depending on the orientation of the insert, the obtained fragment can have two different sizes. Preferably, an enzyme was used that cuts close to one end of the insert to obtain a great difference in size between the two possible fragments. The digestion mixture was analyzed on an agarose gel to determine the size of the fragment as described above.

Insert	**Restriction enzymes**	**Fragment sizes for right orientation (base pairs)**
SNAP23 SNAP25	SacI, ApaI HindIII	1138 and 5203 631 and 5698
Stx17 NSF	BGlII XhoI	983 and 5630 650 and 7292
Alpha-SNAP STAR	SphI XhoI	434 and 6595 571 and 5991

3.1.11 Glycerol stocks

5ml of LB-amp were inoculated with a single *E. coli* colony which had grown to log-phase. 800µl of this culture were given into a sterile tube containing 200µl of autoclaved 80% glycerol. The sample was mixed

briefly by vortexing and then stored at -80 C. To grow cells from the stock, the surface of the glycerol stock was carefully touched with a sterile pipette tip, which was then given into 3ml LB-amp media.

3.2 Induction, expression and purification of recombinant SNARE proteins

3.2.1 Induction of recombinant protein expression in *E. coli* and harvesting the cells (*Bl21DE3*)

SNAP25, SNAP23, α-SNAP, Stx17

A 3ml LB-amp culture derived from glycerol stocks of the recombinant SNARE proteins was grown over night (37°C, 220rpm). In the morning, 1ml of this culture was diluted with 100ml of LB-amp and cultured at 37°C, 220rmp until an optical density at 600nm (OD600) of 0.6 – 0.75 was reached. 1ml of the culture was centrifuged for 3min at 4000g and the pellet was resuspended in Laemmli buffer (200µl /OD; 60mM Tris·HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromphenol-blue) and heated for 5min at 95°C to serve as a negative control before induction. Then the protein expression was induced with 1ml 0.1M isopropyl-β-D-thiogalactopyranoside (IPTG)/100ml culture for 2.5 - 3h at 37°C. IPTG binds to the lac-repressor leading to a conformational change and hence the disinhibition of the operator of the lac-operon. Thus, the inserted SNAP/Stx-sequences downstream of the lac promoter is transcribed and translated.

1ml of the induced culture was taken as a positive control after 1h, 2h and 3h and treated like the negative control (see above). The rest of the cells was spun down at 2500g for 5min at 4°C and stored at -20°C.

The negative and positive controls were separated by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE, see 3.2.3) according to their molecular weights. Then they were coloured with commassie blue (see 3.2.4) to check if induction was successful and which induction time shows the highest yield without degradation of the recombinant proteins.

For STAR and NSF the same protocol was applied with some changes to ensure that STAR is not degraded and that NSF is in the soluble fraction despite its size of 82kDa.

STAR

For StAR, induction was started at OD600 of 0.6 (not higher) for 2.5h. Longer incubation can result in increased degradation of the STAR protein.

NSF

For NSF, a colony from the glycerol stock was grown in 3ml LB-amp for 8h. Then the 3ml culture was diluted with 100ml LB-amp and grown until an OD600 of 0.6 was reached. The culture was induced with 1ml 0.1M IPTG/100ml culture over night at 25°C. Higher induction temperature results in the recombinant protein being locked up in inclusion bodies.

3.2.2 Purification of recombinant proteins expressed in *E. coli* (*Bl21DE3*)

After the recombinant plasmid had been transferred into *E. coli* by heat shock transformation and the expression of the recombinant protein had been induced by IPTG, the cells were resuspended in 5ml of lysis buffer (10mM Tris pH7.5, 130mM NaCl, 1% Triton X100 (for NSF 2%), Protease Inhibitor 1:1000, Phosphatase Inhibitor 1:100). Incubation at 4°C in the overhead shaker for 60min followed. Every 10min the cells were disrupted

by sonication (level 4, 30%, in ice water, for 10sec). The lysed cells were centrifuged at 20,000g for 30min at 4°C.

The soluble recombinant proteins were purified using nickel-nitrilo triacetic acid (Ni-NTA) Agarose beads. The beads were washed 4 times with 6xHis washing buffer (50mM sodium phosphate, 300mM NaCl, 10% glycerol, pH8). While washing the beads were kept at 4°C and centrifuged at 500g for 4min. For 750µl of beads 750µl of 6xHis washing buffer were used for each wash. Then the beads were resuspended in the same volume of lysis buffer. 150-200µl of beads (in lysis buffer) were added to 1ml of sup each and incubated over night at 4°C in the overhead shaker. The His-tag binds the to the nickel ion so that the recombinant protein is connected to the beads.

The next morning the beads were washed 2 times with 6xHis washing buffer (incubation on the overhead shaker for 5min at 4°C and centrifugation at 500g for 4min at 4°C) and 2 times with 6xHis washing buffer containing 20mM imidazole.

Then the proteins were eluted from the beads by incubating consecutively with 0.5ml of 6xHis washing buffer containing 100mM 200mM /300mM /500mM imidazole for 5min each (for NSF 200mM imidazole incubation for 7min) at RT on a rocking platform. The beads were centrifuged down at 500g for 4min and the elution fractions (1 to 4) were collected in eppis, alliquoted and stored at -20°C. The elution fractions were tested for their content of recombinant protein and purity by SDS-PAGE and commassie blue staining (see below).

3.2.3 Sodium dodecyl sulfate -Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separates the proteins under denaturing conditions according to their molecular weights in a one-dimensional way. Concentration of acrylamide and bisacrylamide varied according to the moleculare sizes of the loaded proteins. However, mainly 14% gels were used, which were

self-poured (for exact compositions of the gels see table below). The volume of the loaded protein varied between 5 and 15µl which equals amounts of 5-30µg.

The commercially available proteins mixture SeeBlue® Pre-Stained Standard (Invitrogen) with 4 - 250kDa served as marker. For electrophoresis, 35mA for 60min was used for gels of approximately 14cm x 9cm x 0.1cm. Otherwise 125V were applied for precast gels 9cm x 10cm (PAGEr™ Gold Gels, 9 cm x 10 cm, 4-12%, Lonza) according to the protocol recommendations by Lonza.

	Separating Gel							**Stacking gel**
%Acrylamide	20	10	12.5	14	15	16	17.5	5
30% (w/v) Acrylamide 0.33% (w/v) Bisacrylamide (ml)	6.7	5.7	6.5	7.9	8.5	9.0	9.7	0.83
1M Tris/HCl, pH 8.8 (ml)	3.7	6.5	6.5	6.5	6.5	6.5	6.5	
0.6M Tris/HCl, pH 6.8(ml)								0.5
H2O (ml)	1.6	4.5	3.4	3.0	1.74	1.2	0.5	3.6
10% (w/v) SDS (µl)	100	167	167	167	167	167	167	50
TEMED (µl)	25	10	10	6	6	6	6	5
10%(w/v) APS (µl)	50	100	100	84	84	84	84	25
Total (ml)	10	17	17	17	17	17	17	5

TEMED = N,N,N',N'-Tetramethylethylendiamin, APS = Ammonium persulfate

Electrophoresis buffer: 50mM Tris base, 384mM glycine, 0.1% (w/v) SDS, pH 8.3 without adjustment.

3.2.4 Staining of SDS -polyacrylamide-gels with coomassie blue

For staining SDS-Polyacrylamid-gels with coomassie blue (1g/l coomassie R250, 10% glacial acetic acid, 40% methanol, 50% distilled H2O), the gel was rinsed with distilled water three times. Then, it was put into a plastic box and fully covered with coomassie blue for 30min. Afterwards, the gel was fully covered with destainer (20% methanol, 10% glacial acetic acid, 70% distilled H2O). The gel was heated in the microwave for 15sec and then destained over night with a small paper towel positioned next to the gel to absorb the stain.

3.2.5 Determination of protein concentration via BCA Pierce Protein Assay Kit

For detection and quantification of total protein the Thermo Scientific Pierce BCA Protein Assay was used. In this procedure, Cu2+ is reduced to Cu1+ by proteins in an alkaline environment. Chelatation of BCA with protein-bound Cu1+ leads to a purple colour change. This BCA-Cu-complex exhibits a strong linear absorbance with increasing protein concentrations.

The BSA Standard Curve covered concentrations from 0µg/ml to 2000µg/ml of bovine serum albumin (BSA). The working reagent was prepared according to the kit's protocol with 50 parts of BCA Reagent A and 1 part of BCA Reagent B. The microplate procedure was performed with 25µl of each standard and 5µl and 2µl of each sample. 200µl of the Working Reagent were added. The plate was mixed on a plate shaker for 30sec. Then the plate was covered and incubated at 37°C for 30min. After cooling the plate to RT, the absorbance was measured at 562nm on a plate reader.

3.3 Animal model

Wild-type Spragule Dawley rats with a weight of at least 180g, aged between 3 months and 24 months were used. Rats were maintained in the animal facility at the Veterans Affairs (VA) Palo Alto Health Care System on a 12-h light-dark cycle. All procedures were in accordance with institution guidelines and approved by the institutional animal care and use committee of VA Palo Alto Health Care System. Animal experimentation was conducted in accordance with accepted standards of humane animal care. In brief, rats were killed by decapitation or CO2 intoxication and the adrenal glands and/or ovaries were aseptically removed and put into ice-cold PBS (NaCl 137mM, KCl 2.7mM Na2HPO4*2H2O 10mM, KH2PO4 2.0 MM pH 7.4) and dissected free of fat for isolation of mitochondria.

3.4 Isolation of mitochondria from rat adrenals and ovaries

After sacrificing the rats, adrenals and/or ovaries were removed and put into PBS. From this step onwards all actions were performed on ice. Fat was removed from the organs and the cleaned adrenals/ovaries were cut into very little pieces with scissors. The pieces were put into the glass tube of a dounce homogenizer. For 6 adrenals or ovaries 2ml of sucrose-HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (10mM HEPES, 250mM sucrose, H2O, 10µl/ml protease inhibitor and 10µl/ml phosphatase inhibitor) were used. The mixture was homogenized on ice by douncing it about 15 times until all the lumps had vanished. The homogenized organs were decanted into an eppi and centrifuged at 700g, 4°C for 10min to remove the bulky fragments. The supernatant was then centrifuged at 12000g for 15min at 4°C. The pellet contained

mitochondria and was resuspended in about 1ml of Sucrose-HEPES buffer and washed once while the sup (=cytosol) was stored at 4°C. Then the washed mitochondria were resuspended in a small volume of Sucrose-HEPES buffer (about 400µl for 6 adrenals/ovaries) and kept on ice. The concentration of mitochondria was measured using the Pierce BCA Protein Assay Kit.

3.5 Reconstitution assay and preparation of the lipid emulsion (LE)

The reconstitution assay was performed in glass tubes. For each reaction the total volume was 100µl. The amount of Sucrose-HEPES buffer was calculated in advance and given into the tube first. The other components were added in the same order as listed below.

1) succinate with a final concentration of 5mM
2) 2µl of lipid emlusion if needed
3) 1µg of each recombinant protein needed
4) GTP or guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) with a total amount of 10µg/reaction if needed
5) Cytosol with a total amount of 100µg of protein/reaction if needed
6) 200µg of purified mitochondria

After mixing the reagents by gently shaking the tube, the assay was left at RT for 20min. Then the mitochondria were pelleted down by centrifugation at 1500g for 15min at 4°C. The supernatant was transferred into a fresh glass tube using a pipette and stored at -20C.

The lipid emulsion was prepared one day before it was used for reconstitution assays. 2.7mg cholesterol (Invitrogen) and 307µl

phosphatidylcholine were given into a glass tube. The mixture was dried under a constant flow of gaseous nitrogen to evaporate the chloroform in which phosphatidylcholine was suspended. Afterwards, the dried lipids were resuspended in 500µl KPO4-taurocholic acid buffer (0.02mM KPO4, 0.01mM taurocholic acid, H2O) and sonicated on ice 4 times, 10 beats each time with a 30sec break in-between. Within this buffer, taurocholic acid served as emulsifier. The lipid droplet containing emulsion was stored at 4°C over night.

3.6 Quantitative pregnenolone ELISA

The ALPCO IMMUNOASSAY Pregnenolone ELISA Kit was used.
In this enzyme-linked immunosorbent assay, rabbit anti-pregnenolone antibodies are bound to the 96-well plate. The pregnenolone-containing sample (or standard or control) is given into the well together with a buffer containing a pregnenolone-biotin conjugate so that pregnenolone and conjugate compete for the binding position at the well-bound antibody. After washing, a streptavidin horseradish peroxidase (HRP) conjugate is added, which binds to the biotin-conjugate. Tetramethylbenzidine serves as substrate for the HRP and is oxidized with hydrogen peroxide serving as oxidising agent leading to a colour change. Sulfuric acid is used as stop solution. Hence, colour intensity is indirectly proportional to the pregnenolone concentration in the sample (or standard or control).
The Assay procedure instructions were followed carefully (http://www.alpco.com/pdfs/11/11-PREHU-E01.pdf). Briefly, after preparing all the working solutions, 50µl of each standard, control and sample were given into a well. The controls and the standards were performed in duplicate. 100µl of biotin conjugate working solution were added and the plate was incubated on a shaker (200rpm) for 1h at RT. After washing the wells 5 times with 300µl of diluted wash buffer, the plate was tapped firmly against absorbent paper until all the wells were dry. 150µl of HRP conjugate working solution were pipetted into each well with a multichannel pipette. The plate was incubated on a shaker (200rpm) for 30min at RT. After the same washing procedure as before, 150µl of TMB substrate were given into each well at timed intervals (10sec). The plate was incubated on a shaker (200rpm) at RT for 14min. Then 50µl of stop solution were pipetted into each well at the same timed intervals as before (10sec). The plate was read on a microwell plate reader at 450nm within 5min after addition of stop solution. The standard curve is a semi-log curve.

3.7 Statistics applied

The reconstitution assay was performed with at least three replicates per treatment. All results within the measuring range of the pregnenolone ELISA (0-25ng/ml, sensitivity 0.054 ng/ml) were taken into account. For calculating mean, standard deviation (SD) and confidence intervals (CI) as well as for displaying the results as graphs, the program GraphPad Prism 6 was used. Furthermore, GraphPad Prism 6 was utilized to test for statistical significance between different treatments (tests see below) and to visualize the distribution of variances in a graph. Logarithms of pregnenolone concentration and variances were calculated with Microsoft Excel.

All sets of data showing homoscedasticity were analysed by one-way analysis of variances (ANOVA). To avoid type I errors, for multiple comparisons the unpaired parametric Tukey's multiple comparison test or Sidak's multiple comparison test were utilized. For data sets displaying heteroscedasticity Dunn's multiple comparison test was applied. p-values of <0.05 were considered significant and confidence intervals are shown in square brackets.

4 Results

4.1 Induction, purification and storage of SNAREs and StAR

In order to assess the effect of SNAREs on cholesterol transport via lipid droplets to mitochondria in an *in-vitro* reconstitution assay, recombinant SNAREs and StAR needed to be cloned. The recombinant proteins had to fulfil certain requirements to be functional in the reconstitution assay: 1) They need to be inducible. 2) After purification they have to be in a solute state to be functional for the *in-vitro* assay. 3) The proteins must stay intact and must not be degraded during expression or purification. Before the recombinant SNAREs were used for reconstitution assays, fulfilment of these requirements was tested.

4.1.1 Induction of SNAREs

All recombinant proteins have been expressed in *E. coli*. To test inducibility and rate of induction of recombinant SNAREs and StAR, the proteins expressed by each *E. coli* culture have been separated by SDS-PAGE and visualized by coomassie-blue staining 0, 1, 2 and 3 hours after induction (0, 1, 10, 25 hours after induction for NSF). All recombinant proteins show a prominent band increasing with time of induction at the height predicted by their molecular mass. For NSF the band appears after 10 hours of induction and is smaller than the bands of the other recombinant proteins.

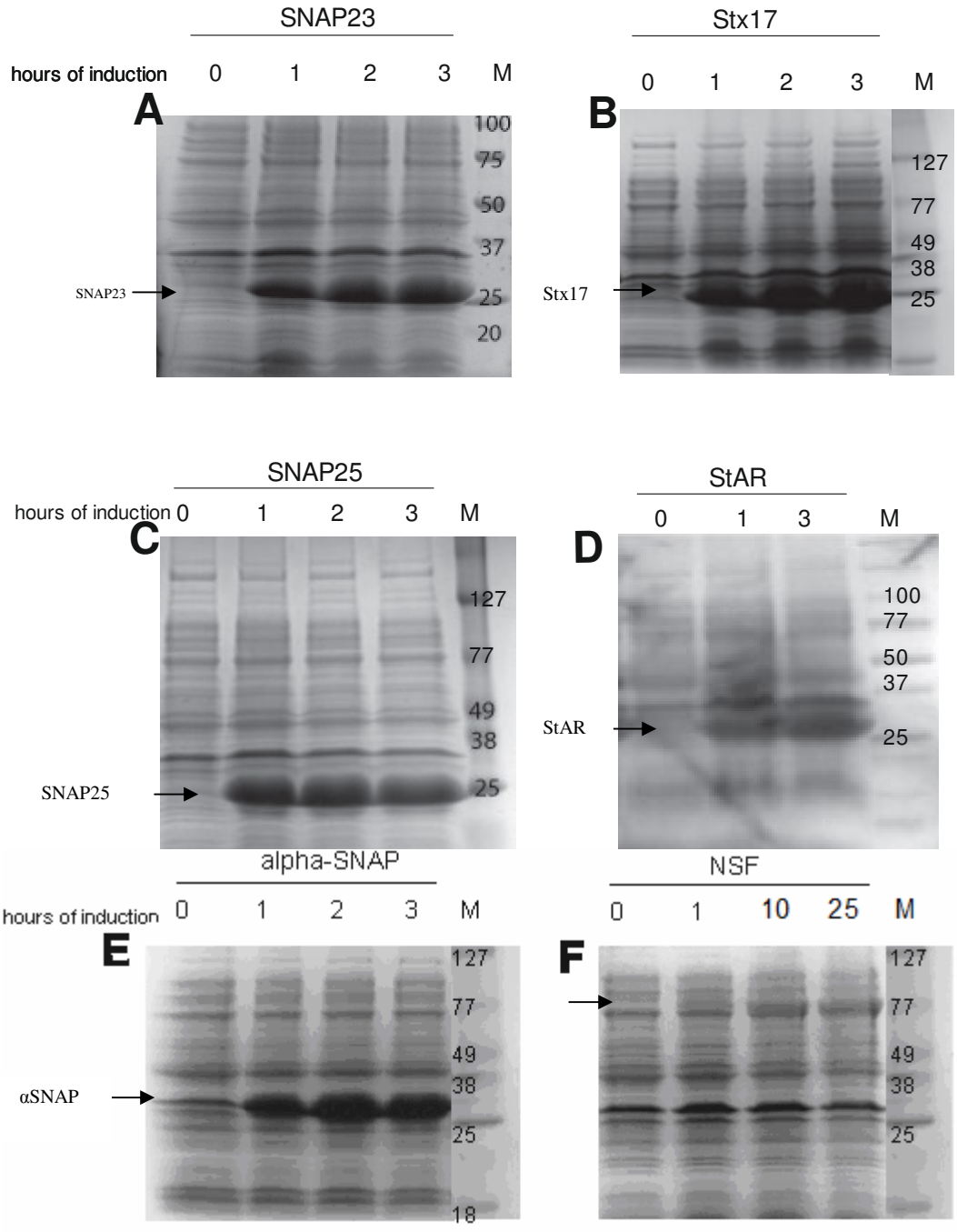


Figure 3 Induction of recombinant SNAREs and StAR
A Coomassie-blue stained sodium dodecyl sulfate -polyacrylamide gel, induction of SNAP23 (23kDA) after 0, 1, 2 and 3 hours ;**B** Coomassie-blue stained sodium dodecyl sulfate -polyacrylamide gel, induction of Stx17 (17kDa) after 0, 1, 2 and 3 hours; **C** Coomassie-blue stained sodium dodecyl sulfate -polyacrylamide gel, induction of SNAP25 (25kDa) after 0, 1, 2 and 3 hours; **D** Coomassie-blue stained sodium dodecyl sulfate -polyacrylamide gel, induction of StAR (37kDa) after 0, 1 and 3 hours; **E** Coomassie-blue stained sodium dodecyl sulfate -polyacrylamide gel, induction of α-SNAP (35kDa) after 0, 1, 2 and 3 hours; **F** Coomassie-blue stained sodium dodecyl sulfate -polyacrylamide gel, induction of NSF (82 kDa) after 0, 1, 10 and 25 hours

4.1.2 Purification and storage of SNAREs and StAR

After induction, the recombinant proteins were purified and collected in elution fractions containing different concentrations of imidazole. The proteins of the elution fractions were separated by SDS-PAGE and visualized by coomassie-blue staining. For each recombinant protein, elution fraction 2 shows a bigger band than elution fraction 1 at the height of the molecular weight of the protein.

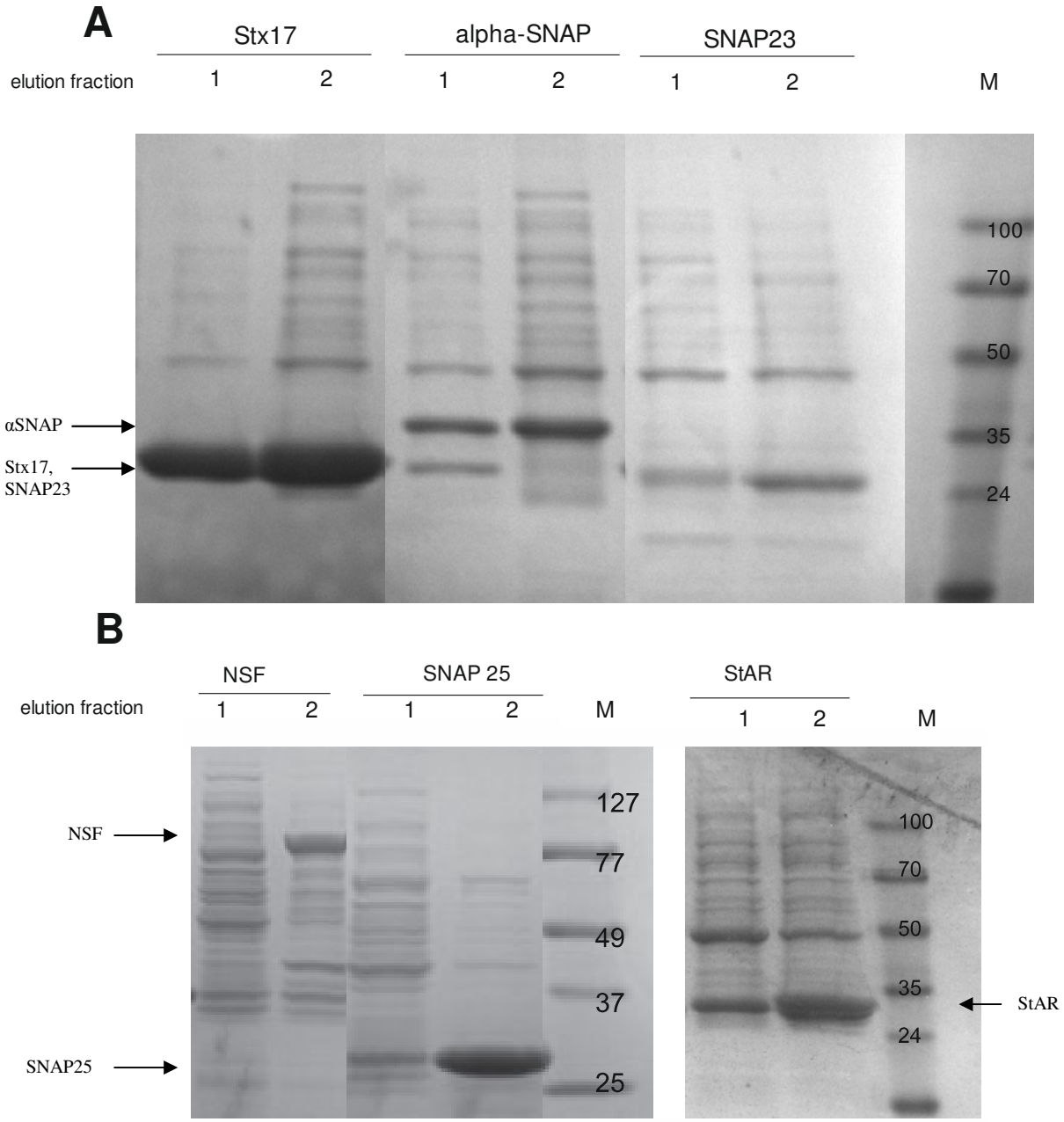


Figure 4 Elution fractions of purified SNAREs and StAR
A Elution fraction 1 (100mM imidazole) and 2 (200mM imidazole) of Stx17 (17kDa), alpha-SNAP (35kDa) and SNAP23 (23kDa); **B** Elution fraction 1 (100mM imidazole) and 2 (200mM imidazole) of NSF (82kDa) and SNAP25 (25kDa) and StAR (37kDa)

4.2 Results of the *in-vitro* reconstitution assay

An *in-vitro* reconstitution assay was used to model cholesterol transport to mitochondria via LDs and to investigate the effect of SNAREs on this process. In the mitochondrial matrix, cholesterol was converted to pregnenonlone, of which the concentration was measured by ELISA. Results were summoned in 8 groups for comparison of pregnenolone concentrations within these sets. The basic constituents of the reconstitution assay - amount of mitochondria, sucrose-HEPES-buffer and succinate– were kept constant. Mitochondria were isolated from rat adrenals and ovaries to test the effect of SNAREs in both organs (group 1 and 2 respectively). Furthermore, the combination of the added recombinant SNAREs was altered to find out which arrangement has the biggest effect on the pregnenolone concentration (group 6). It was also investigated if GTP, GTPγS and cytosol from rat adrenal cells (groups 3-5) have an effect on pregnenolone concentrations. BSA and imidazole served as negative controls (groups 7 and 8 respectively).

Group	**Mitochondria**	**Treatments**
1	ovarian	None
		LE
		LE+StAR
		LE+StAR+SNAREs
2	adrenal	None
		LE
		LE+StAR
		LE+StAR+SNAREs
3	adrenal	LE
		LE+cytosol
		LE+StAR
		LE+StAR+cytosol
		LE+StAR+SNAREs
		LE+StAR+SNAREs+cytosol

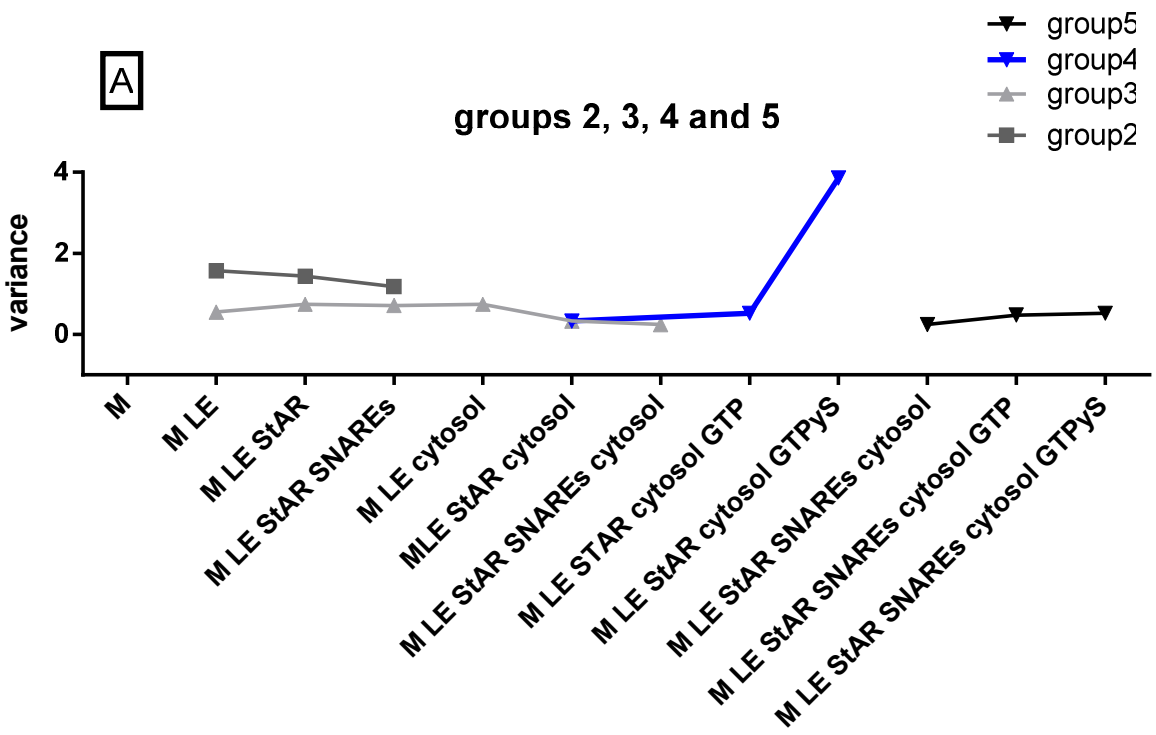
4	adrenal	LE+StAR+cytosol
		LE+StAR+cytosol+GTP
		LE+StAR+cytosol+GTPγS
5	adrenal	LE+StAR+SNAREs+cytosol
		LE+StAR+SNAREs+cytosol+GTP
		LE+StAR+SNAREs+cytosol+GTPγS
6	adrenal	None
		LE
		LE+StAR
		LE+StAR+SNAREs
		LE+StAR+SNAP23
		LE+StAR+SNAP25
		LE+StAR+Stx17
		LE+StAR+NSF+α-SNAP+SNAP25
		LE+StAR+ NSF+α-SNAP+SNAP23
		LE+StAR+ NSF+α-SNAP
		LE+StAR+SNAP25+SNAP23
		LE+StAR+ NSF+α-SNAP+SNAP23+SNAP25
7	adrenal	LE
		LE+StAR
		LE+StAR+SNAREs
		LE+StAR+BSA
8	adrenal	LE+StAR
		LE+StAR+SNAREs
		LE+StAR+60mM imidazole

LE=lipid emulsion, StAR=recombinant StAR, SNAREs= SNAP25, SNAP23, NSF, α-SNAP, Stx17, BSA=bovine serum albumin

4.2.1 Test for homoscedasticity

In order to pick a correct statistical test for evaluating significance between pregnenonlone concentrations of different treatments, the results were scanned for homoscedasticity or heteroscedasticity, respectively.

Data were summoned in groups as described above. The distribution of variances for different treatments is displayed below. For group 1, 6, 7 and 8 variances of the pregnenolone concentrations (ng/ml) are shown. Heterogeneous conditions e.g. concerning age (3 to 24 months old rats) and killing methods (decapitation vs. CO2 intoxication) or degree of purification of recombinant proteins lead to an increased range of variances. Hence, for the affected groups (2, 3, 4 and 5) variances of the natural logarithm (ln) of pregnenolone concentrations are demonstrated in order to preserve homoscedasticity. Therefore, the natural logarithm of the respective pregnenolone concentrations was also used for further statistical testing. Except from group 4, all groups show a homoscedastic distribution of variances.



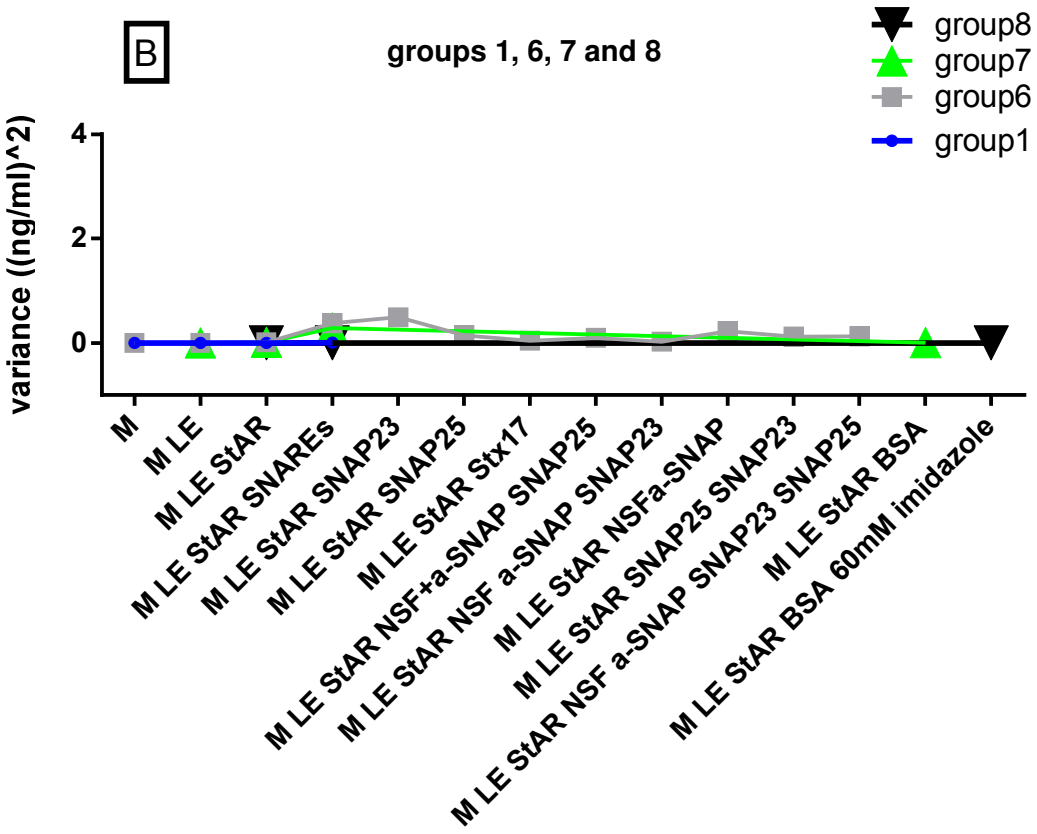


Figure 5 Distribution of variances
A Variances of groups 2, 3, 4 and 5, variance of ln(pregnenolone concentration); **B** variances of groups 1, 6, 7 and 9, variance of pregnenolone concentration ((ng/ml)^2)

4.2.2 Reconstitution assay with mitochondria isolated from rat ovaries (group 1)

Mitochondria were isolated from rat ovaries (see 3.4). The pregnenolone concentration of the reconstitution assay was measured by ELISA under four conditions:

1) mitochondria (M) only,

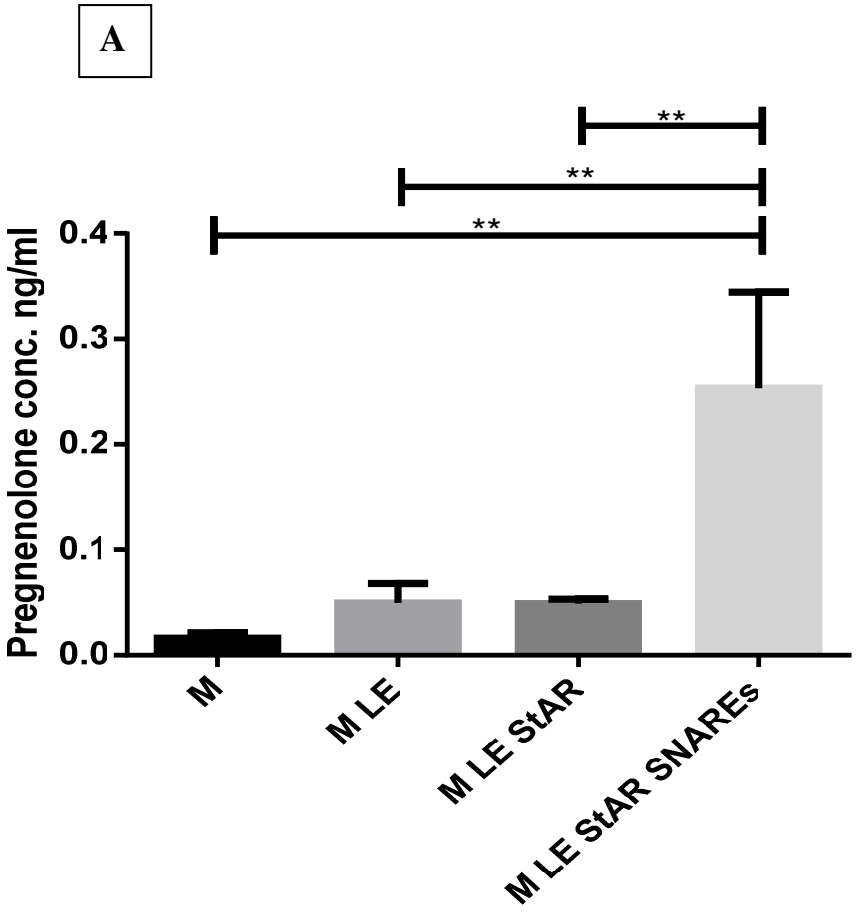
2) addition of LE,

3) addition of LE and recombinant StAR,

4) addition of LE, recombinant StAR and all cloned recombinant SNAREs (SNAP23, SNAP25, Stx17, NSF and α-SNAP)).

The pregnenolone concentration of the fourth condition (M+LE+StAR+SNAREs) was significantly higher in comparison to the three other treatments ($p<0.01$). The mean differences were:

- M vs. M+LE+StAR+SNAREs -0.2370 [CI-0.3596 to -0.1144], p<0.01
- M+LE vs. M+LE+StAR+SNAREs -0.2037 [CI -0.3262 to -0.08108] , p<0.01
- M+LE+StAR vs. M+LE+StAR+SNAREs -0.2043 [CI -0.3269 to -0.08175], p<0.01



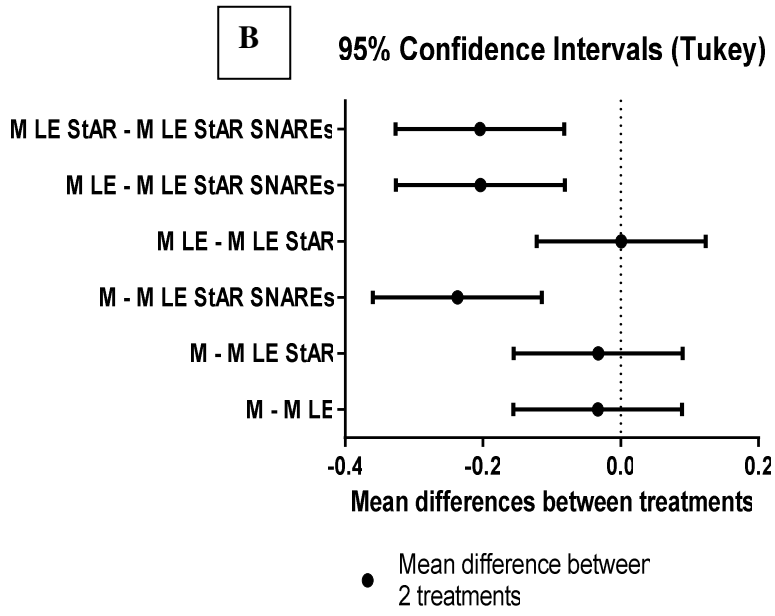


Figure 6 A Pregnenolone concentration (ng/ml) of the reconstitution assay of group 1, mitochondria (M) were treated with LE, LE+StAR and LE+StAR+SNAREs, values are shown as mean +/- SD for three independent experiments, $p<0.01$ (**) Tukey's multiple comparison test **B** Mean difference between two treatments of group 1 with 95% CI, Tukey's multiple comparisons test

4.2.3 Reconstitution assay with mitochondria isolated from rat adrenals (group 2)

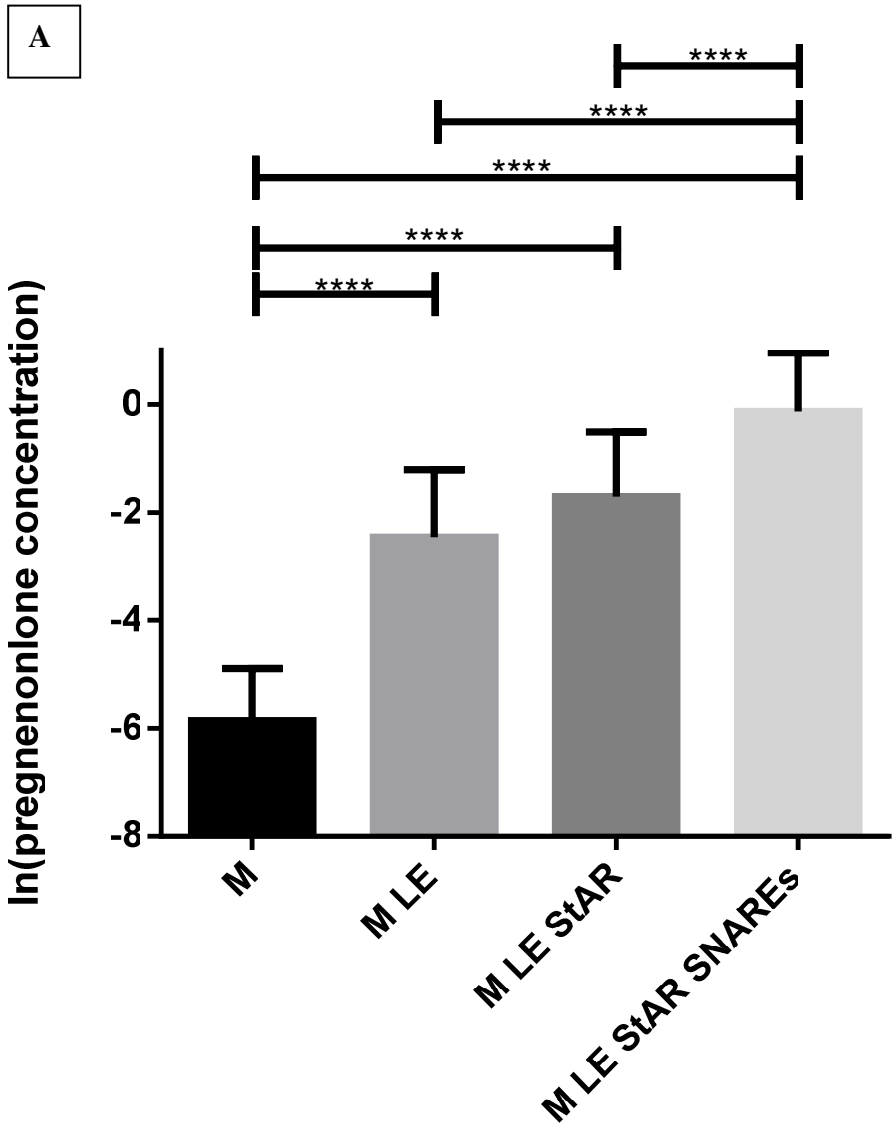
Mitochondria were isolated from rat adrenals (see 3.4). The pregnenolone concentration of the reconstitution assay was measured by ELISA under the same conditions as before (4.2.2):

1) mitochondria only,
2) addition of LE,
3) addition of LE and recombinant StAR,
4) addition of LE, recombinant StAR and SNAREs (SNAP23, SNAP25, Stx17, NSF and α-SNAP).

A significant difference was found between ln (pregnenolone concentrations) of

- M vs. M+LE (mean difference -3.397 [CI -4.684 to -2.110] $p<0.0001$)
- M vs. M+LE+StAR (mean difference -4.145 [CI -5.395 to -2.894] $p<0.0001$),

- M vs. M+LE+StAR+SNAREs (mean difference -5.731 [CI -6.951 to -4.512] p<0.0001),
- M+LE vs. M+LE+StAR+SNAREs (mean difference -2.335 [CI 3.256 to -1.413] p<0.0001)
- M+LE+StAR vs. M+LE+StAR+SNAREs (mean difference -1.587 [CI -2.457 to -0.7168] p<0.0001)



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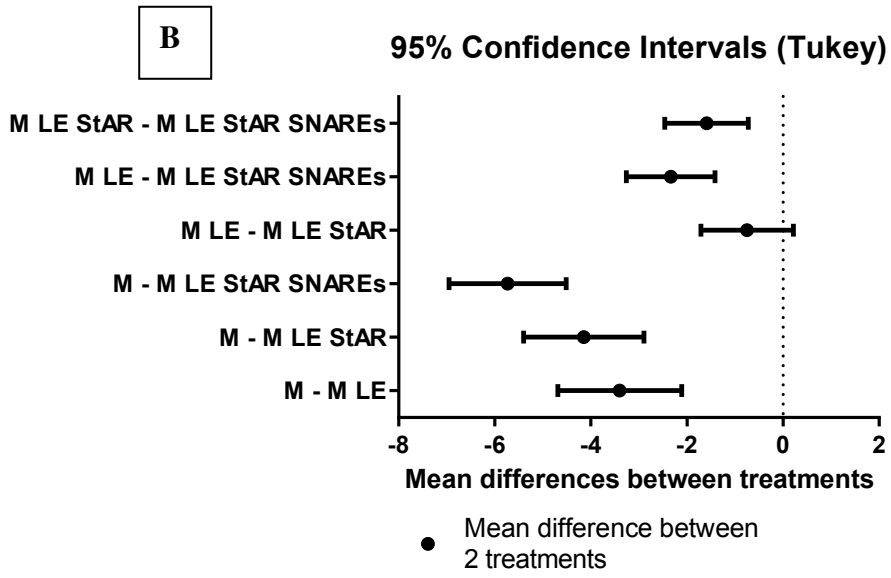


Figure 7 A Natural logarithm (ln) of pregnenolone concentrations (ng/ml) of reconstitution assays of group 2, M were treated with LE, LE+StAR and LE+StAR+SNAREs, values are shown as mean +/- SD for twenty-four independent experiments, $p<0.0001$ (****), Tukey's multiple comparison test **B** Mean difference between two treatments of group 2with 95% CI, Tukey's multiple comparisons test

In summary, pregnenolone concentrations in both assays – with adrenal and ovarian mitochondria – show the same pattern of significantly higher values when SNAREs are added (46fold increase for adrenals). Moreover, with adrenal mitochondria a significant increase of the pregnenolone concentration was found under addition of LE and LE+StAR to mitochondria and significance levels are higher for adrenals than for ovaries ($p<0.0001$ and $p<0.01$ respectively). Furthermore, rat adrenals are bigger than rat ovaries and easier to homogenize for mitochondria isolation, which results in a higher yield of mitochondria and need for less animals. Therefore, the following experiments will be performed with rat adrenals only.

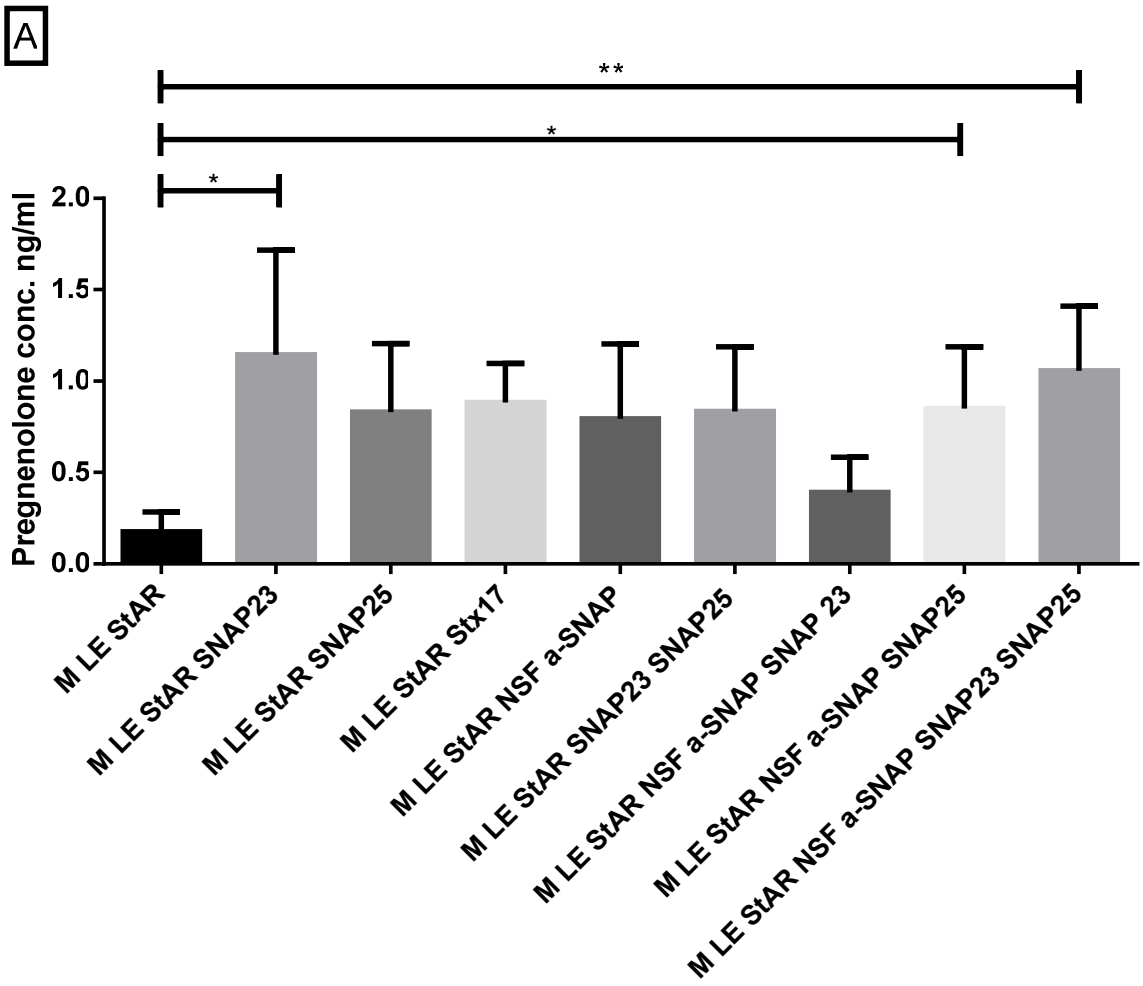
4.2.4 Effect of different combinations of SNAREs on pregnenolone concentrations of reconstitution assays (group 6)

It was shown that all SNAREs together (SNAP23, SNAP25, Stx17, NSF and α-SNAP) lead to an increase in pregnenolone concentration in reconstitution assays. Different combinations of SNAREs were tested to

find out which single SNARE or which combination accounts for the rise in pregnonlone concentration.

A significant difference of pregnenolone concentrations was found between:

- M+LE+StAR vs. M+LE+StAR+SNAP23 ($p<0.01$)
- M+LE+StAR vs. M+LE+StAR+NSF+α-SNAP+SNAP25 ($p<0.01$)
- M+LE+StAR vs. M+LE+StAR+NSF+α-SNAP+SNAP25+SNAP23 ($p<0.01$)
- M+LE+StAR vs. M+LE+StAR+SNAREs ($p<0.0001$)
- M+LE+StAR+SNAREs vs. all other combinations ($p<0.05$, $p<0.01$, $p<0.0001$) except from
- M+LE+StAR+SNAREs vs. M+LE+StAR+NSF+α-SNAP+SNAP25 +SNAP23 ($p>0.05$)



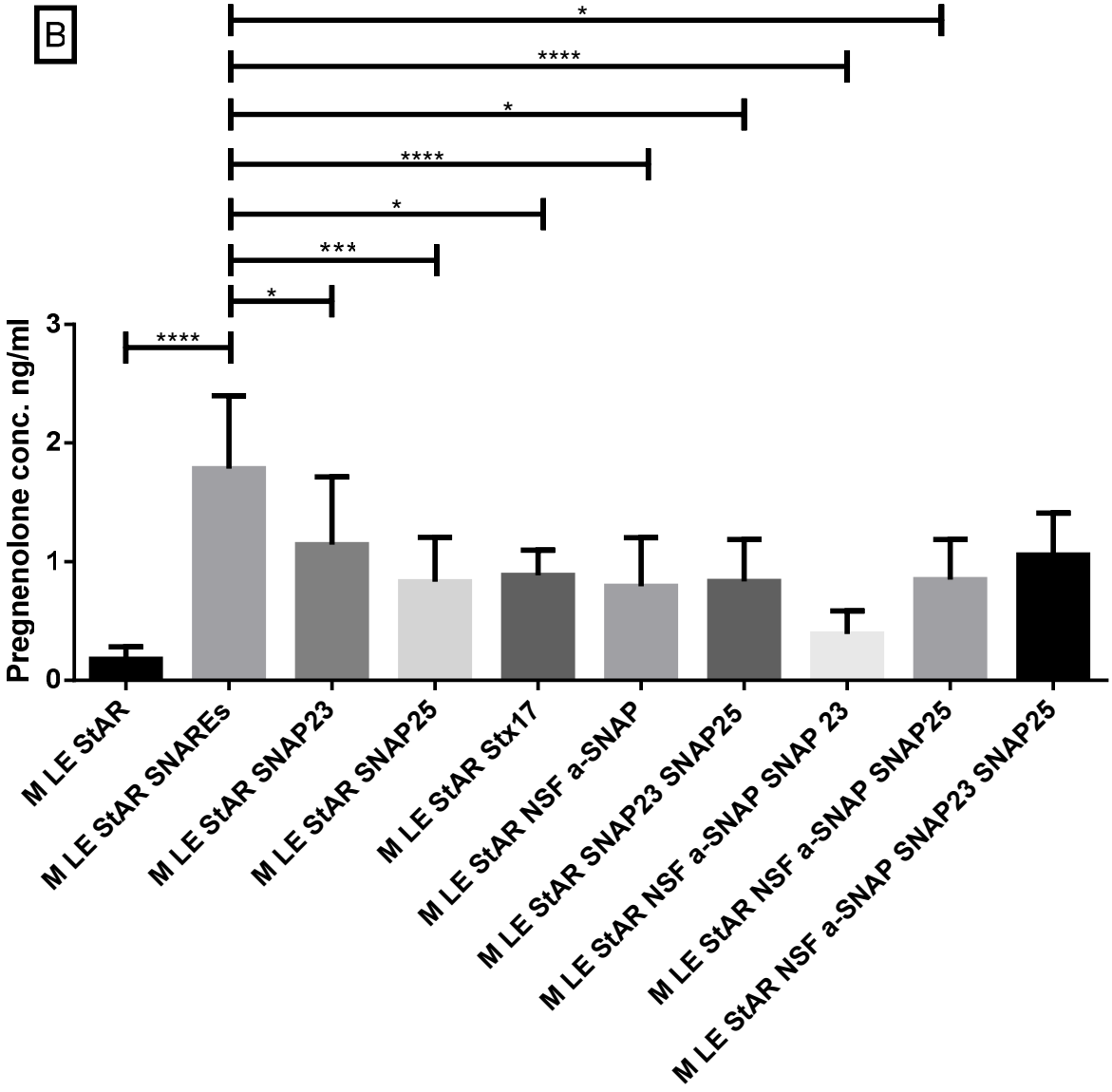
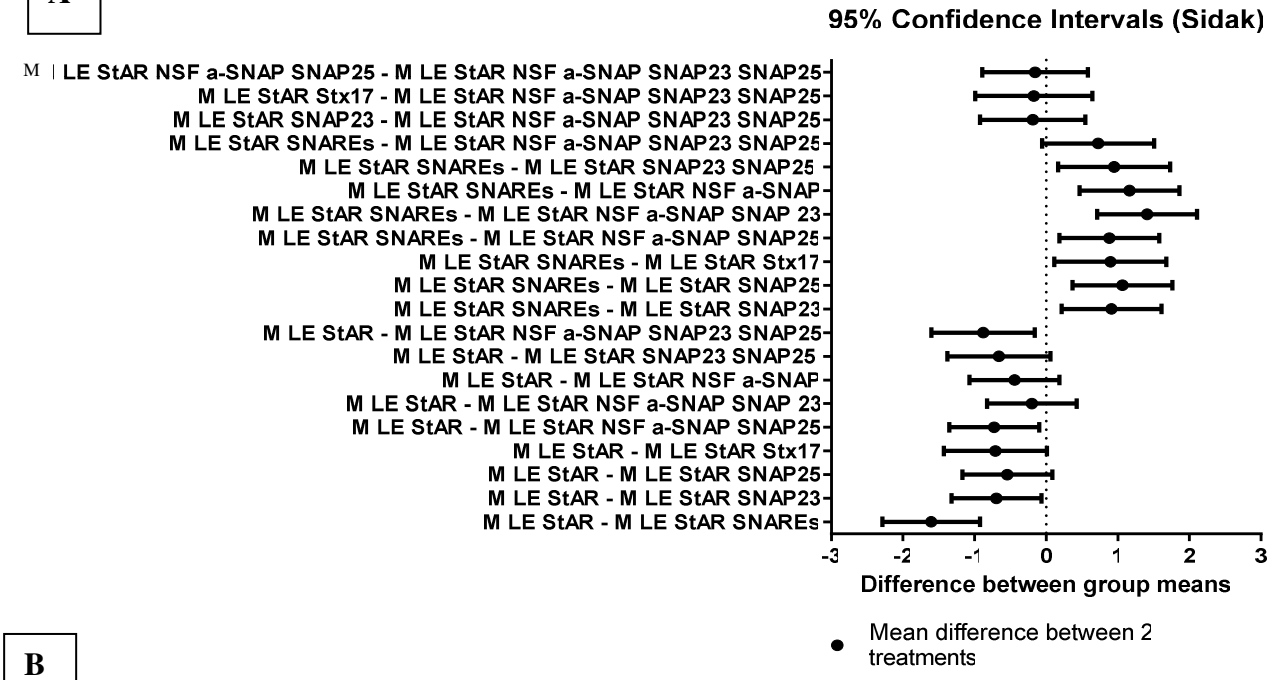


Figure 8 A Pregnenolone concentrations (ng/ml) of reconstitution assays of group 6, M were treated with LE+StAR, LE+StAR+SNAP23, LE+StAR+SNAP25, LE+StAR+Stx17, LE+StAR+ NSF+αSNAP, LE+StAR+ SNAP23+SNAP25, LE+StAR+ NSF+αSNAP+SNAP23, LE+StAR+NSF+αSNAP+SNAP25, LE+StAR+NSF+αSNAP+SNAP23+SNAP25, values are shown as mean +/- SD for six independent experiments, $p<0.05$ (*), $p<0.01$ (**), Sidak's multiple comparison test; **B** Pregnenolone concentration (ng/ml) of the reconstitution assay of group 6, M were treated with LE+StAR, LE+StAR+SNAREs, LE+StAR+SNAP23, LE+StAR+SNAP25, LE+StAR+Stx17, LE+StAR+ NSF+αSNAP, LE+StAR+SNAP23+SNAP25, LE+StAR+NSF+αSNAP+SNAP23, LE+StAR+NSF+αSNAP+ SNAP25, LE+StAR+NSF+αSNAP+SNAP23+SNAP25, values are shown as mean +/- SD for six independent experiments, $p<0.05$ (*), $p<0.01$ (**), $p<0.0001$ (****); Sidak's multiple comparison test

Confidence intervals for mean differences between two treatments are displayed below.

A



B

Comparison of treatments	Mean difference	95% CI, upper end	95% CI, lower end
M LE StAR - M LE StAR SNAREs	-1,606611	-0,929997	-2,283225
M LE StAR - M LE StAR SNAP23	-0,6934028	-0,06959534	-1,31721
M LE StAR - M LE StAR SNAP25	-0,5422028	0,08160466	-1,16601
M LE StAR - M LE StAR Stx17	-0,7083778	0,007683337	-1,424439
M LE StAR - M LE StAR NSF a-SNAP SNAP25	-0,7244028	-0,1005954	-1,34821
M LE StAR - M LE StAR NSF a-SNAP SNAP 23	-0,1986528	0,4251547	-0,8224602
M LE StAR - M LE StAR NSF a-SNAP	-0,4427778	0,1810296	-1,066585
M LE StAR - M LE StAR SNAP23 SNAP25	-0,6581778	0,05788332	-1,374239
M LE StAR - M LE StAR NSF a-SNAP SNAP23 SNAP25	-0,8799778	-0,1639166	-1,596039
M LE StAR SNAREs - M LE StAR SNAP23	0,9132084	1,606532	0,2198852
M LE StAR SNAREs - M LE StAR SNAP25	1,064408	1,757731	0,3710851
M LE StAR SNAREs - M LE StAR Stx17	0,8982334	1,675604	0,1208629
M LE StAR SNAREs - M LE StAR NSF a-SNAP SNAP25	0,8822083	1,575531	0,1888852
M LE StAR SNAREs - M LE StAR NSF a-SNAP SNAP 23	1,407958	2,101282	0,7146352
M LE StAR SNAREs - M LE StAR NSF a-SNAP	1,163833	1,857157	0,4705102
M LE StAR SNAREs - M LE StAR SNAP23 SNAP25	0,9484333	1,725804	0,1710629
M LE StAR SNAREs – M LE StAR NSF a-SNAP SNAP23 SNAP25	0,7266334	1,504004	-0,05073708
M LE StAR SNAP23 – M LE StAR NSF a-SNAP SNAP23 SNAP25	-0,186575	0,5452951	-0,9184451
M LE StAR NSF a-SNAP SNAP25 – M LE StAR NSF a-SNAP SNAP23 SNAP25	-0,155575	0,5762951	-0,8874451

Figure 9 A Mean difference between two treatments of group 6 with 95% CI, Sidak's multiple comparisons test; **B** Table showing 95CI for mean differences between two treatments of group 6, Sidak's multiple comparison test

4.2.5 Effect of adrenal cytosol on pregnenolone concentrations of reconstitution assays (group 3)

In order to investigate if cytosol of adrenal cells contains further components affecting the pregnenolone concentration in reconstitution assays, cytosol was extracted and added to different combinations of reconstitution assays:

1)M+LE

2)M+LE+StAR

3)M+LE+StAR+SNAREs

A significant difference was found between ln(pregnenolone concentration) of:

- M+LE+StAR+SNAREs vs. M+LE+StAR+SNAREs+cytosol (mean difference -0.9564 [CI -1.820 to -0.09301] $p<0.05$)
- M+LE+StAR+cytosol vs. M+LE+StAR+SNAREs+cytosl (mean difference -1.407 [CI -2.336 to -0.4783] $p<0.01$)
- For M+LE+StAR vs. M+LE+StAR+cytosol $p=0.0564$ (mean difference -0.9288 [CI -1.875 to> -0.01781]

In total, the combination of SNAREs and cytosol leads to the highest pregnenolone concentration in reconstitution assays so far.

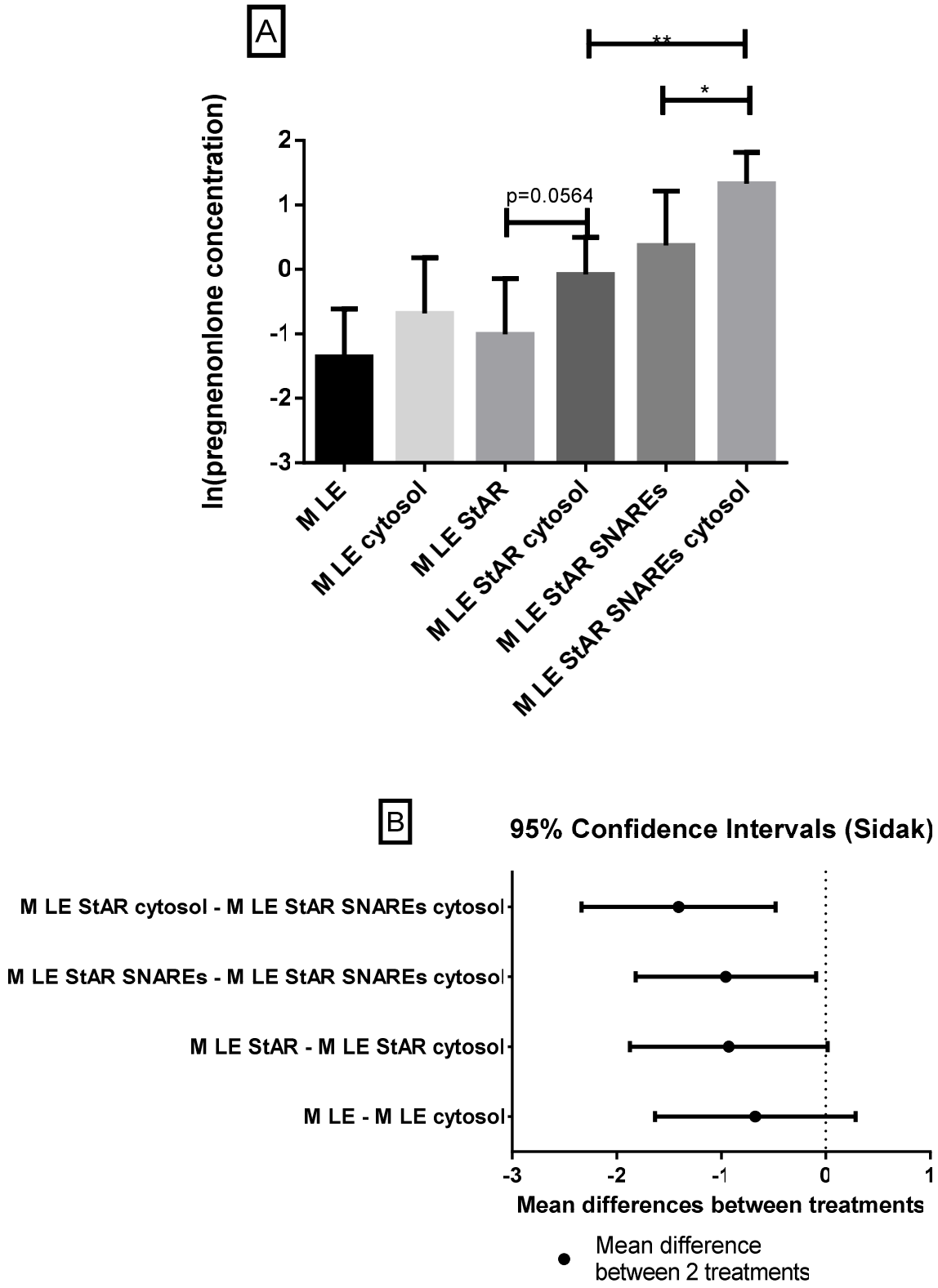


Figure 10 A Natural logarithm (ln) of pregnenolone concentrations (ng/ml) of reconstitution assays of group 3, M were treated with LE, LE+cytosol, LE+StAR, LE+StAR+cytosol, LE+StAR+SNAREs and LE+StAR+SNAREs+cytosol, values are shown as mean +/- SD for eleven independent experiments, $p<0.001$ (**) or $p<0.05$ (*), Sidak's multiple comparison test **B** Mean difference between two treatments of group 3 with 95% CI, Sidak's multiple comparisons test

4.2.6 Effect of GTP/GTPγS and adrenal cytosol on pregnenolone concentrations of reconstitution assays (groups 4 and 5)

Addition of cytosol to reconstitution assays treated with SNAREs has lead to a significant increase in pregnenolone concentration. Now it shall be investigated if GTP or non-hydrolysable GTPγS show an effect on pregnenolone concentration when added to reconstitution assays treated with cytosol.

4.2.6.1 Addition of GTP/GTPγS to M+LE+StAR+cytosol

It was found that GTP added to M+LE+StAR+cytosol leads to a significant increase in pregnenolone concentration ($p<0.05$) while no significant difference was found for addition of GTPγS.

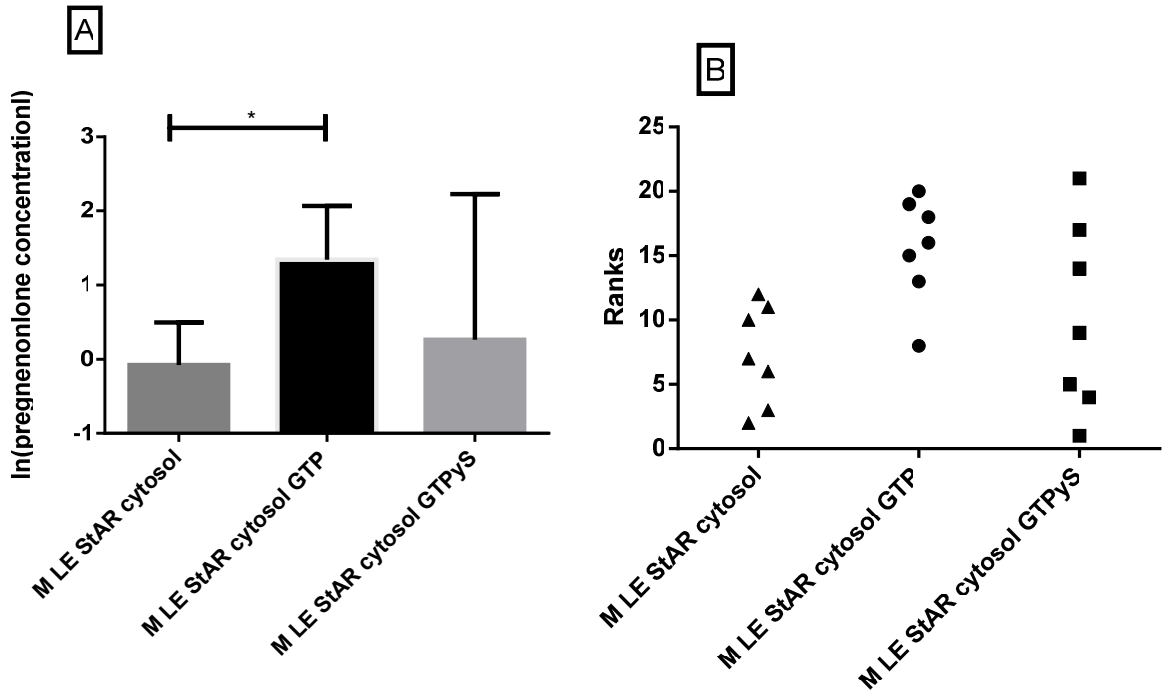


Figure 11 A Natural logarithm (ln) of pregnenolone concentrations (ng/ml) of reconstitution assays of group 4, M were treated with LE+StAR+cytosol, LE+StAR+cytosl+GTP and LE+StAR+cytosol+GTPγS, values are shown as mean +/- SD for seven independent experiments, $p<0.05$(*), Dunn's multiple comparison test **B** Distribution of ranks for the three treatments of group 4, Dunn's multiple comparison test

4.2.6.2 Addition of GTP/GTPγS to M+LE+StAR+SNAREs+cytosol

Addition of GTP or GTPγS to M+LE+StAR+SNAREs+cytosol lead to no significant difference in ln(pregnenolone concentration).

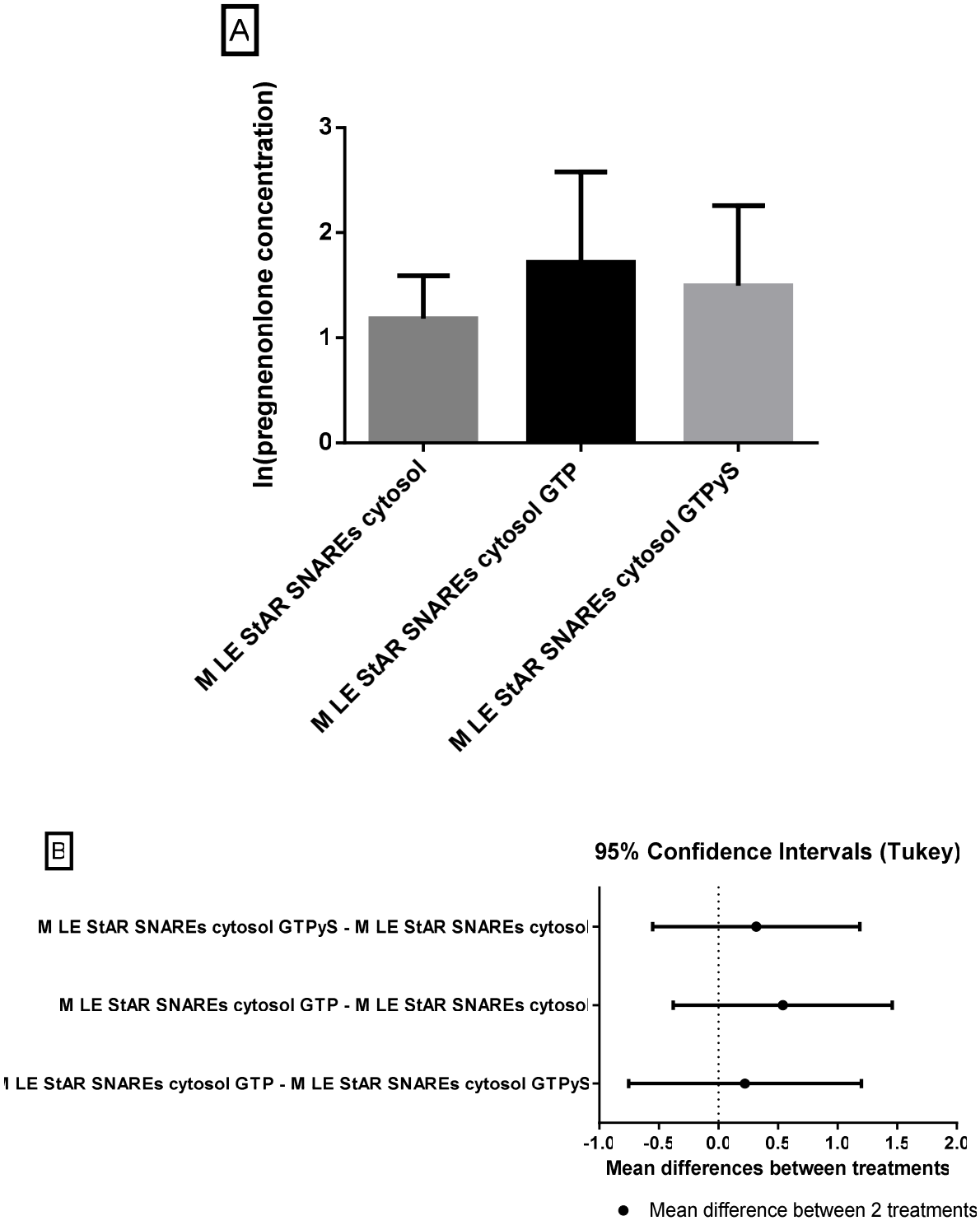


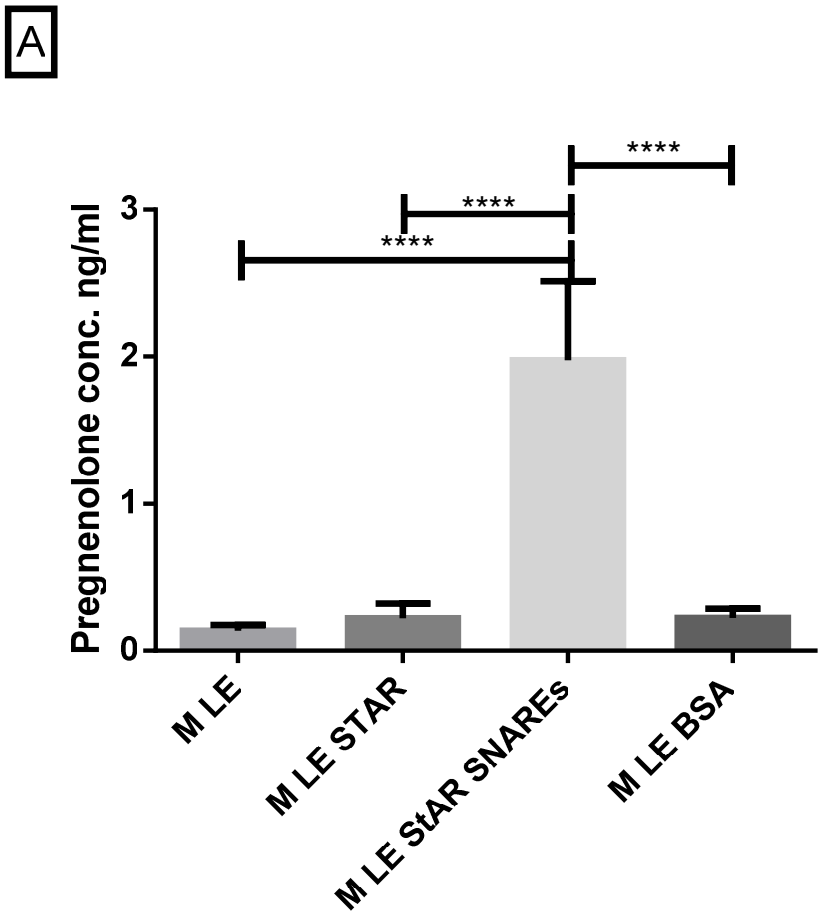
Figure 12 A Natural logarithm (ln) of pregnenolone concentrations (ng/ml) of reconstitution assays of group 5, values are shown as mean +/- SD for six independent experiments, no significant difference was found, Tukey's multiple comparison test; **B** Mean difference between two treatments of group 5 with 95% CI, Sidak's multiple comparisons test

4.2.7 Effect of BSA and imidazole as negative controls on pregnenolone concentrations of reconstitution assays (group 7 and 8)

BSA and imidazole were used as negative controls to ensure that a) the observed effect of SNAREs is not a mere protein effect leading to increased pregnenolone concentrations and b) that increase of pregnenolone concentration is not caused by increasing concentrations of imidazole, in which the SNAREs are dissolved.

4.2.7.1 Effect of BSA

It was found that BSA has no effect on the pregnenolone concentration.



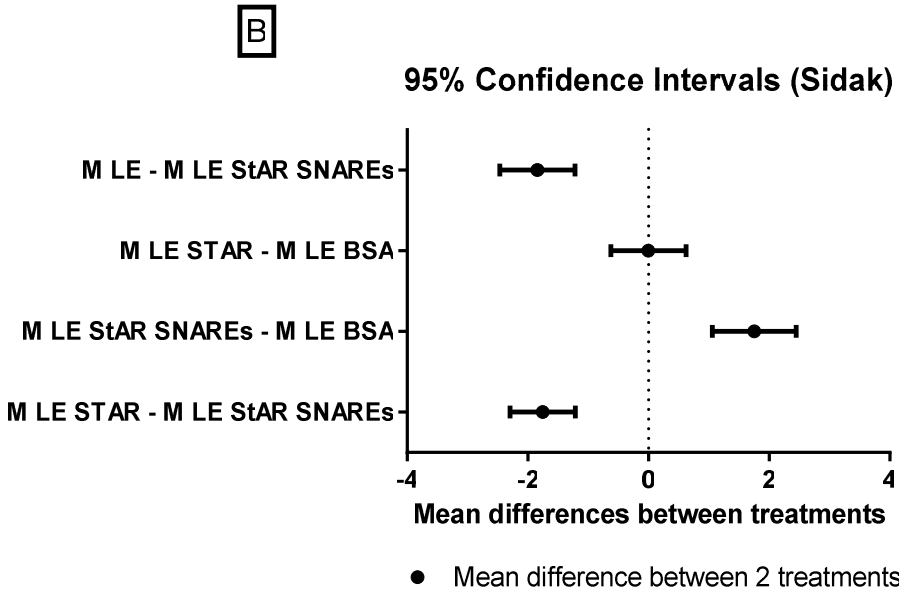
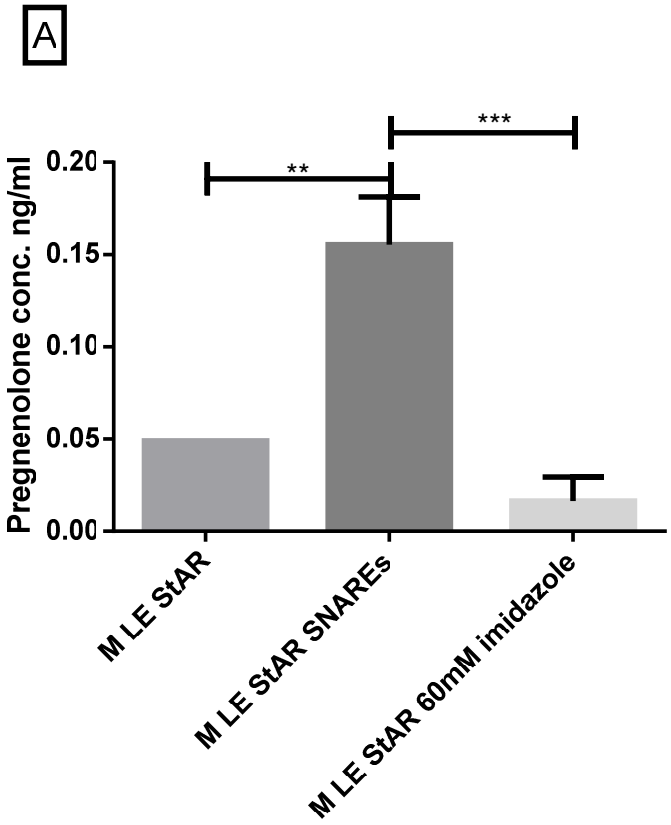


Figure 13 A Pregnenolone concentrations (ng/ml) of reconstitution assays of group 6, M were treated with LE, LE+StAR, LE+StAR+SNAREs, LE+BSA, values are shown as mean +/- SD for three independent experiments, $p<0.001$ (***) and $p<0.0001$ (****), Sidak's multiple comparison test; **B** Mean difference between two treatments of group 6 95% CI, Sidak's multiple comparisons test

4.2.7.2 Effect of imidazole

Treatment with imidazole showed no significant increase of pregnenolone concentration in comparison to M+LE+StAR.



B

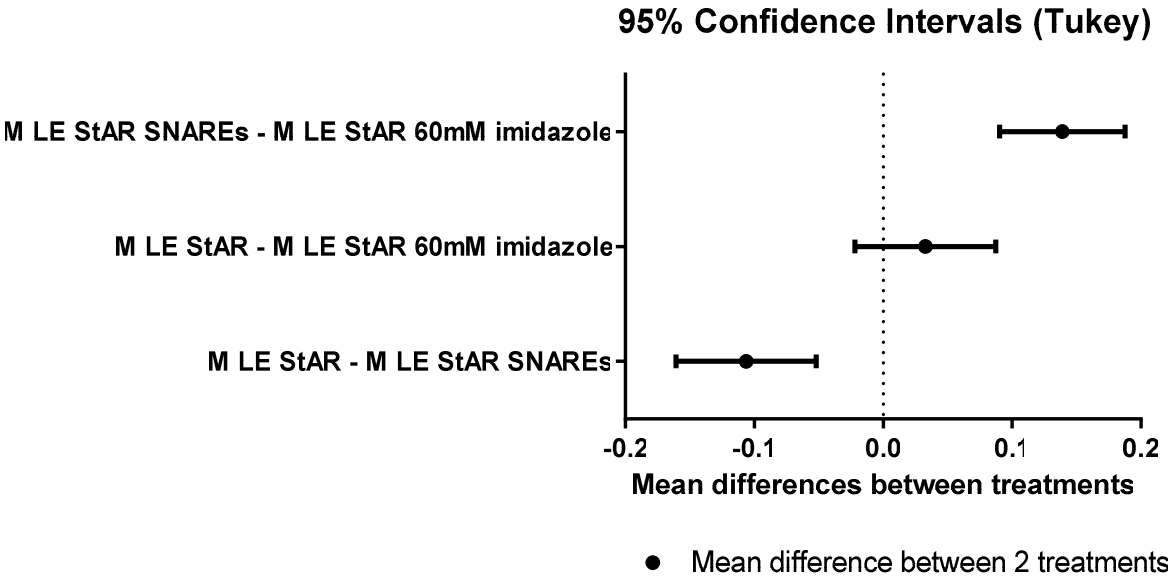


Figure 14 A Pregnenolone concentrations (ng/ml) of reconstitution assays of group 7, M were treated with LE+StAR, LE+StAR+SNAREs and LE+StAR+60mM imidazole, values are shown as mean +/- SD for three independent experiments, $p<0.01$ (**) and $p<0.001$ (***), Tukey's multiple comparison test; **B** Mean difference between two treatments of group 7 with 95% CI, Tukey's multiple comparisons test

5 Discussion

5.1 Intact SNAREs and StAR could be induced, purified and stored in a solute state

All recombinant proteins could be induced (see Figure 3). In contrast to the other induced proteins the band of NSF appears to be weaker on SDS-PAGE. Recombinant proteins exceeding a size of 80kDa often show a varying yield of induction as they are often found to be instable or quickly degraded by *E.coli* proteases.

Furthermore, all recombinant proteins could be purified (see Figure 4). They were eluted with imidazole which keeps the proteins in a solute state so that they keep their functional structure. The better the purification of the recombinant protein, the less other bands can be seen on the sodium dodecyl sulfate -polyacrylamide gel. A sufficient grade of purification was reached for all proteins. The fraction containing the highest concentration of each recombinant protein, which was indicated by the size of the band, was further used. The proteins need to be fully intact to function in the reconstitution assay. Except from StAR, none of the recombinant protein bands shows signs of partial degradation like double bands or the whole band being shifted to a slightly lower molecular size. StAR shows a double band increasing with the duration of induction so that it was only induced for 2.5 hours maximum.

5.2 Results of the *in-vitro* reconstitution assay: Cholesterol is transported from LDs to mitochondria in a SNARE-mediated manner

5.2.1 Distribution of variances and choice of statistical tests

It was tested for homoscedasticity to determine which statistical tests will be used for evaluating the results (Figure 5). One-way ANOVA requires

homoscedasticity, which was fulfilled by all groups except from group 4. For group 4, the heteroscedastic behaviour could possibly be explained by the addition of cytosol as the homogeneity of cytosol and the distribution of factors within the cytosol affecting the reconstitution assay are hard to control. Tukey's and Sidak's multiple comparison tests were applied for groups 1-3 and 5-8 while Dunn's multiple comparison test was used for group 4 showing a heteroscedastic pattern of variances.

5.2.2 Reconstitution assay with mitochondria isolated from rat ovaries (group 1): Cholesterol is transported from LDs into mitochondria for steroidogenesis and SNAREs are involved in mediating this transportation process

It has been observed previously that LDs are in physical contact with mitochondria (Pu et al., 2011). Furthermore, Shen et al. identified the cytoskeletal LD assosicated protein vimentin to be involved in LD movement towards mitochondria for steroidogenesis and SNAREs have been found on LDs mediating LD fusion (Bostrom et al., 2007; Shen et al., 2012). However, it has not been investigated yet if LDs transport cholesterol to mitochondria for steroidogenesis in a SNARE-mediated manner. Within the reconstitution assay, addition of LE or LE+StAR to mitochondria isolated from rat ovaries showed no significant difference in pregnenolone concentration and hence production. However, addition of recombinant SNAREs lead to a significant increase in pregnenolone production ($p<0.01$) (see Figure 6). This indicates that more cholesterol has reached the inner mitochondrial membrane for conversion to pregnenolone. Hence, cholesterol is transported from LDs into mitochondria and the tested SNAREs (SNAP23, SNAP25, NSF, α-SNAP and Stx17) are involved in mediating this transportation process.

5.2.3 Reconstitution assay with mitochondria isolated from rat adrenals (group 2): SNAREs mediate cholesterol transport from LDs to the outer mitochondrial membrane

To verify if involvement of SNAREs in cholesterol transport from LDs to mitochondria is ovary specific, mitochondria isolated from rat adrenals were tested. For adrenal mitochondria, 46 fold increased pregnenolone concentrations were found under addition of SNAREs ($p<0.0001$) confirming the results of ovarian mitochondria (see Figure 7).

Furthermore, a significant difference was discovered between mitochondria only and mitochondria under addition of LE and LE+StAR ($p<0.0001$) supporting that cholesterol is transported from LDs to mitochondria. The mean difference in pregnenolone concentration was bigger for M vs. M+LE+StAR than for M vs. M+LE. This indicates that not only the isolated mitochondria but also recombinant StAR are functional. Addition of recombinant StAR leads to an increased import of cholesterol across the outer mitochondrial membrane within the reconstitution assay. Only recently, Rone et al identified the transduceosome/metabolon, a dynamic mitochondrial protein complex including i.a. VDAC1, P450scc, ATAD3 and TSPO. After initiation by StAR, this complex transports cholesterol across the outer mitochondrial membrane, the intermembranous space and the inner mitochondrial membrane for conversion to pregnenonlone by P450scc (Rone et al., 2012).

Addition of SNAREs has lead to an even higher pregnenolone production demonstrating that in this model transport of cholesterol across the outer mitochondrial membrane is not a speed regulating step but depends on cholesterol supply from LDs. *In vivo*, however, StAR-regulated cholesterol transport across the outer mitochondrial membrane is the speed regulating step of ACTH induced steroidogenesis (Lin et al., 1995; Ohno, Yanagibashi, Yonezawa, Ishiwatari, & Matsuba, 1983; Reinhart, Williams, & Stocco, 1999). Ohno et al. observed that cholesterol accumulates in the outer mitochondrial membrane if corticoidogenic response to ACTH is blocked by cycloheximide (Ohno et al., 1983). Furthermore, Stevens et al.

identified the outer mitochondrial membrane as location for a steroidogenic cholesterol pool which is primarily mobilised by cyclic adenosine monophosphate (cAMP)/hormone dependent signals (Stevens, Xu, & Lambeth, 1992). This suggests that cholesterol from LDs might enter the steroidogenic cholesterol pool of the outer mitochondrial membrane from where it could be mobilised by StAR for trespassing mitochondrial membranes via the transduceosome/metabolon.

Different hypotheses address the question how LDs deliver their cargo to other organelles. While SNAREs mediate homotypic fusion of LDs, Rab proteins (specifically Rab5) regulate the interaction between endosomes and LDs as well as ER and LDs (specifically Rab18) (Bostrom et al., 2007; P. Liu et al., 2007; Ozeki et al., 2005). However, LDs do not seem to fuse with endosomes *in vitro* as ATP, which is required for SNARE-mediated fusion, was found to cause dissociation of bound endosomes from isolated LDs (P. Liu et al., 2007). It was proposed that non-fusion interactions may lead to formation of transient inter-compartmental contact sites for transfer of lipids, ions or small molecules irrespective of ATP-supply (Zehmer et al., 2009). In this work, however, ATP was required for *in-vitro* SNARE-mediated cholesterol transport from LDs to mitochondria. Succinate was added to the reconstitution assay for ATP formation by mitochondria and found to be essential for pregnenolone production. According to the stalk hypothesis, an intermediate step with formation of a stalk between the two membranes precedes total fusion by pore formation (Kozlov & Markin, 1983; Kozlovsky, Chernomordik, & Kozlov, 2002). As LDs are surrounded by a lipid monolayer, stalk formation would theoretically lead to access of the cargo (cholesterol) to the outer membrane and could hence lead to resupply of cholesterol to the steroidogenic cholesterol pool within the outer mitochondrial membrane (summary see Figure 15). Overall, the exact mechanism of SNARE-mediated cholesterol transport to the outer mitochondrial membrane stays matter of further investigation.

5.2.4 Effect of different combinations of SNAREs on cholesterol transport (group 6): NSF, α-SNAP, SNAP25 and SNAP23 work in a rectified manner on cholesterol transport

It was shown that the combination of SNAP23, SNAP25, Stx17, NSF and α-SNAP influences cholesterol transport from LDs to the outer mitochondrial membrane. Different combinations were tested to identify which single SNARE or combination of SNAREs accounts for this effect (see Figure 8).

Addition of SNAP23 to M+LE+StAR has lead to a significant increase in pregnenolone production ($p<0.05$) indicating its role in cholesterol transport to mitochondria. Previously, SNAP23 was identified to mediate LD fusion and insulin stimulated transportation of the glucose transporter Glut4 to the plasma membrane (Bostrom et al., 2007; Bostrom et al., 2005; Kawanishi et al., 2000). Furthermore, Jägerström et al. have observed complex formation between LD and mitochondria via confocal and electron microscopy. They have found that siRNA knock-down of SNAP23 in *NIH 3T3* fibroblasts leads to reduced complex formation and reduced β-oxidation indicating its role in triacyle glycerine transport from LD to mitochondria (Jagerstrom et al., 2009). Interestingly, treatment with NSF+α-SNAP+SNAP23 did not lead to a significant increase in pregnenolone production but showed lower pregnenolone levels than treatment with SNAP23 ($p>0.05$) which could be explained by NSF and α-SNAP inducing disassembly of the SNAP23 mediated complex.

Surprisingly, treatment with NSF+α-SNAP+SNAP25 was followed by augmented pregnenolone production ($p<0.05$) suggesting involvement of these SNAREs in cholesterol transport. However, it remains unclear why SNAP25 by itself does not cause augmented pregnenolone production and why NSF and α-SNAP do not seem to cause disruption of a SNAP25 comprising complex. NSF, α-SNAP and SNAP25 have all been found on LDs but have not been connected with cholesterol transport to mitochondria so far (Bostrom et al., 2007).

However, even though addition of SNAP 23 and NSF+α-SNAP+SNAP25 lead to increased pregnenolone production, the rise in pregnenolone production is still significantly lower than if all five SNAREs are added ($p<0.01$). In contrast, treatment with NSF+α-SNAP+SNAP25+SNAP23 resulted in a rise of pregnenolone production equal to the pregnenolone increase under addition of all five SNAREs ($p=0.08$). Hence, it seems likely that NSF, α-SNAP, SNAP25 and SNAP23 interact or work in a rectified manner on cholesterol transport from LDs to mitochondria. It could be assumed that SNAP25 prevents disruption of a SNAP23 comprising LD-mitochondria complex by NSF and α-SNAP (summary see Figure 15). The detailed role of each SNARE within this process, however, remains matter of further investigation.

Interestingly, Stx17 caused an increase in pregnenolone production that was close to significance level ($p=0.055$). Stx17 is abundantly expressed in steroidogenic cell types and was found to localize to smooth membranes of the ER, which are highly involved in lipid metabolism (Steegmaier et al., 2000). Recently, it has been identified to recruit the autophagosome marker autophagy related 14 (ATG14) to ER-mitochondria contact sites, which seem to be involved in autophagosome formation (Hamasaki et al., 2013). ER-mitochondria contact sites also play an important role in lipid transport and synthesis suggesting that involvement of Stx17 in cholesterol transport is worth further investigation (Rusinol, Cui, Chen, & Vance, 1994).

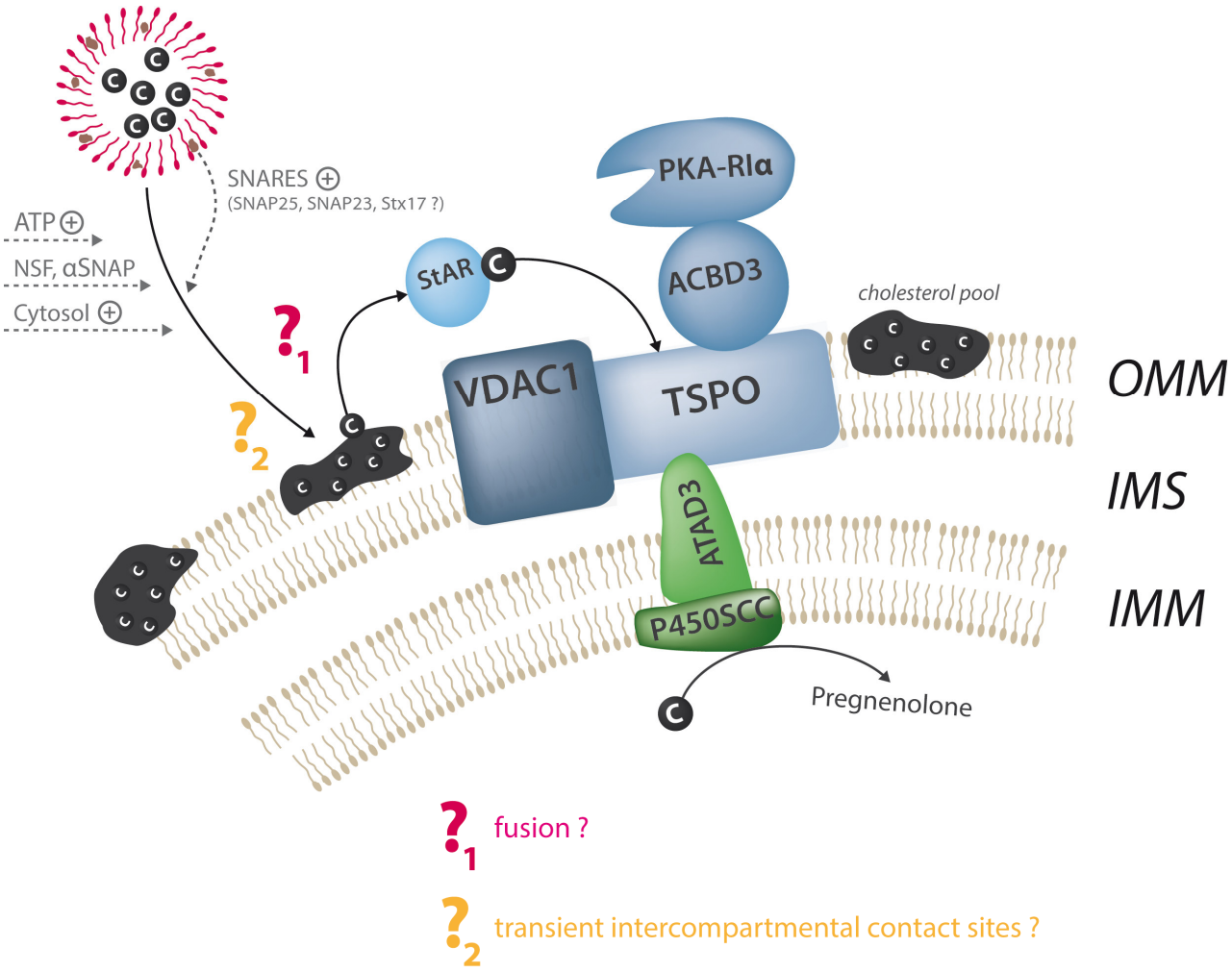


Figure 15 Model of SNARE-mediated transport of cholesterol from lipid droplets into mitochondria for steroidogenesis
Cholesterol (C) transport from lipid droplets (LD) to the outer mitochondrial membrane (OMM) is influenced by the SNARE-proteins SNAP23 and SNAP25 as well as NSF and α-SNAP. The role of Stx17 remains to be determined. ATP and cytosol enhance SNARE-mediated cholesterol transport from LDs to the OMM. C might join pre-existing cholesterol pools in the OMM by hemi-fusion between the LD and the OMM or by transient intercompartmental contact sites before it is bound by StAR and transferred across the OMM, the intermembranous space (IMS) and the inner mitochondrial membrane (IMM) into the matrix. C is converted to pregnenolone by P450scc.

5.2.5 Effect of adrenal cytosol on cholesterol transport (group 3): Cytosolic components enhance SNARE-mediated cholesterol transport from LDs to mitochondria

Jägerström et al. have shown that cytosolic factors are involved in the formation of complexes between LDs and mitochondria (Jagerstrom et al., 2009). Similarly, Pu et al. observed that cytosolic factors influence physical interaction between mitochondria and LDs (Pu et al., 2011). Hence, it was tested if addition of adrenal cytosol has an effect on pregnenolone production to investigate if there are further cytosolic components notably affecting cholesterol transport from LDs to mitochondria.

A significant increase in pregnenolone production was observed under addition of cytosol to reconstitution assays treated with SNAREs ($p<0.05$) but not if it was adjoined to assays without SNAREs ($p=0.2696$ for M+LE vs. M+LE+cytosol and $p=0.0564$ for M+LE+StAR vs. M+LE+StAR+ cytosol) (see Figure 10). This suggests that cytosol might contain components that affect SNARE-mediated transport of cholesterol from LDs to mitochondria rather than StAR-mediated transport. However, the p-value of M+LE+StAR vs. M+LE+StAR+cytosol ($p=0.0564$) is close to significance level (0.05). This could be explained by a low level of SNAREs naturally present in cytosol. Furthermore, a significant increase in ln(pregnenolone concentration) was found between M+LE+StAR+cytosol vs. M+LE+StAR+cytosol+SNAREs ($p<0.01$) which leads to the conclusion that SNAREs are essential for cholesterol transport and cannot be replaced by cytosolic components (summary see Figure 15).

5.2.6 Effect of GTP/GTPγS and adrenal cytosol on cholesterol transport (groups 4 and 5): GTP has no effect on SNARE mediated cholesterol transport from LDs to mitochondria

It has been observed previously that LD associated Rab proteins regulate interaction of LDs with early endosomes (Bartz et al., 2007; P. Liu et al., 2007; P. Liu et al., 2004). Furthermore, Pu et al. have conducted an *in-vitro* reconstitution assay with purified LDs which showed that GTP and cytosol affect interaction of LDs with mitochondria indicating that Rab proteins might be involved in LD-mitochondria interaction (Pu et al., 2011). As the results of this work suggest that cytosolic components are engaged in SNARE-mediated cholesterol transport from LDs to mitochondria, it was investigated if these cytosolic components function on GTP, like GTPases or Rab proteins. Therefore, GTP or non-hydrolysable GTPγS were added to reconstitution assays.

It was found that GTP added to M+LE+StAR+cytosol leads to a significant increase in pregnenolone production ($p<0.05$) (see Figure 11). This might indicate that cytosolic components require GTP for transport of cholesterol to mitochondria. However, addition of GTPγS leads to no significant change in pregnenolone production (see Figure 12). As only addition of GTP+cytosol to M+LE+StAR but not addition of cytosol (see 5.2.5) or cytosol+ GTPγS to M+LE+StAR results in an increased pregnenolone production, it seems likely that cytosolic factors can affect cholesterol transport to mitochondria in a GTP dependent manner.

However, addition of GTP or GTPγS to M+LE+StAR+SNAREs+cytosol did not lead to any significant change in pregnenolone production suggesting that GTP or GTPγS has no influence on SNARE-mediated cholesterol transport in this *in-vitro* assay. It needs to be considered, however, that the LDs in this reconstitution assay were artificially produced and not isolated from cell extracts as in Pu et al.'s experiments (Pu et al., 2011). Hence, they cannot contain any Rab proteins which might be affected by GTP/GTPγS so that no conclusion can be drawn about involvement of Rab proteins in cholesterol transport from LDs to mitochondria. Overall, it can

be summarised that cytosolic, GTP dependent components can enhance cholesterol transport to mitochondria in absence of SNAREs. In presence of SNARE components, cytosolic factor augment SNARE-mediated cholesterol transport to the outer mitochondrial membrane without need for GTP.

5.2.7 Negative controls (group 7 and 8): Neither presence of proteins nor imidazole confound pregnenolone production in the reconstitution assay

To ensure that the observed effects of SNARE proteins on pregnenolone production are not a mere protein effect or caused by imidazole, in which the SNAREs and StAR are dissolved and stored, BSA and imidazole served as negative controls.

It was found that neither BSA nor imidazole have an impact on pregnenolone concentration (see Figure 13 and Figure 14). They do not interfere with the ELISA used for measuring the pregnenolone concentrations nor influence the outcome of the reconstitution assay and thus, transport of cholesterol to mitochondria. It can be concluded that the observed effects of SNARE proteins are truly due to their activity and are not caused by a confounder such as mere presence of proteins or imidazole.

5.3 Summary of conclusions

In this project it was investigated if cholesterol is transported from LDs to mitochondria for steroidogenesis in a SNARE-mediated manner. In summary, addition of SNARE proteins resulted in a 46fold increase in pregnenolone production ($p<0.0001$, Tukey's multiple comparison test), indicating their involvement in cholesterol transport from lipid droplets to mitochondria. In particular, the combination of SNAP23, SNAP25, NSF and

α-SNAP was found to mediate cholesterol transport to the outer mitochondrial membrane ($p<0.05$, Sidak's Multiple Comparison Test) while the detailed role of Stx17 remains unclear. Furthermore, it was observed that cytosolic components enhance SNARE-mediated cholesterol transport without need for GTP ($p<0.05$, Sidak's Multiple Comparison Test).

Overall, these results allow proposing a model of SNARE-mediated cholesterol transport from lipid droplets to the outer mitochondrial membrane and across the mitochondrial membranes into the matrix for steroidogenesis. Cholesterol might join the cholesterol pool in the outer mitochondrial membrane by hemifusion of lipid droplets with the outer mitochondrial membrane. StAR could bind cholesterol from this cholesterol pool before it induces its transport across the mitochondrial membranes via the transduceosome/metabolon. Cholesterol is then converted into pregnenolone as the first step of steroidogenesis in the mitochondrial matrix.

These results raise the question about the exact molecular mechanism of SNARE-mediated cholesterol transport to the outer mitochondrial membrane and the detailed role of each SNARE within this process. Up to now no effect on a known cholesterol transport disease is known. For example, siRNA knock-down of Stx17 and SNAP25 would lead to deeper insight into their specific roles in cholesterol transport *in vivo* and bimolecular fluorescence complementation assays would allow visualising SNARE-complex formation.

A Appendix

A1 Zusammenfassung

Cholesterinspeicher und -transportstörungen liegen einer Vielzahl von Erkrankungen zu Grunde – von der Niemann-Pick Krankheit, über die kongenitale Nebennierenhyperplasie bis zur Schaumzellenbildung bei Atherosklerose. Eine wichtige Rolle spielt Cholesterin in Nebennieren und Ovarien, da es in diesen Organen als Grundbaustein der Steroidhormonsynthese dient.
Intrazelluläres Cholesterin wird in Form von Cholesterinestern in Lipidtröpfchen gespeichert. Es muss jedoch in die mitochondriale Matrix transportiert werden, um dort als erster Schritt der Steroidhormonsynthese in Pregnenolon umgewandelt zu werden. Ein Proteinkomplex – das Transduceosome/Metabolon – ist dabei für den Transport durch die zwei mitochondrialen Membranen zuständig. Der genaue Mechanismus, wie Cholesterin von den Lipidtröpfchen zum Transduceosome in der mitochondrialen Außenmembran gelangt, ist indessen noch unklar. Da *s*oluble *N*-ethylmaleimide-sensitive-factor *a*ttachment *re*ceptor-Proteine (SNARE-Proteine), die vesikuläreren Transport vermitteln können, auf Lipidtröpfchen gefunden werden, war es das Ziel dieser Arbeit zu untersuchen, ob Cholesterin SNARE-vermittelt von Lipidtröpfchen zu Mitochondrien transportiert wird.

Dazu wurden Mitochondrien aus Ovarien und Nebennieren von Ratten isoliert. In einem *in-vitro* Assay wurden dann verschiedene Kombinationen von rekombinanten, aufgereinigten SNARE-Proteinen (SNAP23, SNAP25, NSF, α-SNAP and Stx17) und cholesterinhaltige Lipidtröpfchen zu den Mitochondrien hinzu gegeben. Anschließend wurde mittels *e*nzyme *l*inked *i*mmuno*s*orbent *a*ssay (ELISA) gemessen, wie viel Pregnenolon aus dem in den Lipidtröpfchen enthaltenen Cholesterin synthetisiert wurde. Zusätzlich wurde untersucht, ob GTP und zytosolische Komponenten den Cholesterintransport von den Lipidtröpfchen zu den Mitochondrien beeinflussen.

In diesem *in-vitro* Modell erhöhte die Zugabe von SNARE-Proteinen die Pregnenolonsynthese um das 46fache ($p<0.0001$, Tukey's Multiple Comparison Test), welches die Beteiligung von SNARE-Proteinen am Cholesterintransport belegt. Die Kombination aus den SNARE-Proteinen SNAP23, SNAP25, NSF und α-SNAP ist dabei wesentlich an der Vermittlung des Transportprozesses beteiligt ($p<0.05$, Sidak's Multiple Comparison Test), während die Rolle von Stx17 noch weiterer Aufklärung bedarf. Des Weiteren konnte gezeigt werden, dass zytosolische Komponenten GTP-unabhängig den SNARE-vermittelten Cholesterintransport steigern ($p<0.05$, Sidak's Multiple Comparison Test).

Zusammen mit bereits publizierten Daten lassen sich die Ergebnisse zu einem Modell zusammenfassen, in dem Cholesterin SNARE-vermittelt von Lipidtröpfchen zu Mitochondrien transportiert wird. Dabei könnte das Cholesterin durch Hemifusion des Lipidtröpfchens mit der mitochondrialen Außenmembran einem Cholesterinpool in der mitochondrialen Außenmembran zugeführt werden. An das StAR-Protein (steroidogenesis acute regulatory protein) gebunden, welches Teil des Transduceosomes ist, würde es anschließend innerhalb der Außenmembran zu dem restlichen Transduceosome/Metabolon-Komplex bewegt, welches das Cholesterin in die mitochondriale Matrix zur Steroidhormonsynthese weitertransportiert.

Diese Arbeit liefert Erkenntnisse über den Transport von Cholesterin von Lipidtröpfchen zu Mitochondrien *in vitro* und vereinigt diese mit dem bisherigen Wissensstand in einem Modell über Cholesterintransport von Lipidtröpfchen in die mitochondriale Matrix zur Steroidhormonsynthese. Dabei bleiben der molekulare Mechanismus des SNARE-vermittelten Cholesterintransports und die detaillierte Rolle der einzelnen SNARE-Proteine *in vivo* Gegenstand für weitere Forschungsarbeiten.

A2 References

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A3 List of abbreviations

3HSD	3-hydroxysteroid dehydrogenase
α-SNAP	alpha-soluble NSF-attachment protein
a	adenosine
ACAT (Figure1)	Acyl CoA-cholesterol acyltransferase
ACBD3	Acyl-CoA binding domain containing 3
Acetyl CoA (Figure1)	Acetyl Co-enzyme A
ACSL	acyl-CoA synthetase and long chain family member
ACTH	Adrenocorticotropic hormone
AKAP	A-Kinase anchoring protein
Amp	ampicillin
ANOVA	Analysis of variance
APS	Ammoniumperoxodisulfat
ATAD3	ATPase family AAA Domain-containing protein 3
ATG14	autophagy related 14
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
Bp	Base pair
BSA	bovine serum albumin
c (base)	Cytosine
C (Figure1)	cholesterol
cAMP (Figure2)	Cyclic adenosine monophosphate
CE (Figure 1)	Cholesteryl ester
CI	Confidence interval
Dist.	Distilled
DNA	deoxyribonucleic acid

dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
eppi	Eppendorf tube
ER	Endoplasmic reticulum
g	Guanine
GDI	GDP dissociation inhibitor
GDP	Guanosin diphosphate
Glut4	Glucose transporter 4
GTP	Guanosintriphosphate
GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
H	Hour(s)
H2O	Water
HDL (Figure1)	High density lipoprotein
HEPES	N-2 hydroxyl piperazine-N´-2-ethane sulphonic acid
His	histidine
HMG-CoA-Red (Figure1)	Hydroxy-3-Methylglutaryl Coenzyme A Reductase
HMGCoA-reductase	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
HSL	Hormone sensitive lipase
IMM (Figure2 and 15)	Inner mitochondrial membrane
IMS (Figure2 and 15)	Intermembranous space
IPTG	isopropyl-β,D-thiogalactopyranoside
kDa	kilodalton
LB	lysogeny broth
LD	Lipid droplet
LDL	Low density lipoprotein

LDLR (Figure1)	Low density lipoprotein receptor
LE	Lipid emulsion
LH	Luteinising hormone
Ln	Natural logarithm
M	Mitochondria
Min	Minute(s)
Ml	Millilitre
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
Ni-NTA	nickel-nitrilo triacetic acid
NPC1	Niemann Pick C protein type 1
NPC2	Niemann Pick C protein type 2
NSF	N-ethylmaleimide Sensitive Factor
ODx	optical density at x nm
OMM (Figure2 and 15)	Outer mitochondrial membrane
P450c11β	steroid 11β-hydroxylase
P450c11AS	aldosterone synthase
P450c17	steroid 17 alpha-hydroxylase/17,20 lyase
P450scc	cholesterol side chain cleavage enzyme
PCR	polymerase chain reaction
PKA	Protein kinase A
PKARIα	Riα subunit of cAMP dependent Protein Kinase A
Plin	Perilipin family proteins
Preg. (Figure1)	Pregnenolone
Primer_as	Primer in anti-sense direction
Primer_s	Primer in sense direction
Prog. (Figure1)	Progesterone
Q	glutamine
R	Arginine

Rab	Ras related in brain
RNA	Ribonucleic acid
Rpm	Rounds per minute
RT	room temperature
SD	Standard deviation
SDS -PAGE	sodium dodecyl sulphate – polyacryle gel electrophoresis
sec	second
siRNA	Small interfering RNA
SNAP	soluble N-ethylmaleimide sensitive factor adaptor proteins
SNAP23	(synaptosomal-associated protein of 23 kDa
SNAP25	synaptosomal-associated protein 25
SNARE	*s*oluble *N*-ethylmaleimide-sensitive-factor *a*ttachment *re*ceptor
SR-BI	scavenger receptor class B, type 1
StAR	Steroidogenic acute regulatory protein
STARD4	StAR-related lipid transfer (START) domain containing 4
STARD5	StAR-related lipid transfer (START) domain containing 5
Stx17	Syntaxin 17
sup	Supernatant
t (base)	Tyosine
t (-SNARE)	Target-membrae
Taq	*Thermus aquaticus*
TaqHifi	Platinum Taq DNA Polymerase High Fidelity
TEMED	N,N,N`,N`-tetramethylene diamine
Tim50	translocase of the inner

	mitochondria membrane 50
Tris	tris-(hydroxymethyl)-aminomethane
TSPO	translocator protein, 18kDa
v (-SNARE)	Vesicular membrane
VA	Veteran Affairs
VAMP4	vesicle-associated membrane protein 4
VDAC1	voltage dependent anion channel 1
v/v	volume per volume
w/v	weight per volume

A4 List of figures

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Eidesstattliche Versicherung

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