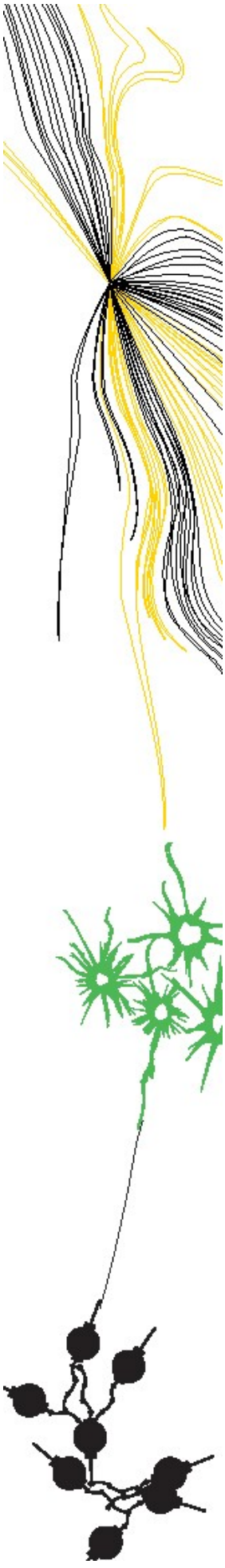

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Master Thesis

In vitro studies of the interaction between α-synuclein and SNAREs

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Abstract

Previous studies have hinted at the role of α-synuclein on the SNARE mediated release of synaptic vesicles and one of these studies has shown an interaction between α-synuclein and one of these SNAREs, synaptobrevin[1-4]. Thus far no information is available on the affinity of α-synuclein with the SNAREs in terms of dissociation constant. No data is known about the strength of the interaction between α-synuclein and synaptobrevin or if any interaction takes place between α-synuclein and the SNAREs SNAP25 and syntaxin at all. The affinities between α-synuclein and these three SNAREs have been investigated in this report.

The SNAREs have been produced and purified untill the desired concentrations and purity of protein has been achieved. Using the first plasmid constructs to produce these SNAREs resulted in low production yield and a high concentration of impurities. Attempts have been made to optimize the production of the SNAREs but these results did not fulfill the requirements. The requirements have been met with the acquisition and production of new plasmid constructs of the desired SNAREs.

The affinity for the interaction between α-synuclein and the SNAREs was analyzed using microscale thermophoresis. One of the interacting partners was labelled covalently and the other binding partner was used to create a serial dilution series to find the dissociation constant of the interaction between the α-synuclein and the SNAREs.

The α-synuclein protein was shown to interact with all three of the SNAREs however the interaction between α-synuclein and synaptobrevin proved to be of higher affinity than the interaction of α-synuclein with SNAP25 or syntaxin. The interaction between α-synuclein and synaptobrevin is not dependent on the isoelectric point of the molecules but an increase in Ca^{2+} concentration increases the affinity between these proteins. Both the α-synuclein and the synaptobrevin interact with Ca^{2+}-ions and upon monitoring the binding between these proteins in conditions of increasing Ca^{2+} concentration the dissociation constant drops.

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Introduction

Parkinson's disease

They symptoms of Parkinson's disease were first described by the English doctor James Parkinson in 1817 as uncontrolled body movements of patients with a shaking palsy. Later the cause of this condition was named after James Parkinson, the first to describe its symptoms with remarkable accuracy, as Parkinson's disease. Currently Parkinson's disease is the second most common degenerative disease of the nervous system, only surpassed by Alzheimer's disease. Parkinson's disease is characterized by motor symptoms such as uncontrollable tremors during rest, rigidity and bradykinesia, and neuropsychiatric symptoms including disorders in speech, cognition, mood, behaviour, and thought[8]. The tremor is the most apparent and common symptom of Parkinson's disease in which the tremor is most severe at rest and has been known to disappear during voluntary motion and sleep. Rigidity of movement is caused by an excessive and continuous contraction of muscles which results in stiffness and resistance to limb movement. Bradykinesia is a symptom of Parkinson's disease which results in difficulties during the whole process of the movement of a limb. These processes range from initiation to execution of the movement and result in slowness of movement. This effect depends on the activity and/or emotional state of the patient[8]. These symptoms of Parkinson's disease are a result of degeneration of the neuronal network in the brain.

Parkinson's disease and α-synuclein

Pathologically Parkinson's disease is recognized by the patient's gradual loss of dopamine producing neurons in the brain, especially in the substantia nigra section of the brain which is among other things associated with movement. Lewy bodies have been formed in the dopamine producing regions of the brain of Parkinson's disease patients. These Lewy bodies are mainly made up of the α-synuclein protein which has been shown to play an influential role in Parkinson's disease. Mutations in the α-synuclein protein have been observed in the familial forms of Parkinson's disease in which the mutation results in an early age onset of Parkinson's disease, rapid development of Parkinson's disease, and high occurrence of dementia associated with Parkinson's disease. The mutated α-synuclein protein induces cell death in the familial form of Parkinson's disease, and elevated concentrations of normal or mutated α-synuclein can result in Lewy bodies and Parkinson's disease.

The α-synuclein protein has a length of 140 amino acids and is highly expressed by the dopaminergic neurons in the substantia nigra pars compacta[8]. In these neurons the α-synuclein is concentrated in the nerve terminus close to the synaptic vesicles. The α-synuclein protein belongs to the 'natively unfolded' proteins because of the absence of secondary configuration in aqueous solution resulting in a seemingly random-coil structure[9]. In the α-synuclein molecule three regions are distinguished. The N-terminal part (amino acids 1-60) contains most of seven imperfect eleven residue repeats which acquire an α-helical configuration when associated with phospholipids[9,14]. The central part (amino acids 61-95) is named the NAC (Non-Aβ Component) and is hydrophobic. This NAC region confers the β-sheet potential and contains a sequence of 12 amino acids which are essential for α-synuclein aggregation. The last part is the C-terminal region (ranging from amino acid 96 up to 140). This region is very acidic due to the glutamate and aspartate amino acids in this region. This domain is responsible for the natively unfolded structure of the protein due to a combination of a large net charge of the protein region and due to the low hydrophobicity; typical properties for unstructured proteins. Despite the N-terminal part and the NAC region both having hydrophobic and charge

properties typical for folded proteins, the properties of the C-terminal region dominate the properties of the whole protein; resulting in α-synuclein being a natively unfolded protein.

In a healthy person the α-synuclein protein is mostly present in the brain and typically located at the end of neuronal cells. The role of α-synuclein in the process of neuronal function and synaptic transmission is not fully understood, however there is evidence that α-synuclein plays an important role in neurotransmitter release and presynaptic vesicle control. Knockout of α-synuclein has resulted in an altered dopamine release and increased vesicle refilling rate of the presynaptic vesicles[2]. Sudden increase of α-synuclein concentration by injecting α-synuclein in the neuron results in increased and long lasting synaptic transmission and can recover α-synuclein knockout in mice[4]. It is clear that α-synuclein has an effect on the release of neurotransmitters, however how α-synuclein affects the mechanism of synaptic transmission is still to be investigated.

Synaptic transmission

Synaptic transmission is the process by which a neuronal signal is transmitted from one neuronal cell to the next. The site of signal transmission is called a synapse which are the functional contacts between neurons. There are two types of synapses which can be distinguished based on the mechanism of transmission. There is the electrical synapse where current flows through the neurons by specialized membrane channels called gap junctions. These synapses allow for direct passive ionic current flows from the upstream neuron into the downstream neuron by direct linking the gap junction in both neurons. Synaptic transmission by electrical synapses is bidirectional allowing the ionic current to flow in either direction, this depends in which of the neurons the action potential origionates. The neuron where the action potential arrives is the upstream neuron while the neuron on the other side of the gap junctions is the downstream neuron to which the action potential is transmitted. The gap junctions are composed of pores from both the upstream and downstream neurons aligned to form a channel. The other form of synaptic transmission is achieved by the chemical synapses in which transmission is achieved by secretion of chemical agents, neurotransmitters, into the synaptic cleft between the upstream and downstream neurons. Upon crossing the synaptic cleft these neurotransmitters will activate receptors in the downstream neuron to trigger an action potential in the downstream neuron.

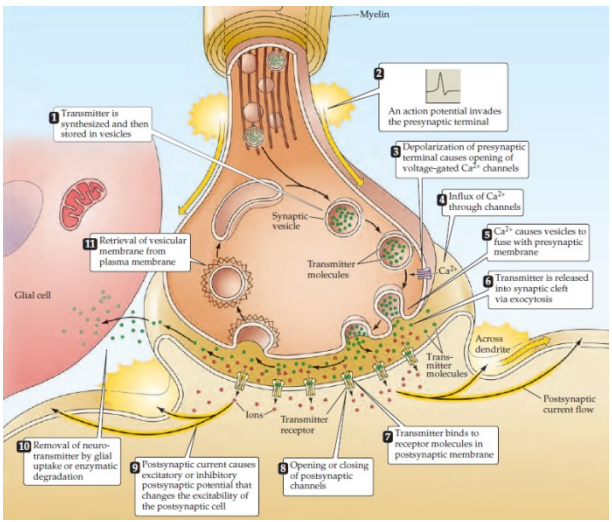

Figure 1: Sequence of events involved in transmission at a typical chemical synapse[7]

The steps in signal transmission for chemical synapses are shown schematically in Figure 1[7]. In the rest state of neurons synaptic vesicles, spherical lipid bilayers of phospholipids with a radius of 40 nm are formed and docked near the synapse of the neuron[10]. Upon the arrival of an action potential, change of membrane potential of the normally negatively charged neuronal cell as a result of influx of Na^+, K^+ or Ca^{2+} gated ion channels, the Ca^{2+} channels at the terminus of the neuron open. The influx of Ca^{2+} ions increases the Ca^{2+} concentration from 0.1 μM to 100 μM, causing the docked presynaptic vesicles to fuse with the cell membrane, which is composed of a phospho-

lipid bilayer with a protein-to-phospholipid ratio of 1:3[2,7,10]. Fusion of the presynaptic vesicle with the cell membrane releases the contents of the presynaptic vesicle into the synaptic cleft between the neurons. Upon crossing the synaptic cleft the neurotransmitters bind to the receptors of the postsynaptic neuron resulting in an excitatory or inhibitory change in membrane potential. The excitatory change causes the ion channels to open and increases the membrane potential, while an inhibitory change causes a drop in membrane potential. If the resulting change in membrane potential, as a result of multiple neurotransmitters binding to receptors and opening ion channels, exceeds the activation threshold an action potential is created in the postsynaptic neuron. The neurotransmitters in the synaptic cleft are taken up by glial cells or degradated by enzymes to limit the time neurotransmitters are effective. Synaptic vesicles are recycled or new synaptic vesicles are formed from the cell membrane and once again docked for a next action potential.

The fusion of two phospholipid bilayers is no easy feat to accomplish. In order to fuse the synaptic vesicles with the cell membrane an energy barrier needs to be overcome. The SNAREs are a protein family which facilitate docking of synaptic vesicles to the cell membrane and lowers the energy barrier to overcome before fusion occurs[2].

SNAREs

Fusion of two separate negatively charged phospholipid bilayers in an aqueous environment requires overcoming a major activation energy barrier. Upon arrival of an action potential multiple synaptic vesicles need to be fused with the cell membrane. This requires multiple synaptic vesicles to be ready and waiting for the action potential to arrive upon which this triggers simultaneous fusion of the docked synaptic vesicles with the cell membrane. Phospholipid bilayers are formed when amphipatic phospholipids, which have both a hydrophilic and a hydrophobic region, try to deal with these conflicting affinities towards water. These phospholipids aggregate in a lipid bilayer with the hydrophilic heads exposed to the outside while the hydrophobic tails are shielded inside of the lipid bilayer. This structure, combined with the different affinities for water, makes these lipid bilayers resilient to changes in structure as disruptions will be repaired by rearrangement of the phospholipids. Close proximity of two intact lipid bilayers alone is not enough to induce fusion, instead both these phospholipids bilayers also need to be disrupted to favour fusion of both membranes over restoration of both membranes. The bilayer disorder requires the naturally shielded hydrophobic regions in the bilayers to be exposed in such a way that allows rearrangement of individual lipids or lipid clusters between the adjacent lipid bilayers and leads to eventual fusion of the lipids[2,11].

The SNAREs (SNAP (Soluble NSF Attachment Protein) Receptor) are a family of proteins which mediate the fusion of vesicles with their target membranes. The SNAREs contain a characteristic sequence of about 60 amino acids, the SNARE-motif, which can assemble with other SNAREs to form a SNARE-complex composed of four SNARE-motifs. The four SNARE-motifs in the SNARE-complex are folded into a tight four-helical bundle containing four types of SNARE-motif, classified R, Qa, Qb and Qc based on sequence structure and central region. Upon formation of the SNARE-complex some of the SNAREs are incorporated into the membrane of the vesicle (v-SNAREs) while other SNAREs are located in the target membrane (t-SNAREs)[12]. The formation of the SNARE-complex forces the v- and t-SNAREs to be in close proximity and this drags the attached cargo of the v-SNARE close to the cell membrane to whicht the t-SNARE is embedded[12]. Both negatively charged lipid bilayers are now close together and this creates tension between the bilayers resulting in easy disruption of the

bilayers.

The t-SNARE used in release of neurotransmitters is synaptobrevin (or VAMP, Vesicle Associated Membrane Protein) which is incorporated in the vesicle by the C-terminal transmembrane region and has the R-SNARE-motif exposed in the cytosol to allow for SNARE-complex formation. The t-SNAREs corresponding to neurotransmitter release are syntaxin and SNAP-25. The syntaxin is connected with the cell membrane by a C-terminal transmembrane region exposing the SNARE-motif exposed in the cytosol. The SNAP-25 protein is docked onto the cell membrane by forming four covalent bonds between cysteine residues in the middle of the protein and palmitoyl side-chains of the cell membrane. Two of the four SNARE-motifs of a SNARE-complex are found in SNAP-25, both SNARE-motifs are exposed in the cytosol of the cell but one of those regions is to be found close to the docking site of the protein. This forces the whole SNARE-complex to be located near the cell membrane and hereby ensures that the vesicle and the cell membrane are close together[2,11].

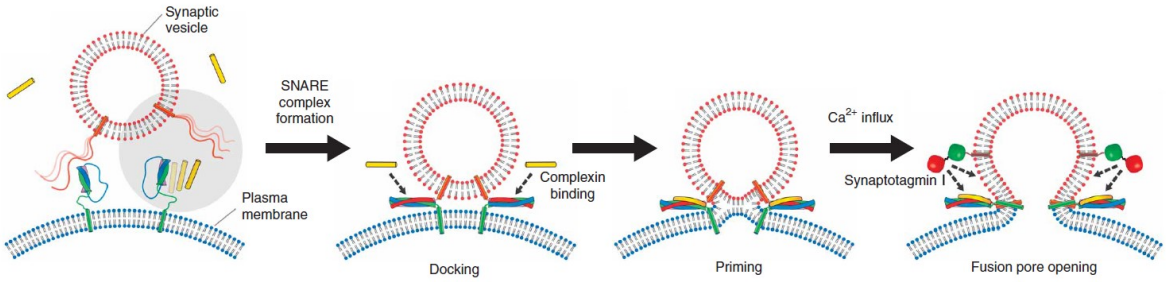

Figure 2: Schematic diagram of presynaptic vesicle docking, priming and SNARE mediated Ca^{2+}-triggered neurotransmitter release.

The steps leading to formation of the SNARE-complex and the fusion of the vesicle with the cell membrane are illustrated in Figure 2. Synaptic vesicles filled with an incorporated synaptobrevin molecule approach the cell membrane as a result of diffusion and Van der Waals interactions. When the v-SNARE and the t-SNAREs interact to form the SNARE-complex, the vesicle is docked to the cell membrane. Upon binding of the protein complexin with the SNARE-complex , the docked vesicle is primed. Addition of the complexin lowers the energy barriers for vesicle fusion by changing the structure of the lipid bilayers of both the vesicle and the cell membrane in such a way that both bilayers are disrupted without forcing negatively charged phospholipids close together[2,13]. Influx of Ca^{2+} ions, upon arrival of the action potential, triggers the protein synaptotagmin to further lower the fusion energy barrier to achieve fast fusion-pore opening to release the neurotransmitters into the synaptic cleft[2,13,14].

As synaptotagmin is a Ca^{2+}-sensitive trigger for vesicle fusion, the triggered release of neuro-transmitters is dependent on the arrival of an action potential and the following influx of Ca^{2+} ions[15]. The synaptotagmins are a family of single membrane proteins which occur predominantly in neurons. The synaptotagmins are characterized by their transmembrane region, a variable linker and two C-terminal C2-domains. The C2-domains, in most members of the syntaxin family, are capable of binding Ca^{2+}-ions. This makes most members of the synaptotagmin family a Ca^{2+}-sensor which couples the Ca^{2+}-influx upon arrival of an action potential to the fast release of neurotransmitters. This has been confirmed by experiments showing that deletion of the synaptotagmin 1 gene in fruit flies and mice results in a loss of neurotransmitter release upon arrival of an action potential[16,17]. Many details of the molecular mechanism, in which synaptotagmin affects other processes such as

the SNARE vesicle fusion machinery, are still unclear, but it is expected that the binding of Ca^{2+} to the C2-domains results in forcing the different membranes together[2]. Bringing the membranes of the vesicle and the plasma membrane together may facilitate formation of a transition state or destabilize the membrane during transition states. Hereby synaptotagmin 1 is expected to mediate the neurotransmitter release upon the arrival of an action potential at the end of the terminus of the presynaptic neuronal cell[11].

Studying the fundamentals of interactions occurring during synaptic transmission requires the experimental conditions to be as simple as possible to be able to determinatine which particles interact and exactly how these particles interact with each other. In these simplified experimental conditions the interactions need to be studies outside of the cell to prevent cellular components affecting the interaction one way or another. To achieve pure proteins outside of a cell, these proteins need to be produced in a high concentration and purified.

Protein production

In eukaryotic cells the genetic information (e.g. instructions for development, functioning and reproduction) is stored in the chromosomes of the cell. The chromosomes are located in the cell's nucleus and are a densely packaged and organized structure containing most of the DNA of the organism, this is schematically visualized in Figure 3. The DNA (Deoxyribonucleic acid) itself consists of two complementary strands of polynucleotide chains in an α-helical configuration held together by hydrogen bonds between the nucleotides of the chains. Several different molecules play a role in condensing the DNA molecule into the chromosome structure of the cells. After interacting with other proteins and forming hydrogen bonds between the complementary strands, the DNA is safely protected from damages but is also hindered in the production of proteins from its genetic information. The road from the genetic information stored in the DNA to the resulting proteins is known as gene expression and this is tightly regulated. The two main steps in gene expression are transcription and translation[12].

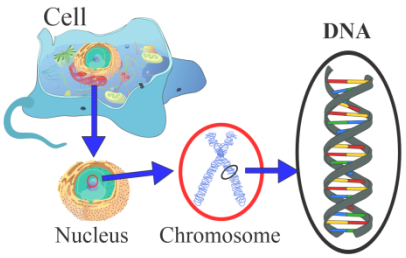

Figure 3: Schematic representation of the location of the genetic information in an eukaryotic cell.

The first main step in the gene expression is transcription of the double stranded DNA into the single stranded mRNA (messenger ribonucleic acid). This step occurs in the cell nucleus and is tightly regulated by a number of different factors before the chromosome reveals its information. As the need for a specific protein arises only the corresponding location of the genetic information is uncoiled and made available for transcription. Once the DNA strand is freed from most proteins and uncoiled an enzyme called RNA polymerase attaches itself to the promotor site on one of the DNA strands to initiate transcription of DNA into RNA. In this process the enzyme RNA polymerase breaks the hydrogen bonds between the two strands and starts attaching RNA nucleotides complementary to the DNA strand. The RNA polymerase runs along the gene of the DNA and keeps elongating the single RNA strand untill the RNA polymerase reaches the stop site of the gene upon which it releases from the DNA. As the RNA polymerase moves along the DNA strand, the RNA strand is formed and elongated as the RNA polymerase continues. All the while only 8 nucleotides of one RNA strand are able to form a RNA-DNA hybrid at the same time. As the RNA polymerase moves further the RNA-DNA hybrid is broken and the growing RNA strand is released from the DNA. After passing of the RNA

polymerase the DNA reestablishes its double stranded form and other proteins attach themselves to the DNA again to reform its densely packed form[12].

After transcription of double stranded DNA into single stranded RNA, the RNA is exported out of the cell nucleus and into the cytoplasm for translation, displayed in Figure 4. During translation the linear sequence of the nucleotides is translated into a linear sequence of amino acids, the building blocks of proteins. This translation is enabled by a ribosome, a complex of over 50 molecules, which work together to translate the sequence of mRNA into a sequence of amino acids. Every combination of three RNA nucleotides is called a codon and every codon corresponds to an amino acid. In the ribosome the amino acid corresponding to that codon is covalently linked to the previous amino acid to form the protein. The amino acid chain is produced starting with the N-terminus of the protein and stops once the ribosome reaches the stop codon. The stop codon itself does not encode for an amino acid and upon reaching the stop codon, the ribosome dissociates and releases the newly formed protein into the cytosol. Proteins are not only a strand of amino acids but have a three dimensional structure which is formed by polar interactions, hydrophobicity, hydrogen bonds (results in α-helixes and β-sheet) and disulphide bonds. In some cases the structure of a protein is affected by other proteins, multiple protein subunits interact together, non-covalently, to form a single complex with a characteristic configuration[12].

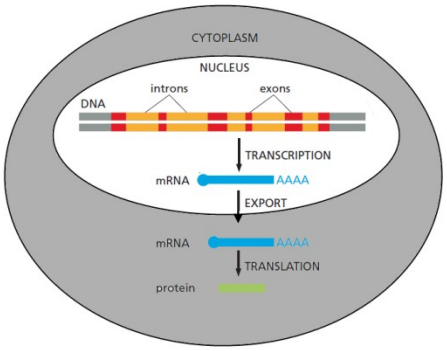

Figure 4: Schematic representation of gene expression

The road from transferring the genetic information in the DNA into a functional protein is complicated as there are many factors involved in the regulation of gene expression. While this is a successful method to produce proteins, this typically results in low protein concentrations as the host's own expression mechanism regulates the production of proteins. Acquiring a high protein concentration can be achieved using recombinant DNA encoding for the desired protein and increasing the promotor concentration to induce overexpression of the recombinant DNA.

Recombinant protein biosynthesis

With the understanding how proteins are produced in normal eukaryotic cells, the proteins can also be expressed in other cells. By using genetic manipulation many types of cell can be manipulated to make proteins which normally are not present in those cells or overexpress proteins to concentrations above the standard. Overexpression of proteins is used as a method to acquire proteins of interest. Insulin was one of the first proteins which has been produced by transfecting a cell with non-native DNA and overexpressing this protein.

Transfection of cells occurs in multiple steps, see Figure 5. The first step is to prepare the plasmid (small circular DNA molecules) for transfection by opening the foreign DNA vector just after the promotor and inserting the DNA encoding for the desired protein and closing it afterwards. Next the DNA is inserted into the host cell by applying stress (electric or heat) on the cells after which the cell membrane is temporarily disrupted and the plasmid can enter the host cell[12].

The plasmid will be expressed in the host cell by the processes occurring naturally in the host cell, this will result in a small concentration of the inserted DNA. It is common practice, when using bacteria as host cells, to use nutrients with an antibacterial agent and incorporate a resistance to that specific antibacterial agent in the inserted DNA. This addition will result in only maintaining the hosts for which the transfection has been successful, as only successfuly transfected bacteria will be able to survive from the nutrient source and expand. When adding an activator protein to the hosts, the promotor sequence the will be fully functional and transcription will be triggered. These activators (e.g. IPTG) are used to induce overexpression of the inserted DNA segment and result in high concentrations of the corresponding protein in the host cell[12,18].

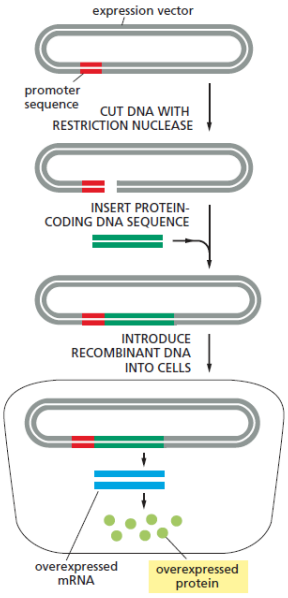

Figure 5: Creation of a plasmid, transfection of a cell and overexpression of the recombinant protein coding DNA in the plasmid.

The produced proteins are trapped in the host cells, these host cells will have to be broken down by cell lysis to extract the protein. During cell lysis of the host cells the produced proteins must be protected from oxidation, degradation of the protein by enzymes and contamination. Cell lysis occurs by means of disrupting the host's cell membrane as a result of viral, chemical (osmotic or detergents), enzymatic (lysozyme) or mechanical (sonification) mechanisms. During lysis other agents can be added to break down large non-protein structures in the cell, stop enzymes from harming proteins and allow proteins to acquire their natural conformation by binding with ions. Typical examples of these are DNAse to break down DNA, PMSF to block protease and urea to extract and protect proteins from inclusion bodies[12,19].

The proteins must be purified from the mixture of all the cellular components. This can be done in several ways; size exclusion, charge separation, affinity chromatography and hydrophobicity. For proteins which do not have properties which makes it easy to separate them using these techniques, a recognizable sequence is added to the inserted DNA in such a way that the extra sequence is attached to the protein. The extra sequence contains a region by which the protein is easy to recognize, such as a polyhistidine region, antibodies or an antigen. Antibodies and antigen bind to one another, using a column with the opposite partner will result in the protein attaching to the column. Using a cleavage enzyme the added sequence can be cleaved from the main protein to release the proteins from the column. The polyhistidine region, or HisTag, is a region with a sequence of 6 to 8 histidines at the N- or C-terminus of the protein. The amino acid histidine has a strong affinity to divalent metal ions such as nickel. Passing a solution with HisTagged proteins through a column with divalent metal ions will result in accumulation of these proteins in the column. Using high concentrations of imidazole the HisTagged proteins can be eluted from the column because imidazole, with a higher affinity for divalent metal ions, competes with the HisTag for the surface area in the columns[20-22].

Purified proteins need to be characterized to observe the purity, concentration and type of protein. Depending on the type of purification used multiple types of protein can end up being purified as all these protein properties for purification are similar[20]. Protein characterization can be performed using mass chromatography or SDS-PAGE to analyze the contents of the samples upon elution of the purification[22]. Using mass spectroscopy the protein sample will be reduced to charged fragments and

the ratio between the charged fragments will provide critical information on the composition of the sample analyzed, e.g. types of protein, purity and concentration. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is a method to compare the molecular weight of a sample of proteins against reference proteins. During SDS-PAGE the SDS molecules aggregate at the hydrophobic sites of the protein hereby inducing a "reconstructive denaturation". In this induced conformation the protein has a mix of α-helical and random-coiled structures. Applying an electrical field, during the electrophoresis, will separate proteins based on their mass-to-charge ratio. The charge will be the result of attachment of the anionic SDS molecules to the protein and the of protein's natural charge. As both the proteins under investigation and the proteins used for molecular weight calibration undergo the same treatment, this method proves to be a good indicator for the molecular weight of the sample proteins with respect to the calibration proteins[23]. Using a dye (e.g. Coomassie Brilliant Blue which interacts with positive amine groups and forms Van der Waals interactions[24,25]) to stain the protein will provide an estimate of the purity of the sample and give an indication of the size of the proteins in the purified sample. The concentration of the full protein mixture can be determined by means of UV absorption[20].

After the production and purification of the proteins, these proteins can be analyzed to discover their properties. There are several powerful methods suitable to monitor interactions occurring to proteins, each method with its own capabilities, strengths and weaknesses.

Microscale Thermophoresis

There are several successful methods to determine the affinity of an interaction between biomolecules, however these types of detection typically require large sample quantities, controlled conditions and significant changes to allow detection. This leads to some disadvantages to these methods of analysis of interaction, first because large quantities of sample proteins are expensive to produce in high purities. Secondly, the requirement of controlled conditions in some methods of detection limits the effectiveness of these methods. Putting restraints on the conditions used for the determination for interaction between biomolecules, limits the representability of the determined results in biological liquids. And last, interaction between biomolecules often results in small changes as the molecules involved can be very small and the interaction will result in a slight change in e.g. mass, charge or configuration change making it hard to detect these changes[26-28]. Microscale thermophoresis is a very sensitive all-optical technique, able to detect small changes requiring only very small sample quantities even in complex solutions by making use tracking of fluorescent particles moving as a result of thermodiffusion.

Thermodiffusion is the effect of molecules behaving differently if affected by a small temperature change (typically 1-7 K). Upon a change of temperature, thermophoresis induces a movement onto particles or molecules to move into or out of the heated area resulting in a accumulation or depletion of molecules as a result of thermodiffusion. Typically molecules deplete from regions with an increased temperature but the reversed effect can also occur. The amount and direction of thermodiffusion depends on the size, charge, hydration shell, conformation of the particles or molecules and the temperature gradient used for thermodiffusion. The thermodiffusion is balanced by ordinary diffusion resulting in a steady state between these types of diffusion[26,27].

In an experimental microscale thermophoresis setup an infrared (IR) laser is coupled into the optical path of a fluorescence excitation/emission, a schematic overview is provided in Figure 6-A. The

optical path is focused onto the sample by an objective allowing excitation of the particles in the focus. At least one of the particles or molecules involved in the interaction should be traceable by the fluorescence excitation. Tracing of molecules can be obtained by using the intrinsic fluorescence of a protein, using a fluorescent fusion protein or attaching a fluorescent label to the protein. The photons emitted from the sample travel through the filter and the dichroic mirrors and can be recorded, e.g. by a CCD camera. The IR laser creates a small temperature change to induce the migration of small particles and molecules on a microscale as a result of thermodiffusion until a steady-state between normal diffusion and thermodiffusion is reached.

A schematic time trace of a MST measurement is displayed in Figure 6-B and five different phases can be identified in the MST measurement. The first phase is the initial fluorescence (I) recorded by emission of particles or molecules before the IR-laser has been initiated. Any change in signal in this region is the result of bleaching of fluorescent particles and normal diffusion of molecules. After initiation of the IR-laser two phases are observed, first the T-jump (II) is the change (within 100 ms after activation of the IR-laser) in fluorophore emission as a result of temperature change by the activation of the IR-laser. After the initial change in signal upon activation of the IR-laser, thermophoresis (III) is the driving force in signal change. During the thermophoresis phase a balance is reached between the thermodiffusion and normal diffusion resulting in a steady-state. Upon deactivation of the IR-laser a fast inverted T-jump (IV) is observed before gradually a steady-state is restored by backdiffusion (V) as a result of normal diffusion.

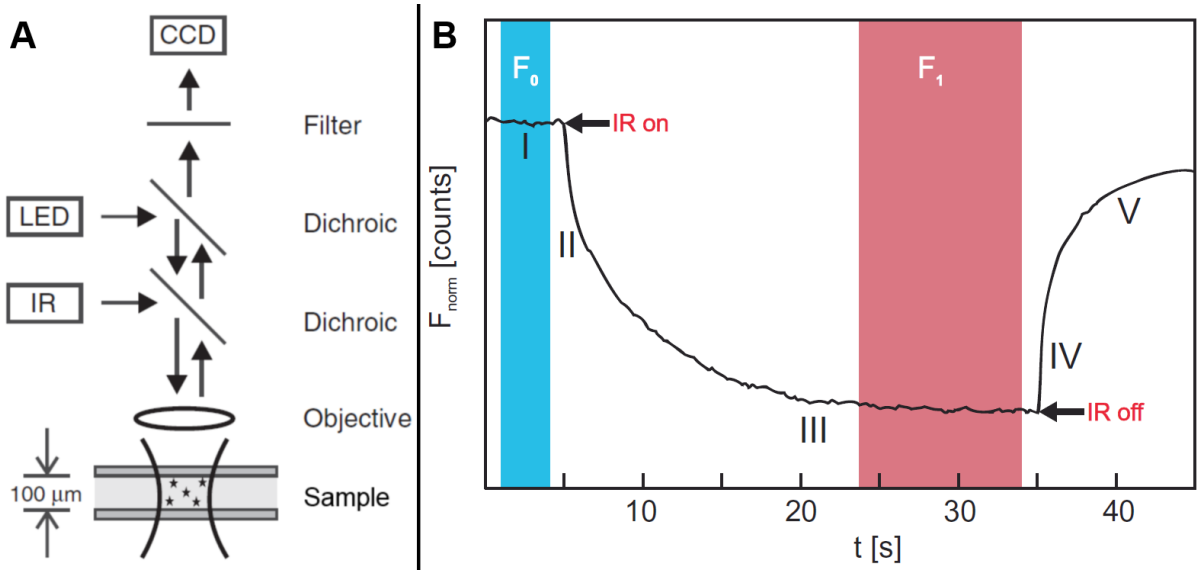

Figure 6: A) Schematic overview of a MST setup in which an IR-laser and a LED are coupled into the same optical path to ensure that the sample heated by the IR-laser is at the same spot as the area monitored by the LED excitation/emission of traceable particles. B) Schematic representation of a fluorescence time trace recorded by MST. The time trace is composed of five processes happening over time. The initial fluorescence ($F_{initial}$ in phase I) is measured before the IR-laser is activated. Once the IR-laser is initiated the in the first 100 ms the T-jump (II) is observed while afterward the thermophoresis ($F_{steady-state}$ in phase III) dominates the signal where thermodiffusion and normal diffusion reach a steady-state. Upon deactivation of the IR-laser an inversed T-jump (IV) is observed followed by backdiffusion (V) of particles since thermodiffusion no longer affects the steady-state.

The MST measurements measures the equilibrium between binding events using a low but constant concentration of the traceable binding partner and using a serial dilution series to change the concentration of an unlabelled interacting molecule or particle. By using a constant concentration of the traceable molecule this ensures that the initial fluorescence (F_0 inFigure 6-B) stays constant, as

the initial fluorescence is controlled by normal diffusion in a homogeneous solution. In the cases that the initial fluorescence does not stay constant, this is an effect of having fluorophore emission affected by the change in conformation as a result of its proximity to the binding site or there are problems with surface adsorption or aggregation in the solution. Inhomogeneous solutions as a result of surface adhesion and aggregation can be prevented by adding BSA to the solution to coat the surface[29] and use Tris (Tris(hydroxymethyl)aminomethane)[30] and Tween 20 (Polysorbate 20)[31] to prevent protein aggregation.

During the steady-state in thermophoresis (F_1 in Figure 6-B) the normal diffusion and the thermodiffusion reach an equilibrium. As the thermodiffusion of a molecule depends on properties as size, charge, hydration shell and conformation, these properties also affect the steady-state of diffusion. Any interaction between molecules changes these conditions, which results in a change in the steady-state signal of the sample depending upon the degree of interaction. The steady-state does not have to be completely reached before the differences between different samples can be observed[26]. Using different ratio's of binding partners will result in a specific steady-state signal for each specific ratio, ranging from completely unbound to fully interacted (this can be observed in Figure 7-A.

The degree of interaction between in the sample for different ratios can be determined by the steady-state fluorescence. As the initial fluorescence should remains constant, the ratio between the steady-state and initial fluorescence shows the degree of binding if the full extent of the binding has been observed. As only the ratio between initial and normalized fluorescence matters, the data points of each time trace are normalized by dividing all the data points by the value found at t=0 for that specific trace to have all the time traces of a full binding curve start at a value of 1. Figure 7-A an example of the normalized time traces in a complete range of binding events. Dividing the time trace by the value at t=0 does not affect the ratio between the initial fluorescence (highlighted blue in Figure 7-A and the fluorescence at the steady state (highlighted red in Figure 7-A for an individual trace, but instead allows an easy overview of the change in a binding experiment covering the full extent of binding. In the red highlighted area of Figure 7-A it the steady state signal rises for an increasing concentration of the unlabelled binding partner. Traceable particles move out of the focus upon activation of the IR-laser but increase in the unlabelled binding partner in the solution results in a higher steady-state signal. This indicates that the unlabelled particles interact with the labelled particles and keeps the labelled particles in the focus. The ratio is the normalized fluorescence (F_{norm}) and is calculated as presented in Equation 1.

$$F_{norm} = \frac{F_1}{F_0}$$

Equation 1 Formula to calculate the normalized fluorescence (F_{norm}) as the ratio between the initial fluorescence (F_0) and the fluorescence during the steady-state of thermophoresis (F_1).

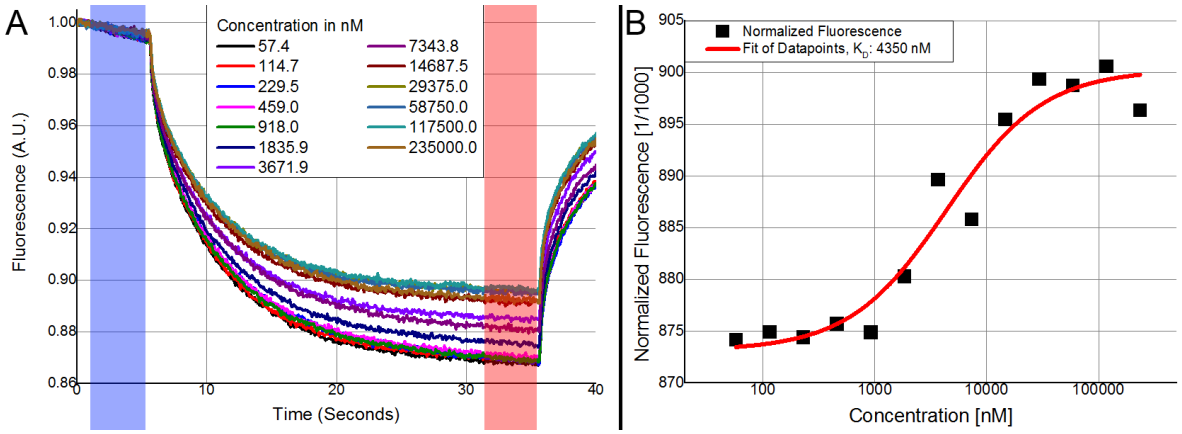

Figure 7: A) Normalized MST measurements for different concentrations of unlabelled protein with the initial fluorescence recorded in the blue highlighted area and the steady-state fluorescence recorded in the red highlighted area. B) F_{norm} plotted against the unlabelled protein concentration and fitted to determine the K_D of the binding measurement.

When plotting the values of F_{norm} from Figure 7-A against its corresponding concentration of the titrated binding partner this results Figure 7-B. Near the highest and the lowest concentrations of titrated binding partner, the signals measured at steady-state start to overlap more with directly higher or lower concentrations used. In these cases the traceable particles are completely free or have fully interacted with the titrated binding partner. Changing the concentration beyond these extremes will not affect the steady-state much. Any interaction occuring has already been completed as a result of a surplus of titrated binding partner (high concentrations) or not occurring at all because of a limited availability of the unlabelled binding partner (low concentrations). The affinity between both binding partners is determined by analyzing the change in F_{norm} as an effect of the concentration of titrated binding partner. The fraction of the traceable binding partner which is bound can be derived from the F_{norm} by Equation 2, this makes use of the linearity of the thermodiffusion between the bound and the unbound state of the proteins.

$$F_{norm} = (1 - FB) * F_{norm,unbound} + (FB) * F_{norm,bound}$$

Equation 2: Formula to derive the fraction of traceable binding partner (FB) from the recorded fluorescence (F_{norm}) using the fluorescence of the bound and the unbound state, $F_{norm,bound}$ and $F_{norm,unbound}$ respectively.

For an equilibrium reaction in which two different binding partners, A and B, lead to a complex (AB) the binding process is characterized by $A + B \rightleftharpoons AB$. The binding affinity of this reaction is defined by the equilibrium constant K_D as presented in the first part of Equation 3. In MST measurements the concentrations of free binding partners are unknown, however the total concentrations are know as these are what the solution started with. The total concentration of each parameter is the sum of the concentration of free partner and the concentration of bound partner $[A] = [A]_{free} + [AB]$ and $[B] = [B]_{free} + [AB]$. Using these total concentrations instead of free concentrations of binding partners, the equilibrium dissociation constant can be determined by the right part of Equation 3.

$$K_D = \frac{[A]_{free} * [B]_{free}}{[AB]} = \frac{([A] - [AB]) * ([B] - [AB])}{[AB]}$$

Equation 3: The equilibrium dissociation constant for an equilibrium binding reaction $A + B \rightleftharpoons AB$. The dissociation constant is defined as ratio of free binding partners $[A]_{free}$ and $[B]_{free}$ over the bound complex [AB]. For unknown

concentrations of free binding partners but known total concentrations and known bound concentrations, the first part has been rewritten into the second part of the equation.

Equation 4 describes how the equilibrium dissociation constant K_D can be derived from the MST data using the fraction bound FB. In this formula the titrated partner is represented by A while the fluorescence will be measured from the traceable protein B for which the concentration is kept constant. The fraction bound is expressed as the total concentration of the free parameters and the K_D is the only free parameter. The fraction bound is directly reported by the F_{norm} in Equation 2 to derive the K_D from the MST measurements. Plotting the F_{norm} linearly against the logarithmic concentration of the titrated partner results in a characteristic S-shaped curve with a plateaus for the unbound and the saturated states of the traceable partner, as has previously been displayed in Figure 7: A) Normalized MST measurements for different concentrations of unlabelled protein with the initial fluorescence recorded in the blue highlighted area and the steady-state fluorescence recorded in the red highlighted area. B) F_{norm} plotted against the unlabelled protein concentration and fitted to determine the K_D of the binding measurement.-B.

$$FB = \frac{[AB]}{[B]} = \frac{[A] + [B] + K_D - \sqrt{([A] + [B] + K_D)^2 - 4 * [AB]}}{2 * B}$$

Equation 4: Formula to determine the K_D from the fraction bound as derived from the MST data.

Research Goal

It is known that α-synuclein is naturally mainly is present in the brain and especially at the end of the neurons. The location of α-synuclein is similar as the location of the SNARE proteins for the end of the neurons are the locations where synaptic transmission takes place[32]. The role of α-synuclein in synaptic transmission is still unclear however it has been observed that α-synuclein plays a role in synaptic transmission and is able to bind to the cell membrane[32]. The α-synuclein protein has been shown to bind with the SNARE-complex and has an affinity for the SNARE protein synaptobrevin[1]. Over expression of α-synuclein inhibits neurotransmitter release whereas knock-out of α-synuclein has been shown to result in a loss of docked synaptic vesicles[3], another process where SNAREs are involved. All in all, it has been observed that the α-synuclein has an effect on synaptic transmission, but what exactly the role is, that is still unknown.

The goal of this research is to investigate the interaction between α-synuclein and the SNAREs; SNAP25, synaptobrevin 2B and syntaxin 1a. The SNAREs assemble into the SNARE-complex in the cytosol of the neurons and also α-synuclein is located in the cytosol of the neuronal cells. Already studies have shown that the α-synculein has an affinity for the soluble C-terminal part of synaptobrevin[1]. The interaction between α-synuclein and the SNAREs will be investigated using only the soluble parts of the proteins. The hydrophobic transmembrane region will result in the protein to behave differently than natural conditions and leaving out regions of the protein embedded into a lipid bilayer will make the observes results more representable for the in vivo processes.
The SNAREs will be produced and the production will be optimized in such a way that the SNAREs can be acquired in sufficiently pure concentrations and high protein yields. The interaction between the SNAREs and α-synuclein will be studied in vitro using microscale thermophoresis under different conditions to investigate what affects the binding affinity of α-synuclein with these SNAREs and how the binding affinity is affected.

Results

Protein Production

Production of the SNAREs using the method as described in the 'Materials and Methods' has resulted in the desired proteins, however the SDS gel shows a relatively low protein production and a high level of impurities for both methods of cell lysis. A typical example of such a SDS gel is shown in Figure 8 here different fractions of syntaxin 1a are separated by the SDS-PAGE. The His_6-tagged syntaxin 1a protein has a molecular weight of 30.6 kDa and the location of this protein should be comparable to the position of the 31.0 kDa protein used in the ladder as a reference. Instead of finding a clear band at the height of the ladder, several bands representing different kDa's are found over several fractions.

The SDS gel displayed in Figure 8 shows that out of the complete protein solution the expressed protein has been successfuly filtered out of the protein mixture by the Ni^{2+}-affinity chromatography. The clear bands spread out over the different fractions are not abundantly present in the flow through, otherwise the flowthrough would have shown intense bands for these proteins. The bands in the gel which are smaller than the expected 30.6 kDa are impurities which can have been the result of degradation of the protein into smaller fragments by enzymatic activity or just natural breakdown of the protein itself. Bands larger than the expected 30.6 kDa can be the result of aggregation of multimers of the syntaxin 1a protein and complexes of degradation products with each other or with the protein.

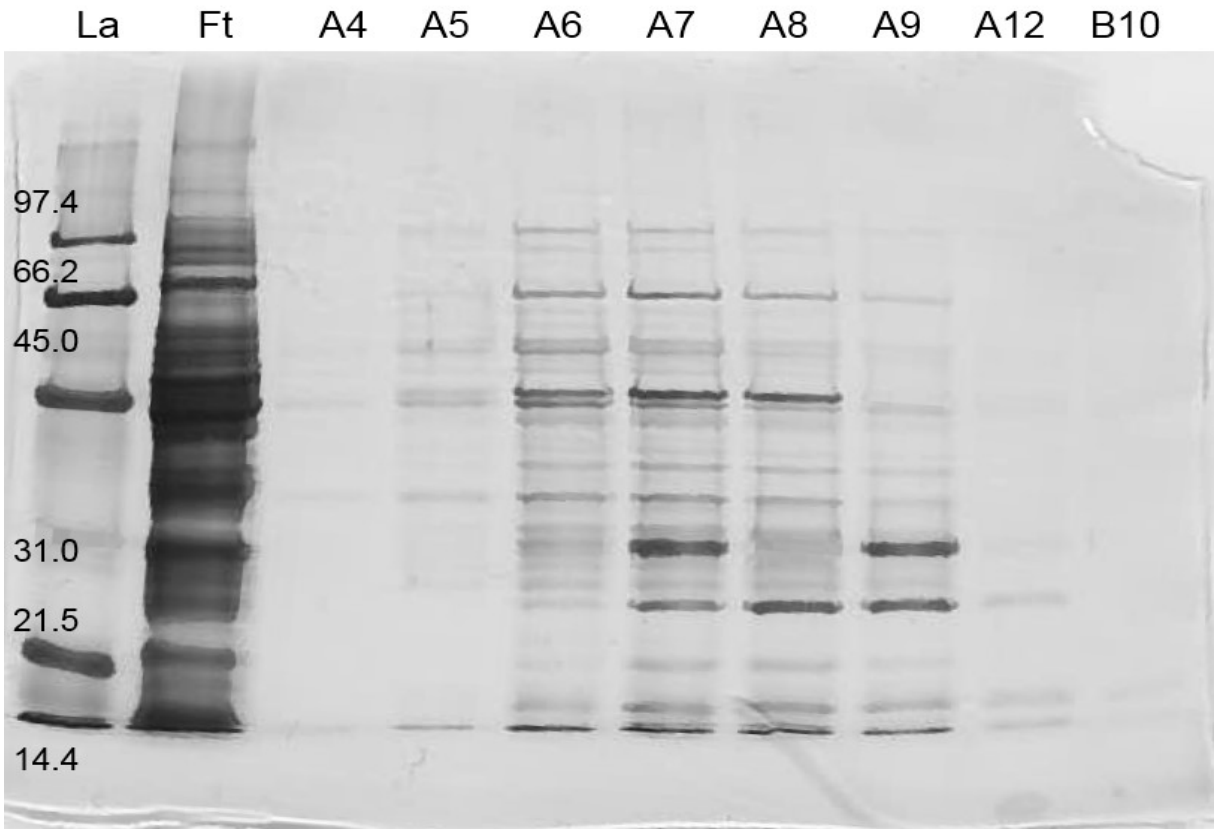

Figure 8: SDS gel (10% SDS) from SX1a purified by nickel affinity purification. The different columns loaded with samples are filled from left to right with the LA (ladder for size comparison of the proteins, MW in kDa),FT (flow through from nickel affinity chromatography) and A4-B10 (protein fractions as collected from nickel affinity chromatography)

The SDS gel in Figure 8 is representable for all the produced proteins using this method of protein production and purification for the initial plasmids as provided by DNF from the University of Lausanne. All the produced proteins showed a low protein yield and a high number of impurities. This method has resulted in a low concentration and insufficient purity of the proteins to perform experiment with. The results of the protein production needed to be improved to ensure better and more reliable method of SNARE production before these proteins can actually be used in further experiments.

Improving concentration and purity of SNAREs

Step by step, the road to acquiring the desired SNAREs has been analysed and improvements for purity and protein yield have been implemented. This has led to a series of repeated processes of protein production in which each time only one aspect of the protein production has been changed from the initial experiment in an attempt to reach better SNARE production. The outcome of these attempts for improvement are compared by the SDS-PAGE gel with additional controls.

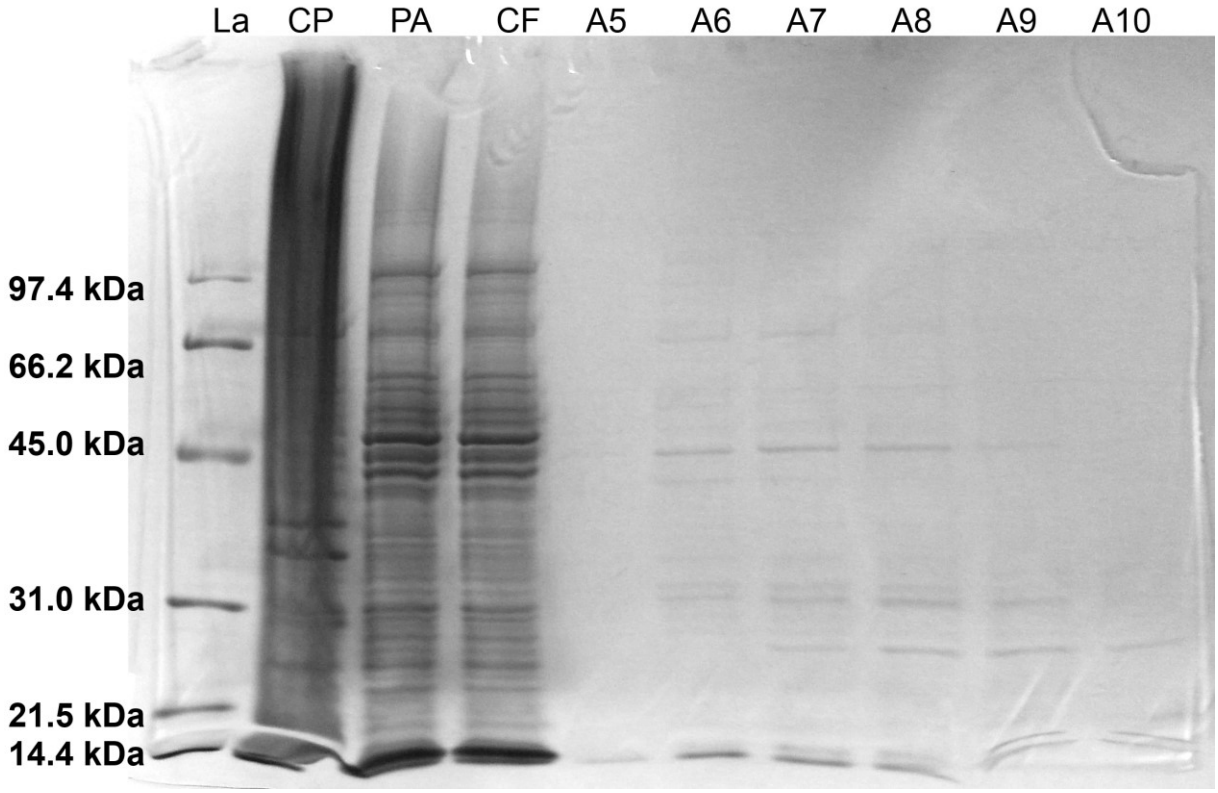

Figure 9: SDS-PAGE (10% SDS) gel of syntaxin 1a. In this gel the above mentioned control samples are added to see if protein acquisition from the cell lysis suspension has been successful and to monitor the effectiveness of the Ni^{2+}-affinity chromatography. From left to right La (ladder), CP (cell pellet after cell lysis), PA (solution before Ni^{2+}-affinity chromatography), CP (collected fluid, after Ni^{2+}-affinity chromatography) and A5-A10 are the fractions likely to contain the syntaxin 1a protein. The bands visible in the PA sample are only slightly visible in the CP solution, this indicates that the cell pallet does not contain high concentrations of proteins. The effectiveness of Ni^{2+}-affinity chromatography is hard to determine based on the PA and CF samples due to the low concentration of proteins in the fractions.

These controls are a samples of the cell pellet after cell lysis, the solution before it is purified by nickel affinity chromatography and the flowthrough from the nickel affinity chromatography. A sample of the cell lysis pellet is taken and used observe if any proteins still reside in the cell pellet instead of the extracted supernatant, this is shown in Figure 9. The samples of the solutions before and the flowthrough after the nickel affinity chromatography are used to monitor its effectiveness in

filtering the His_6-tagged proteins out of the protein solution, also shown in Figure 9. With these extra controls the following attempts to improve protein production and purification can be monitored better.

- The first step where things could have gone wrong has been during the transfection of the bacterial DNA with the plasmid. Complications with the transfection of the SNARE plasmids can have resulted in transferring damage plasmids into the bacterial DNA resulting in production of these damaged plasmids. To rule out that something has gone wrong with the transfection of the bacteria with the provided plasmids this process of bacterial transfection is repeated. After control of the protein production by SDS-PAGE the proteins still showed low yield and a relatively high amount of impurities. Repeated transfection of bacteria with the provided plasmids has shown no improvement from the initial protein production. This means that the first time transfection of the bacterial cells with the plasmids went successful and no further problems have arisen during transfection of the bacteria.

- As the transfected bacteria have shown no improvement in protein production the next step in the protein production process is induction of the bacteria to produce the desired proteins. Improvement of this step has been attempted by using a fresh batch of promotor. If there was something wrong with IPTG promotor used this could also have resulted in a reduced protein production. Using a new batch of IPTG the quality of initially used IPTG can be compared by running a SDS-PAGE gel with the new proteins. The gel showed no significant effect on protein purity or yield using the new batch of IPTG.

- The conditions during the protein production affect the outcome. Initially a temperature of 37 °C had been used for a duration of 4 hours. This temperature had been selected because at this temperature the enzymatic activity in the bacteria has been increased to stimulate production of proteins. However the increased enzymatic activity as a result of the elevated temperature is not restricted to enzymes taking part in the production of proteins, all enzymatic activity is increased. This includes enzymes playing a role in removal of proteins in the bacteria which can result in degradation of the desired SNAREs and affect purity and protein yield. To reduce the degradation of proteins by enzymes a colder temperature would be preferable but this would in turn reduce the production of proteins by other enzymes. In an attempt to shift the balance of produced proteins in favour of protein production the bacteria are induced at 20 °C and to compensate for reduced productive activity the induction period has been increased to 16 hours. Running a SDS-PAGE gel for this adaptation in protein production has resulted in similar production and quality as initially achieved. Reducing the temperature over an extended period of time has not resulted in an increase in protein production or increase in quality.

- After induction of the bacteria the protein needs to be extracted from the bacterial cells without damaging the protein itself. Initially a commercial mixture, BugBuster® Master Mix, has been used to stop degradation of the proteins from enzymatic activity in the cells and destroy the cells without damaging the protein itself. However it is unknown what exactly is in the BugBuster® Master Mix and there might be too few ingredients to protect the proteins or even contain components which can damage the SNAREs. To see if the BugBuster® Master

Mix has a negative effect on protecting the SNAREs a homemade lysis mixture is used to destroy the bacterial cells and easily extract the SNAREs. After confirmation of the concentration and the purity by SDS-PAGE, no improvement in these aspects has been observed. The method of cell lysis by adding the BugBuster® Master Mix has not turned out to be harmful for the SNAREs, has sufficiently protected the proteins and disabled any enzymes from harming the proteins.

New constructs

Each attempt of improvement took two weeks of time if all went well, taking into account all the steps from planning the adjustments to the procedure all the way up to evaluation of the results. After three months of trying to find out how to improve the production yield and protein quality have been in vain and no results have led to an improvement up to this point. All that could be concluded thus far was that the purification of the proteins was successful. All the steps from transfection up to cell lysis have been subject to attempts for improvement and only the quality of the constructs used for transfection have not been changed. These construct might have been damaged prior to the start of the experiments during transport or constructs unsuitable for our method of protein production might have been provided.

New plasmids were once again acquired from the DNF group from the University of Lausanne and with these plasmids BL21 bacterial cells are transfected. The proteins are produced using the initial method of protein production; standard induction of protein expression and the commercial BugBuster® Master Mix method of cell lysis to extract proteins from the bacterial cells. SDS-PAGE has been performed to function as a control for the purity and yield of protein production.

A typical example of the SDS gels is displayed in Figure 10 in which the SNAP25 protein is visualized. These new constructs result in a much higher yield and purity of proteins. The increase in protein yield can be observed by comparing the intensity of the ladder with the intensity of the protein bands in the collected fractions as the concentration of the ladder has remained the same. In the previous gels the bands in the ladder were of higher intensity when compared with the collected proteins but with the new proteins the bands of the proteins are much higher than the bands of the ladder. The ladder used contains the same concentration of proteins and only the concentration of protein in the fractions has changed.

In Figure 10 the role of the CP, PA and FT samples are also visualized better than with the low protein yield SDS gels. Amongst all the components residing in the cell pellet after cell lysis the SNAP25 protein can still be recognized to be present in the cell pellet. It is near impossible to extract all of the proteins out of the pellet so trace amounts will always be present here. The supernatant after cell lysis is the solution right before the Ni^{2+}-affinity chromatography, but not filtered with a 0.2 µm membrane. In this solution the presence of the SNAP25 is clearly visible and even higher then in the cell pellet. Comparing the samples before and after Ni^{2+}-affinity chromatography (PA and FT) the band around 20 kDa has greatly diminished indicating that the protein has been extracted successfuly from the solution. In the collected fractions the intensity of the SNAP25 protein is greatly higher than for the solutions before Ni^{2+}-affinity chromatography.

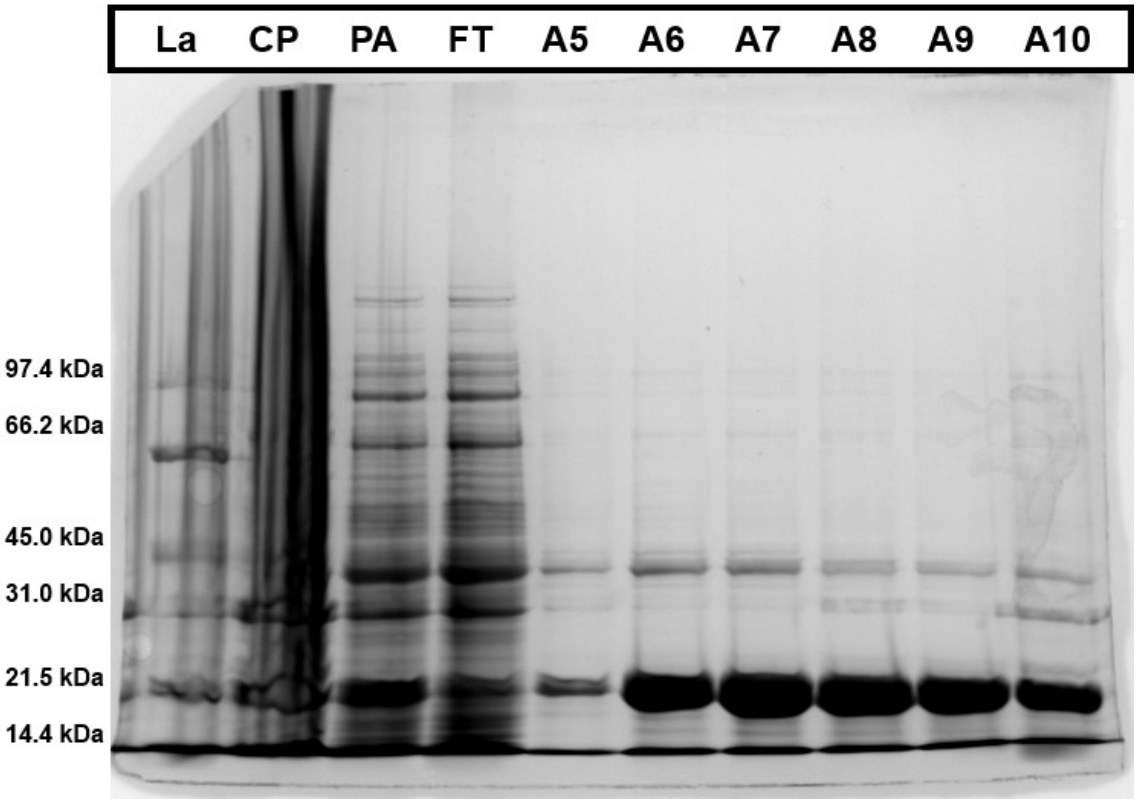

Figure 10: SDS gel (15% SDS) of SNAP25. The different columns loaded with samples are filled from left to right with the LA (ladder for size comparison), CP (sample of the cell pellet after cell lysis), PA (sample before nickel affinity chromatography), FT (flow through from nickel affinity chromatography) and A5-A10 (protein fractions as collected from nickel affinity chromatography).

With these new constructs production and purification of the SNAREs is successful and results in high yield and purity of the protein. The fractions showing the best yields and purity are pooled together and the protein yield is displayed in Table 1.

Table 1: Overview of the quantity of produced proteins using the new constructs

Protein	Volume (ml)	Concentration
SNAP25b	10	26.6 µM
Syntaxin 1a (aa 1-262)	12	37.0 µM
Syntaxin 1a (aa 1-262)	10	47.2 µM
Synaptobrevin (aa 1-96)	11	19.9 µM
Synaptobrevin (aa 1-96)	11	35.2 µM

Binding affinity determination

The protein interaction between α-synuclein and the SNAREs has been studied using microscale thermophoresis (MST). The used proteins are produced, purified, labelled and further concentrated by means of centrifugation as described in the 'Materials and Methods'. Due to the nature of the MST experiments and the use of a serial dilution the concentration of the produced proteins needed to be increased before it was high enough to be used in the binding affinity experiments as shown below. All the MST binding affinity measurements are performed at 20% of the MST IR-laser at buffer conditions of 10 mM Tris pH 7.4, 0.5 mg/ml BSA and 0.05% Tween dissolved in MilliQ-water unless specified differently.

α-Synuclein with Synaptobrevin B2

Previous studies have indicated that there is a stronger interaction between the binding of α-synuclein and the synaptobrevin B2 protein then with the other SNAREs involved in neurotransmitter release in presynaptic terminals[1]. How strong these interactions between α-synuclein and the SNAREs are has never been reported previously, while this will provide crucial information regarding the role of α-synuclein on the SNARE-complex. The dissociation constant (k_D) is commonly used to describe the affinity for protein interactions[33]. Because it has been indicated that the interaction between α-synuclein and synaptobrevin B2 is stronger than with other SNAREs the binding between these two proteins will be worth looking into to discover the strength of the interaction.

Examples of the resulting dissociation equilibria for different ratios of labelled and unlabelled protein can be found in Figure 11 and Figure 12. In the results of Figure 11 α-syncuclein has been labelled while synaptobrevin has been used for the series dilution and for Figure 12 synaptobrevin has been labelled and a series dilution of α-synuclein has been used. Using both proteins as the labelled and unlabelled partner investigates effect of the choice of labelled binding partner on the calculated dissociation constant. The choice of labelled protein should not have an effect on the resulting dissociation coefficient in the interaction as the calculated k_D is dependent on the ratio of free binding partners to the complex[33].

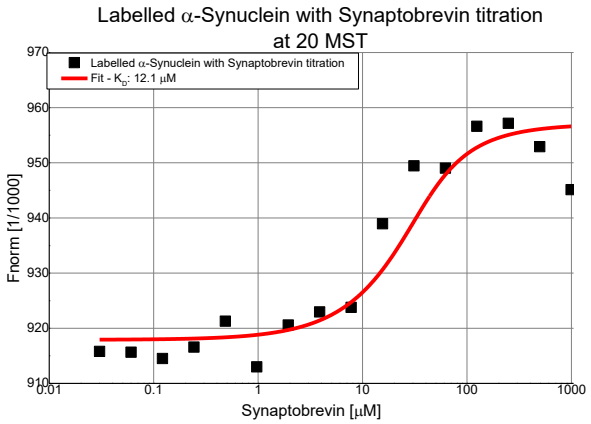

Figure 11: Determination of the binding affinity between labelled α-synuclein and using a synaptobrevin titration series at 20% of the IR-laser for MST results in a dissociation coefficient of 12.1 µM.

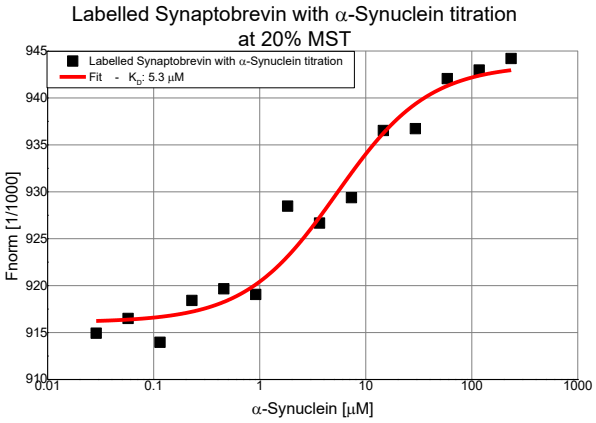

Figure 12: Determination of the binding affinity between labelled synaptobrevin and using a α-synuclein titration series at 20% of the IR-laser for MST results in a dissociation coefficient of 5.3 µM.

The results of Figure 11 and Figure 12 show that MST is an effective method to investigate the affinity between α-synuclein and synaptobrevin and derive the dissociation constant of these experiments. Both the binding curves in Figure 11 and Figure 12 show plateau formation, where the effects of further concentration change has diminished effects on the resulting equilibrium, below 0.1 µM and above 100 µM of the titrated partner. Fitting of these curves results in a dissociation constant, k_D, in the range of 5-15 µM, this goes for the results displayed in Figure 11 and Figure 12 and for results not displayed.

While the range in k_D from 5 µM to 15 µM seems broad, this is only a slight change when realizing that the scale of the x-axis is logarithmic and a change from 5 to 15 µM on these axis will only result in a slight shift in position of the fitted curves in Figure 11 and Figure 12. The determined binding affinity for the reaction between these binding partners is in a range typical for most intrinsically disordered proteins like α-synuclein[34]. Besides documenting the affinity for α-synuclein with synaptobrevin for the first time, these results also confirm that the choice in labelling one of the binding partners does not affect the resulting dissociation constant.

Interaction under denatured conditions

The protein solutions have been denatured, using a solution of 2% v/v SDS and 20 mM DTT, and their binding affinity for these proteins have been determined once again. During the denaturing process, SDS forces the proteins to lose its secondary structure while DTT breaks covalent disulphide bonds in and among proteins. Without secondary structure and covalent bonds only smaller forces (e.g. electrostatic attraction and Van-der-Waals forces) affect the interaction between these two proteins. With only small forces aiding the interaction between these two protein, it is expected that no binding takes place in the denatured solution. Any change in signal still remaining is not the result of typical protein binding, so denaturing of the protein solutions is performed to confirm that the observed signal change is the result of protein interactions.

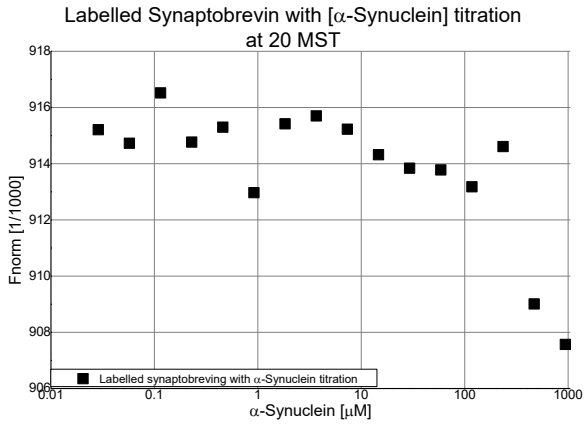

Figure 13: Determination of the binding affinity of a denatured solution of labelled synaptobrevin and using a α-synuclein titration series at 20% of the IR-laser for MST

A typical example of the MST measurement on a denatured sample has been shown in Figure 13, this is the denatured solution from the experiments shown in Figure 12. Comparing Figure 13 with Figure 12, it can be observed that in the denatured solution there is no change in F_{norm} to indicate an interaction which resembles the effect observed in Figure 12. In fact the F_{norm} observed in the denatured solution has a value comparable to the F_{norm} found at the plateau formed for low concentrations of α-synuclein in Figure 12. At these low concentrations in Figure 12 there are little interactions occurring between the two proteins and nearly no complexes of these two proteins are present. This shows that in the denatured solution no interactions between proteins occur as there is no change in F_{norm} upon increasing the concentration of α-synuclein. The few slight variations in F_{norm} for the highest concentrations of α-synculein in Figure 13 can be contributed to pipetting errors or sedimentation of the labelled partner. Since not interactions between proteins are shown in denatured solutions, all

change in F_{norm} upon increase in unlabelled protein in natural conditions can be contributed to interaction between the proteins and thus resulting in complex formation.

For all other experiments performed the experiment has been repeated under denatured conditions with similar results. No interactions have been monitored under denatured conditions and a F_{norm} comparable to the lower plateau is recorded. The signal change of F_{norm} in natural conditions upon binding has been confirmed to originate from binding of the proteins, as denaturing the solution prevents the binding reaction to take place and results no F_{norm} change can be observed.

Charge screening effects on α-synuclein and synaptobrevin interaction

As binding reaction between α-synuclein and synaptobrevin has been confirmed using MST, the details of this binding reaction can be investigated more in detail. In this section this interaction will be monitored for charge dependence of the proteins by investigating charge screening by adding NaCl to the solution.

Ions of similarly charge repel each other in solution which results in small regions with fewer ions of that particular charge. This local depletion of ions results in a screening hole for oppositely charged particles such as proteins. Increasing the number of ions in the solution reduces the effect in which the charged particles can repel each other,hereby reducing in a reduced electric field created by an individual ion as there another ion is in range to counteract the created electric field. Increasing the concentration of ions in the solution will result in effectively reducing the distance at which all charged particles can attract or repelling each other. This allows the all charged particles to approach each other unhindered to closer distances which in turn increases the binding between charged particles without being repelled.

Using labelled synaptobrevin and an α-synuclein dilution series the binding affinity has been observed under different conditions of NaCl (1, 10 and 100 µM) determine the effect of charge screening on the binding affinity of these proteins. The resulting equilibria of binding for the different concentrations of α-synuclein under different conditions can be observed in figures Figure 14-16. An overview of the found k_D's at the different NaCl concentrations is shown in Figure 17.

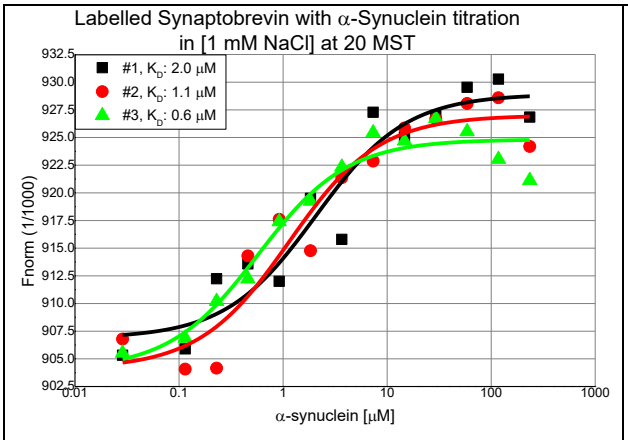

Figure 14: Repeated experiments to determine the binding affinity between labelled synaptobrevin and using an α-synuclein titration series under 1 mM NaCl conditions show very similar curves with clear plateau formation for α-synuclein concentrations higher than 50 µM. The α-synuclein concentrations used are too high to observe the lower plateau but based on the fitting it is expected that the plateau is located around 0.05 µM of α-synuclein. The binding affinity has been studied using MST at 20% of the IR-laser and has resulted in dissociation constants ranging from 0.6 to 2.0 µM.

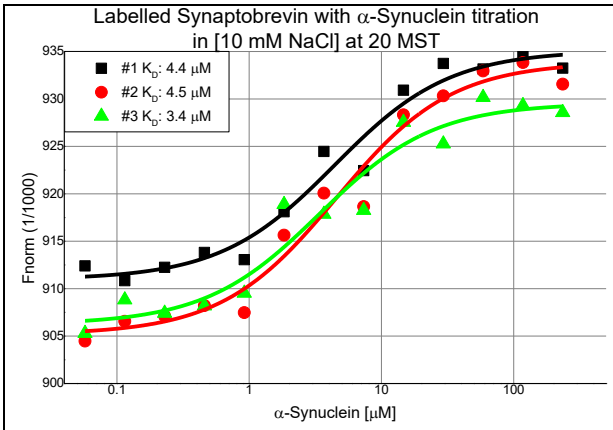

Figure 15: Triple repeated measurements of labelled synaptobrevin and using an α-synuclein titration series at 10 mM NaCl conditions show similar and overlapping curves with clear plateaus formed. The lower plateau and the higher plateau starting to occur around 0.1 µM and 100 µM of α-synuclein respectively, further increasing the concentration of α-synuclein has no effect on the recorded F_{norm} beyond these plateaus. The binding affinity of all three experiments has resulted in dissociation constants ranging from 3.4 to 4.5 µM.

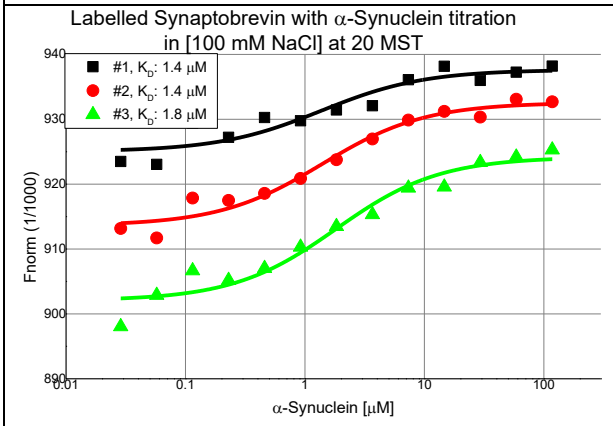

Figure 16: Triple repeated between labelled synaptobrevin and using an α-synuclein titration series at 100 mM NaCl conditions show similar trends in the F_{norm} upon increasing α-synuclein concentration. Formation of plateaus occurs below 0.2 µM and above 20 µM of α-synuclein with no overlap between the similar curves. While the trend in F_{norm} upon increasing α-synuclein remains similar for all three experiments, the F_{norm} drops steadily for each repeat. Despite the drop in F_{norm} for every next experiment this does not result in a large change in dissociation constants as these range from 1.4 to 1.8 µM. The steady drop in F_{norm} for next concentrations can be a result of starting self-aggregation of α-synuclein which has been known to occur more rapidly at high NaCl concentrations[5,6] or an error in sample preparation resulting in less labelled protein in the MST LED focus.

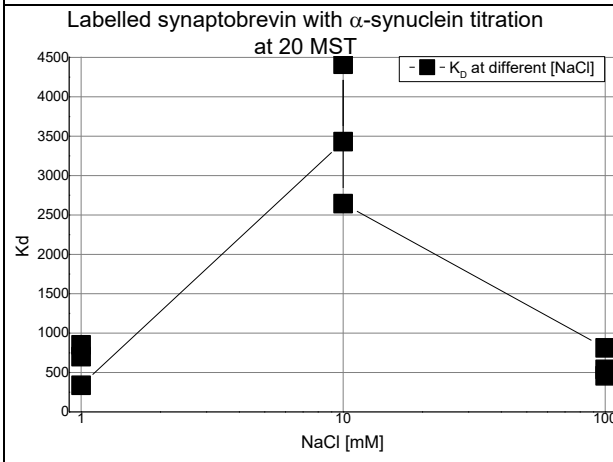

Figure 17: Overview of the dissociation coefficients found at the different NaCl concentrations used on the binding reaction between synaptobrevin and α-synuclein.

The effects of NaCl on the binding affinity are shown in an overview in Figure 17. From this figure it can be observed that there is no significant trend in the binding affinity as a result of charge screening. The discovered k_D's for the 10 mM NaCl conditions are all higher than the other buffer conditions, but this effect only occurs at this specific condition while any affect of charge screening on the binding affinity would result in a trend increasing with NaCl concentration instead of a sudden peak at one point. While the change in dissociation constant is seems significant compared to the other measured NaCl concentrations, however the change in is minor considering the amount of

NaCl added to the solution. Even when comparing the results of binding affinity under 100 mM NaCl conditions with 10 mM NaCl conditions the change is less then an order of magnitude.

When comparing the binding affinity of α-synuclein without any added NaCl (5-15 µM) to measurements with NaCl added (0.6-4.5 µM) only a small change is detected, while for interactions sensitive to charge screening typically larger changes are observed. The binding of α-synuclein to synaptobrevin is not very sensitive to NaCl changes in the solution, there are some changes but nothing to indicate a strong influences as a sensitivity for charge screening.

Interaction of α-synuclein with SNAP25 and syntaxin 1a

The binding of α-synuclein with synaptobrevin has thus far been investigated in detail because previous studies have shown that these two proteins bound together[1]. The same study has not presented any evidence that the other two SNAREs involved in presynaptic neurotransmitter release has an interaction with α-synuclein. It is important to rule out any interactions between α-synuclein and these other SNARE proteins to allow further studies to focus solely on the interaction of α-synuclein with synaptobrevin.

To confirm or rule out affinty of α-synuclein with SNAP25 and sytaxin the interaction has been observed using MST. During these measurements the α-synuclein protein was labelled and the SNARE proteins, either SNAP25 or syntaxin 1a, were used in a serial dilution series to derive the affinity of binding by acquiring the dissociation constant. The results of these measurements are displayed in Figure 19 and Figure 18.

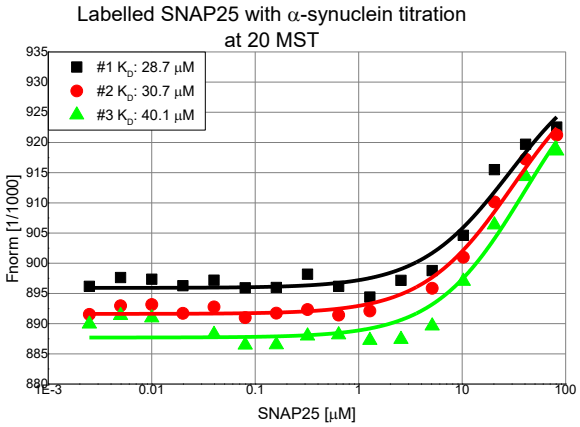

Figure 19: Repeated experiments to determine the binding affinity between labelled α-synuclein and using a SNAP25 titration series at 20% of the IR-laser for MST have resulted in a dissociation coefficients ranging from 28.7 to 40.1 µM.

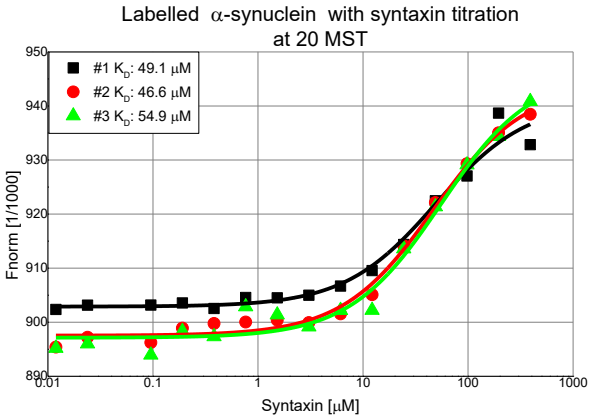

Figure 18: Repeated experiments to determine the binding affinity between labelled α-synuclein and using a syntaxin titration series at 20% of the IR-laser for MST have resulted in a dissociation coefficients ranging from 46.6 to 54.9 µM.

The binding affinities of binding interactions of α-synuclein with SNAP25 and syntaxin 1a are displayed in Figure 19 and Figure 18, respectively. For both interactions the lower plateau is clearly visible, below the concentration of 1 µM of the titrated binding partner, whereas the upper plateau is not visible for the concentrations used. The repeated experiments clearly show similar curves showing consistency for each of the experiments. Without a clearly defined upper plateau the dissociation constants cannot be determined accurately, however it is clear that the k_D's for both interactions are significantly higher than the α-synuclein-synaptobrevin binding as shown earlier (Figure 11 and Figure 12). The determined dissociation for these interactions are at least an order of magnitude higher resulting in a lower affinity between the proteins. Most likely the dissociation

constants are even higher then indicated as the previous experiments have shown that the upper plateau is typically 10^4 times as high as the lower plateau. If this usual spread also goes for the interaction between α-synuclein and these SNAREs this would result in an expected dissociation constant around 100 µM.

The interaction between α-synuclein and the SNAREs SNAP25 and syntaxin 1a has significantly higher dissociation constant than between α-synuclein and synaptobrevin b2. Without having reached the upper plateau, and thus fully binding the labelled protein, the dissociation constant is estimated to be at least an order of multitude higher but this can become even higher depending on where exactly the upper plateau is positioned. This confirms previous studies in which it also has been shown that α-synuclein has an interaction with synaptobrevin, in addition here it is shown that α-synuclein also has an interaction with SNAP25 and syntaxin 1a but a much weaker one[1].

Effect of Ca^{2+}-ions on the binding of α-synuclein with synaptobrevin

Now that it has been determined that α-synuclein interacts much stronger with synaptobrevin 2B than with the SNAP25 or the syntaxin 1a protein, the role of α-synuclein on the SNARE complex can be investigated. The SNARE complex is a coiled-coil complex which regulates the release of neurotransmitters in the presynaptic vesicles. The neuronal signal transduction occurs by an action potential, the propelling a positive charge through the normally negatively charged neuronal cell. This propelling charge throughout the neuronal cell is the action potential and the directional movement is a result of opening of voltage-gated ion channels once the charge in the cell has exceeded the action potential threshold. These ion channels can be based on calcium, allowing Ca^{2+} ions to enter the neuronal cell and hence pass on the action potential. The once the calcium driven action potential reaches the end of the axon the presynaptic vesicles are released into the synaptic cleft to pass on the signal from one cell to another. At the end of the axon the Ca^{2+} concentration regulates the release of presynaptic vesicles which are docked and primed by the SNARE-complex.

The SNAREs, including synaptobrevin, are closely involved in the Ca^{2+}-triggered synaptic transmission[2,13,14] and α-synuclein has been shown to be able to affect the synaptic transmission[1,4,32], it might be that Ca^{2+} has an affinity with either one or perhaps both of these proteins. To determine whether Ca^{2+} has an interaction with these proteins, proteins are labelled and $CaCl_2$ is used for the serial dilution so the concentration of Ca^{2+}-ions in the solution increases.

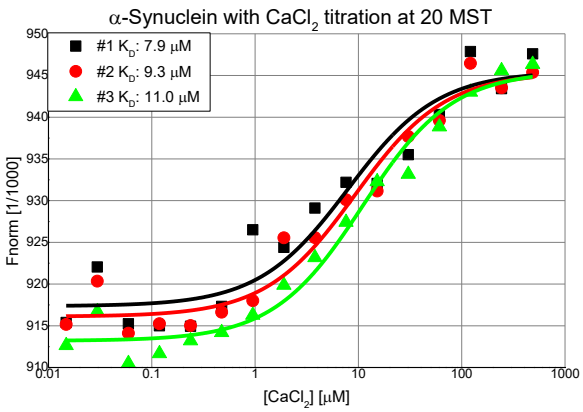

Figure 21: Experiments to determine the binding affinity between labelled α-synuclein using a $CaCl_2$ titration series. The binding affinity has been studied using MST at 20% of the IR-laser and has resulted in dissociation coefficients ranging from 7.9 to 11.0 µM.

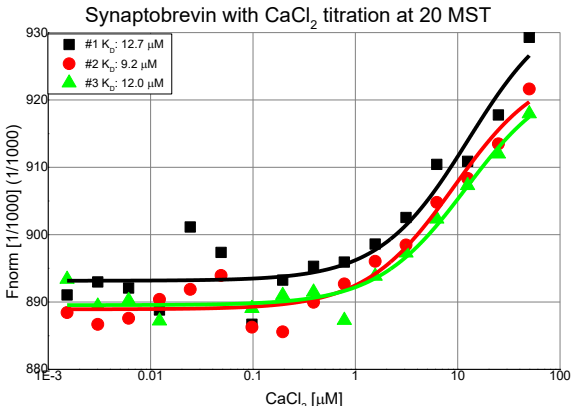

Figure 20: Experiments to determine the binding affinity between labelled synaptobrevin using a $CaCl_2$ titration series. The binding affinity has been studied using MST at 20% of the IR-laser and has resulted in dissociation coefficients ranging from 9.2 to 12.7 µM.

In Figure 21 and Figure 20 the results of MST measurements of Ca^{2+} binding with respectively α-synuclein and synaptobrevin are displayed. For both experiments the lower plateau can be observed below 0.1 µM of $CaCl_2$ however in both experiments the upper plateau has not been reached. For the interaction between α-synuclein and $CaCl_2$ it looks like the upper plateau is nearly reached, however this cannot be said for sure as not enough measurements have been performed for the higher concentrations of Ca^{2+}. Both proteins clearly show an affinity for Ca^{2+} and the binding affinity is currently estimated to be at least in the range of 10 µM and probably even higher depending on the location of the upper plateau. These results show an affinity between Ca^{2+} and α-synuclein and synaptobrevin, the next experiments have been performed to investigate what role Ca^{2+} plays on the interaction between these two proteins.

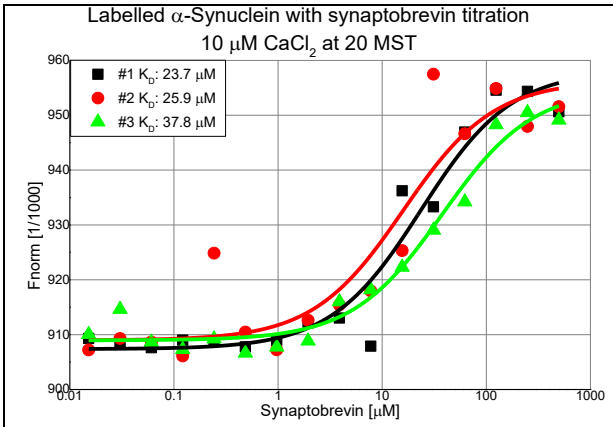

Figure 22: Experiments to determine the binding affinity between labelled α-synuclein and a synaptobrevin titration series at 10 µM $CaCl_2$ buffer conditions. The lower plateau, where further decreasing the concentration has no effect on the F_{norm}, is located near 1 µM while the upper plateau starts around 200 µM. The curves of the repeat experiments are very similar to each other, however all these repeated experiments have a few data points which deviate from the general trend. These points need not to be taken into account when deriving the k_D as these points are clearly faulty as they do not appear in the other measurements. The fitting this data has resulted in dissociation constants in the of 23.7 to 37.8 µM and the fitter curves correspond with the data points (not taking into account the deviating points).

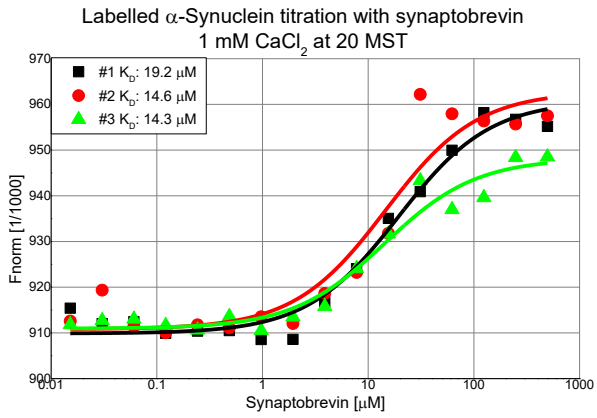

Figure 23: Experiments to determine the binding affinity between labelled α-synuclein and a synaptobrevin titration series at 1 mM $CaCl_2$ buffer conditions. The repeated experiments result in similar curves with a lower plateau fully formed below 0.5 µM and the upper plateau being formed above 250 µM. Each of the repeated experiments has some data points which do not agree with the general trend set by the other points, these points are likely the result of pipetting errors and should not be taken into account when determining the k_D. Fitting of these data points has resulted in dissociation constants in the range of 14.3 to 19.2 µM.

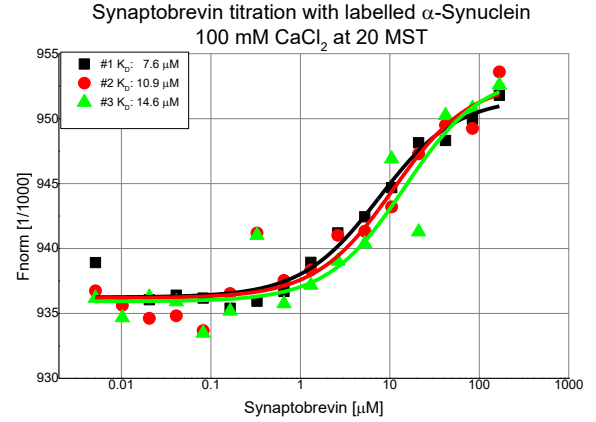

Figure 24: Experiments to determine the binding affinity between labelled α-synuclein and a synaptobrevin titration series at 100 mM $CaCl_2$ buffer conditions. The repeated experiments result in similar and overlapping curves with a lower plateau fully formed below 0.5 µM and the upper plateau still not fully formed at 100 µM. Each of the repeated experiments has some data points which do not agree with the general trend set by the other points, these points are likely the result of pipetting errors and should not be taken into account when determining the k_D. Fitting of these data points has resulted in dissociation constants in the range of 7.6 to 14.6 µM and even though the upper plateau has not been fully formed the fitted curves match the trend set by the data points. This makes the derived dissociation constant reliable despite the absence of the fully formed upper plateau.

The measurements to monitor the effect of Ca^{2+} on the binding of α-synuclein with synaptobrevin are performed using labelled α-synuclein and using a serial dilution of synaptobrevin under conditions with a different concentration of $CaCl_2$ (10 µM, 1 mM and 100 mM) added to the solution. This results in different concentrations of Ca^{2+} present when the two proteins bind together and any affect of Ca^{2+}-ions on the interaction between α-synuclein and synaptobrevin will result in a change in the dissociation constant for the different concentrations of $CaCl_2$.

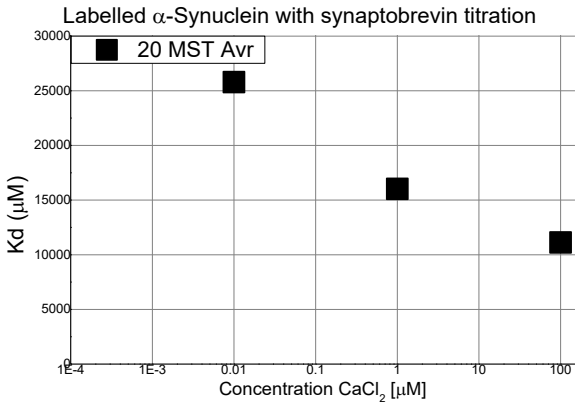

Figure 25: Overview of the average dissociation coefficients found in Figure 22-25 at the different $CaCl_2$ concentrations used on the binding reaction between synaptobrevin and α-synuclein.

The effects of the $CaCl_2$ concentration on the binding affinity between α-synuclein and synaptobrevin are shown in Figure 22-24. In the overview of Figure 25 the effect of Ca^{2+} on the binding of these two proteins is shown. For low concentrations of Ca^{2+} the dissociation constant for interaction between α-synuclein and synaptobrevin is around 25 µM and increasing the Ca^{2+} concentration results in lowering of the dissociation constant and thus increasing the affinity between these proteins.

In neuronal cells the concentration of Ca^{2+} is typically very low and only during the propagation of the action potential the voltage-gated ion channels open up to allow the Ca^{2+} to enter the cell. Typical calcium concentrations during neuronal rest are around 0.1 µM while during the action potential the Ca^{2+} concentration rises up to 100 µM[7]. When comparing this information with the overview in Figure 25, it appears that the dissociation constant of interaction between α-synuclein and synaptobrevin drops upon increasing the Ca^{2+} concentration. During resting conditions of a neuron these two proteins are less likely to bind while upon arrival of the action potential these proteins have a higher affinity for each other.

Both α-synuclein and synaptobrevin proteins have shown to have an affinity for Ca^{2+} (Figure 21 and Figure 20) with an dissociation constant of at least 10 µM and likely higher, depending on the exact location of the upper plateau. Upon interaction of a proteins with an ion this is likely to induce a configurational change upon the protein. Under conditions of neuronal rest neither α-synuclein nor synaptobrevin are likely to have bound to Ca^{2+} as they have a low affinity for the ion. However upon concentrations comparable to arrival of an action potential at the end of a neuron (100 µM), both proteins are likely to have bound Ca^{2+} ions. Under these conditions the interaction between α-synuclein and synaptobrevin is also more likely to occur as seen in Figure 25. This makes it likely that an interaction of Ca^{2+} with the proteins α-synuclein and synaptobrevin induces a configurational change on these proteins which in turn increases affinity between α-synuclein and synaptobrevin upon the arrival of an action potential.

The increased affinity between α-synuclein and synaptobrevin upon Ca^{2+} concentration might imply that α-synuclein binds to synaptobrevin upon arrival of the action potential and thus starts affects the SNARE-complex also upon the arrival of the action potential. The timing of α-synuclein binding to synaptobrevin, and thus likely affecting the SNARE-complex, corresponds to the timing of the release of presynaptic vesicles into the synaptic cleft. Based on these findings α-synuclein might play a

regulatory role in cell-cell signaling by affecting the controlled release of the neurotransmitters in the presynaptic vesicles. These findings are in line with other studies which have shown that α-synuclein affects the signal transduction in neuronal cells[2,4]. However more research is required before this direct interaction can be confirmed.

Discussion

Protein production and purification

During the initial production of the SNAREs a lot of time has been invested in attempts to improve the protein production both in protein purity and protein yield. With these constructs all the attempted adjustments (repeated transfection of new bacterial cells, new promotor activator, other method of induced protein production and different bacterial lysis) have not resulted in the desired improvements in protein production. Using new bacterial constructs resulted in a successful protein production which met the standards previously attempts failed to reach. Why protein production with the new constructs was more success is unknown and, as the requirements were met, no further optimization has been attempted. However production of SNAREs might have had even better results by putting some effort in optimizing the production of these new constructs.

The produced SNAREs still contain the HisTag region required for purification attached to the original protein. While this region does not typically hinder the interaction of the protein, it can't be ruled out that this this region influences the interaction and functionality of the SNAREs.

Binding affinity determination

For several experiments to determine the dissociation constant of the interactions for protein-protein interactions or protein-ion interactions some of the lower or upper plateaus have not been fully formed and observed. These plateaus are used for fitting the dissociation constant to the data points and without both plateaus fully formed the exact dissociation constants cannot be determined. For many measurements the plateau is not fully formed but still the trend is there and this allows for a good indication where the plateau is located and an accurate estimation of the dissociation constant can be acquired. However there are some measurements for which no plateau has been formed and for these measurements it's hard to fit a k_D to these data points.

Some of the experiments are performed using α-synuclein for the serial dilution resulting in high concentrations of α-synuclein in the solution. The α-synuclein proteins has been known to self-aggregate under some conditions, especially under high NaCl conditions[35,36]. Even as the measurements have shown not indication self-aggregation of α-synuclein, it cannot be ruled out for sure that the α-synuclein has not started to aggregated. For low concentrations of α-synuclein the self-aggregation of α-synuclein is not likely to happen.

There are some measurements which show large differences between the three repeats of each experiment. These repeats seem to deviate more and more for increasing number of repeats of the experiment, thus how much has time between the first and the next measurements. While for these measurements the trend in the measurements is still comparable, the value for F_{norm} drops as the time between the measurements increases. This can be an effect of sedimentation of the labelled protein or adhesion of these proteins to the wall of the eppendorfs. All in all this has not shown to have an effect on the observed dissociation constants, however the observed change over time cannot be ignored and should be observed critically.

It is important to keep in mind that these experiments are performed in vitro and under costum conditions. These conditions are not comparable to the in vivo situation as under these conditions other proteins and ions are present. Natural conditions need not to have different results on the interactions observed in this work, however the possibility should be kept in mind.

Conclusion

The goal of this research was to study the interaction of α-synuclein with the soluble parts of the SNARE proteins under different conditions. The results from these in vitro studies will provide insight on the role of α-synuclein on the fusion mechanism of presynaptic vesicles occurring during synaptic transmission.

The soluble parts of the SNAREs have been produced in our own labs at the University of Twente. The first production rounds resulted in the desired proteins but very little of it and mixed with a lot of impurities making these products unsuitable for further experimentation. Attemprs were made to improve the purity and yield of the proteins produced but these changes did not result in meeting the desired standards. With the acquisition of new plasmid constructs the protein production has greatly been improved, both in purity and production yield of a single production cycle, and the results were well above the required standards. The reason why these protein production with these new plasmids was so successful is unknown, but confirmation by SDS-PAGE showed that the produced proteins were the SNAREs and the protein purity and quantity was sufficient for further experimentation.

Determination of the dissociation constant for protein interactions has been performed using MST. This technique has resulted in similar results in dissociation coefficient for the interaction between α-synuclein and synaptobrevin. For the MST measurements of protein-protein interactions the choice of which of the binding partners to label has no effect on the resulting detected dissociation coefficient. Preventing the proteins to interact by denaturing the proteins (SDS and DTT) results in no change in MST signal over the titration series, thus confirming that the MST signal only changes upon binding of the proteins. These effects have shown that to determine the affinity between proteins, MST proves to be a reliable tool, even allowing the user to determine which of the binding partners to label, to monitor the affinity for the interaction.

The out of all the one-on-one interactions of α-synuclein and one of the SNAREs, the α-synuclein proved to have a higher affinity with synaptobrevin than with SNAP25 or syntaxin. These findings confirm other studies which were able to detect interaction between α-synuclein and synaptobrevin but did not find interaction between α-synuclein and SNAP25 or syntaxin[1]. The determination of the affinity for the interaction between α-synuclein and synaptobrevin has resulted in a dissociation constant in the range of 5-15 µM. This dissociation constant corresponds to the range of dissociation constants for interactions with intrinsically disordered proteins[34].

The interaction between α-synuclein with synaptobrevin has been analyzed for charge screening by monitoring the dissociation constant at several concentrations of NaCl. Increased concentrations of NaCl resulted in a change in dissociation constant for the interaction but this effect did not discriminate between low or high concentrations of NaCl. Using a buffer containing any NaCl had an effect on the dissociation constant while further increase of the NaCl concentration did not affect the affinity of the interaction. Because further increasing the ion concentration does not affect the dissociation constant, charge screening does not affect the interaction between α-synuclein and synaptobrevin. The interaction between α-synuclein and synaptobrevin is not dependent on the isoelectric point of the proteins involved.

The interaction of α-synuclein and synaptobrevin is affected by the Ca^{2+} conditions in the environment. Both the α-synuclein and the synaptobrevin protein have shown to have an interaction

with Ca^{2+}. The dissociation constant for the interaction between these proteins shows a steady drop when increasing the Ca^{2+} concentration from 0.1 μM to 100 μM, these are the Ca^{2+} conditions comparable to neuronal rest and to the arrival of an action potential respectively. These results imply that upon increasing the Ca^{2+} concentration a change occurs in both proteins resulting in an increased affinity for each other.

Recommendations

Protein production and purification

With the new constructs arriving from Lausanne the initial requirements in protein yield and purity were met and no further attempts have been made to optimize the concentration or purity of the protein. For further and more extensive experimentation, it can be desirable to try optimize the protein production to result in a higher yields for every cycle of protein production.

The SNAREs are fragile proteins and ideally these proteins would be protected as much as possible from degradation by storing the proteins on ice as much as possible. The Äkta used during the purification process has been located in a laboratory under room temperature conditions during which the SNAREs are vulnerable to degradation. Allowing Ni^{2+}-chromatography purification of the SNAREs to occur at lower temperatures can reduce protein degradation during purification, while still resulting in an effective purification of the protein mixture[1].

The produced SNAREs are purified using Ni^{2+}-affinity chromatography and these proteins have been stored before directly being used in the further experiments. These proteins still have the HisTag-region, used in Ni^{2+}-affinity chromatography, attached to the original protein structure. While the HisTag may not affect the results of further experiments this has not been ruled out. Using trombine cleavage has previously been used to remove the HisTag from the SNAREs without harming the SNAREs[1]. Further purification of the SNAREs can be achieved by using ion exchange which will filter the proteins from solution using the protein's isoelectric point for adsorption to a purification columns, elution from this column occurs using a mobile phase of high ionic strength, e.g. NaCl.

After elution of the Ni^{2+}-affinity column the SNAREs are collected in several fractions, each with a volume of 2 ml, with the SNAREs are spread out over several fractions, typically 6 to 7. The protein concentration in each of these fractions are too low concentrations to be used in the MST binding affinity measurements. By means of centrifugation using filter tubes with a molecular weight cut-off, the concentration can be increased however this results in a loss of proteins as some protein stay behind in the filter tubes. Collecting the eluted proteins in less volume (e.g. by using a steeper elution gradient) the proteins can be collected in less volume hereby maintaining higher protein concentrations removes the need for, and thus the loss during, centrifugation.

Binding affinity determination

In some of the binding affinity experiments performed the lower and upper plateaus have not, or not fully, been reached making it hard to provide a reliable estimate of the dissociation constant based on these measurements. For further and future experiments it is of importance to clearly observe both the plateaus for a more reliable determination of the dissociation constant. In the performed experiments the range of a series of measurements consisted of a serial dilution of 16 steps using a 1:1 ratio of labelled solution to titrated solution. These conditions limit the reach which can be monitored for a single measurement and these limitations need to be circumvented to acquire a broader range. This can be achieved by using a different ratio of labelled solution to titrated solution so that for each next step in the concentration of the serial dilution is less than half of the previous concentration and a broader range can be covered in just 16 samples.
In the performed experiments the number of steps in each dilution series was 16 for that was the maximum number of capillaries to be held at once by the Monolith NT.115 MST device. A measurement does not need to be limited to just 16 steps, loading the Monolith NT.115 multiple

times can greatly increase the concentrations range for a measurement and hereby there are more data points available to determine the locations of the lower and upper plateau. This in turn leads to a better determination of the dissociation constant and thus a better determination of the affinity of the protein-protein and protein-ion interactions.

The α-synuclein protein has been known to self-aggregate. The performed measurements have indicated no self-aggregation, however it is still worth to look into measurements using α-synuclein for the serial dilution. Repetition of these experiments and switching of the labelled and unlabelled binding partners will confirm or deny α-synuclein aggregation occurring.

Some of the measurements have shown a change in F_{norm}, without affecting the trend and maintaining comparable dissociation constants, for measurements taken on a later point of time. These effects over time can be reduced by attempting to reduce the time between the measurements, perhaps even performing them simultaneously. The cause of the decrease in F_{norm} is still unknown and could be an effect of dehomogenization of the solution as a result of adhesion to the wall of the storage capsule or sedimentation of the solution. The effects of sedimentation can be reduced by continues mixing of the solution to keep the solution homogeneous.

Further measurements of the interaction between α-synuclein and the SNAREs using a wider range of Ca^{2+} conditions might provide more information on the role of α-synuclein on the SNAREs. Will further decreasing or increasing the Ca^{2+} concentration further decrease or increase the affinity of α-synuclein for synaptobrevin? And will increase of Ca^{2+} concentration increase the affinity for SNAP25 or syntaxin? These questions can be answered using these experiments and this help will answer the question of the role of α-synuclein on synaptic transmission.

The interaction of α-synuclein with the individual SNAREs has been investigated, but so far the interaction between the SNAREs themselves is still unknown. It is known that the SNARE-complex itself is SDS resistant but so far no numbers have been able to be put on the interaction between the fully formed SNARE-complex or sub-units of the SNARE-complex[37]. Any information about the strength of the interaction between the individual SNAREs and of synaptobrevin with the sub-SNARE of SNAP25-syntaxin will provide great information about the likelihood of interaction and thus formation of the SNARE-complex without the aid of the other proteins which have been shown to play a role on the SNARE-complex[2,7,13].

So far the experiments have been focused on the interaction between α-synuclein and the SNAREs, one SNARE at a time. The interaction with synaptobrevin has been studied in more detail and these results show that α-synuclein has a higher affinity for synaptobrevin at Ca^{2+} conditions resembling the arrival of an action potential in a neuronal cell. Other factors influencing this interaction occurs and what this implies for the role of α-synuclein upon synaptic transmission is still undefined. This role of α-synuclein can be further defined in vitro by looking at the interaction of α-synuclein with partially or fully formed SNARE-complexes.
During the vesicle docking phase in preparation of synaptic transmission synaptobrevin interacts with a complex of SNAP25 and syntaxin to form the SNARE-complex[2]. For the role of α-synuclein on synaptic transmission, it would be interesting to know if α-synuclein interacts with synaptobrevin before or after formation of the full SNARE-complex. This information can be acquired by comparing the affinity α-synuclein for synaptobrevin, the partially formed SNAP25-syntaxin SNARE-complex and the fully formed SNARE-complex. In this research the current data has shown that α-synuclein has

more affinity to synaptobrevin upon the arrival of an action potential, during the arrival of an action potential the presynaptic vesicles are already primed for release. Priming of the presynaptic vesicles requires the full SNARE-complex to be formed and thus increased affinity is expected of α-synuclein with the fully formed SNARE-complex

If the affinity experiments of the soluble parts of the SNARE-proteins have yielded positive results, it might be time to look deeper into the role of the full SNARE-proteins in the synaptic transmission mechanism and the role of α-synuclein under these conditions. These additional regions will induce changes on the protein conformation as the transmembrane regions in syntaxin and synaptobrevin will be highly hydrophobic and thus move away from the solution. This will complicate the measurements as the hydrophobicity can result in micelle-like aggregation of these transmembrane regions in solution complicating determining the interaction. To prevent transmembrane regions to affect the interaction of the complete protein, it might be simple to dock the proteins in lipid bilayers resembling presynaptic vesicles and cell membranes. With the membrane docked parts of the proteins present (either embedded in or palmitoylated to a lipid bilayer), these regions will no longer affect the interaction by clustering the hydrophobic regions together, but instead larger vesicles will be attached to the proteins. Any information on the interaction among SNAREs (individual, sub or fully formed SNARE-complex) and the interaction of α-synuclein on the various stages of these SNAREs, will be useful to fully understand the mechanism and the role of α-synuclein in synaptic transmission. Using different buffer concentrations, especially ranging the Ca^{2+} concentration from resting to action potential conditions, will provide great insight in these matters.

If all goes well, the in vitro experiments can be expanded by gradually involving the other proteins known to play an important role in synaptic transmission (e.g. syntaxin, CSP, Munc18, Rab3A and complexin) and observing what effect these proteins have on the SNARE-complex and how this changes under different conditions (preferably under different Ca^{2+} concentrations)[2,7,13]. The research can be even further expanded to monitoring the interaction between the SNAREs themselves and with α-synuclein in situ. However tempting these in situ experiments may seem, it is important to realize that these studies are still miles away and a lot of ground breaking work is required before that stage of experimentation is reached.

Materials and Methods

Expression of SNAREs

The proteins used in the experiments to investigate the role of α-synuclein with the SNARE proteins are produced at the NanoBioPhysics (NBP) group of the University of Twente. The process of production of the SNAREs and further improving production yield and protein quality has been performed by myself while the α-synuclein protein has been produced by K. van Leijenhorst-Groener and N. Schilderink. All the proteins have been produced using expression of the proteins in E. coli (Escherichia coli) BL21 (DE3) bacteria and purification using a His_6-tag based system to produce recombinant proteins.

Production of SNARE proteins

The proteins required to investigate the role of α-synuclein on the SNARE complex are the three SNAREs involved in neuronal exocytosis (SNAP25b, Syntaxin1a and SynaptobrevinB2) and of course α-synuclein itself. The rat SNAREs are encoded in pET28a plasmids, with a kanamycine resistance site and NHe1-Xh01 cutting sites, encoding His_6-tagged fusion proteins, kindly provided by D. Fasshauer (DNF, University of Lausanne, Switzerland). The SNAP25 is the full length protein (1-206) with its cysteines on positions 85, 88, 90 and 92 replaced for alanines[38]. The Syntaxin 1a is truncated at the C-terminus resulting in the first 262 amino acids, only the hydrophilic part, of the protein. The Synaptobrevin B2 protein is also truncated at the C-terminus, leaving only the first 96 amino acids.

Transfection of bacteria

The pET28a plasmids are transformed into E.coli BL21 (DE3) cells by adding 325 ng of DNA to a tube containing 100 µl of the BL21 (DE3) competent cells. The BL21 (DE3) cells have been chosen for their high level of protein expression[21]. The solution was left to incubate for 20 minutes on ice to allow the DNA to attach to the cell surface. Next the bacteria solution was heat shocked by taking the solution from ice and placing it for 45 seconds in a water bath of 42°C before placing the bacteria on ice again for 1 minute. The heat shock was applied to create disruptions in the cell membrane allowing the DNA to enter the cell through the temporary openings in the membrane. After the cooling step 450 µl of LB medium (25 gram of LB Broth (high salt), Sigma-Aldrich Chemie, Munch, Germany, in 1000 ml of MQ water) is added to the bacteria solution and placed in an incubator (Centomat® BS-1) set at 37°C and 150 RPM for 45 minutes. After the incubation time, 100 µl of the bacteria solution is spread over an petridish covered with agar (20 ml of LB medium, 0.3 grams of agar (Sigma-Aldrich) and 0.05 mg/ml of kanamycin (Invitrogen) per petridish) and incubated overnight at 37°C, 5 % CO_2 in an incubator to allow successfuly transfected bacteria to form colonies. From a single bacterial colony a sample is taken and placed in LB medium for cell expansion overnight before creating a glycerol stock in a 1:1 volume ratio of autoclaved glycerol and bacterial cell suspension. The glycerol stocks are stored at a temperature of -80°C until needed for further use.

Expression of recombinant SNAREs

Only bacteria resistant to kanamycine were able to survive on the agar plates. Successfully transfected bacteria acquire the kanamycine resistance from the pET28a plasmids and in turn contain the sequence of the SNARE proteins.

Normal induction of expression

A sample of the transfected bacteria was taken from the glycerol stocks and placed in a growth medium (LB medium with 0.05 mg/ml of kanamycine (Invitrogen), incubated at 37°C at 150 RPM) to

expand the bacterial solution to an optical density of 0.6 (measured at 600 nm by WPA Biowave CO800 Cell Density Meter). The T-7 promotor was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside, 1 mM) and allowing protein expression for 4 hours at 37 °C at 150 RPM. The cells are harvested at 10000 x g (Sorvall 5C Plus, precooled to 4 °C) for 10 minutes. The resulting cell pallet is resuspended in 1/40th of the culture volume in washing buffer (500 mM NaCl, 20 mM Tris pH 8.0 and 8 mM imidozole).

Overnight induction of expression

The only differences between the normal expression of recombinant SNAREs and the overnight induction of recombinant SNAREs are the temperatures and the time of induction. Instead of inducing protein production for 4 hours at 37 °C, this time the recombinant proteins are expressed overnight (16 hrs) in an incubator set to keep the temperature at 20 °C.

Bacterial lysis

The harvested bacteria contain the overexpressed SNARE proteins enclosed in the bacterial cells. To allow purification of the SNAREs from the content of the cells, the plasma membrane of the bacterial cells needs to be broken down by means of lysis of these bacterial cells.

BugBuster® method of lysis

Lysis of the E. Coli cells has been achieved by adding 10 ml BugBuster® Master Mix (Novagen®) and 250 mM PMSF (phenylmethanesulfonyl fluoride, Sigma-Aldrich) for each liter of LB medium used for protein expression. This results in a total volume of 15 ml of lysis solution, cell pellet and BugBuster®, for each liter of LB medium used. The BugBuster® is a mixture of ingredients to obtain the soluble fraction of the bacterial cells.

The lysis mixture is mixed slowly for 20 minutes before the insoluble material is spun down in 30 minutes at 15000 x g at 4 °C (Sorvall 5C Plus). The supernatant, containing the soluble cell contents, is removed from the pellet and filtered through a 0.2 µm membrane (Nalgene™ Thermo Scientific™) to remove large bits of cell debris from the remaining mixture.

Home-made method of lysis

Lysis of the E. Coli cells by using a home-made method of lysis, has been achieved by adding 25 mmol PMSF, 100 mmol $MgCl_2.(H_2O)_6$ (Merck KGaA, Germany), 1 ml of Triton X-100 20% (20%, Sigma-Aldrich), 50 mg of DNAse (Sigma-Aldrich) and 50 mg of lysozyme (Sigma-Aldrich) to each liter of LB medium used for protein expression. The resulting solution undergoes cell lysis on ice by means of sonification (4x 30 seconds with 45 seconds interval) after which) and 6 gram of urea (Sigma-Aldrich) was added for every liter of of LB medium used during protein expression. The insoluble material was separated from the soluble material, by centrifugation for 30 minutes at 15000 x g at 4 °C, and filtered through a 0.2 µm membrane (Nalgene™ Thermo Scientific™) to remove large pieces of cell debris.

Purification of expressed SNARE proteins

The production of the SNARE proteins has so far resulted in a mixture of all the soluble components of the E. coli cells in which the recombinant protein has been expressed. From this mixture the proteins of interest, His_6-tagged fusion proteins, are purified from the mixture of proteins by Ni^{2+}-affinity chromatography. The purified SNAREs are checked for purity and are quantified.

Ni^{2+}-affinity chromatography

The filtered solution is loaded onto a 6 ml ResourceQ column using an Äkta Purifier system (GE Healthcare, Little Chalfort, United Kingdom) using a loading buffer (500 mM NaCl, 20 mM Tris pH 8 and 8 mM imidozole). The His_6-tagged SNARE proteins are eluted from the ResourceQ column using a linear gradient of imidozole (8-500 mM) in 500 mM NaCl, 20 mM Tris pH 8 at a flow-rate of 5 ml/min over 8 column volumes.

SDS-PAGE purity determination

The fractions collected during Ni^{2+}-affinity chromatography are checked for the SNARE proteins by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) followed by Coomassie staining of the gel. SDS-PAGE is a universally used method to determine the molecular weight of a protein. The proteins are stained by Coomassie Brilliant Blue (Bio-Rad). The SNAREs are compared with a SDS-PAGE ladder Molecular Weigth Standards for Low Range molecules (Bio-Rad Laboratories, Hercules, California, United States of America). The fractions which contain the SNAREs are pooled together.

Concentration determination

The protein concentrations were determined by measuring absorbance at 280 nm using the extinction coefficient belonging to each specific SNARE. The amino acids tyrosine, tryptophan, and to some extend phenylalanine, contain a phenol group which absorbs light in the UV range. Quantifying the amount of protein makes use of the amount of these UV absorbing amino acids in the protein sequence[39]. For the versions of SNAP25, synaptobrevin b2 and syntaxin 1a used in these experiments the resulting absorption coefficients at 280 nm are 6990 $M^{-1}cm^{-1}$, 12490 $M^{-1}cm^{-1}$, and 7450 $M^{-1}cm^{-1}$, respectively.

Expression and purification of α-synuclein

The α-synuclein has been produced as previously described in several papers and resently most explicate by V. V. Shvadchak et all in Biophysics Journal (2006)[40-42]. The supporting information describes how the human wild-type α-synuclein protein has been expressed by E. coli bacteria by means of a pT7-7 based expression system. The α-synuclein has been produced using IPTG to induce protein production afterwards the protein is harvested by centrifugation and cell lysis has occured by means of sonification.

The resulting solution was purified by means of a 6 ml ResourceQ column using an Äkta Purifier system (GE Healthcare). The resulting fractions were checked for α-synuclein using SDS-PAGE and the fractions containing α-synuclein are pooled and concentrated by centrifigation (Vivaspin 20, 10 kDa; GE Healthcare). The concentration of the pooled fractions was determined by Tyr absorption at 275 nm using the absorption coefficient, ε=5600 $M^{-1}\,cm^{-1}$.

Labelling of proteins

The proteins are labelled using an amine-reactive labelling kit, Monolith NT™ Protein Labelling Kit BLUE-NHS (NanoTemper® Technologies GmbH, München, Germany). This label has a molecular weight of 800 Da and contains a N-hydroxysuccinimide (NHS) ester which binds covalently with the primary amines (-NH_2) present in the side chains of the amino acids or on the protein's N-terminus. Every amino acid contains at least one potential binding site for the NHS-ester and typically more. The exact position of the label is of no importance for tracking of the thermophoresis and random labelling of proteins minimizes the effect of the label's position on the binding reaction.

The proteins are labelled using a 1:1 label-protein ratio and the unreacted dye is removed from the solution. The degree of labelling has not been determined, however since we only track the labelled proteins the unlabelled fraction does not affect the results of the experiments. The labelled proteins will behave similar to the fraction of unlabelled proteins, making the traceable labelled proteins representative for the behaviour of the total population of that protein under those conditions.

Microscale Thermophoresis

The buffer used for the microscale thermophoresis (MST) experiments consists of 10 mM Tris pH 7.4 (tris(hydroxymethyl)aminomethane, Sigma-Aldrich Chemie, Munch, Germany), 0.5 mg/ml BSA (NanoTemper® Technologies GmbH, München, Germany) and 0.05% Tween (NanoTemper® Technologies GmbH) dissolved in MilliQ-water. To this buffer is added NaCl or $CaCl_2$ respectively to investigate the effect of charge screening or calcium binding on the protein binding reactions. The labelled proteins are dissolved in the buffer solution resulting in a concentration of 30 nM of the labelled protein. A 1:1 serial dilution is made for the unlabelled proteins using the concentrated proteins and diluting the proteins with the buffer solution (10 mM of Tris pH 7.4, 0.5 mg/ml BSA and 0.05% Tween). To each step of the serial dilution of the unlabelled protein an equal amount of labelled protein is added and the solution is left to incubate for at least 10 minutes, allowing the labelled and the unlabelled proteins to interact and form an equilibrium. Capillaries (MO-K002 Monolith™ NT.115 Standard Treated Capillaries by NanoTemper® Technologies GmbH) have been filled evenly with the different steps of the serial dilution through capillary action.

The microscale thermophoresis has been measured using a Monolith NT.115 MST (NanoTemper® Technologies GmbH) equipped with Nano –BLUE/GREEN with LED-filter combinations blue (excitation 460-480 nm, emission 515-530 nm) and green (excitation 515-525 nm- emission 560-585 nm). An IR-laser with a wavelength of 1480 nm is coupled into the optical path of the blue LED-filter combination so the excitation and emission is used to record the exact same spot where the IR radiation creates a temperature gradient[26]. For the measurements the Monolith NT.115 MST has been set to use the blue settings. At this spot the fluorescence is recorded for 5 seconds before activating the IR laser for 30 seconds and the fluorescence is recorded for 5 more seconds after turning off the IR laser. Each set of the serial dilution is measured from the lowest to the highest concentration of unlabelled protein and each dilution series is measured three times.

Conformation of observed change in signal upon activation of the IR-laser being the result of protein interactions is performed by denaturing the protein solutions. To do this the protein solution is mixed in a 1:1 ratio with a denaturing solution (4% v/v SDS and 40 mM DDT) and heated to 95 °C for 5 minutes. From these denatured samples capillaries are filled and MST measurements are made as described previously.

The data is recorded using NT Control 2.0.2.29 (NanoTemper® Technologies GmbH) and processed using NT Analysis 1.5.41 (NanoTemper® Technologies GmbH) and Origen (OrigenPro® 9.0.0 32-bit SR2 by OrigenLab Cooperation, Northhampton, USA).

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