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Studies on Synaptic Processing of Amyloid Precursor Protein - an Important Protein in Alzheimer's Disease

Amirah Khan

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Biology Education Centre, Uppsala University, and Department of Neurobiology, Care Sciences and Society, Karolinska Institute
Supervisor: Susanne Frykman

SUMMARY

In Alzheimer's disease (AD) the nerve synapses are degenerated. There is a lot of speculation on the underlying molecular mechanisms behind the degeneration, and currently the role of amyloid precursor protein (APP) and its proteolytic derivative amyloid β-peptide (Aβ) is much investigated. The normal physiological role of APP is in synapse formation, function and maintenance. Aβ is its proteolytic derivative, and is formed through the sequential cleavage of APP by β- and γ-secratases. Recent studies on APP trafficking show, contrary to the dogma accepted in the literature, that APP is a protein of the synaptic vesicles. Another current research indicates that APP might be processed in the synaptic vesicles, yet it has not been validated in any study. Preliminary results from this thesis indicate that Aβ is generated in purified synaptic vesicles.

Several ongoing clinical trials are using drugs that inhibit γ-secretase activity in order to limit the production of Aβ in the brains of AD patients. However, there are harmful side-effects linked to the indirect inhibition of a γ-secretase substrate, Notch. The results gained in this thesis indicate that Notch processing is absent from the synaptic vesicles, and therefore in the future it might be feasible to produce γ-secretase inhibitors that specifically target the synaptic vesicles. These preliminary results are based on γ-secretase activity assays, ELISA and western blot detection.

Furthermore, though a recent study claims that APP is a synaptic vesicle protein, there is currently no established method to investigate whether Aβ can be secreted from the neurons through normal neurotransmitter release. I investigated possibilities of developing a method of isolating pinched-off nerve endings (called synaptosomes) that still retain the ability to release neurotransmitters upon stimulation. The ultimate aim is to use synaptosomes to study Aβ release

in response to neuronal stimulation. In the scope of this thesis such a method was not developed but nevertheless knowledge was generated in refinement of both the synaptosome isolation procedure and the assay for monitoring the neurotransmitter release.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent cause of dementia with an estimated >35 million people suffering from it worldwide (Finder, 2010). The hallmarks of the disease include the formation of extracellular senile plaques, intraneuronal neurofibrillary tangles, and a rapid degeneration of the nerve synapses; all correlating to a progressive loss of cognition and memory in the affected. Naturally, there is a lot of interest in inhibiting and reversing the underlying molecular mechanisms behind the degeneration, and currently the role of amyloid precursor protein (APP) and its proteolytic derivative amyloid β-peptide (Aβ) is much investigated (for a review see Zhang et al., 2011; see Figure 1 for detailed description of APP processing).

Amyloid precursor protein (APP)

APP is a 120 kDa type I transmembrane glycoprotein that is expressed throughout the mammalian cells (Sisodia and Price, 1995). Its normal physiological role is in synapse formation, function and maintenance, though involvement in cell adhesion, axonal protein trafficking, transmembrane signal transduction, and calcium metabolism have also been suggested (Zheng and Koo, 2006).

APP can be cleaved to generate Aβ peptides of various lengths but predominately the residues are 40 and 42 peptides long (Selkoe, 2008). According to the amyloid hypothesis, an increase in the production or a decreased in the clearance of Aβ causes amyloidosis (condition in which amyloids are abnormally deposited on tissues and organs) which culminates into AD (Hardy and Selkoe, 2002). In a concentration-dependent manner these Aβ peptides polymerize into fibrils that deposit extracellularly to form the characteristic senile plaques found in the AD-affected brains (Master et al., 1985; Walsh and Selkoe, 2007). However, recent studies indicate that fibril

formation is not essential for neurotoxicity, and even soluble species of Aβ oligomers can lead to the synaptic dysfunction and the neuronal loss seen in AD (Walsh and Selkoe, 2007).

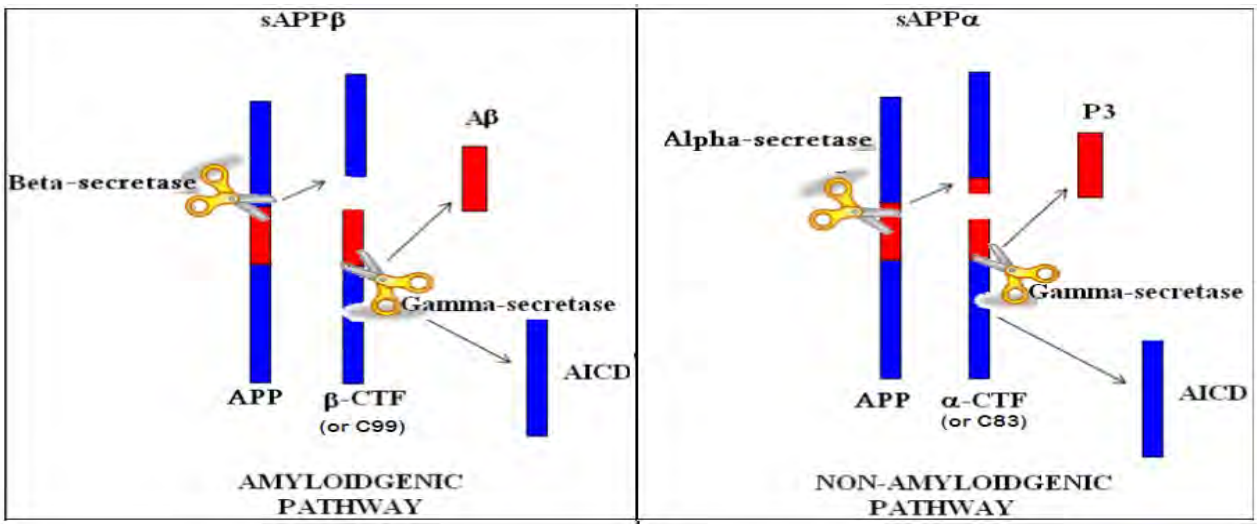

Figure 1. APP processing. APP can be metabolized through either the amyloidgenic or the non-amyloidgenic pathways. In the former, APP is initially cleaved by β-secretase to release a soluble fragment (sAPPβ). Thereafter the remaining membrane-bound C-terminal fragment (β-CTF, also called C99) is cleaved by γ-secretase to release the APP intracellular domain (AICD) as well as a soluble Aβ peptide. Instead in the non-amyloidgenic pathway, APP is cleaved initially by α-secretase to release a soluble sAPPα fragment. Thereafter the remaining membrane-bound C-terminal fragment (α-CTF, also called C83) is cleaved by γ-secretase to produce the P3 and AICD fragments. Both α and β-secretases are believed to cleave APP in the same region, therefore the cleavage by one eliminates the possibility of cleavage by the other.

As shown in Figure 1, it is γ-secretase that mediates the cleavage of APP to generate Aβ peptides through the amyloidgenic pathway (Selkoe, 2008). γ-secretase is an intramembrane aspartyl protease (Hardy and Selkoe, 2002) consisting of at least four subunits: anterior pharynx defective-1 (Aph-1), nicastrin, presenilin (PS), and presenilin enhancer-2 (Pen-2) (Kimberly et al., 2003). Besides APP, it has more than 50 other substrates, including Notch (Beels and Sanders, 2008). The cleavage of Notch by γ-secretase releases a Notch intracellular domain (NICD) which translocates into the nucleus and is there involved in transcriptional regulation (De Stooper et al., 1999). Due to the role of γ-secretase in AD pathogenesis it has been a target of clinical trials aimed at treating AD (for a review see Imbimbo et al., 2011). However, several

clinical trials based on γ-secretase inhibition have been discontinued due to detrimental cognitive and functional effects of the drug, which were ascribed in large part to the indirect inhibition of Notch processing.

APP trafficking

The dysregulation of APP trafficking in neurons is believed to have a central role in AD pathogenesis (Sisodia and Price, 1995). The current view of APP trafficking is that the protein is constitutively synthesised in the endoplasmic reticulum (ER) of the pre-synaptic neurons and then transported through the Golgi network to the neuronal cell surface (Bell et al, 2008). A small fraction of the protein is believed to be cleaved on the cell surface by the α-secretase, releasing a 100-110 kDa soluble fragment (sAPPα). However, most of APP is believed not to be cleaved at the cell surface, instead it is thought that the full-length APP is internalised through clathrin-mediated endocytosis to early endosomal compartments (Cirrito et al., 2008). Therein sequential cleavage by β- and γ-secretases generates 37-49 amino acid Aβ peptides (De Stooper et al., 1998). The APP fragments, including Aβ, are then trafficked back to the cell surface and secreted out of the neurons (Cirrito et al., 2005).

It is not known exactly what organelle(s) traffic APP to the synapse. In the late 1990's, the synaptic vesicles were investigated as potentially being the endocytotic organelles that traffic APP in the neurons. Studies by Ikin et al. (1996) and Marquez-Sterling et al. (1997) were among the seminal research that laid the basis for the currently accepted dogma that APP is not processed in the synaptic vesicles. Instead it is hypothesised that Aβ is released primarily through exosomes (Cirrito et al., 2008). Exosomes are endosome-derived vesicles that are released upon the fusion of multivesicular bodies with the plasma membrane (Théry, 2011). However, the amount of Aβ released through the exosomes accounts only for 1% of the total Aβ released from neurons (Rajendran et al., 2006), leaving the vast percentage of the peptide release unaccounted

for. In 2005, Cirrito and colleagues demonstrated a correlation between increased neuronal stimulation (synaptic activity) and increased Aβ levels in the extracellular brain fluid. Like exosomes, synaptic vesicles are also exocytosed in response to neuronal stimulation (ex. Koenig and Ikeda, 1996). Moreover, the synaptic vesicle recycling pathway and Aβ release share sensitivity to tetrodoxin, tetanus toxin, and dynamin inhibitors (Cirrito et al., 2005 & 2008). Even when considering the sheer number of releasable synaptic vesicles per neuron (between 100 to 200, ex. Ikeda and Bekkers, 2009) and the rate of vesicle recycling, it seems plausible that the vast majority of Aβ secreted out of the neuron could be ascribed to the synaptic vesicles. However, due to the accepted 'dogma', the synaptic vesicles are still not considered to traffic APP. Recently Frykman and collegues (2010) found high enrichment of APP, APP derivatives, and γ-secretase components in crudely purified synaptic vesicles. Also Groemer et al. (2011) published a study showing that APP is endogenously present in a small but relevant number of synaptic vesicles. So it seems prudent to revise the current view of APP trafficking and reinvestigate the role of synaptic vesicles as an APP trafficking organelle.

A potential method to study Aβ release in synaptosomes

If APP is processed in the synaptic vesicles through the amyloidgenic pathway then it should be possible to release Aβ from the neurons together with other neurotransmitters.

Synaptosomes are pinched-off nerve-ending that have all the essential components to store, release and retain neurotransmitters (Whittaker, 1973). They have been extensively studied in the literature and are believed to closely mimic the nerve terminals *in vivo* in their ability to recycle synaptic vesicles upon stimulation. Figure 2 outlines a potential method to investigate whether Aβ can be released from synaptosomes through normal neurotransmitter release.

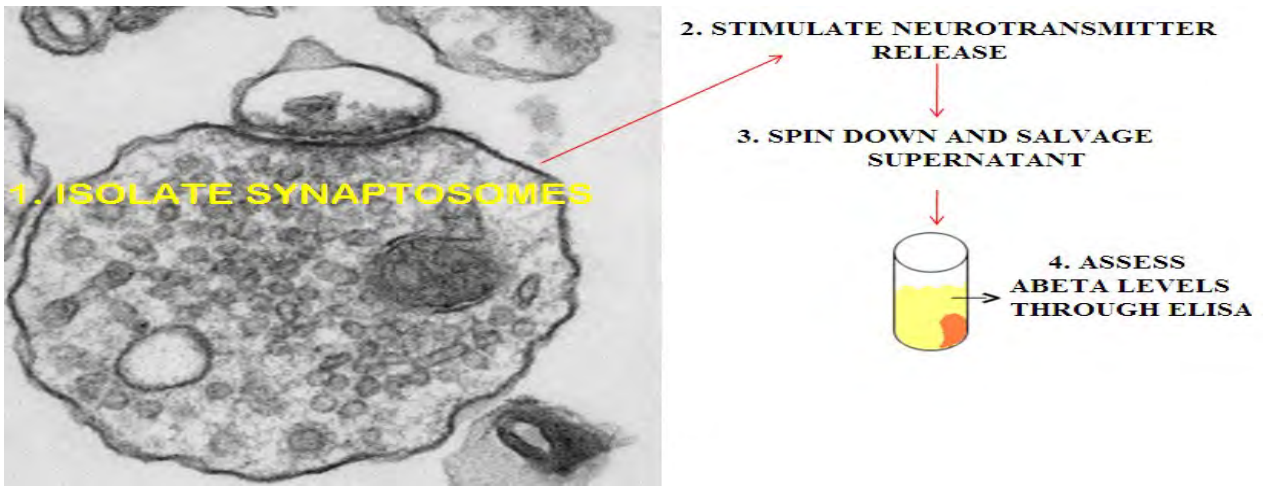

Figure 2. Crude sketch of a potential method to investigate Aβ secretion from synaptosomes (pinched-off nerve endings). 1) An EM image of an intact synaptosome filled with synaptic vesicles. It is of utmost importance that the isolated synaptosomes remain functional and retain the ability to release neurotransmitters in response to stimulation. **2)** Once functional synaptosomes are isolated then the next step is to stimulate these to trigger the synaptic vesicles to fuse with the plasma membrane and release their contents into the extracellular space. **3)** A spin to pellet down the synaptosomes, while the lighter neurotransmitters and Aβ float in the supernatant. **4)** Finally through ELISA detect and access the concentration of Aβ (if present).

In the scope of this thesis I have focused on Steps 1-2 of the method outlined in Figure 2:

Step 1. Isolate synaptosomes

Briefly, the procedure of isolating the synaptosomes is based on homogenizing fresh brain tissue in an isotonic buffer solution to separate the nerve-terminals from the axons. The homogenate is thereafter subjected to a number of specific centrifugation steps that distinguish the homogenate contents (cellular debris, myelin, mitochondria, synaptosomes and plasma membranes, etc.) according to their buoyancy densities. The fraction enriched in synaptosomes is then collected.

Step 2. Stimulate neurotransmitter release

A Ca^{2+}-dependent neurotransmitter release can be evoked in the synaptosomes with the addition of a depolarising agent (Dunant and Israël, 1998). The major excitatory neurotransmitter in vertebrates is the amino acid L-glutamate (McMahon and Nicholls, 1993). Therefore, one would expect an ample detectable amount of glutamate to be released from stimulated synaptosomes.

In 1986, an assay for indirectly monitoring endogenous glutamate release from synaptosomes was described by Nicholls and Sihra. Their glutamate release assay (GRA) is based on the principle that the enzyme glutamate dehydrogenase (GDH) reduces $NADP^+$ to NADPH in the presence of glutamate (Glutamate + $NADP^+$ → Ketoglutarate + NADPH), and since NADPH is a fluorescent molecule it can be detected in real-time through florescence monitoring. Thus, in their GRA the synaptosomes are stimulated to release glutamate with the addition of a depolarising agent. The choice of the depolarizing agent used is based on the mode of exocytosis that it is suggested to induce. In the literature two modes of synaptic vesicle recycling are mentioned: 1) a kiss-and-run mode, in which the vesicles transiently fuse with the plasma membrane and release their contents through pores in the plasma membrane; or 2) a full fusion mode, in which the vesicles completely fuse with the plasma membrane (Koeing and Ikeda, 2006). The stimulation with high concentrations of 4-AP is suggested to give rise to both fusion modes and the synaptosomes are expected to respond to the stimuli in the same manner as they would respond to action potentials in physiological settings (McMahon and Nicholls, 1991). Whereas, high K^+ concentration is suggested to give rise to full fusion mode, eliciting a comparatively stronger stimulus and could therefore provoke exocytosis in instances where 4-AP is unable to do so.

Aims

In this thesis I had three basic aims: 1) investigate whether Aβ production occurs in synaptic vesicles, 2) investigate whether Notch-processing occurs in synaptic vesicles, and 3) attempt to isolate synaptosomes that retain the ability to release neurotransmitters.

RESULTS

AIM 1. Does APP Processing occur in the Synaptic Vesicles?

In this thesis, I investigated Aβ production in a fraction of purified 'Controlled Pored Glass synaptic vesicles' (CPG-SVs) obtained through Dr. Matthew Holt (Max Planck Institute for Biophysical Chemistry, Department of Neurobiology). Figure 3 depicts the scheme of purifying CPG-SVs from rat brain homogenate.

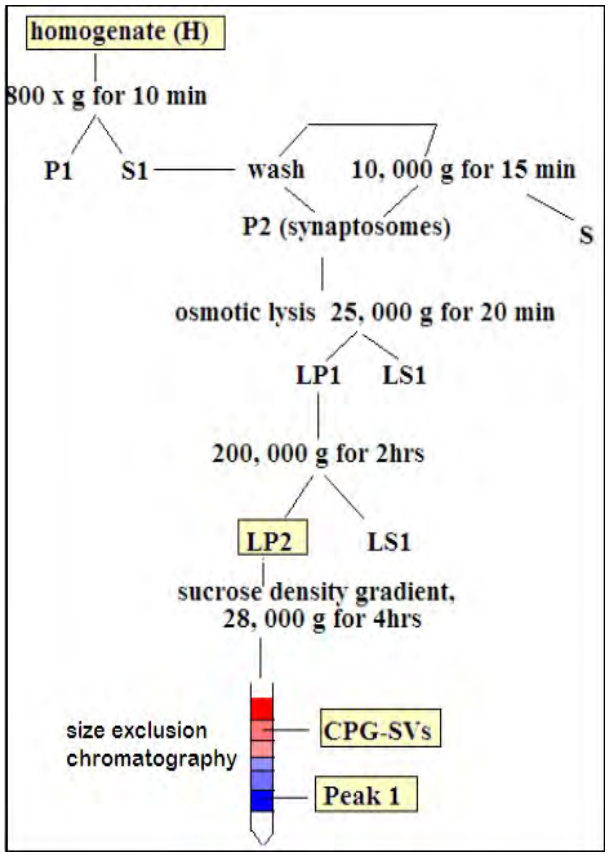

Figure 3. A flow chart depicting the procedure of purifying 'Controlled Pored Glass synaptic vesicles' (CPG-SVs) from a homogenate of rat brain tissue. The yellow boxes indicate the fractions used in this study: the Homogenate (H), Lysed Pellet 2 (LP2), the purified synaptic vesicles (CPG-SVs) and Peak 1 (I).

Purity assessment of 'Controlled Pored Glass synaptic vesicles'

The actual purification was done at another lab and the following fractions were sent to us: the Homogenate (H), Lysed Pellet 2 (LP2), the purified synaptic vesicles (CPG-SVs) and Peak 1 (I). Before looking at Aβ production in the CPG-SVs, I assessed the purity of these fractions by probing them with markers specific for different organelles and membranes, including: the Golgi (GM130), the ER-Golgi intermediate compartments (ERGIC53), the synaptic vesicles (Synaptophysin) and the endosomes (Syntaxin-13). Since synaptic vesicles are endocytosed therefore Syntaxin-13 could be also present in the synaptic vesicles.

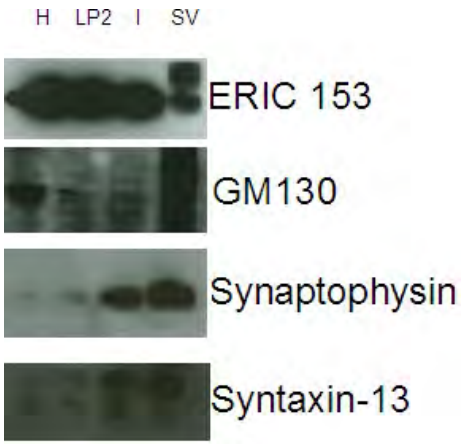

Figure 4. Purity assessment through Western blotting. Western blot detection of markers specific for ER-Golgi intermediate compartments (ERGIC53), Golgi (GM130), Synaptic vesicles (Synaptophysin) and Endosomes (Syntaxin-13) in the following fractions: the Homogenate (H), Lysed Pellet 2 (LP2), Peak 1 (I), and the purified synaptic vesicles (SV).

The apparent smear from GM130 probing in the synaptic vesicle fraction (Figure 4), could be indicative of possible contamination. However, the Electron microscopic (EM) imaging of the same fraction, shown in Figure 5 (below), shows uniformly small round bodies of around 40 nm (which is the size of synaptic vesicles). It would therefore suggest that the CPG-SVs fraction was sufficiently pure for analysis.

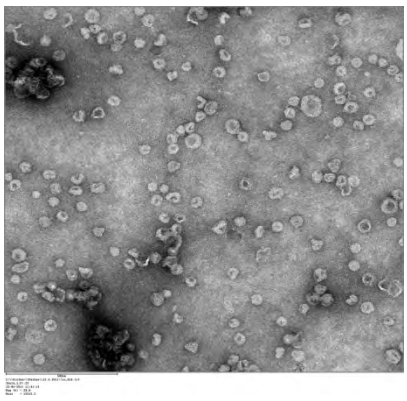

Figure 5. Electron microscopic image of Controlled Pored Glass synaptic vesicles (CPG-SVs). Image provided by Dr. Matthew Holt (Max Planck Institute for Biophysical Chemistry, Department of Neurobiology).

After assessing the purity of the CPG-SVS fractions, I investigated whether Aβ production occurs in these purified synaptic vesicles.

Assessment of Aβ production in synaptic vesicles

To investigate Aβ production in the CPG-SVs, I performed an assay that targets γ-secretase activity. Thereafter I determined the Aβ40 levels through a sandwich ELISA and calculated the *in vitro* production of Aβ40 in the CPG-SVs.

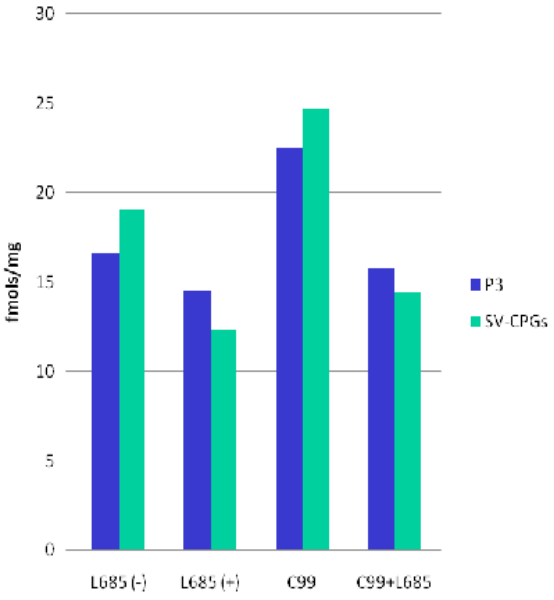

Figure 6. Aβ40 levels in CPG-SVs and P3. The assay was done under four conditions: **1)** inhibition of γ-secretase activity by an L685-inhibitor (+L685); **2)** no inhibition of γ-secretase activity (-L685); **3)** no inhibition of γ-secretase activity but with C99 enrichment (-L685; +C99); and **4)** C99 enrichment and inhibition of γ-secretase activity by L685-inhibitor (+C99; +L685;). Basically, samples of CPG-SVs were incubated in the presence and absence of the γ-secretase activity inhibitor (+/- L685) for 16 hrs at 37°C. Then through a sandwich ELISA kit, I investigated the *in vitro* generation of Aβ40 in rat brain CPG-SVs. As a positive control for Aβ40 generation, the assay was simultaneously also performed on microsomal pellets spun down from rat brains (P3). Since I did not know if the endogenous APP in CPG-SVs was enough for detection, so in two samples I also added the exogenous substrate C99 (in the amyloidgenic pathway, γ-secretase cleaves C99 to release Aβ).

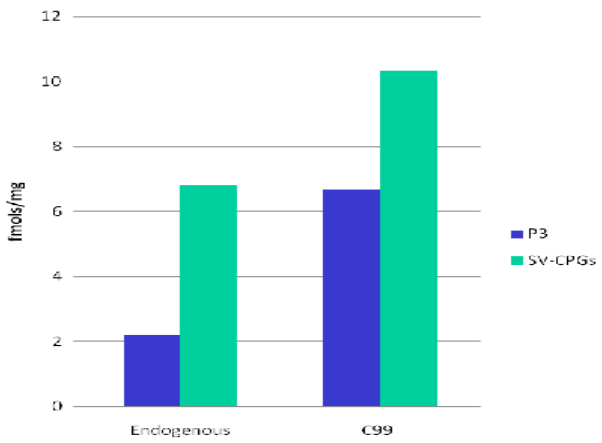

Figure 7. Aβ40 production in CPG-SVs and P3. The endogenous Aβ40 production in CPG-SVs and P3 was calculated as the difference between the Aβ40 levels in the presence and absence of the γ-secretase inhibitor (L685). Aβ40 production was also assessed in samples of CPG-SVs and P3 enriched with C99 (γ-secretase substrate yielding Aβ40). The Aβ40 production in C99 enriched samples was calculated as the difference between the Aβ40 levels in presence and in the absence of the γ-secretase inhibitor (L685).

The results, depicted in figure 6 and 7, indicate Aβ40 generation in CPG-SVs. However, as these results are based on single samples they will have to be repeated for a conclusive result.

To further characterise the synaptic vesicles, I probed the CPG-SV fractions (and fractions from the purification steps depicted in yellow in Figure 3) with markers specific for γ-secretase components: presenilin (PS), nicastrin, and anterior pharynx defective-1 (Aph-1). All three

components were highly enriched in the CPG-SV fraction. Figure 8 (below), shows a representative western blot for one of the components (Aph-1).

Figure 8. Detection of γ-secretase component through Western blotting. Probing of the Homogenate (H), Lysed Pellet 2 (LP2), Peak 1 (I), and the purified synaptic vesicles (CPG-SVs) with marker specific for the γ-secretase component anterior pharynx defective-1 (Aph-1).

AIM 2. Does Notch Processing occur in the Synaptic Vesicles?

Since Notch is a substrate of γ-secretase, hence I investigated Notch processing in the CPG-SVs through a γ-secretase activity assay. A western blot is shown in Figure 9 probed with a marker that specifically binds to the NICD-fragment without binding to full-length Notch. The result shows an absence of Notch cleavage products in the CPG-SVs, and thus indicates the absence of Notch processing in the synaptic vesicles.

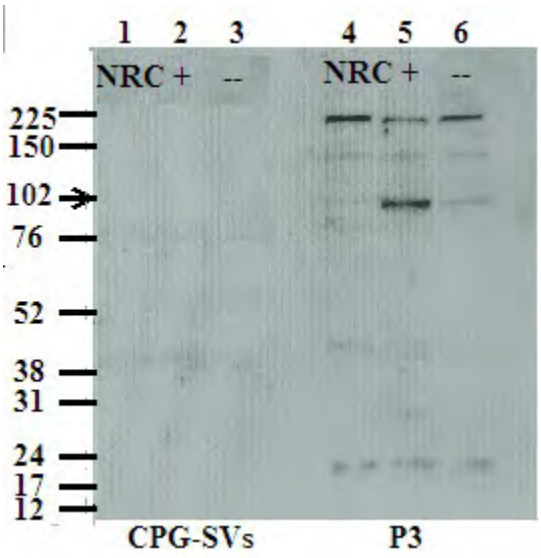

Figure 9. Probing for the Notch cleavage product NICD (Notch intracellular domain) through Western blotting. CPG-SVs and P3 samples, shown in lanes 2-3 and 5-6 respectively, were incubated in the presence (+) and absence (-) of the γ-secretase inhibitor (L685) at 37°C for 16 hrs. As a control to the validity of the assay, Non-reaction controls (NRCs) were also included, in which the CPG-SVs and P3 samples, shown in lanes 1 and 4 respectively, were incubated at 4°C for 16 hrs in the absence of the γ-secretase inhibitor. The samples were probed with markers specific for the NICD-fragment and not the full-length Notch. The arrow at 102 kDa indicates the region where bands for NICD should be detectable if present.

AIM 3. Attempts to isolate synaptosomes that retain the ability to release neurotransmitters.

Current literature findings (Frykman et al., 2010; Groemer et al., 2011) as well as results reported in this thesis seem to indicate Aβ production in the synaptic vesicles. It should therefore be possible to release Aβ from the neurons together with regular synaptic vesicle exocytosis. A potential method to study Aβ release through normal neurotransmission was outlined in the INTRODUCTION section. It is a method that heavily relies on the isolation of functional synaptosomes. In the scope of this thesis, I isolated synaptosomes and assessed their functionality through a glutamate release assay (GRA).

Preparations for the GRA

Before performing a GRA on synaptosomes, I did simple glutamate assays in which I mixed known concentrations of L-glutamic acid, GDH, and $NADP^+$ and tried to generate standard curves under different fluorescence and absorbance wavelengths. It was through these assays that the optimal reagent concentrations and basic parameters for monitoring NADPH generation were

determined. Notably I decided to monitor NADPH generation through fluorescence using an excitation wavelength of 350 nm and an emission wavelength of 440 nm. Moreover, it could be determined that the assay could also be performed in the Sodium buffer (the buffer in which the isolated synaptosomes would be dissolved in for the GRA).

Presented below in Figure 10, is the method standardized for generating a glutamate standard curve for the GRA.

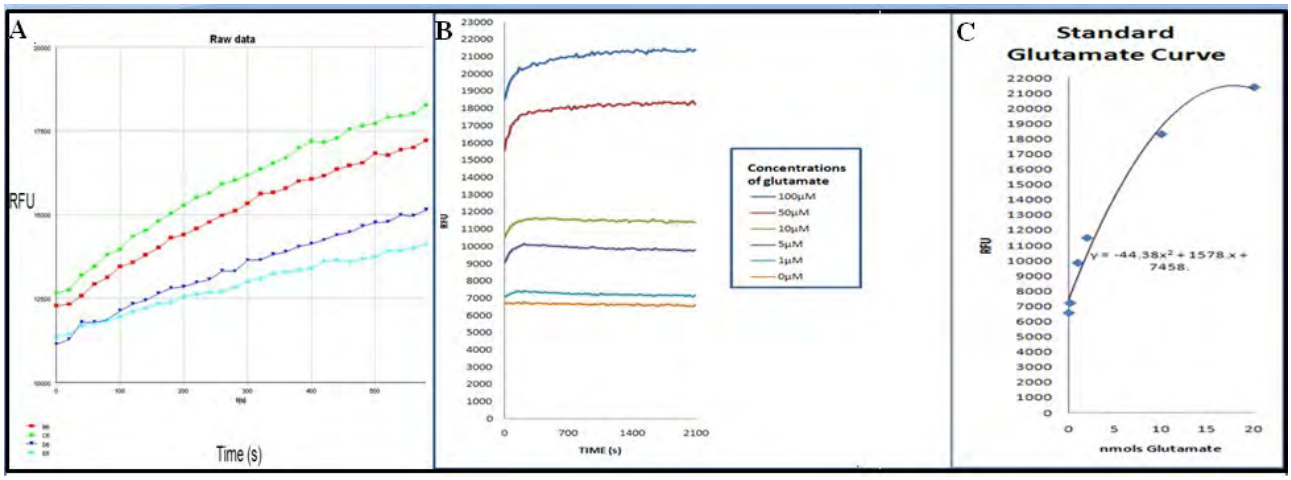

FIGURE 10. GLUTAMATE STANDARD CURVE. The processing of raw data from the glutamate release assay. **A)** The data collected from the assay was initially in Relative Fluorescence Units (RFU). In order to convert it into glutamate levels, standard curves had to be generated. **B)** The standard curves consisted of serial dilutions of known L-glutamic acid concentrations (0-100 µM) and were generated under the same conditions as the actual glutamate release assay with the synaptosomes. **C)** Later a common time point was chosen where all the generated curves had reached a plateau and the enzyme glutamate dehydrogenase (GDH) had saturated the available L-glutamic acid. The L-glutamic acid concentrations were then plotted as a function of RFU to yield a graph from which RFU could be converted into glutamate levels.

Pilot experiment

Next I performed a pilot experiment to assess the functionality of synaptosomes purified through a Ficoll-gradient based method (Figure 11). To stimulate the exocytosis of synaptic vesicles from synaptosomes, I added a depolarizing agent to the synaptosomes (either 4-AP or KCl).

Since the release of neurotransmitters is Ca^{2+}-dependent, the synaptosomes had to be supplemented with extracellular Ca^{2+} ions. In the assays, I added either $CaCl_2$ or a Ca^{2+}-chelating compound (EGTA) to the synaptosomes before adding the depolarising agents.

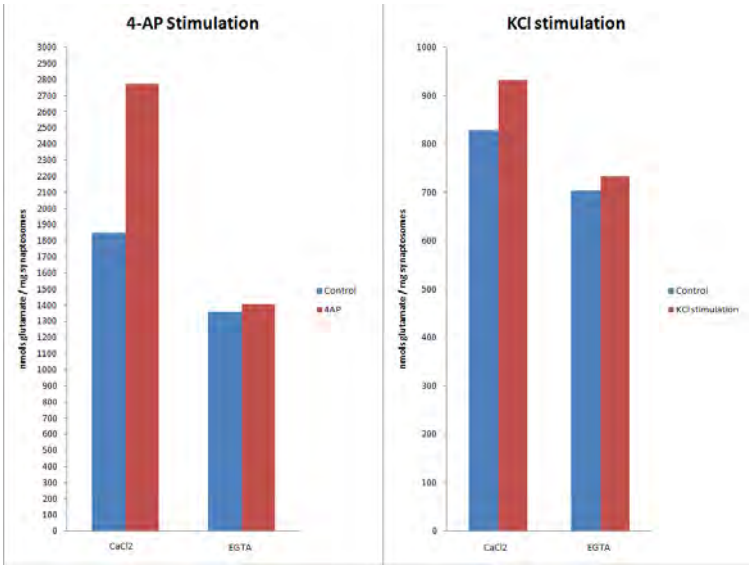

Figure 11. Pilot experiment with Glutamate Release Assay. The synaptosomes in this experiment had been purified through the Ficoll-based procedure. In brief, 100 µg of synaptosomes were pre-incubated in an isotonic buffer for 15 min. Thereafter, 1 mM $NADP^+$, 37 units GDH and either 1.3 mM $CaCl_2$ or 0.5 mM EGTA was added to the synaptosomes. The synaptosomes were transferred to a 96-well microtiter plate and incubated on shake for 5 min. Then either 1 mM 4-AP or 50 mM KCl was added to two of the synaptosome samples (nothing was adding to the remaining two samples). All the samples were incubated for 10 min and an endpoint fluorescence reading was taken (Ex=350 nm and Em=440 nm). The results are based on duplicate samples. (All incubation were done on shake at 37°C).

As expected, there is a higher amount of glutamate in the samples supplemented with $CaCl_2$ than in EGTA; also strangely more glutamate is observed in 4-AP stimulated samples than in KCl stimulated samples. However, the glutamate levels observed in Figure 11 are much higher than stated in the literature; in comparison Sim et al (2006) observed less than 16 nmols per mg when they assayed the glutamate release from an equivalent amount of synaptosomes.

A plausible explanation could be that the synaptosomes were ruptured (either before, after or during the GRA) spilling the total glutamate contents into the extracellular space. Figure 12 (below) shows an electron microscopic image of the synaptosomes isolated from a Ficoll-gradient based method.

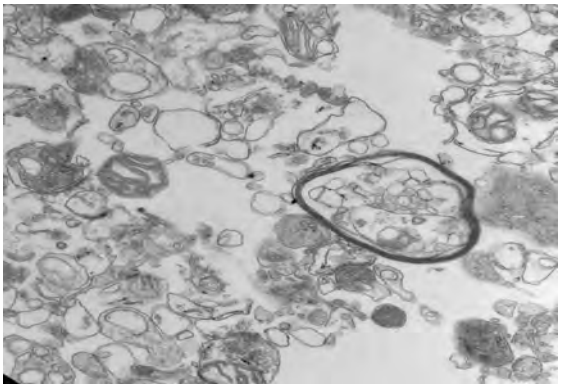

Figure 12. Electron microscopic image of synaptosomes isolated from the Ficoll-based method. The synaptosomes were fixed in 25% glutaraldehyde (the EM was processed by Associate Prof. Kjell Hultenby at Karolinska University Hospital, Emil unit).

However, the EM-image is too congested and it is difficult to distinguish whether the individual synaptosomes were ruptured or intact, likewise it is also difficult to assess sample purity. Thus, in successive experiments, I refined the synaptosome isolation protocol.

Synaptosome isolation

I tried to determine what factors could lead to improved quality and purity of the synaptosomes isolated from the Ficoll-based method (described by Nicholls and Sihra, 1986). Initially I optimized the handling of the brain tissue from which the synaptosomes were isolated. I took precautions to keep the samples as fresh as possible throughout the isolation protocol. So all the steps and material were kept at 4°C when feasible. Also I tried to reduce the time it took from

sacrificing the animals to homogenizing the tissue by concentrating only on one animal per experiment, and bringing the homogenizer down to the animal lab (altogether saved roughly 30 min). Furthermore, care taken to pre-chill the buffers and the allotted utensils (including the Teflon-glass homogenizing pestle), and adding a protease inhibitor (PI) to the buffer, seemed likely to enhance the availability of fresher (less degraded) material to work with. Then instead of working with whole rat brain, I dissected out the brain cortex and removed most of the visual myelin/white matter to yield as pure and uniform samples as possible. Also after the final isolated products had been pelleted down, I tried to remove all the liquid surrounding the pellets to avoid degradation. Next I switched over to a Percoll-based isolation protocol (described by Dunkeley et al., 2008). Figure 13 contrasts both methods of isolation. The precautions described in handling the brain tissue as well as the final washing step were transferred further to the Percoll-gradient based method.

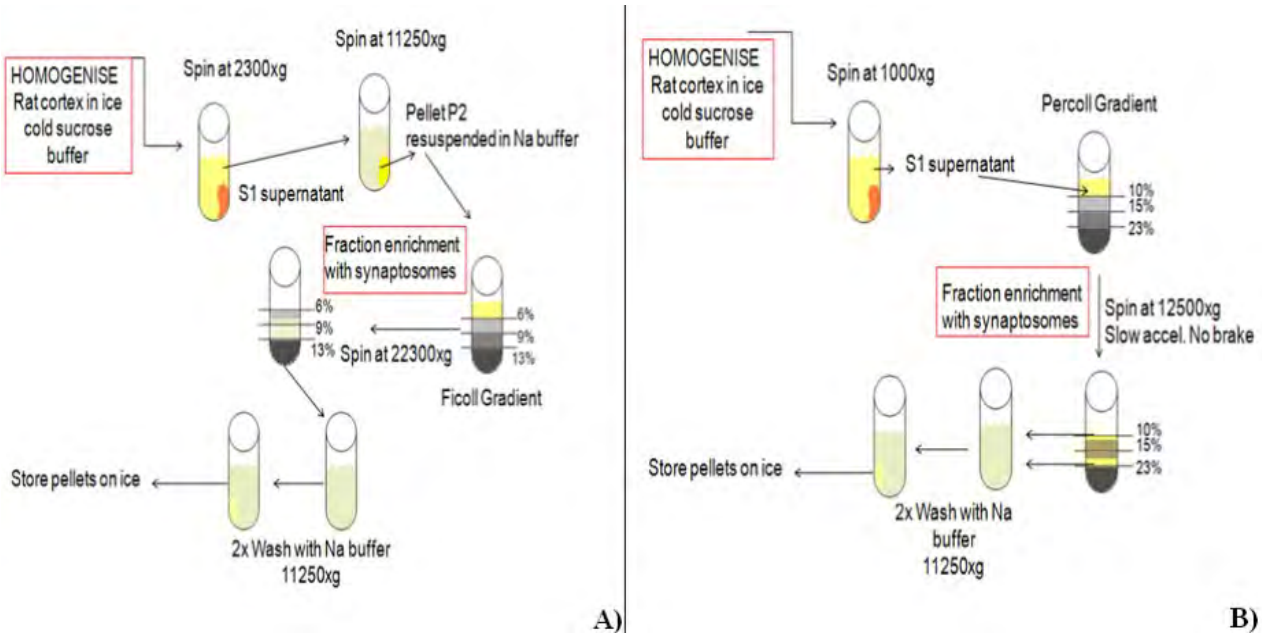

Figure 13. Ficoll- vs. Percoll- based synaptosome isolation procedure. The flowchart contrasts **A)** the Ficoll-based synaptosome isolation (modified protocol of Nicholls and Sihra, 1986) with **B)** the Percoll-based procedure for synaptosome isolation (modified protocol of Dunkley et al., 2008).

Figure 14 (below) shows EM-images of synaptosomes isolated through a Percoll-gradient based method. Individual synaptosomes can be distinguished and evidence of both intact and ruptured synaptosomes are present. Even sample purity can be assessed through the EM-images to relatively pure (some traces of myelin are present, in Figure 14A).

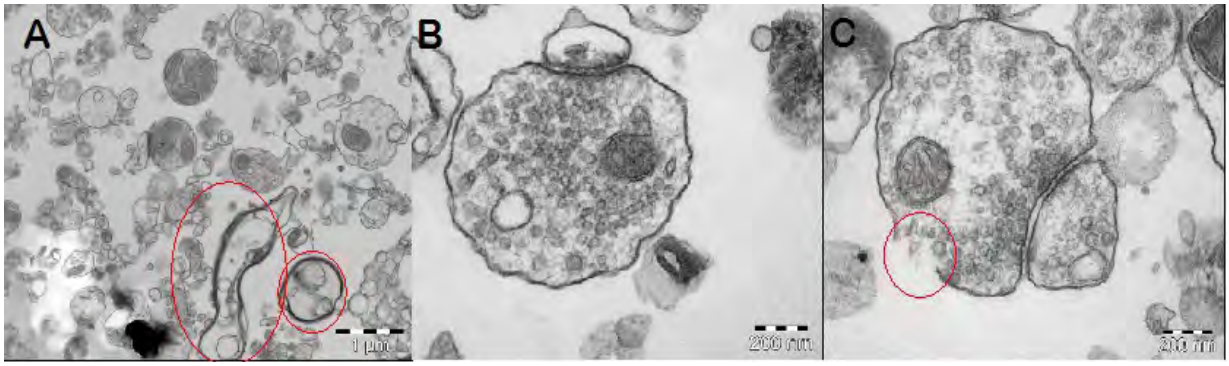

Figure 14. Electron microscopic images of synaptosomes isolated from the Percoll-based method. The synaptosomes were fixed in 25% glutaraldehyde (the EM was processed by Associate Prof. Kjell Hultenby at Karolinska University Hospital, Emil unit). In **A)** an overview of synaptosome fraction (scale: 1 µm). Encircled in red are myelin contaminants. In **B)** a zoom-in of a synaptosome with intact membrane (scale: 200 nm). In **C)** a zoom-in of a synaptosome with a 'tear' in its membrane (scale: 200 nm). Encircled in red is the area of the membrane that is 'torn' and what seems like synaptic vesicles that are leaking out (small round black bodies).

Assessing the functionality of isolated synaptosomes through glutamate release assay

Compared to how I performed the pilot experiment, the assay described in Figure 15 is more in line with how Nicholls and Sihra (1986) had originally described it.

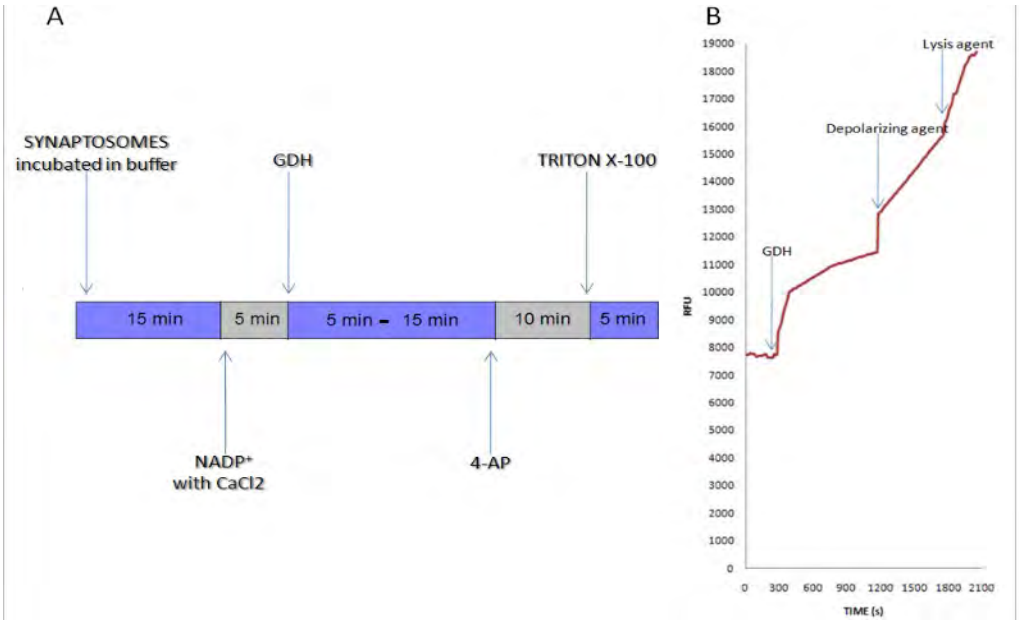

Figure 15. Scheme of Glutamate Release Assay. In **A)** the scheme of the assay is depicted: purified synaptosomes should be pre-incubated in an isotonic solution for 15 min at 37°C. Thereafter the synaptosomes should be transferred to a microtiter plate reader and $NADP^+$ added together with $CaCl_2$; at this point the basal fluorescence should be monitored (Ex = 350 nm and Em = 440 nm) for 5 min. Next the enzyme glutamate dehydrogenase (GDH) should be added to the samples and fluorescence recorded until the relative florescence units (RFU) starts leveling off. Synaptosomes should then be stimulated to release neurotransmitters with the addition of a depolarizing agent (for example, 4-AP) and fluorescence recorded for an additional 5-10 min. The total glutamate content of synaptosomes should be determined with the addition of 1% Triton X-100. In **B)** a theoretical graph shows a glutamate release assay in which the synaptosomes have responded to the depolarizing agent. The assay starts after the addition of $NADP^+$ and $CaCl_2$ to the synaptosomes. Next the enzyme GDH is added to the synaptosomes. With the addition of GDH we would ideally like to observe an increase in glutamate levels as the enzyme converts the available glutamate into ketoglutarate. However, gradually we would like to see the graph leveling off as GDH saturates the glutamate available in the extracellular space. At this point, the depolarizing agent should be added. A peak of glutamate release should be observed if the synaptosomes have been stimulated and fluorescence monitored for at least 5-10 min post-depolarization. Then at the end of the assay, 1% Triton X-100 should be added to lyse the synaptosomes and release all the enclosed glutamate. The lysis of the synaptosomes should yield a markedly visible increase in glutamate release if the majority of the synaptosomes have remained intact until the end of the assay. The arrows define the "jumps" observed in the graph as the addition of the enzyme glutamate dehydrogenase (GDH), depolarizing agent (such as 4-AP) and the lysis agent (Triton X-100). The graph is shown in a raw data form, in which the Relative Fluorescence units (RFU) have not been translated into glutamate.

I assessed the glutamate levels in the synaptosomes in accordance with the scheme described above in Figure 15; the representative results are shown in Figure 16.

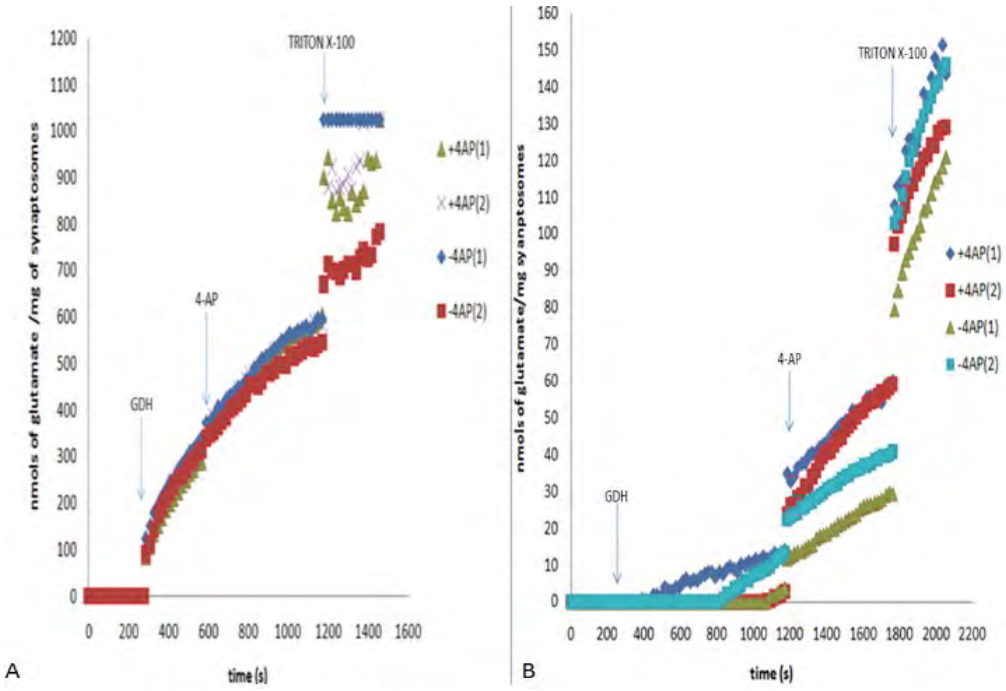

Figure 16. Two Glutamate Release Assays representative of the different variables tested. In **A)** the assay was performed on synaptosomes isolated from the Ficoll-based method. 100 µg of synaptosomes were pre-incubated for 15 min. Then the synaptosomes were transferred to a florescence reader and 1 mM $NADP^+$ and 1.3 mM $CaCl_2$ was added and florescence monitored for 5 min. Next 37 units of enzyme glutamate dehydrogenase (GDH) was added to the synaptosomes and incubated for 5 min. Then in two of the synaptosome samples 1 mM 4-AP was added (nothing was added to the remaining two samples) and florescence monitored for 10 min. At the end of the assay, the synaptosomes were lysed with the addition of 1%-Triton to all samples; florescence was monitored during the next 5min. (All incubation were on shake at 37°C). And in **B)** the assay was performed on synaptosomes isolated from the Percoll-based isolation procedure. 25 µg of synaptosomes were pre-incubated for 15 min. Then the synaptosomes were transferred to a florescence reader and 1 mM $NADP^+$ and 2.5 mM $CaCl_2$ was added to the synaptosomes and florescence monitored for 5 min. Next 5 units of enzyme glutamate dehydrogenase (GDH) was added to the synaptosomes and incubated for 15 min. Then in two of the synaptosome samples 1 mM 4-AP was added (nothing was added to the remaining two samples) and florescence monitored for 10 min. At the end of the assay, the synaptosomes were lysed with the addition of 1%-Triton to all the samples; florescence was monitored during the next 5 min. (All incubations were on shake at 37°C).

In assessing the functionality of isolated synaptosomes, different variables were tested, including the protocol for synaptosome isolation (Ficoll vs. Percoll), amount of synaptosomes assayed (25-

100 µg), $CaCl_2$ concentration (1.3-2.6 mM), amount of GDH added (5-37 units) and relative incubation times with GDH (5-15 min), the concentration of the depolarizing agent (30-50 mM KCl; 1-2 mM 4-AP) and the duration of incubation times with the depolarizing agent (5-10 min). Despite the several optimizing efforts made in the isolation protocol and GRA, the method for the isolation of functional synaptosomes was not developed (see figure 16). The effect of the depolarizing agent on glutamate release was sometimes seen to be higher than in the controls, however, after the addition of the lysis agent it could then be seen that the total glutamate levels were also higher in the controls than in the stimulated samples.

DISCUSSION

Assessing APP and Notch processing in synaptic vesicles

The preliminary results reported in this thesis indicate that Aβ is generated in the synaptic vesicles through the cleavage of γ-secretase. They also indicate the absence of another γ-secretase substrate, Notch. The relevance of these findings, when validated, is in the novel design of γ-secretase inhibitors that could specifically target the synaptic vesicles and thus bypass the harmful side-effects linked to the indirect inhibition of Notch.

Assessing the functionality of isolated synaptosomes through glutamate release assay

Notably the amount of glutamate observed in the assays were much higher than those stated in the literature, for example, for 100 µg of synaptosomes Sim et al. (2006) reported less than 160 nmols per mg glutamate released. I suspect that this is due to ruptured synaptosomes. With the addition of the lysing agent at the end of the assay, there was a notable peak in glutamate levels which would indicate that not all the synaptosomes were ruptured until the end of the assay. I speculate that glutamate might constantly be leaking out from the synaptosomes during the assay (look at Figure 14b, synaptosomes with “a tear” in its membrane). This hypothesis could gain support from the fact that after the addition of the enzyme GDH I was unable to see the typical 'leveling off' phase even after monitoring for 15 min. Whereas in the literature (Nicholls and Sihra, 1986; Sim et al, 2006) this point is usually reached after 5 min. I tested the assay with different amounts of GDH (5-37 units), however, it would appear that GDH was unable to saturate the extracellular glutamate and would therefore not plateau. Though I do not rule out the possibility of synaptosomes also getting ruptured during the assay, I think that it is the conditions prior to the assay (the isolation protocol) that should be further optimised in order to reduce the high glutamate levels observed. When I switched from the Ficoll-based to the Percoll- based method of synaptosome isolation, a notably lower amount of glutamate was observed in the assays. This could be because the Percoll-based method is faster than the Ficoll-based method, and is also much gentler on the synaptosomes with less centrifugation steps at lower g-forces and the homogenization step being less rigid with 8 stokes at 700 rpm compared to the 10 stokes at

900 rpm in the Ficoll-based method. A possible improvement in the isolation protocol would be to find a balance between the number of up-and-down strokes of the pestle and the rate of pestle rotation that would reduce the damage to the synaptosomes without compromising the homogeneity of the tissue.

Refinement of the assay used to assess synaptosome functionality (GRA)

Typically when glutamate release assays are reviewed in the literature (ex. Nicholls and Sihra, 1986; or Sim et al., 2006) a known concentration of glutamate is added at the end of each experiment to quantify the amount of glutamate. However, in the case of ruptured or 'leaky' synaptosomes, the amount of glutamate at any given time is merely a reflection of how much glutamate the enzyme has already processed, and not how much glutamate is available. Therefore, I generated standard curves (figure 11) based on serial dilutions of known concentrations of L-glutamic acid (0-100 μM) and used them to assess the amount of glutamate in the assays.

ACKNOWLEDGEMENT

This is not a recommendation for someone to make any mistake but if someone by chance were to make one, then I do wish for them that they do it under the supervision of Dr. Susanne

Frykman. Because in return she will give them far more knowledge than what they were lacking up to that point, without having them feel guilty for their inexperience. She has a very open and kind personality, and through her experience and knowledge she made this research work both a learning and enjoyable experience for me. I thank her for the independence she gave me in this research work, and for her constant support and guidance. She is a very professional and dedicated supervisor. I would also like to acknowledge Dr. Matthew Holt (at Max Planck Institute for Biophysical Chemistry, Department of Neurobiology) for providing the CPG-SVs used in this study, and Associate Prof. Kjell Hultenby (at the electron microscopy unit Emil, Karolinska University Hospital) for processing the EM-images of my synaptosome fractions.

MATERIALS AND METHODS

Isolation of membrane fractions (P3) from rat brain

20 frozen rat (Sprague-Dawley, 8-12 week old) brains were obtained from Rockland. In groups of 3-4 the rat brains were homogenized (24 stokes at 1500 rpm) in buffer A (EGTA, 2 mM; KCl, 50 mM; HEPES, 20 mM; pH 7.5) containing protease inhibitor cocktail. The homogenates were centrifuged at 1000xg for 10 min at 4°C. The supernatant was centrifuged at 10 000xg for 30 min in the JA 25,50 rotor at 4°C. The resulting supernatant was centrifuged in a SW32 rotor at 100,000xg for 1hr, at 4°C. The pellets were re-suspended in buffer A and aliquoted into 1ml fractions and frozen in liquid nitrogen.

Activity Assay- APP processing

In vitro generation of Aβ40 in CPG-SVs was analysed using the WAKO sandwich ELISA kit. This kit utilizes the monocolonal antibody BNT77 which binds to Aβ amino acids 11-28.

65 µg/sample rat brain CPG-SVs were centrifuged at 100,000xg for 1hr, at 4°C. The pellet was re-suspended in H Buffer (NaCl, 150 mM; EDTA, 5 mM; HEPES, 20 mM; pH 7,0) containing protease inhibitor cocktail and with 0,4% CHAPSO, 1,10-Phenanthroline (5 mM) and Thiorphan (10 mM), with either DMSO (100 µM), L685 (100 µM), C99 (0,2 nM) or a combination of L685 (100µM) + C99 (0,2 nM), and incubated (37°C) on shake for 17 hrs. As positive control, 100 µg/sample rat brain membrane fractions (P3) was processed in parallel to the CPG-SVs.

The reactions were stopped after 17 hrs with 1 x RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM TRIZMA, pH 8.0). The samples were then incubated at 95°C for 5 min followed by a 5 min centrifugation at 1000xg at room temperature. The resulting supernatants were analysed through the WAKO sandwich ELISA kit.

Activity Assay- Notch Processing

90 µg/sample rat brain CPG-SVs were centrifuged at 100,000g for 1hr, at 4°C. The pellet was re-suspended in H Buffer (NaCl, 150 mM; EDTA, 5 mM; HEPES, 20 mM; pH 7,0) containing protease inhibitor cocktail with either DMSO (100 µM) or L685 (100 µM) and incubated for 17 hrs at 37°C, on shake. The negative controls were kept for 17 hrs at 4°C. As positive control, 90 µg/sample rat brain membrane fractions (P3) was processed in parallel to the CPG-SVs.

SDS-PAGE and Western Blotting

90 µg protein samples (1 x Laemmli sample buffer; DTT, 50 mM; to yield a final protein concentration of 4 µg/µl) were denatured at 95°C for 5 min. The samples were separated by SDS-PAGE on 4–12% Bis-Tris gel (run at 130V, 400mA, and 100W until the dye run out). Proteins were transferred to PVDF membranes and probed for 17 hrs with NICD primary antibodies at room temperature, on shake. The membrane was washed 5-6 times with PBST. Next the membrane was incubated for 2 hrs with Horseradish peroxidase-coupled secondary Anti-rabbit antibody, on shake. The proteins were visualised through DURA chemiluminescence kit and detected through CCD-camera and/or X-ray film exposure.

Detection of different cellular organelles and γ-secretase components

5 µg of rat brain homogenate (dissolved in Sucrose, 320 mM), Peak-1 (dissolved in Sucrose, 320 mM), Lysis Pellet 2 (dissolved in Sucrose, 40 mM) and CPG-SVs (dissolved in DTT) were mixed with 4 x Laemmli sample buffer with 50 mM DTT added. The samples were heated at 95°C for 5 min and then separated by SDS-PAGE on 4–12% Bis-Tris gel (run at 130V, 400mA, and 100W until the dye ran out). Proteins were transferred to PVDF membranes and probed overnight with the primary antibodies: GM130, ERG1C53, Synaptophysin, Syntaxin-13, and

anterior pharynx defective-1, at room temperature, on shake. The membrane was washed 5-6 times with PBST. Next the membrane was incubated for 2 hrs with Horseradish peroxidase-coupled Anti-rabbit or Anti-donkey antibody at room temperature, on shake. The proteins were visualised through DURA chemiluminescence kit and detected through CCD-camera and/or X-ray film exposure.

Synaptosome Isolation, Ficoll-based method

Synaptosomes were prepared by modification to the protocol described by Nicholls and Sihra, (1986). Briefly, two rats were sacrificed with carbon dioxide gas. The cerebral cortices were dissected out from the brain and cleared of visible myelin and arteries. The isolated tissue was then homogenized (10 stokes at 900 rpm) in 30 ml chilled sucrose solution (Sucrose, 0.32 M; HEPES, 5 mM, pH 7.4) with protease inhibitor cocktail. The homogenate was spun at 2,300xg for 2 min at 4°C. The supernatant was collected and centrifuged in a JA 25.50 rotor at 11,250xg for 12 min, at 4°C. The resulting pellets were re-suspended in 4 ml chilled Sucrose solution (320 mM; HEPES, 5 mM, pH 7.4) containing protease inhibitor cocktail. 2.4 ml of this mixture was loaded onto a discontinuous Sucrose:Ficoll (top-down) gradient, consisting of 13% (3.5 ml), 9% (0.9 ml), and 6% (3.5 ml) dissolved in sucrose solution. The gradients were spun for 35 min at 24,000xg in a SW40 rotor at 4°C. The bands between the 9% and 13% Ficoll boundaries were collected and diluted up to 20-ml with chilled Sodium buffer (NaCl, 140 mM; KCl, 5 mM; Sodium HEPES, pH 7.4, 20 mM; $NaHCO_3$, 5 mM; Na_2HPO_4, 1.2 mM; $MgCl_2$, 1 mM; glucose, 10 mM). These crude synaptosome suspensions were centrifuged in a JA 25.50 rotor at 11,250xg for 12 min, at 4°C. The pellets were re-suspended in 6 ml chilled Sodium buffer and fractionated into aliquots of 1 ml. These aliquots were centrifuged in the small lab bench at 11,200xg for 12 min, at 4°C. The resulting pellets were stored on ice and used within 8 hrs.

Synaptosome isolation, Percoll-based method

Synaptosomes were prepared by modification to the protocol described by Dunkeley et al. (2008). One rat was sacrificed through carbon dioxide gas and decapitation. Within minutes the cerebral cortex was dissected out from the brain and homogenized (8 stokes at 700 rpm) in 9 ml chilled homogenizing buffer (sucrose, 0.32 M; EDTA, 1 mM; DTT, 0.25 mM; Tris (Trizma base), 5 mM ; pH 7.4) containing protease inhibitor cocktail. The resulting homogenate was spun at 1,000xg for 4 min at 4°C. Supernatant (2 ml/ gradient) was loaded onto 4x discontinuous sucrose:Percoll (top-down) gradients consisting of 2 ml of each: 23%, 15%, and 10% Percoll dissolved in homogenizing buffer. The gradients were spun in a SW40 rotor, at 32,000xg (4°C), with 5 min at full speed; the centrifuge was started at vacuum <20, run at max acceleration, whereas the last 44.3 g was with slow deceleration. The bands between the 10% - 15% borders and between the 15% - 23% borders were pooled from all the four gradients and diluted 10 folds in chilled Sodium buffer (NaCl, 140 mM; KCl, 5 mM; sodium HEPES, pH 7.4, 20 mM; $NaHCO_3$, 5 mM; Na_2HPO_4, 1.2 mM; $MgCl_2$, 1 mM; glucose, 10 mM). These synaptosome suspensions were centrifuged in the JA 25,50 rotor at 11,250g for 12 min, at 4°C. The formed pellets were re-suspended in 6 ml of chilled Sodium buffer and fractionated into aliquots of ca 1 ml. The fractions were then centrifuged in the small lab bench at 11,200xg for 12 min, at 4°C. The resulting pellets were stored on ice and used within 8 hrs.

Electron Microscopy

One of the synaptosome pellets was used for protein determination through the Bradford Protein Assay kit with bovine serum standards. Two of the synaptosome pellets (0.98-1.14 mg protein)

were fixed in 1 ml 25% glutaraldehyde and sent for electron-microscopic processing (Sent to Associate Professor Kjell Hultenby at Karolinska University Hospital, Emil-Unit).

Pilot Experiment

100 µg of synaptosomes isolated from the Ficoll-based method was dissolved in Sodium buffer (NaCl, 140 mM; KCl, 5 mM; sodium HEPES, pH 7.4, 20 mM; $NaHCO_3$, 5 mM; Na_2HPO_4, 1.2 mM; $MgCl_2$, 1 mM; glucose, 10 mM) to yield final concentration of 1 mg/ml synaptosomes. The synaptosomes were pre-incubated for 15 min. Thereafter, 1 mM $NADP^+$, 37 units GDH and either 1.3 mM $CaCl_2$ or 50 mM EGTA was added to the synaptosome samples. The samples were transferred to a 96-well microtiter plate and incubated on shake for 5 min. Then either 1 mM 4-AP or 50mM KCl was added to two of the samples (nothing was added to the remaining two samples). All the samples were incubated for 10 min and an endpoint fluorescence reading was taken. The generation of NADPH was monitored by excitation at 350 nm and emission at 440 nm; Bandwidth (emission and excitation): 20 nm; Gain (manual): 91; FlashMode: High sensitivity; Valid temperature range: 35-38°C. The experiment was done with duplicate samples. (All incubation were done on shake at 37°C).

Glutamate Release Assay

25-100 µg of synaptosome was dissolved in sodium buffer (NaCl, 140 mM; KCl, 5 mM; Sodium HEPES, pH 7.4, 20 mM; $NaHCO_3$, 5 mM; Na_2HPO_4, 1.2 mM; $MgCl_2$, 1 mM; glucose, 10 mM) to yield a final protein concentration of 1 mg/ml. The synaptosomes were incubated on shake for 15 min at 37°C . The synaptosomes were then transferred to a 96-well microtiter-plate with a clear bottom. Next $NADP^+$ (1 mM) and $CaCl_2$ (1.3-2.6 mM) were added and fluorescence monitored for 15 cycles. Then GDH (5-37 U) was added and fluorescence monitored for 15- 45

cycles. In order to stimulate glutamate release from the synaptosomes, 4-AP (1-2 mM) was added to the synaptosomes and fluorescence monitored for 30 cycles. In the negative controls, 4-AP was not added. Then at the end of the assay, 1% Triton X-100 was added and fluorescence monitored for 15 cycles.

The generation of NADPH was monitored by excitation at 350 nm and emission at 440 nm; Bandwidth (emission and excitation): 20 nm; Gain (manual): 91; Number of reads: 10; FlashMode: High sensitivity; Time between move and flash: 300 ms; Valid temperature range: 35-38°C; Shake duration (orbital medium): 10 s; Shake settle time: 1 s; Shake duration between cycles (Orbital medium): 3 s; Shake settle time between cycles: 1 s; Kinetic interval: 20 s.

Glutamate Release Assay, Standard Curve

A glutamate standard curve (0-100 µM) was also set up alongside the glutamate release assay. In a 96-well microtiter-plate: L-glutamic acid (serial dilution to yield: 0 – 100 µM), $NADP^+$ (1 mM), GDH (5-37 U) and $CaCl_2$ (1.3-2.6 mM) was added and fluorescence monitored for 75-100 cycles. All dilutions were made using Sodium buffer. The generation of NADPH was monitored by excitation at 350 nm and emission at 440 nm; Bandwidth (emission and excitation): 20 nm; Gain (manual): 91; FlashMode: High sensitivity; Number of kinetic cycles: 75-105; Time between move and flash: 300 ms; Valid temperature range: 35-38°C; Shake duration (orbital medium): 10 s; Shake settle time: 1s ; Shake duration between cycles (Orbital medium): 3 s; Shake settle time between cycles: 1 s; Kinetic interval: 20 s.

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