5-2017

Exploration of the Interactions between amyloid-Beta Protein and Insulin in Various Ionic Conditions

Quade Goldenberg
University at Albany, State University of New York

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Exploration of the interactions between amyloid-β protein and insulin in various ionic conditions

An honors thesis presented to the
Department of Biological Sciences,
University at Albany
State University of New York
in partial fulfillment of the requirements
for graduation with Honors in Biochemistry and Molecular Biology
and
graduation from the Honors College.

Quade Sinclair Goldenberg

Research Advisor: Ewan McNay, Ph.D.
Second Reader: Gabriele Fuchs, Ph.D.

May, 2017
Abstract

Alzheimer’s disease (AD) is characterized by tau tangles and amyloid-β (Aβ) peptide aggregates amyloids in the brain, specifically in the hippocampus. Aβ plaques are known to form primarily in the extracellular space around neuronal cells, interfering with synaptic transmission. The aggregation of Aβ in the hippocampus is associated with a decline in hippocampally-mediated cognitive abilities, which is a symptom of AD. AD has a strong correlation to type 2 diabetes mellitus (T2DM), which is defined by hyperinsulinemia and dysregulated glucose metabolism. It has been shown that insulin plays a role in both memory and learning in the hippocampus. The simultaneous elevation in insulin and Aβ suggests a connection between the molecular effectors of both T2DM and AD, insulin and Aβ (monomeric, oligomeric and fibril), respectively. Moreover, both zinc and calcium are required for the stabilization of insulin and AD patients show signs of both zinc and calcium dysregulation. Thus, I propose that destabilization of insulin facilitates Aβ oligomerization. To investigate the relationship between Aβ and insulin, I varied concentrations of Aβ, insulin, zinc, calcium and glucose and observed amyloid formation by performing dot blot immunoassays. I have found that at low concentrations of calcium and zinc and high concentrations of glucose, increased formation of Aβ oligomers were observed. I discuss future experimentation to further characterize these complexes on the molecular level.
Acknowledgements

I would like to graciously thank my advisor Dr. Ewan McNay for bringing me into his lab and providing me with his expertise, understanding and freedom to explore a topic that is of great interest to me, for without him, none of this research or precious experiences would have been possible.

I am eternally grateful to Greg Fitzgerald and both graduate and undergrad members of the lab, whose constant support, time and immense interest in my growth and success cannot be thanked enough. I would like to show my appreciation for their constant support and knowledge and for always being a consistent and reliable source, for without them, I would have never found many valuable links and resources.

No amount of words can express how indebted I am to Dr. Gabriele Fuchs for providing me with the necessary materials to complete my research. Her overwhelming generosity and kindness is irreplaceable. She always seemed to have time in her busy schedule to let me into restricted areas, guide me during times of confusion and be the second reader of my undergraduate honors thesis.

I would like to thank my parents for their constant motivation and undivided support and attention through it all. They helped me focus on my immediate task at hand and were always accessible regardless of the time.

I would like to show my enormous appreciation to my girlfriend Lena, for her ridiculous understanding towards my focus on academics and her persistent support. Both her and my parents were my true pillars and without them, none of this would have been possible.
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Background

Alzheimer’s disease (AD) is a devastating incurable neurological disorder that is seen to affect more than 5.4 million people in the United States, with a vast majority being over the age of 65. Today, someone develops AD on average every 66 seconds with forecasted estimates to reach 13.8 million in the United States by 2050. AD is the 6th leading cause of death in the United States, killing more than breast and prostate cancers combined. AD is characterized by intracellular tau tangles and extracellular aggregation of amyloid-β (Aβ) peptides in the brain, primarily in the hippocampus. The hippocampus is located in the brain’s medial temporal lobe, and is known primarily for its association in memory formation, emotion, and spatial navigation. Aβ peptides congregate in the extracellular space between neurons, causing a decline in synaptic transmission, in turn decreasing hippocampally-mediated cognitive abilities which is symptomatically seen in AD. AD is the leading form of senile dementia, accounting for roughly 80 percent of cases.

There are two main hypotheses, the tau hypothesis and the amyloid hypothesis, that provide some possible pathways and framework for this amyloidogenic disease, but the pathology is still not well understood. Rather, a large variety of mechanisms contribute simultaneously to the disease, causing its complexity. The first major breakthrough in understanding the neuropathology of AD came from Glenner and Wong (1984), who purified Aβ from senile plaques (SP) associated with the disease. Aβ is a 36-43 residue protein that is derived from the amyloid-β precursor protein (APP) after it is cleaved by β-secretase and γ-secretase. Aβ40 and Aβ42 are the most common isoforms found in SP, with Aβ42 being more fibrillogenic and neurotoxic, and are therefore the main focus of AD research. The insoluble Aβ plaques are mainly composed of misfolded Aβ proteins, although a SP has been observed to
contain up to 488 unique proteins.\(^6\) Aβ is considered an intrinsically unstructured protein due to its ability to take on many different conformations in solution, allowing for promiscuous interactions with many substrates.\(^7\) There are three conformation forms for Aβ: monomeric, oligomeric and fibrillar. Soluble oligomeric Aβ is reputed to be the most neurotoxic form,\(^8\) and is seen to be incorporated in multiple facets of the dementia aspect of the disease.

Another disease that has reached epidemic levels in the United States is Type II Diabetes Mellitus (T2DM) which is an incurable metabolic disorder characterized by dysfunctional insulin signaling. Insulin is a hormone that regulates blood sugar levels by promoting the uptake of glucose into cells. In a T2DM patient, the body has an abnormally high resistance to insulin, and the beta cells of the islets of Langerhans in the pancreas cannot produce enough insulin to compensate for the inadequate signaling. This results in heightened blood glucose levels, or hyperglycemia. The insulin resistance also leads to hyperinsulinemia, or excess insulin in the blood.\(^9\) Though insulin has been established as a peripheral glucose regulator, studies have suggested that responsive insulin receptors (IR) are expressed in the central nervous system, including the hippocampus.\(^10\) Additionally, it is seen that insulin plays a role in hippocampally-mediated tasks, such as memory.\(^11\)

Many studies suggest that T2DM patients are at an increased risk for AD,\(^12\) and AD has been coined the term “Type 3 diabetes” due to the commonality of insulin dysregulation and resistance.\(^13\) Clinical studies have supported the strong correlation between T2DM and AD via hyperinsulinemia and insulin resistance.\(^14\) These characteristics, along with hyperglycemia, connect T2DM and AD through common symptoms such as inflammation, oxidative stress and cognitive impairment, or directly through Aβ and tau.\(^15\)-\(^17\) The primary molecular effectors of both AD and T2DM, Aβ and insulin, respectively, are also linked. For instance, insulin
encourages Aβ production by stimulating the activity of γ-secretase in Aβ cleavage and by the release of intraneuronal Aβ. Insulin was also found to promote APP metabolism. Additionally, insulin and Aβ are degraded by the same insulin-degrading enzyme (IDE), suggesting that hyperinsulinemia may elevate Aβ through competitive degradation. Conversely, Aβ also functions in insulin’s regulation and pathways. Aβ competitively antagonizes insulin by binding to IRs, which further exacerbates insulin resistance by blocking the signal transduction of the receptor.

Aβ and insulin are both amyloidogenic proteins that share similar structural motifs, and can aggregate with one another. Due to their similarities and connections via IDE, APP and overall integration within each other’s regulation, it is possible that insulin is related to the accumulation of Aβ plaques. It is also not unreasonable for a third-party substrate to be involved in this connection. This is further supported by the multitude of molecular components found in a SP. One of three additional components that is explored is zinc. Zinc is an essential metal for homeostasis and physiologically found in cationic form. Zinc is integrated throughout the body, playing roles in prosthetic groups, protein structure, co-enzyme, signaling, etc. Zinc ions are a cofactor for the hexamerization of insulin. Usually two ions are needed, otherwise insulin will typically remain as a dimer or tetramer. Disputably, dysfunctional homeostasis of specific
transition metals, one of which being zinc, are believed to play a role in the pathogenesis of AD. In more recent research, this zinc AD pathology model involving increased localization of zinc in cortical grey matter and pathological lesions has been supported. The increased localized zinc levels are accompanied with a decrease in systemic and cellular zinc levels. Zinc’s association with AD, along with its strong association with insulin and IRs followed by the cascading effect of insulin resistance and hyperinsulinemia, lead to a more supportive need for the exploration of zinc in this context.

An additional constituent that is explored is calcium. Calcium is an essential metal found in all cells in the body, playing roles from cell signaling to muscle contraction and hormone regulation. Like zinc, calcium is also involved in insulin hexamer assembly, but plays a much larger role in insulin stabilization. Destabilized insulin is more likely to take the monomeric or dimeric form, and is more amyloidogenic. Even partial destabilized insulin is enough for its aggregation. Small insulin aggregates act as nucleation sites for further growth. This initial seed of insulin amyloid can hypothetically interact with Aβ, creating an alternative pathway for the initiation of Aβ plagues. Additionally, calcium dysregulation is correlated to AD via the calcium hypothesis. This hypothesis explains calcium ion signaling dysregulation leading to neurodegeneration and apoptosis via the amyloidogenic Aβ pathway. In brief, this hypothesis accuses Aβ oligomers for increasing the concentration of calcium ions pumped into the endoplasmic reticulum creating a cascade that ultimately ends with the release of internal calcium storages leading to calcium dyshomeostasis. The crux of this hypothesis revolved around the possibility of Aβ40 and Aβ42 peptides forming an ion channel that incorporates into the bilayer, allowing an influx of calcium ions.
The third constituent under exploration is glucose. The human brain’s main energy source is glucose and it accounts for roughly 20% of total glucose-derived energy consumption in the body. Glucose’s main metabolite is ATP, which is used for neuronal function, maintenance, neurotransmitter production and other processes. Dysglycemia is a standard characteristic seen in both T2DM and AD, with hyperglycemia being a major connection between the two diseases as previously discussed. Additionally, research has shown in rat models that extracellular glucose in the hippocampus is depleted during cognitive activity (spatial working memory), and that task performance is enhanced by the administration of exogenous glucose. Furthermore, it is also reported that hyperglycemia is associated with a decline in cognitive performance in diabetic patients. This reinforces the importance of tight glucose regulation and supports the association of cognitive impairment during dysglycemia as seen in both T2DM and AD. Glucose is also regulated by insulin as previously mentioned. The necessity of glucose for proper brain function along with the strong association of glucose dysregulation during T2DM and AD diseased states are just some of the reasoning for the exploration of glucose in its interactions with Aβ plaque formation.

**Materials and Methods**

*Dot blot*

The dot blot technique is used to determine the presence of monomeric/oligomeric/fibril Aβ. Dot blotting is usually done by spotting small samples volumes onto a nitrocellulose membrane in a grid like fashion. Marks are made in pencil at the top and side of the membrane to pinpoint spot locations. Each sample is normalized by the present protein mass concentrations to allow uniform signaling. Two microliters of a sample are spotted onto the membrane at the corresponding location. Spots are blotted in succession for each sample set, each being a dilution
of 1:2 from the preceding spot. Multiple sample sets can be spotted on one membrane sheet. As an improvement for manual dot blotting, a dot blot apparatus (e.g. by Bio-Rad) can be used, which allows for up to 96 samples in a 8x12 fashion, each with volumes between 200 and 500 μL to be spotted with the help of a vacuum.

<table>
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<th>Aβ (pg/mL)</th>
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Table 1: Five different sets with varying concentrations of Aβ, insulin, calcium, zinc, and glucose were tested by dot blotting.

Two sheets of filter paper, which were dampened with DI water, and a nitrocellulose membrane with a pore size of 0.2 μm (Amersham Protran 0.2 NC, GE Healthcare Life Sciences, prod. code 10600004) were cut (7.5cm x 11.5cm) and assembled in the dot blot apparatus. Serial dilutions of the stock solutions (table 1) were initially prepared in PBS, but then later only in 150 mM sodium chloride (Sigma) and 50 mM Tris-HCl, pH 7.5 (Sigma) and applied to the membrane by using one master stock solution and varying the volumes applied to the membrane. The highest concentration was achieved by spotting 6 mL of the solution onto the membrane, then 5 mL, 4 mL, etc. This was done with successive applications of 500μL, after each was fully passed through with the help of the full vacuum appendage and valve. The membrane was cut into four strips, destined for the respectively labeled antibody or loading control.
Aβ protein fragment 1-42 (Sigma, cat. 107761-42-2), zinc-free insulin (Apidra insulin glulisine 100 U/mL), calcium chloride (Sigma), zinc chloride (Sigma) and D-(+)-glucose (Sigma) were all used in their respective dosages in the creation of a master stock solution (100 mL) for each respective set seen in table 1. The dose values for each constituent are based off of estimated physiological levels pulled from the literature, with a deviation low (blue) and high (red) from these levels for each respectively. In attempt to create conditions closely mocking those seen physiologically, cerebrospinal fluid (CSF) or even mouse extracellular fluid (ECF) values were used, since no human brain ECF values are seen to be reported. Aβ CSF values fluctuate greatly in literature, and the correlation between increased, decreased or unchanged Aβ levels in AD patients compared to healthy controls is seemingly unestablished. Nonetheless, Aβ<sub>42</sub> CSF levels were seen to reside at ~500 pg/mL.<sup>41,42</sup> This value was thus used as a baseline for Aβ concentration, but due to the minimum sensitivity of the dot blot assay, the low concentrations used are seen to be three times of the estimated baseline values. The loose knowledge between CSF Aβ levels and those actually seen in the human brain allow for this small deviation. Additionally, it would be expected that in an AD diseased state, the Aβ levels, especially those around SPs, would be at a much higher concentration. From this, and the mere exploration of Aβ in these experiments leads to the deviation of the high Aβ value of 50,000 pg/mL or 100x greater of the previously established estimated baseline. Likewise, this was done for insulin, with an estimated baseline or roughly 3 pg/L<sup>43</sup>, with 100x deviations for both high and low concentrations. The exact values of 0.349 and 349 pg/L (low and high respectively) were used due to the simplicity of dilutions found from the stock Insulin (Apidra). Additionally, calcium concentrations of 0.7 mM<sup>44</sup> are reported in mouse hippocampus ECF; zinc concentrations of roughly 0.2 mg/L<sup>45</sup> are reported in CSF levels in AD patients; and glucose ECF
concentrations are reported to be 1.25 mM\textsuperscript{44}. These concentrations were used as rough estimates to mock physiological concentrations as expectedly seen in a T2DM/AD diseased brain. These values once again, were used as a baseline, and both high and low concentrations were established from these values. The magnitude for deviation for calcium, zinc and glucose were 10x that from baseline, opposed to the 100x for Aβ and insulin due the increased interest in the involvement and interactions between the proteins. Lastly, though not recorded, it should be known that there are also chloride ions in each of the solutions due to the use of CaCl\textsubscript{2} and ZnCl\textsubscript{2}.

Each solution set as seen in table 1 was incubated at 37°C for 1 hour, followed by a 20-minute cool off period, after creation. The spotting was done in sets of two (1&2, 3&4, etc.) as blotting immediately followed.

The Aβ stock was sonicated in a cold water bath for 1 minute after being suspended in 10 mM sodium hydroxide solution and later frozen at -20°C for storage. All other reagents used were stored as recommended.

\textit{Loading control}

One strip was rinsed with Milli-Q water, then stained with approximately 20 mL of Silver Stain (40% Sodium Citrate, 20% Iron (II) Sulfate-heptahydrate, 20% Silver nitrate weight per volume solutions mixed in a 90:5:4:1 Water: Sodium citrate: Iron sulfate: Silver nitrate ratio). This loading control sample was incubated on a rocker at room temperature (RT) for 30 minutes, then rinsed with DI water.

\textit{Immunoblot detection}
The three.remaining membranes were blocked for 1 hour at room temperature with 10-15 mL of blocking buffer (10% milk-PBST [PBS + 0.1% Tween20]). Following three 15-minute washes with DI water, the membranes were incubated with a 1:5000 dilution of LOC antibody in PBST (host rabbit, cat. AB2287, EMD Millipore), a 1:2000 dilution 6E10 antibody in 5% milk-PBST (host mouse, cat. 803001, Jackson ImmunoResearch Inc.), or a 1:1000 dilution of A11 antibody in 5% milk-PBST (host rabbit, cat. AHB0052, ThermoFisher). Incubation in primary antibody was done overnight at 4°C on a nutator. Following incubation in primary antibody, each membrane was washed four times with PBST for 10 minutes at RT. Anti-rabbit HRP or anti-mouse HRP secondary antibodies were diluted 1:1000 in PBST and 1:10000 in 5% milk-PBST, respectively. Membranes were then incubated on a rocker in the appropriate secondary antibody at room temperature for 1 hour. Following three washes with PBST standard chemiluminescence detection was performed using a Bio-Rad Chemidoc XRS+ instrument. Exposure times and signal intensities were chosen to maximize image quality (typically between 30s-120s).

Results

![Antibody control test](image)

Figure 2: Antibody control test. 2 μL of diluted 6E10, LOC and A11 antibodies were spotted straight onto the nitrocellulose membrane and incubated with the appropriate secondary antibody prior to imaging.

While the volume that can be spotted onto a membrane via traditional dot-blotting is limited to volumes of up to 10 μL or less, a dot blot apparatus (e.g. by Bio-Rad) allows for spotting of up to 96 samples with volumes of up to 500 μL with the help of a vacuum. To use physiological concentrations of protein samples, I first established a protocol to ensure that volumes of up to 6 mL can be applied to the nitrocellulose membrane.
Prior to testing the proposed model (figure 1), antibody activity (figure 2) and specificity (figures 3 and 5) were tested in several control experiments. To ensure that all antibodies can be used for immunoblotting, 2 μL of 6E10, LOC and A11 primary antibodies were spotted in their respective working dilutions and detected with their corresponding secondary antibodies by immunoblotting. The LOC and A11 primary antibodies are detected with HRP-conjugated anti-rabbit IgG secondary antibody, while only the 6E10 primary antibody is specifically detected by HRP-conjugated mouse IgG secondary antibody (Figure 2). Furthermore, the signal from the LOC antibody appears to be stronger than the A11 and 6E10 signal. This is not concerning since this test was merely to show that the antibody is active. Generally, signal strengths can vary greatly based off differences in exposure times, changes in binding affinities to the nitrocellulose membrane due to the presence/absence of the recognizable protein, or differences in antibodies’ respective working dilutions.

Since Aβ and insulin are both potentially amylogenic proteins, the A11 antibody, which recognizes amino acid sequence-independent oligomers, may potentially cross-react and recognize insulin oligomers in addition to the Aβ oligomers. In the A11 antibody reference sheet, it is mentioned that A11 antibody recognizes other oligomeric species, one of which being human insulin. To determine if any oligomeric insulin present contributes to a background signal, a serial dilutions of high (349 pg/L) and low (0.349 pg/L) insulin were spotted on a nitrocellulose membrane and an immunoassay with the A11 antibody was performed (Figure 3). While the A11 antibody does
detect some insulin signal, the low signal-to-noise ratio indicated that the levels of insulin has to be increased far beyond the levels used here to significantly impact the observed A11 signals.

I then tested how the concentration of glucose impacts Aβ detection. This initial experiment (figure 4) revealed that an increase in glucose concentration (same conditions as seen in Table 1, sets 1 and 2 [in PBS not NaCl Tris-HCl buffer]) resulted in increased binding and detection of Aβ with the LOC antibody. In attempt to potentially mock a diseased state of hyperglycemia and with the results seen from figure 4, high glucose conditions were used in most of the dot blot assays.

I confirmed the conformational form of Aβ detected from each of the three different antibodies that are used in the immunoassays. The antibody A11 should detect only oligomeric Aβ and minimally cross-reacts with insulin (figure 3). The LOC antibody has been described to mainly detect fibrils of Aβ and non-specifically detect monomeric Aβ but to a much lesser extent. The 6E10 antibody should detect all conformational forms of Aβ. The addition of 1% SDS followed by a 5-minute boil will cause Aβ to become monomeric. In contrast, oligomeric Aβ was formed by incubating Aβ in PBS for 4 days at room temperature (RT) or 37°C. Next, serial dilutions of monomeric and oligomeric Aβ (both RT and 37°C samples) were tested at starting concentrations of 50000 pg/mL (high) or 1500 pg/mL (low) with all three primary antibodies. Surprisingly, only high concentrations of monomeric Aβ were robust enough for detection by the 6E10 antibody at a low (30s) exposure time, while the lower concentrations were only weakly detected with a long (120s) exposure time (figure 5, left panel). As expected,
although the LOC antibody detects monomeric Aβ, the signal is weaker compared to the 6E10 signal (figure 5, middle panel), and the A11 antibody does not detect monomeric Aβ even during a long exposure (figure 5, right panel).

Interestingly, the signal for oligomeric Aβ was much stronger in samples incubated at physiological temperatures (37°C), and all three antibodies detect signals (figure 5). While it was expected for the A11 antibody to detect oligomeric Aβ, the oligomerization of Aβ was rather inefficient, leaving monomeric Aβ to be detectable by the LOC antibody. Because the LOC antibody has a lower affinity for monomeric Aβ, the signal intensity for the LOC antibody is decreased compared to the 6E10 antibody. This comparatively lower signal intensity supports the

Figure 5: Detection of monomeric and oligomeric Aβ with 6E10, LOC and A11 antibodies. Serial dilutions of 50000 pg/mL (high) and 1500 pg/mL (low) Aβ were spotted and immunoblotting was performed.

Interestingly, the signal for oligomeric Aβ was much stronger in samples incubated at physiological temperatures (37°C), and all three antibodies detect signals (figure 5). While it was expected for the A11 antibody to detect oligomeric Aβ, the oligomerization of Aβ was rather inefficient, leaving monomeric Aβ to be detectable by the LOC antibody. Because the LOC antibody has a lower affinity for monomeric Aβ, the signal intensity for the LOC antibody is decreased compared to the 6E10 antibody. This comparatively lower signal intensity supports the
rational that the Aβ is still monomeric and not fibril, where if it were fibril, you would expect to see a stronger signal from LOC. Overall, these results confirm the properties described for these antibodies.

Once I had established the antibody properties, I performed the experiments described in table 1. Because my earlier experiments indicated that glucose might increase Aβ binding potentially due to crowding, I tested whether higher concentrations of glucose also facilitate Aβ oligomerization. At low concentrations of Aβ, insulin, calcium and zinc, glucose does not appear to alter Aβ oligomerization (figure 6, sets 1 and 2). At high concentrations of Aβ, insulin, calcium and zinc, when comparing high concentrations of glucose to low concentrations, the A11 antibody detects oligomerized Aβ at lower dilution steps in high glucose concentrations (figure 7, sets 3 and 4). This indicates that high glucose concentrations...
concentrations meekly facilitate Aβ oligomerization.

Because destabilized insulin has been suggested to form a seed for Aβ to oligomerize, and calcium plays a large role in insulin stabilization, I next tested the effect of varying calcium concentration on Aβ oligomerization. At low calcium concentrations A11 detects Aβ at lower dilution steps and with a stronger signal in both low Aβ and zinc, high insulin and glucose (figure 8, compare panels A and B and set 5 and 7), and in high Aβ, insulin and glucose, low zinc conditions (figure 8, compare panels A and B and sets 6 and 8). This indicates that in lower calcium concentrations, destabilized insulin may indeed form, causing Aβ to aggregate and oligomerize. Interestingly, while the 6E10 antibodies shows similar detection with varying calcium concentrations, the signal detected by the LOC antibody is stronger at low calcium concentrations compared to high calcium concentration (figure 8). This indicates that under low calcium conditions some Aβ fibrils might also be forming, which do not form at high calcium concentrations.

**Figure 8: Low concentrations of calcium facilitate some Aβ oligomerization.** Low (1500 pg/mL, sets 5 and 7) and high (50000 pg/ml, sets 6 and 8) concentrations of Aβ were tested in the presence of 349 pg/L insulin, 0.02 mg/L zinc, 12.5 mM glucose and varying concentrations of calcium. A. 0.07 mM calcium B. 7 mM calcium.
Lastly, I tested the effect of varying zinc concentrations has on Aβ oligomerization. Zinc preferentially stabilizes insulin, so it is expected that Aβ oligomerization is more efficient at low concentrations of zinc. To shift the equilibrium further, low concentrations of calcium were used. Overall, good Aβ binding to the membrane was observed under these conditions. In addition, at low zinc concentrations, oligomerized Aβ has a greater detection at lower dilution steps when compared to oligomeric Aβ at high zinc concentrations (figure 9). This indicates that low zinc concentrations also facilitates Aβ oligomerization, albeit to a much lesser extent than low calcium concentrations.

Interestingly, it appears that lower concentrations of calcium overall facilitate better binding of Aβ to the nitrocellulose membrane (figures 8, compare panels A and B 6E10, and figure 9), possibly due to the increased interactions between Aβ and the greater amount of destabilized insulin under low calcium concentrations. This observation can be confirmed by directly comparing all sets at the same exposure (figure 10). Aβ signals are strongest in the presence of high Aβ, insulin and glucose concentrations with low calcium concentrations and either high or low zinc concentrations, supporting the observation. The effect of varying zinc concentrations seems to have a smaller effect on overall Aβ binding and Aβ oligomerization (figures 9 and 10).

**Figure 9: Low concentrations of zinc facilitate some Aβ oligomerization.** High (50000 pg/ml) concentrations of Aβ were tested in the presence of 349 pg/L insulin, 0.07 mg/L calcium, 12.5 mM glucose and varying concentrations of zinc (0.02 mg/L and 2 mg/L zinc in set 9 and 10, respectively).
Finally, I also tested the suspended Aβ stock solution. Serial dilutions of 5000 pg/mL (high) and 1500 pg/mL (low) Aβ directly from the suspended stock solution were spotted on a nitrocellulose membrane and detected with all three primary antibodies. Unpredictably, all three antibodies detected a signal suggesting that even in the presence of 10 mM sodium hydroxide some Aβ oligomers have formed (figure 11). This result is quite unexpected and stresses the need to use other biochemical assays to determine the nature of these Aβ aggregates in the future.

Discussion

In this study, I investigated the interactions between Aβ and insulin in various ionic conditions. To perform these studies with near physiological concentration of Aβ, insulin, calcium, zinc and glucose I first established a high-throughput variation of the dot blot assay. Using Bio-Rad’s dot blot apparatus, which is recommended for usage of volumes between 200 and 500 μL, I first optimized the assay conditions that allowed me to use volumes up to 6 mL. After testing and overall data analysis and comparisons, I found that Aβ oligomers are better detected at high concentrations of Aβ, insulin and glucose and low concentrations of calcium and zinc.
The initial glucose comparison tests were done in PBS. However, because calcium phosphate and zinc phosphate are insoluble, a buffer containing 150 mM sodium chloride and 50 mM Tris-HCl, pH 7.5 were used in all additional assays. The sodium concentration in this buffer is close to the physiological plasma levels of sodium, which vary between 135 – 145 mmol/L\textsuperscript{47}. More importantly, this buffer is frequently used for co-immunoprecipitation experiments, where interactions between proteins are preserved making this buffer suitable for the assay I established here.

Varying glucose concentrations suggested that glucose improved overall Aβ binding to the nitrocellulose membrane. This could be due to a crowding effect. Macromolecular crowding has been shown to affect protein folding and stability \textit{in vitro}.\textsuperscript{48,49} It is possible that binding of Aβ to the nitrocellulose membrane is increased due to effects of molecular crowding on Aβ’s structure/stability in solutions containing high glucose concentrations. Since it is not unreasonable for glucose to potentially change the structure of Aβ via these crowding effects, it is possible that Aβ under these effects, could increase the exposure of specific residues, mainly residues 15-17 and 34-36. These regions of Aβ are known to be involved in Aβ self-aggregation\textsuperscript{50} and have been reported to be potential sites of preferential insulin interactions.\textsuperscript{23} Additionally, the more crowded environment seen in the intra-/extracellular space within the brain can be somewhat replicated by the increased levels of glucose in the context that these
higher glucose concentrations provide a higher number of total molecules in the samples. Although this effect appeared to be minor in later experiments, glucose concentrations were kept constant at (high) 12.5 mM in subsequent experiments.

I further found that low concentrations of calcium, which is known to destabilize insulin, facilitated better Aβ binding to the nitrocellulose membrane. This finding is consistent with my model (figure 1). Although this effect has not yet been described in the literature, it is possible that destabilized insulin aggregates generally improve binding of Aβ to the nitrocellulose membrane via increased interactions and increases Aβ oligomerization. While this finding supports my model, it is not yet known whether the Aβ and insulin bound to the membrane at low calcium concentrations have formed homo- or heteroligomers, or even interact at all. In contrast to calcium, the effect of varying zinc concentrations is rather modest; low zinc concentrations meekly increase Aβ oligomer formation. The weaker effect of zinc when compared to calcium can be explained when looking at insulin conformations and stabilization. Generally, the presence of calcium has a greater effect on the stabilization of insulin monomers/dimers and preventing destabilized aggregates, while zinc effect has a greater association with insulin hexamerization. This supports the disparity in effect strength between calcium and zinc.

Since the LOC antibody preferentially detects fibrils, but also monomeric Aβ, data interpretation is challenging without further experiments. Although the LOC signal correlates well with the signal obtained with the 6E10 antibody, the signal appears to be weaker, which is expected for detection of monomeric Aβ (figure 8B). However, it cannot be excluded that some Aβ could formed fibrils at low concentrations of calcium (figure 10, sets 6, 8 and 10). Further
support for this hypothesis comes from Aβ detection with the A11 antibody, which shows increased signal intensities for sets 6 and 10.

To determine antibody specificity towards monomeric, oligomeric and fibril forms of Aβ, Aβ was treated to obtain monomeric or oligomeric conformation (figure 5). Interestingly, monomeric Aβ was only weakly detected with the 6E10 antibody, even at a concentration of 50000 pg/mL and long exposure time. The solution of monomeric Aβ contains 1% SDS with the presumed use of preventing homodimer formation. With protein binding to nitrocellulose via hydrophobic interactions, the addition of SDS decreased protein hydrophobicity, which likely reduced the protein binding affinity towards the nitrocellulose membrane. To optimize the assay and increase binding of monomeric Aβ a nitrocellulose membrane with a pore size of 0.1 μm could be used in the future. Furthermore, the addition of 20% methanol could also increase protein binding to the nitrocellulose membrane.

Detection of oligomeric Aβ revealed a signal for all three antibodies. This indicates that the oligomerization of Aβ was a rather inefficient reaction, and a mixture of monomeric and oligomeric Aβ most likely is present in the samples. Interestingly, the LOC antibody required a higher Aβ threshold detection than the A11 antibody (figure 5). With the LOC antibody having a lower affinity towards monomeric Aβ, and the A11 antibody recognizing oligomeric Aβ only, this result suggests that the LOC antibody mainly recognized a large amount of monomeric Aβ and that the presence of oligomers are also abundant, or that there could be a small amount of fibril detection which overwhelms any monomeric signal detection from the LOC antibody, in tandem with a large portion of the sample Aβ being oligomeric. Furthermore, by increasing the incubation temperature to 37°C all antibodies detected lower thresholds Aβ suggesting that the formation of Aβ oligomers is a kinetically unfavorable reaction, but by increasing the
temperature the activation energy required for Aβ oligomerization can be partially overcome. In future testing, the use of a different Aβ oligomerization protocol would advised. The use of DMSO and phenol-free F-12 cell culture medium\textsuperscript{51} may have better oligomeric Aβ results.

Lastly, the antibody test of the Aβ stock surprisingly against expectations revealed that 10 mM sodium hydroxide solution does not prevent the formation of some Aβ oligomers. This finding and the fact that the LOC antibody detects Aβ fibrils and monomers emphasized the need to use additional biochemical methods in the future to investigate what Aβ conformations were detected using the dot blot assay. One possible method to explore the nature of the Aβ conformations is a modified dot blot assay: Following separation of the Aβ conformations on a gel filtration column, the different fractions can then be tested using immunoblotting. However due to the use of near physiological concentrations the loss of material might be too significant to purify these complexes. As an alternate approach, direct mass spectrometry can be performed on the complexes. In a top-down mass spectrometry approach the mass of intact proteins and complexes will be determined.\textsuperscript{52} Specifically, following electrospray ionization (ESI) of the liquid sample, the sample composition can be determined with a LTQ-Orbitrap Velos mass spectrometer.\textsuperscript{53} Because mass spectrometry is highly sensitive, composition of potential homo- and heteromeric Aβ/insulin complexes along with the association of calcium, zinc and/or glucose can be examined in greatest detail.

Additionally, it would be interesting to investigate the interactions between post-incubated destabilized insulin aggregates with monomeric Aβ, or oligomeric Aβ and insulin in similar ionic conditions as those seen in this experimentation. The resulting interactions under specific conditions seen here can be somewhat extrapolated and show some insight into ideal \textit{in vivo} conditions that promote Aβ oligomerization. For instance, increased Aβ concentrations,
hyperinsulinemic and hyperglycemic concentrations are a common characteristic in newly
diabetic patients to late stage AD patients. Under conditions of low calcium and/or zinc
concentrations in the brain, the ability of Aβ oligomerization is optimized, potentially speeding
up the onset of AD pathology.

References
and characterization of a novel cerebrovascular amyloid protein.
5. Klein, A.M., Kowall, N.W., Ferrante, R.J. (1999) Neurotoxicity and oxidative damage of
beta amyloid 1-42 versus beta amyloid 1-40 in the mouse cerebral cortex.
Plaques Isolated by Laser Capture Microdissection.
Between the Alzheimer Disease Amyloid-β Peptide and Other Amyloid Proteins: A
Further Aspect of the Amyloid Cascade Hypothesis*.
and other neurodegenerative disorders: the emerging role of oligomeric assemblies.
10. Dore, S., Kar, S., Rowe, W., Quirion, R. (1997) Distribution and levels of 125I-IGF1,
125I-IGF2 and 125I-insulin receptor binding sites in the hippocampus of aged memory-
unimpaired and -impaired rats.
11. McNay, E.C., Ong, C.T., McCrimmon, R.J., Cresswell, J., Bogan, J.S., Sherwin, R.S.
(2010) Hippocampal Memory Processes are Modulated by Insulin and High-Fat-Induced
Insulin Resistance.
Dementia in Diabetes Mellitus: A Systematic Review.
Reviewed.
14. Schrijvers, E.M.C., Witteman, J.C.M., Sijbrands, E.J.G., Hofman, A., Koudstaal, P.J.,
An Epidemiological Perspective.
Metabolism Disturbances in the Development of Alzheimer Disease: Mini Review.


