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Neuroprotective Effects of Selective Estrogen Receptor Modulators Against Amyloid Beta Toxicity and the Pathways that Provide Protection

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Neuroprotective Effects of Selective Estrogen Receptor Modulators Against Amyloid Beta Toxicity and the Pathways That Provide Protection

BY MAGGIE ROSE

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ABSTRACT

Alzheimer's disease is the sixth leading cause of death in the United States. The leading hypothesis to explain the prevalence of the disease in the brain is the aggregation of Amyloid Beta peptides in the brain, which form senile plaques and suppress neuronal function. Selective estrogen receptor modulators (SERMs) have been found to provide protective effects against the neurotoxic effects of Amyloid Beta. This experiment was conducted in two distinct phases: the experimental phase and the literature review phase. The experimental phase sought to determine if Amyloid Beta was neurotoxic to SH-SY5Y neuroblastoma cells, and if STX—an SERM—was able to rescue cell viability after exposure to Amyloid Beta toxicity. During the literature review phase, potential pathways already described in the literature were identified, which might be able to account for how STX was able to rescue cell viability after exposure to Amyloid Beta toxicity. Amyloid Beta was found to have a significant toxic effect on cell viability when compared to a control assay of cells grown in DMSO. STX, however, was not found to have a significant rescue effect against Amyloid Beta toxicity. The literature review identified several pathways that were likely candidates for neuroprotection, including MAPK, ERK1, ERK2, and PI3K. The future direction of this experiment would be to determine if STX is as effective at providing neuroprotection against Amyloid Beta as previously suggested by similar experiments. the next stage of investigation would then be to inhibit different pathways that have been identified as possible pathways for neuroprotection.

INTRODUCTION

Alzheimer's disease is a progressive brain disorder that causes impairment in memory and thinking skills (1). It was first described by Dr. Alois Alzheimer in 1906, who noted memory loss, paranoia, and psychological changes in his patient. Today, Alzheimer's disease is the sixth leading cause of death in the United States (2). The factors that lead to Alzheimer's disease are still unknown, though there are many hypotheses which seek to explain the etiology of cognitive impairments associated with the disorder. One of the leading explanations is the Amyloid Cascade hypothesis, proposed by J.A. Hardy and G.A. Higgins in 1992. Amyloid precursor protein (APP) is expressed by neuronal cells and extra-neuronal tissues. Hardy and Higgins argue that the proteolytic cleavage of the membrane glycoprotein amyloid precursor protein into neurotoxic amyloid beta-peptide (Amyloid β) fragments was the cause of the cellular death process in the central nervous system (3).

In the brain, APP is expressed in three isoforms: APP_{695}, APP_{751}, and APP_{770}. APP_{695} is the most abundant isoform in neurons and is expressed at relatively high levels (4). As mentioned previously, APP is a membrane-bound glycoprotein. It is processed by membrane bound aspartic proteinases: α and β secretases. Each of the products generated by these proteinases is processed by γ secretase and the fragments are secreted into the cytoplasm. When the Amyloid β domain is processed by α secretase, it is cleaved within the Amyloid β domain, between residues Lys16 and Leu17; this is referred to as the non-amyloidogenic pathway, as it inhibits the generation of intact Amyloid β peptides. When processed by β secretase, it is cleaved at Asp1, which is referred to as the amyloidogenic pathway, as it forms intact Amyloid β peptides (40-42

amino acids long) that are released into the cytosol. These peptides will frequently aggregate in the brain and cause the development of senile plaques (5).

Research into the function of APP secretases, specifically γ secretase, has been of interest to many investigators. By understanding this mechanism, it is though that we could stop the production and release of Amyloid β peptide, which would prevent plaque formation all together. Unfortunately, γ secretase plays a critical role in the cleavage of other membrane proteins, such as the Notch1 receptor. Because of this, research into alternative methods of neuroprotection from Amyloid β peptide is necessary (6).

Estrogens have been shown to have neuroprotective effects against brain ischemia, as well as reduce memory impairment after an ischemic event (7). This makes estrogen a compelling compound of study for other conditions that cause cognitive impairment (7,8). There are significant risks, however, associated with 17-β estradiol (E2) treatments; possible side effects include but are not limited to: cardiovascular disease, thrombosis, cerebral vascular accident, and hormone-sensitive cancers, among others. Selective estrogen receptor modulators (SERMs) are synthetic or natural compounds that provide the positive neuroprotective effects of estrogen without the negative side effects. STX is one example of a SERM. STX is able to bind to the G protein coupled estrogen receptor Gq-mER in the plasma membrane instead of the nuclear estrogen receptors. This eliminates the negative transcriptional responses generated by E2 that lead to negative side effects (9).

Building on the research developed by Gray *et al.* (9), my research will seek to understand the pathway that produces the protective effects of STX against Amyloid β toxicity. My project will be undertaken through a mixed methodology of primary research and literature review. In the research phase of my experiment, I will establish that Amyloid $\beta$is toxic to SH-SY5Y neuroblastoma cells, which I will use as my cell culture assay of amyloid toxicity (9). My literature review will be focused on previous research conducted on the different signaling mechanisms of STX in the cell. This combined approach will address my research question: what is the pathway that provides STX its neuroprotective abilities against the harmful effects of Amyloid β?

METHODOLOGY

Experimental phase

SH-SY5Y neuroblastoma cells were cultured in DMEM/F12 medium (GIBCO/Life Technologies) supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were plated in 15,000 cells per well in 96 well plates to assess cell viability. Three days after plating, the cells were washed with phosphate-buffered saline (PBS; Sigma-Aldrich) and switched to serum-free DMEM/F12 medium containing 1% N-2 growth supplement (GIBCO/Life Technologies) and 100nM STX (synthesized by our colleague Dr. Martin Kelly, Dept of Physiology and Pharmacology at Oregon Health and Science University). The next day, the cells were treated with 50 µM oligomeric A$\beta_{1\text{-}42}$ (prepared using the methods described by Stine *et al* (10)) and incubated at 37°C for 72 hours. Cell viability was assessed 48 hours after incubation period using a Bio-Rad xMark microplate spectrophotometer.

Literature review phase

The effects of STX on G-protein coupled receptors (GPCRs) will be the focus of this study. Articles of focus will be peer reviewed journal articles that address the effects of STX on two different membrane estrogen receptors—G-protein coupled receptor 30

(GPR30) and Gq-coupled membrane estrogen receptor (Gq-mER)—specifically with regards to the intracellular signaling cascades that are engaged, following their activation.

RESULTS AND DISCUSSION

Experimental phase

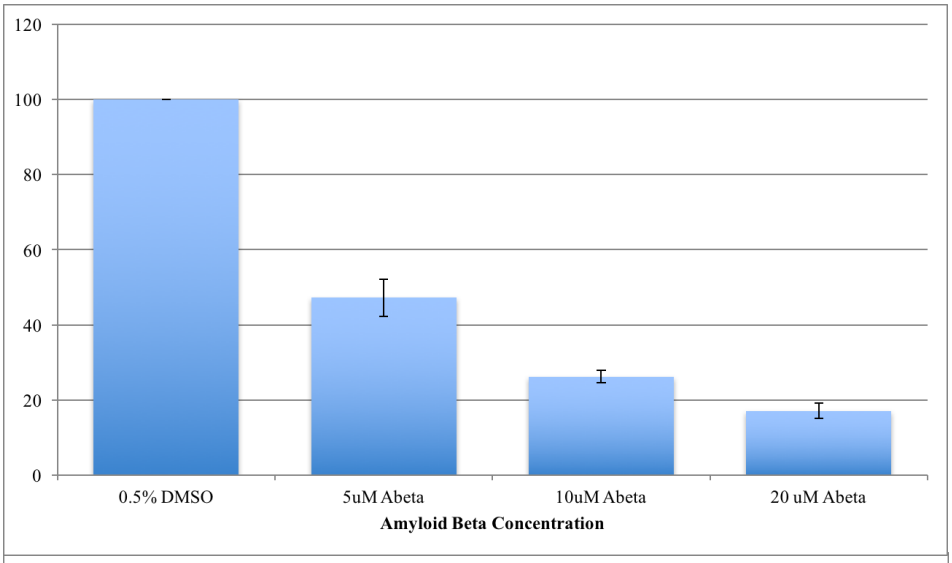
In this experiment, Amyloid β toxicity was evaluated after SH-SY5Y cells were treated with A$\beta_{1\text{-}42}$ and allowed to incubate for 72 hours. When cells treated with A$\beta_{1\text{-}42}$ were compared to cells treated with DMSO, cell viability was found to be significantly decreased (Figure 1). From these results, it was determined that 50 µM oligomeric A$\beta_{1\text{-}42}$ was found to be a sufficient concentration to reduce cell viability.

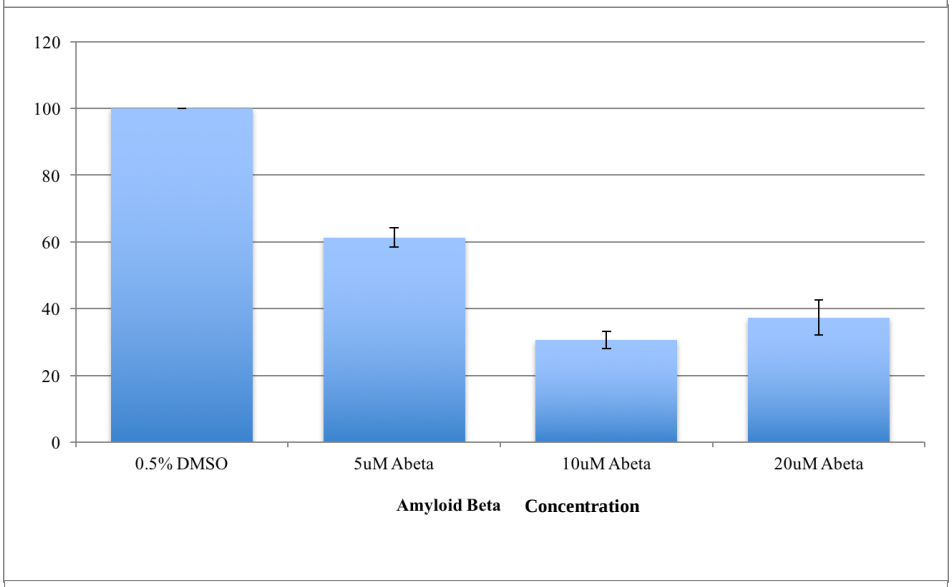
To evaluate the protective effects of STX, cells were pre-treated with STX and then treated with A$\beta_{1\text{-}42}$. STX concentration was titrated to determine if there was a correlation between STX concentration and cellular rescue. When compared to cells treated with DMSO, there was no consistently observed statistically significant difference in cell viability between SH-SY5Y cells treated with A$\beta_{1\text{-}42}$ and STX and those without STX (Figure 2). During one trial, a statistically significant difference was observed between cells treated with A$\beta_{1\text{-}42}$ without STX and those with STX, however, these results have not been able to be reproduced. This observation suggests that there may be confounding sources of error that might be impeding the experimental process.

Possible explanations for the variation in the experimental results could be the preparation of A$\beta_{1\text{-}42}$ itself. The incubation period suggested for A$\beta_{1\text{-}42}$ is 4-7 days. During this time, A$\beta_{1\text{-}42}$ oligomerizes into a clear film, which will be resuspended in solution. However, because the film is clear, there is no good indication that the A$\beta_{1\text{-}42}$ has oligomerized or can bind to cells. It is also possible that the time window for aging is insufficient. A$\beta_{1\text{-}42}$ has been allowed to age for 4 days, but it is possible that increasing the time allotted for oligomerization would increase the concentration of A$\beta_{1\text{-}42}$ that actually forms.

It is also possible that the difference between cellular concentration of each well has an allosteric effect on cellular viability. It is possible that the cellular network produced between larger concentrations of cells is better able to resist the toxic effects of A$\beta_{1\text{-}42}$. Decreasing the concentration of cells per well could potentially decrease the risk of a cellular network forming a resistance to A$\beta_{1\text{-}42}$, but this is not a change that can be made in living organisms, and therefore might decrease the effectiveness of this model.

We have not yet seen conclusive data that STX can reproducibly rescue SH-SY5Y cells from A$\beta_{1\text{-}42}$, however, we will continue to make changes to the experimental methodology to account for discrepancies in results gathered between trials.





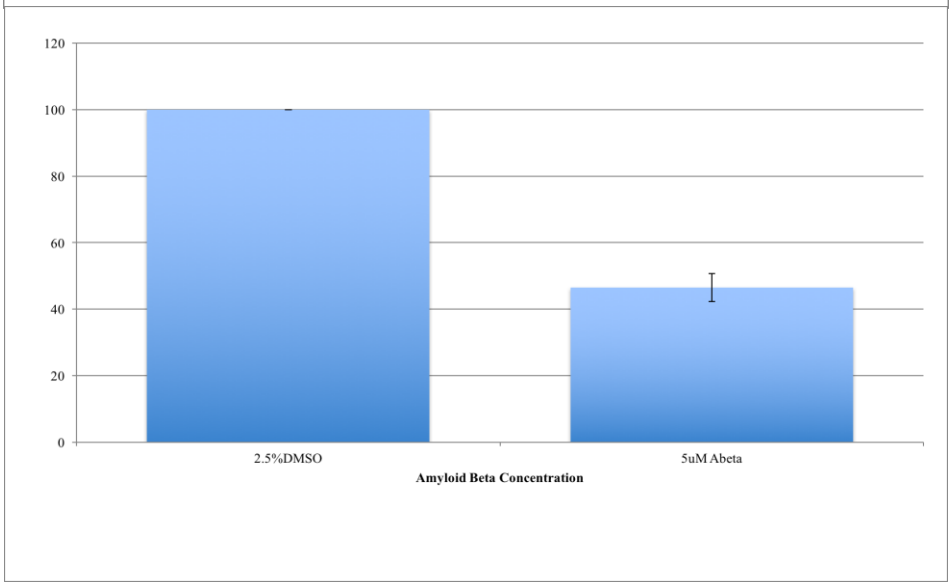
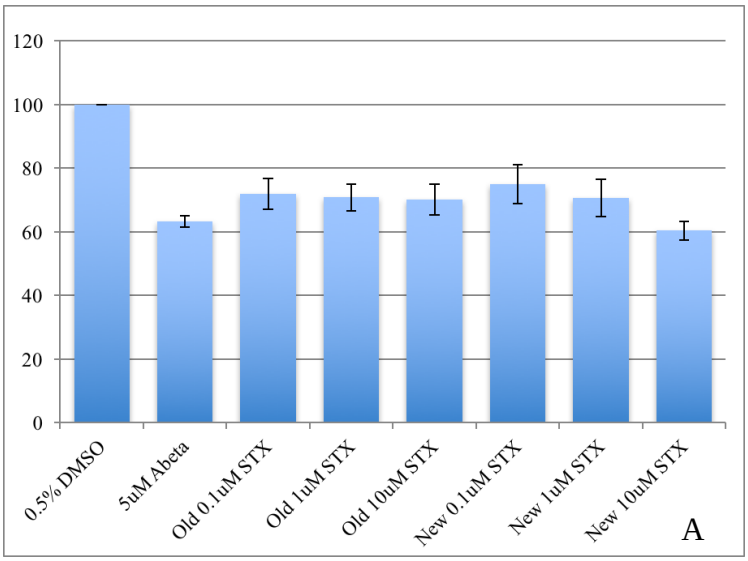
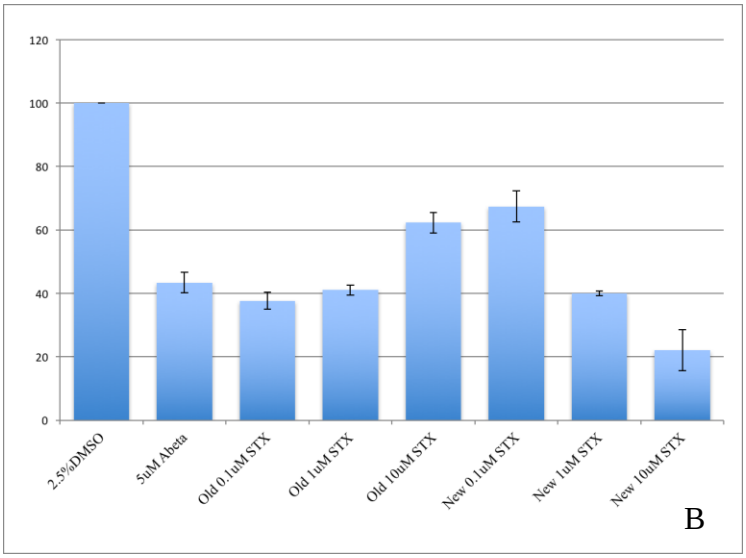


Figure 1. When cell viability after $A\beta_{1\text{-}42}$ exposure was compared to cells exposed to 2.5% DMSO, cell viability decreased by a statistically significant amount ($p=0.05$). Fig1A shows a relationship between the concentration of $A\beta_{1\text{-}42}$ and cell viability, while Fig1B shows that there was not demonstrate this correlation. Fig1C showed that 0.5uM $A\beta_{1\text{-}42}$ was sufficient to decrease cell viability a statistically significant amount. Y-axis of each graph has been scaled to be proportional to the cell viability of cells exposed to 0.5% DMSO.





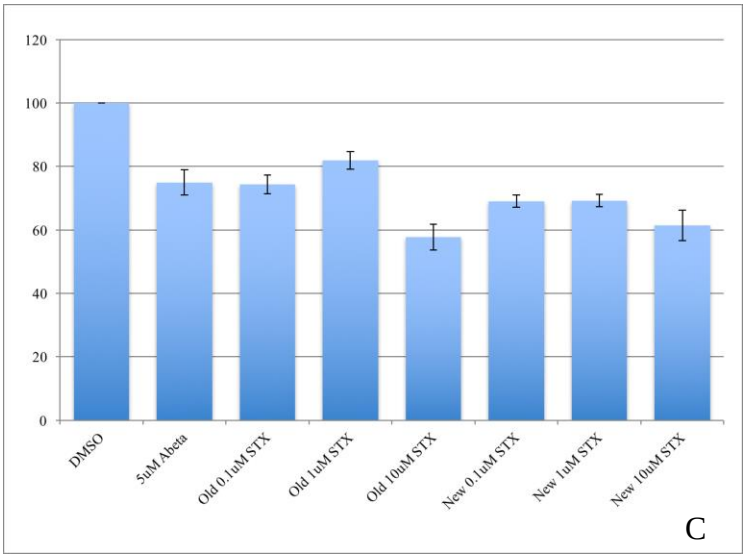


Figure 2. When cell viability of STX pre-treated cells after $A\beta_{1\text{-}42}$ exposure was compared to cells exposed to 2.5% DMSO, there was no consistently significant difference between cells treated with STX and those without. Fig2A shows the results of an experiment performed in December 2018. No statistically significant difference was seen. Fig2B shows the results of an experiment performed in January 2019. A statistically significant difference was seen between cells treated with STX and those without (p=0.05). Fig2C shows the results of an experiment performed in April 2019. No statistically significant difference was seen.

Literature review

Understanding the pathways involved in the neuroprotective effects of selective estrogen receptor modulators is extremely complex. Several pathways have been identified as likely candidates for neuroprotection, and they are not mutually exclusive. E2 is a useful reference compound, as it has very similar effects to SERMs in the body. Initiation of signaling pathways in hypothalamic arcuate neurons begins when E2 binds to the Gq-mER and activates $G\alpha_q$, a G-protein subunit. $G\alpha_q$ dissociates from the membrane and activates phospholipase C (PLC) and initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). PLC then hydrolyzes PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG actives protein kinase C (PKC), which activates adenylate cyclase VII, which increases cAMP production and stimulates protein kinase A (PKA). PKA phosphorylation uncouples the inhibitory $GABA_B$ and μ-opioid receptor from activation of G-protein coupled, inward rectifying K^+ (GIRK) channels. Activation of PKA also phosphorylates cAMP-responsive element binding protein and control gene expression through the CREB response element. IP_3 activates Ca^{2+} release from the endoplasmic reticulum that can activate calcium-dependent signaling. E2 also has been found to bind to the nuclear receptors and activate estrogen response element-dependent transcription (11, 12).

This latter finding highlights the important difference between E2 and SERMs: SERMs do not bind to nuclear receptors. This has been demonstrated in multiple studies. Indeed, it has been shown that the relative binding affinity of SERMs was reduced by about 1 million-fold when compared to E2. In addition to this, STX has been shown to activate the $G\alpha_q$ signaling pathway in mice that lack either $ER\alpha$, $ER\beta$, or both nuclear receptors (11, 12). This suggests that there might be other differences between the behavior of STX and E2 within the cell, including their neuroprotective pathways.

Compelling evidence has shown that STX binds to the Gq-mER rather than GPR30 (13, 14). When STX was introduced into neurons that had the GPR30 genes silenced through an siRNA treatment, the molecule was still fully efficacious (8, 15). This suggests that GPR30 was not essential for the function of STX within the cell. Similar experiments, which block the release of $G\alpha$ from Gq-mER have been performed after cells were treated with E2 (13) and showed that blocking the release of the G-subunit is sufficient to block the effects of E2 in the cell. Reproduction of this experiment with STX would be the first step to understanding the method of action of STX in the cell.

Inhibition of mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways as well as phosphatidylionsitol 3-kinase (PI3K) pathways were shown to block the ability of STX to activate the steroidogenic factor 1 (SF-1) in endometrial cells (15). Inhibition of ERK was also found to block the effects of STX *in vivo* (16). STX was found to attenuate the $GABA_B$ response in cells, and the inhibition of $GABA_B$ by estrogen was found to be required for the activation of PKA intracellularly. In addition to this, PKC δ inhibitor was found to block the function of $GABA_B$ in cells treated with E2. PLC was not found to be an essential inhibitor of $GABA_B$, but it does not rule out its possible significance in the STX pathway (13).

It is possible that each of these pathways, MAPK/ERK, PI3K, PKA, PLC, or PKC δ play a role in the effect of STX within the cell. As each of these pathways are non-genomic, they are the most likely candidates. It is also possible that this list is incomplete and that we do not have enough information about intracellular kinase pathways to yet answer

this question. The next stage of research will be to treat neuroblastoma cells with Aβ, STX, and each of these inhibitors and measure the cellular rescue response. Combinations of these inhibitors would also be interesting, to explore if more than one of these pathways is involved in the response initiation of STX. This is likely, given that this is the case for E2.

Understanding this mechanism of cellular rescue will allow us to design more effective treatments for Amyloid β toxicity and ischemic events. Wider applications to this research include but are not limited to: Alzheimer's disease, Parkinson's disease, stroke, and other cerebral vascular accidents.

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