

Using a synthetic toggle switch to prevent hyperphosphorylation in Alzheimer's

Anna Chatterji, Ishaani Sharma

BioBuilderClub, Andover High School, Andover, Massachusetts, USA

Reviewed on 2 May 2020; Accepted on 22 June 2020; Published on 26 October 2020



Alzheimer's Disease (AD) impacts 46.8 million people worldwide. As a form of dementia, AD causes a progressive loss of memory and hinders thinking processes. Biogen has a monoclonal antibody treatment for AD known as Aducanumab, which is controversial due to the fact that testing was halted after the first phase of trials. Although there is still no definitive way of treating AD, there are a variety of pathways that can be explored to treat the disease. For this project, the focus is on the tau protein pathway. The tau protein is necessary for supporting microtubules in neurons in the brain, which help transfer messages from the brain to the body and vice versa. AD is caused by the formation of tau clusters. Tau clusters, caused by hyperphosphorylation, gather away from microtubules, resulting in a lack of support for the neurons and a damaged messaging system. Although phosphorylation is a necessary process for mitosis, hyperphosphorylation leaves a negative impact on the human brain. Hyperphosphorylation could potentially be a result of high kinase activity and low phosphatase activities in the brain. To combat this, we plan on creating a synthetic toggle switch to prevent hyperphosphorylation. The switch would serve as a regulatory system to be used for cases in which it is clear that abnormal kinase and phosphatase activity is the cause of the symptoms. However, there can be a variety of factors behind symptoms of AD, so kinase and phosphatase activity should not be the sole focus. Components of this system would initially be tested in SH-SY5Y, a human derived cell line that is often used as in vitro models of neuronal function. This will provide an opportunity to test the device in an environment that is similar to the anticipated environment in the human brain.

Keywords: Alzheimer's Disease, tau hypothesis, synthetic toggle switch, kinase, phosphatase, hyperphosphorylation

Mentors: Lindsey L'Ecuyer, Alberto J. Donayre Torres

Direct correspondence to adonayre@utec.edu.pe; llecuyer@k12.andoverma.us

This is an Open Access article, which was copyrighted by the authors and published by BioTreks in 2020. It is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background

Alzheimer's Disease (AD), is a disease that has remained notoriously hard to cure. There is no known cure for AD, and as it continues to impact millions across the world, the need for a cure has become more recognizable than ever before.

There are a variety of different pathways that could be influencing AD patients, such as genetic variations, or the older cholinergic hypothesis, which suggests that AD is the result of dampened activity of the cholinergic system in the brains of AD patients (Teipel, Grinberg, Hampel, et al. 2009). Reports of substantial neocortical deficits in the enzyme responsible for the synthesis of acetylcholine, a neurotransmitter at neuromuscular systems, in AD patients was seen as an indication of a possible pathway to resolve AD in patients (Purves, Augustine, Fitzpatrick, et al. 2001, Francis, Palmer, Snape, et al. 1999). However, the cholinergic hypothesis reveals unsatisfactory results, as attempts to raise acetylcholine levels have not provided a cure to AD (Martorana, Esposito and Koch 2010). Another potential cause of AD could be the accumulation of a different protein, amyloid-beta ($A\beta$). Not only does evidence show that $A\beta$ in higher concentrations impairs blood flow within the brain, but it accelerates neuronal dysfunction (Sadigh-Eteghad, Sabermarouf, Majdi, et al. 2015). Another pathway is the tau hypothesis, which is receiving more attention in the scientific world as evidence suggests that abnormalities in the tau protein plays a role in the progression of AD (Gong and Iqbal 2009). There is a connection between $A\beta$ and tau; $A\beta$ activates tau kinases, which induce neurofibrillary (NFT) formation. NFTs are filaments, which make up hyperphosphorylated tau proteins, and the formation of these filaments is directly associated with neuronal dysfunction (Sadigh-Eteghad, Sabermarouf, Majdi, et al. 2015). Our experiment focuses on the latter portion of neuronal degeneration in AD patients by finding a methodology to reduce the effect of hyperphosphorylated tau on the human brain.

In humans, the tau protein is a microtubule associated protein (MAP) that is crucial in maintaining the structure of axonal microtubules in the brain. Tau is engaged in signaling functions between neurons (Mietelska-Porowska, Waski, Goras, et al. 2014). The tau hypothesis establishes that in AD patients, the tau protein becomes hyperphosphorylated. Normally, phosphorylation plays a critical role in the regulation of cells; the addition of a phosphoryl group is crucial for glycolysis and protein interaction and it helps maintain homeostasis by regulating energy-dependent reactions (Helmenstine 2019). In cases of hyperphosphorylation, however, the phosphorylation sites located on the tau protein become fully saturated. Hyperphosphorylation is generally

used to indicate the next step in the process of mitosis in the cell cycle, yet in context with the tau protein, neurodegeneration occurs by means of toxic functions rather than by a loss of normal activity (Gong and Iqbal 2009). During cases of abnormal hyperphosphorylation, clusters of tau form and contribute to the collapse of cytoskeleton. This causes the deterioration of motor proteins and decreases their effect on microtubule support (Mietelska-Porowska, Waski, Goras, et al. 2014). While hyperphosphorylation is necessary in order to promote mitosis, abnormal cases involving the tau protein are potentially linked to increased kinase activity and decreased phosphatase activity (Gong and Iqbal 2009).

Our experiment will use a synthetic toggle switch to regulate levels of kinases such as glycogen-synthase kinase-3 β (GSK-3 β) and phosphatases like protein-phosphatase 2A (PP2A). To begin with, the switch will be assembled in vitro and then encapsulated in a nanocarrier to replicate the human blood-brain-barrier, which the switch will encounter in human patients. In instances of abnormal GSK-3 β activity, a detection device will notify the synthetic toggle switch, which will then trigger the pathway that produces insulin (Cross, Alessi, Cohen, et al. 1995). Although no specific coding sequence for a detection device was found by us during our research process, there are certain ways to detect kinase activities. The standard method is to detect phosphorylation sites of known kinases to act as an indicator of activity. A research team in Japan found a method to purify the kinases found by the standard method; they did so by re-naturing kinases in an in-gel phosphorylation assay and adding ATP to identify phosphorylation sites (Biotechniques.com 2020). However, in this experiment, the switch is meant to detect abnormal kinase activity internally, so this standard method would not work, but it is still something to consider while the new detection device is being devised. The aforementioned insulin stimulates protein-kinases which in turn inhibit GSK-3 β , thus regulating abnormal kinase activity. In regards to decreased phosphatase activity, the use of transcriptional factors, particularly ETS Proto-Oncogene 1 for this experiment, positively regulates the promoter of the PP2A gene (Chen, Han, Deng, et al. 2009).

Due to the complex nature of this experiment, conditions will initially be tested using the SH-SY5Y cell line. Meant to represent an in vitro model of human neuronal cells, the cell line will best replicate the environment in which this device will be tested. The device will be injected into the cells once it is coated in a nanocarrier. By transcribing the ETS-1 and insulin genes during the first stage of experiments in the SH-SY5Y cell line with decreased PP2A and increased GSK-3 β activity, we will promote the production of the PP2A

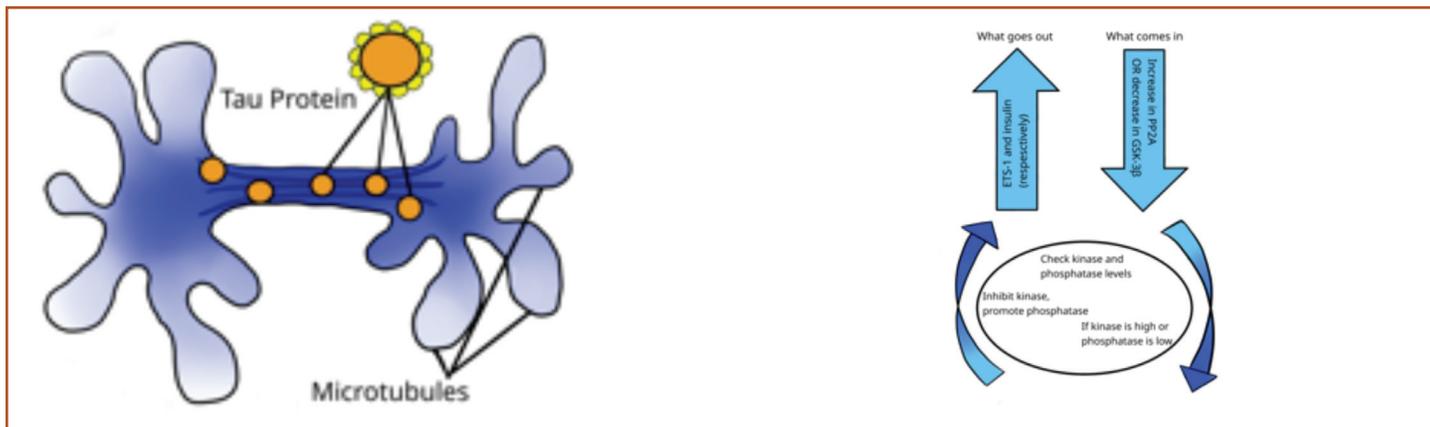


Figure 1. Depiction of the systems level. On the left is a depiction of tau protein present on the microtubules of human neuronal cells. On the right is a portrayal of how the synthetic toggle switch system will work. Detective devices check for abnormal PP2A and GSK-3 β levels and will then regulate these levels by producing ETS-1 and insulin.

gene, thus increasing phosphatase levels in patients with AD and decreasing GSK-3 β activity, respectively. These levels will be regulated in the cell line manually to determine the extent to which tau must be regulated. Then, in order to test the switch physiologically, the next stage of experiments will be in mice. Due to the phylogenetic similarities and relatedness and physiological similarities to humans, testing in mice will provide a structured period to determine how the system will work in living creatures (Perlman 2016). The nanocarrier will be tested in a model meant to replicate the human blood-brain-barrier (BBB): the Transwell apparatus blood-brain-barrier model. Using this apparatus, brain capillary endothelial cells are seeded on a permeable support under static culture conditions (Kaisar, Sajja, Prasad, et al. 2018). This allows us to perform cell migration and drug transport assays across the BBB endothelium, an aspect of this experiment that is imperative to its success in human patients (Kaisar, Sajja, Prasad, et al. 2018). Lipid-soluble molecules naturally pass the BBB, but it has proven difficult to replicate this in drug delivery systems. However, using nanocarriers that consist of polybutylcyanoacrylate, poly lattice-co-glycolic acid, and polylactic acid have traditionally worked in prior experiments. For instance, the model drug loperamide was injected into the central nervous system, and the drug delivery was successful (Vergoni, Tosi, Tachhi, et al. 2009). The findings of the experiment show that polymeric nanoparticles can ensure a strong, long-lasting pharmacological effect (Vergoni, Tosi, Tachhi, et al. 2009). Solutes are able to travel through the semipermeable inserts that the cells are grown on in the Transwell apparatus. By using in vitro models of the human brain, we will be able to predict the extent to which the synthetic toggle switch will function in the actual human brain (Saravia, Praca, Ferreira, et al. 2016).

Systems level

Our proposed system will initially be tested in the SH-SY5Y cell line. The human derived neuronal cell line works well with creating an environment similar to one that the device will eventually experience once testing is complete. By using this cell line, we can test if the device will work in human cell lines. Then, experiments will be performed on mice to test the physiological aspects, and then clinically in humans. We will purify the synthetic toggle switch from the chassis in order to place the system in a nanocarrier. Then, creating an in vitro simulation of the brain will provide an environment to test covers for the synthetic toggle switch. The synthetic toggle switch would function as a negative feedback loop in the SH-SY5Y cell line and, eventually, in the human brain (Figure 1). A toggle switch, however, is separate from a standard negative feedback loop. With the loop, the scientist is unable to alter the proteins or hormones being regulated. Since the device is one that is experimental and untested, by making the device a toggle switch, we can fluctuate the levels to which there are abnormalities in the GSK-3 β and PP2A levels. This allows us to understand how tau, an already complex protein, should be regulated. Additionally, toggle switches have been found in specialized gene circuits of bacteriophages; in 2000, a genetic toggle switch outside of these bacteriophages was created (Gardner, Cantor and Collins 2000). It presented the behaviors of the original switch: bistability and gene-regulatory. Bistable means that a system has two equilibrium states which is crucial to this experiment. In order for one part of the switch to work, there must be abnormal cases of increased kinase and decreased phosphatase. It is not one or the other; both sequences must no longer be on the equilibrium state in order for the switch to work. It is important to use a switch rather than a negative feedback loop in a system as complex as

the human brain because there are a variety of other factors influencing kinase and phosphatase levels. By ensuring that a change in equilibrium for both kinase and phosphatase, or an increase and decrease, respectively, occurs, the switch cannot regulate without the bistable changes. This provides further stability to the system as a whole while functioning in the human brain.

Device level

The synthetic toggle switch system will be placed in an SH-SY5Y. As it is a neuronal cell line, we can manipulate kinase and phosphatase levels to replicate neurodegenerative environments as seen in AD patients. This will allow us to use the synthetic toggle switch in a scenario as similar to the anticipated location as possible. Influencing the levels of kinase and phosphatase in the chassis will provide a glimpse as to the inner mechanisms of the human brain in order to determine the influence of insulin and ETS-1 on GSK-3 β and PP2A levels, respectively.

The two major components of the synthetic toggle switch are the sequences inhibiting GSK-3 β and promoting PP2A. The hormone insulin, encoded on chromosome 11 in humans, can be used to inactivate amounts of GSK-3 β in the human body, while ETS-1, a transcription factor, will be used to regulate PP2A levels in cells. These components would be used in tandem in the same system to detect the changing levels of GSK-3 β and PP2A respectively. Together, they will provide real-time feedback to the current balance which will continuously update the detection devices at the beginning of the system. In order for the system to work, the shift from both equilibrium states must occur, thus ensuring the stability of the toggle switch in a complex environment.

Parts level

The synthetic toggle switch is a two-fold system, as seen in Figure 2. At the beginning, there are two detection devices to determine the hyperphosphorylation situation in the system's environment. Hyperphosphorylation is influenced by a variety of factors, but this system focuses on increased GSK-3 β levels and decreased PP2A levels. In the event of normal phosphorylation, the sequences to influence the aforementioned levels are blocked, and the synthetic toggle switch is not turned on. However, in the case of hyperphosphorylation, the system is turned on. First, it regulates the PP2A level in the environment by transcribing the ETS-1 transcription factor which promotes PP2A. Then, the system moves to regulate GSK-3 β levels by encoding insulin, which can be located on the Standard Registry of Parts using the code BBa_M1877.

A T7 promoter can produce high levels of transcription in an E. coli cell with the presence of T7 RNA polymerase. Ribosomes will bind to the RBS of any transcript in the device, whether it be the kinase-inhibiting or the phosphatase-promoting. We propose using BBa_J61101 because of its relative strength to other ribosome binding sites. Then, the coding sequences are individualized for each aspect of the system, as mentioned earlier, and a transcription terminator is used to terminate the transcription of each coding sequence (CDS). It is important to note that while each system is being coded for separately, it is important to view the kinase-phosphatase relationship as a whole rather than individually. There are a multitude of other factors influencing the relationship between the tau protein and its function to microtubules, but we chose to focus on this relationship in particular for this system.

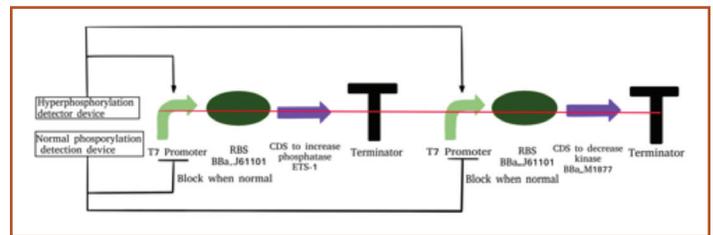


Figure 2. Depiction of parts used in the system. The structure of this depiction is meant to show the synthetic toggle switch design. The boxes with detection devices are abstract concepts, not yet explored, but are meant to trigger the promotion of phosphatase or the inhibition of kinase. The red line indicates that the device is considered as one plasmid, not as two separate plasmids. Not included in this image due to the lack of space are the reporters. For the experimental trials, we will use GFP after the CDSs for ETS-1 and insulin in order to ensure that both coding sequences were transcribed. These reporters will be removed after trials in the cell line and data will be recorded and compared to when the reporters were still left in to determine if the synthetic toggle switch is still regulating GSK-3 β and PP2A levels as deemed necessary. Removing the reporters will minimize the size of the plasmid and maximize the opportunity for it to enter the BBB with the nanocarrier.

Safety

Although the synthetic toggle switch will be tested in the SH-SY5Y cell line, the neuronal cell line is separate from what the environment of the human brain is like. There are a variety of factors influencing human neurons that are not present in the first stage of testing, so this must be considered during experimental trials. Additionally, due to the fact that this design will be placed in human beings at a later time, a high and rigorous level of testing will need to occur prior to human testing. It must be considered safe to enter humans for this to even be considered an option for helping to prevent the spread of AD. Another concern is the possibility that the synthetic

toggle switch will influence kinase and phosphatase levels to a degree that is unwarranted for the sake of this experiment. Deciding to use insulin to inhibit GSK-3 β may have an unprecedented effect; an increase in insulin could desensitize cells to its presence, thus potentially making patients susceptible to diabetes. How the switch will impact other biochemicals in the brain is currently unknown, which is why, once again, the rounds of testing is crucial to guaranteeing the success of the experiment in the future. Another important aspect to consider is that there is currently no way to determine if the insulin or ETS-1 were produced. Adding reporters to each individual pathway would serve as a method to indicate if the CDS for each were transcribed, ideally by putting the CDS for the reporters, perhaps green fluorescent protein, after the CDS for insulin and ETS-1. The reporters would be used during initial testing to ensure that the necessary CDSs are being transcribed. Removing the reporters for the final stage of testing in humans will assist in minimizing the size of the plasmid passing through the BBB in the human brain, thus facilitating the process in an expedited manner. However, the BBB remains a difficult system to penetrate, which is why extreme precaution and testing must be taken prior to human injection. The BBB remains impregnable for the reason that the human brain is complex. Tampering with the brain by adding or removing foreign objects into it carries potential risks that would leave severe consequences. While tracers are routinely placed in the brain, adding a synthetic toggle switch that is larger in size than these tracers can have an unpredictable result on the brain, which is why testing in mice is imperative to see the physiological responses.

Discussions

In the future, the design could potentially include other gene sequences that would seek to regulate the regulators; essentially, additional genes could work to form a checks-and-balances system in the synthetic toggle switch system. Noting the complexity of the human brain, using other regulators to monitor the levels of insulin and ETS-1 in the human brain, even after extensive testing, will allow the design to work as safely as possible inside the human subject. Additionally, to build on the checks-and-balances system, there should be a secondary gene sequence to ensure that the insulin and ETS-1 are not overproduced or underproduced. For example, a kill switch can be included to stop the sequence all together if the effects of the device were negative. This would ensure the safety of the patient and prevent unintended consequences from wreaking havoc on the patient's brain. Also, the effects of excessive insulin in the human body are known, leading to detrimental diseases if left untreated for too long, which is why additional steps to regulate the insulin levels will

be beneficial to the system. Excessive insulin can be toxic to cells, resulting in insular shock. Another aspect of introducing insulin in AD patients is that the insulin pathway is already altered in many of these patients (Liu, Liu, Grundke-Iqbal, et al. 2011). In the experiment cited, inhibiting GSK-3 β by producing insulin could reverse the intent of the experiment, which in turn will promote the production of GSK-3 β , thus increasing tau clusters. An alternate pathway to this design could then potentially be the heat shock pathway which increases the activity of GSK-3 β (Calderwood and Murshid 2017). Heat shock proteins can be utilized to deter tau hyperphosphorylation by unfolding and aggregating GSK-3 β that forms in the clusters. Lastly, AD research is a continuously-advancing field with the possibility of new designs and experiments being likely to influence other designs and experiments. It must be considered that the tau hypothesis is not the only cause of AD, and there are a variety of other causes. This is only a theoretical experiment that would work to determine the extent to which the tau protein influences AD patients.

Acknowledgements

Our team would like to thank our mentor, Mr. Alberto J. Donayre Torres, for taking the time to communicate with us and share his advice in regards to our project. Special thanks to Mrs. Lindsey L'Ecuyer, as well, for her guidance throughout this process, whether it be through the discussion of possible resources for research or by teaching us the critical aspects of synthetic biology. Lastly, our team would like to express our gratitude to the BioBuilder organization which provided us with the opportunity to conduct this research in the first place.

This project was accomplished through participation in the BioBuilderClub, an after-school program organized by BioBuilder Educational Foundation. BioBuilderClub engages high school teams around the world to combine engineering approaches and scientific know-how to design/build/test their own project ideas using synthetic biology.

References

- Anderson J. Part:BBa J61101 [Internet]. Boston (MA): International Genetically Engineered Machine, Registry of Standard Biological Parts; 2007 Jan 28 [cited 2020 Apr]. Available from: https://parts.igem.org/Part:BBa_J61101
- Biotechniques.com. Masumi Eto on a new method for the detection of kinase activity [Internet]. London (UK): BioTechniques; 2020 Jan 8 [cited 2020 May 28]. Available from: <https://www.biotechniques.com/omics/>

- masumi-eto-on-a-new-method-for-the-detection-of-kinase-activity/. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5521006/>
- Calderwood SK and Murshid A. Molecular Chaperone accumulation in cancer and decrease in Alzheimer's Disease: the potential roles of HSF1 [Internet]. United States: Front. Neurosci.; 2017 Apr 21 [cited 2020 May 29]. Available from: <https://www.frontiersin.org/articles/10.3389/fnins.2017.00192/full>
- Chen H-G, Han W-J, Deng M, Qin J, Yuan D, Liu J-P, Xiao L, Gong L, Liang S, Zhang J, Liu Y and Li DW-C. Transcriptional regulation of PP2A-A α is mediated by multiple factors including AP-2 α , CREB, ETS-1, and SP-1. PLoS One [Internet]. Don-Yan Jin, editor. 2009 [cited 2020 Apr 23];4(9):e7019. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2736573/>
- Cross DA, Alessi DR, Cohen P, Andjelkovich M and Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature [Internet]. 1995 Dec 21 [cited 2020 Apr 23];378(6559):785-9. Available from: <https://pubmed.ncbi.nlm.nih.gov/8524413/>
- Francis PT, Palmer AM, Snape M and Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. Journal of Neurology, Neurosurgery & Psychiatry [Internet]. 1999 [cited 2020 May 27];66(2):137-47. Available from: <https://jnnp.bmj.com/content/66/2/137.citation-tools>
- Gardner TS, Cantor CR and Collins JJ. Construction of a genetic toggle switch in Escherichia coli. Nature [Internet]. 2000 Jan 20 [cited 2020 May 27];403(6767):339-42. Available from: <https://pubmed.ncbi.nlm.nih.gov/10659857/>
- Gong CX and Iqbal K. Hyperphosphorylation of Microtubule-Associated Protein Tau: a promising therapeutic target for Alzheimer Disease. Curr Med Chem [Internet]. 2009 Mar 16 [cited 2020 Apr 10];15(23):2321-8. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2656563/>
- Helmenstine, AM. What is phosphorylation and how does it work? [Internet]. New York (NY): ThoughtCo.; 2019 Feb 11 [updated 15 Aug 2019; cited 2020 May 27]. Available from: <https://www.thoughtco.com/phosphorylation-definition-4140732>
- Kaisar MA, Sajja RK, Prasad S, Abhyankar VV, Liles T and Cucullo L. New experimental models of the blood-brain barrier for CNS drug discovery. Expert Opin Drug Discov [Internet]. 2018 Jan 1 [cited 2020 May 28]. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5521006/>
- Liu Y, Liu F, Grundke-Iqbal I, Iqbal K and Gong C-X. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. The Journal of Pathology [Internet]. 2011 Sep [cited 2020 May 29];225(1):54-62. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/path.2912?casa_token=woyQQ8mVf5wAAAAA%3AxmHeBXL0IdvvOJr0_on-QVhdw3gYNyxlHpsCOyaojm290LC_LEkpurbTz10pBNaAtK6r4YemVCxf
- Martinez E. Part:BBa M1877 [Internet]. Boston (MA): International Genetically Engineered Machine, Registry of Standard Biological Parts; 2012 Mar 27 [cited 2020 Apr]. Available from: https://parts.igem.org/Part:BBa_M1877
- Martorana A, Esposito Z and Koch G. Beyond the Cholinergic Hypothesis: do current drugs work in Alzheimer's Disease? CNS Neurosci Ther [Internet]. 2010 Aug [cited 2020 Apr 20];16(4):235-45. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6493875/>
- Mietelska-Porowska A, Wasik U, Goras M, Filipek A and Niewiadomska G. Tau Protein modifications and interactions: their role in function and dysfunction. Int J Mol Sci [Internet]. 2014 Mar [cited 2020 May 28];15(3):4671-713. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3975420/>
- Perlman, RL. Mouse models of human disease: An evolutionary perspective. Evol Med Public Health [Internet]. 2016 [cited 2020 May 28];2016(1):170-6. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4875775/>
- Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia A-S, McNamara JO and Williams SM, editors. Neuroscience [Internet]. 2nd ed. Sunderland, MA: Sinauer Associates; 2001 [cited 2020 May 28]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK11143/>
- Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M and Mahmoudi J.. Amyloid-Beta: a crucial factor in Alzheimer's Disease. Med Princ and Pract [Internet]. 2015 Jan [cited 2020 May 28];24(1):1-10. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5588216/>
- Saraiva C, Praca C, Ferreira R, Santos T, Ferreira L and Bernardino L. Nanoparticle mediated brain drug delivery: overcoming blood-brain barrier

to treat neurodegenerative diseases. *Journal of Controlled Release* [Internet]. 2016 Aug 10 [cited 2020 Apr 11];235:34-7. Available from: <https://www.sciencedirect.com/science/article/pii/S0168365916303236>

Teipel SJ, Grinberg LT, Hampel H and Heinsen H. *Encyclopedia of Neuroscience* [Internet]. [place unknown]: Elsevier; 2009 [cited 2020 May 28]. p. 857-

68. Available from: <https://www.sciencedirect.com/science/article/pii/B9780080450469020416>

Vergoni AV, Tosi G, Tacchi R, Vandelli MA, Bertolini A and Costantino L. Nanoparticles as drug delivery agents specific for CNS: in vivo biodistribution. *Nanomedicine: Nanotechnology, Biology, and Medicine* [Internet]. 2009 Dec [cited 2020 May 28];5(4):369-77. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S1549963409000598>

