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Investigating the Mammillary Bodies as an Early Target of Alzheimer's Disease

by
Cole Martinson

An undergraduate honors thesis submitted in partial fulfillment of the requirements for
the degree of Bachelor of Science

in
University Honors
and
Biochemistry

Martin Kelly

Portland State University

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Abstract:

Alzheimer's disease (AD) is the most common form of dementia. With minimal treatment options and no cure, developing a deeper understanding of the pathology of the disease is crucial. For nearly four decades, the accumulation of beta-amyloid ($A\beta$) plaques has been correlated with the disease and its progression. Previous studies mapping this accumulation show that the mammillary bodies (MB) are an early target of the disease. Glutamatergic neurons are of key interest due to their prevalence in the central nervous system, specifically the MB. To understand the effects of AD on the glutamatergic system, we used vesicular glutamate transporter 2 (VGLUT2) immunocytochemical as an indicator of glutamate activity. In the MB, we identified VGLUT2 as being overexpressed alongside the production of $A\beta$. Additionally, through patch clamp electrophysiology we determined the MB to be in a hyperexcited state.

Introduction:

Since the first characterization of the disease in 1906 by Alois Alzheimer (Goedert & Ghetti, 2007), a cure for the disease has been pursued with little success (Joe & Ringman, 2019). A high prevalence of the disease, at 6 million people in America (*What Is Alzheimer's Disease?*, 2023), warrants a detailed investigation of the pathology of the disease to find a treatment or cure.

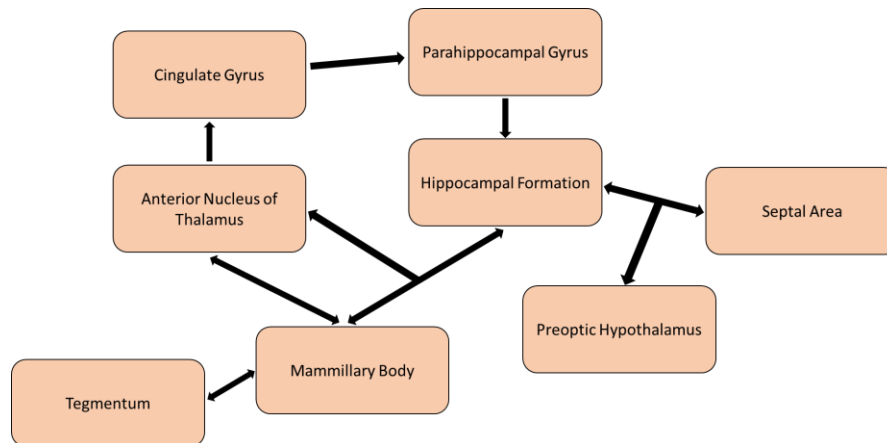


Figure 1: Block Diagram detailing neuroanatomy of the Limbic System.

Symptoms of Alzheimer's disease vary and can be vast due to the extensive nature of the disease in the brain. The main symptoms of the disease are cognitive disorders (Lyketsos et al., 2011). This can include everything from delusions, hallucinations,

psychosis, anxiety, and depression (Patterson et al., 1990). One cause of memory deficits and cognitive disorders is a result of the pathologic effects of AD on the hypothalamus and Entorhinal cortex (Van Hoesen et al., 1991). Additionally, there can be effects on metabolism, circadian rhythms, and aggressive behaviors (Ishii & Iadecola, 2015). Many of these symptoms are related to the Limbic system centering around the Papez circuit (Swaab, 1997), which has been used as a pathway to address the function of memory. The Papez circuit details a neural loop that describes intricate connections between the subiculum, a region on the hippocampus, the MB, the anterior thalamic nuclei, the cingulate gyrus, and then the parahippocampal region before returning to the subiculum in that order (Aggleton et al., 2022; Vann & Nelson, 2015).

While the hypothalamus is generally not the focus of research on memory, the MB has begun to receive more attention for their role in the formation of spatial memory (Dillingham et al., 2019, 2021; Vann, 2010a).

Many papers address that while the MB was one of the first structures to be classified in this system their role has been mostly overlooked (Vann, 2010b; Vann & Nelson, 2015, 2015). Instead, other regions of the limbic system have garnered attention. The thalamus is important for verbal memory, cognition, and encoding stimuli (Van der Werf et al., 2003). The hypothalamus, a central hub for the nervous and endocrine system, is important for spatial and episodic memory and is affected in Alzheimer's disease, Creutzfeldt-Jacob's disease, Parkinson's disease, Pick's disease, Korsakoff's disease and PSP (Swaab, 1997).

The MB, named by early anatomists for its likeliness to breasts, are located posteroinferiorly to the hypothalamus and are an important nucleus for recollective memory (Peterson et al., 2022). As an integral part of the Papez circuit the MB, known

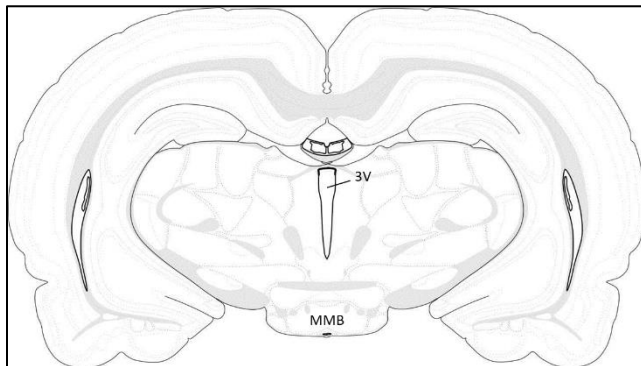


Figure 2: Coronal Section detailing the location of the mammillary bodies (MB). The third ventricle(3V) is also shown for reference.

to consist entirely of projection neurons, connects other regions of the brain associated with memory as previously described in the Papez circuit.

Important fiber tracts directly associated with the MB in the Papez circuit include the mamillothalamic tract and the fornix. The mamillothalamic tract is an integral part of the MB output within the limbic system (Carlesimo et al., 2007; Dillingham et al., 2015, 2021; Vann, 2010b). The mamillothalamic tract

stems from the pars medialis, pars lateralis, and the lateral regions of the mammillary body. Each region projects to different nuclei of the thalamus. The pars medialis innervates the anteromedial thalamic nucleus, and the pars lateralis innervates the anteroventral thalamic nucleus. The lateral MB connects to the anterodorsal thalamic nucleus. Lesions of this tract have been directly connected to amnesia and spatial memory deficits (Dillingham et al., 2019).

The effects of isolated damage to the mamillothalamic tract highlight its importance in cognition. One report showed loss of verbal memory as a result of damage to the thalamus in conjunction with the mamillothalamic tract (Mori et al., 1986). An overview of clinical data and animal research data reveals a connection between thalamus memory and cognition. Further research shows that lesions of the mamillothalamic tract disrupt sensory stimuli (Van der Werf et al., 2003). A case study that looked at these previous implications of the mamillothalamic tract found that damage leads to a loss of recollection but did not affect familiarity in episodic memory tests (Carlesimo et al., 2007). Such symptoms are like those experienced by some Alzheimer's patients.

This provides insight into whether this tract is an integral part of the development of Alzheimer's disease.

The tegmental nuclei of the midbrain are other target structures to be considered when referring to the MB. Different from other structures associated with the MB, the tegmental nuclei both send and receive stimuli from the MB (Dillingham et al., 2021). Connecting them is the mammillotegmental fasciculus. This connection stems from the pars posterior of the mammillary body. Indeed, recent studies have begun to look at the tegmental nuclei as an early target of AD (La Barbera et al., 2022; Nobili et al., 2017).

The exact pathology of the disease has remained elusive with numerous theories proposed including the mitochondrial hypothesis, the cholinergic, and the A β hypothesis (Liu et al., 2019). The mitochondrial hypothesis describes the disease as stemming from the degeneration of mitochondrial activity, leading to the degeneration of neurons (Swerdlow et al., 2010). The cholinergic hypothesis describes the disease as a dysfunction of acetylcholine-containing neurons (Terry & Buccafusco, 2003). Aside from these, since its characterization in 1986, the A β hypothesis has become the main area for research on AD (Glennner & Wong, 1984). AD is marked by the progression of plaque formations consisting of A β and tau protein (Braak & Braak, 1996). While the presence of A β plaques in the hypothalamus has been a major focus point, recent studies have shown that the development of AD begins in the MB before moving to other areas (Gail Canter et al., 2019).

The glutamatergic system is important to understand the dysfunction found in AD. Glutamate, the main fast neurotransmitter of the central nervous system, has been shown to lead to neurotoxicity at high concentrations (Choi, 1987, 1988; Regan & Choi, 1991). This is due to leakage out of the synaptic cleft and through the activation of extrasynaptic N-methyl-D-aspartate (NMDA) receptors (Hardingham & Bading, 2010; Parsons & Raymond, 2014). Research suggests that this difference in extrasynaptic and intrasynaptic receptor neurotoxicity results from conditions differing around the receptors such as glutamate uptake systems rather than the receptors themselves (Hardingham & Bading, 2010; Sattler et al., 2000). Responsible for the packaging, and therefore the release of glutamate is VGLUT2. VGLUT2 is responsible for the transportation of glutamate into synaptic vesicles in preparation to be released into the synaptic cleft. High levels of VGLUT2 have been connected to increased glutamate release (Daniels et al., 2004; Moechars et al., 2006). VGLUTs in AD have been studied and have been shown to have decreased levels in the prefrontal cortex (Kashani et al., 2008).

An understanding of the start and progression of the disease will help in the development of drugs to treat and possibly cure the disease. Our group seeks to do this by analyzing the MB, an early target of the disease. The expression of VGLUT2 was analyzed showing a significant increase in the positive AD specimens. In addition, increased neuron activity was documented utilizing whole-cell patch recordings. This

information will help establish the MB as a point of interest in the development of the disease.

Methodology

Animals

All procedures conducted with animals were according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals with approval for all of the animal use procedures from the Oregon Health and Science University (OHSU) Animal Care and Use Committee.

The transgenic AD mouse model used in this experiment was the 5xFAD line (034848-JAX). This transgenic line carries five mutations related to the inheritance of AD. The mutations that it contains include three APP gene mutations, the KM670/617NL/Swedish, 1716V Florida, and V717/London. It also includes two mutations to the PSEN1 gene, M146L and L286V(Oakley, 2006). Mice were bred with wild-type C57(000664-JAX) mice to produce heterozygous offspring. Each mouse was genotyped to confirm negative and positive carrying mice. Negatives were used as controls.

To image the paths of the glutamatergic neurons from the MB VGLUT2-Cre mice were used. Mice were homozygous and confirmation of genotype was obtained through PCR genotyping.

The ages of the mice were grouped as the following: 1.5-2 months, 2.2-2.9 months, 3.5-4 months, and 5.5-6.9 months.

Tissue Preparation

Female 5xFAD mice were prepared for ICC as follows: coronal hypothalamic blocks (2–3 mm each) were fixed by immersion in 4% paraformaldehyde, cryoprotected in 30% sucrose solution, frozen at -55°C , sectioned coronally on a cryostat at 20 μm , and thaw-mounted on Superfrost Plus slides (Thermo Fisher Scientific).

Immunocytochemistry

5XFAD sections were reacted to visualize both A β 1-40 as well as VGLUT2. Initially, sections were rinsed in PB solution (0.1 m phosphate buffer, pH 7.4 with 5% normal serum and 0.3% Triton X-100) for at least 30 min. Sections were then incubated with rabbit polyclonal A β (1-40) primary antibody (1:2000; ThermoFisher, Waltham, MA) or rabbit polyclonal affinity purified vGlut2 primary antibody (1:1000; Synaptic Systems, Gottingen, Germany). Both primary and secondary antisera were diluted in an antibody diluent solution composed of 0.7 % λ -carrageenan (type IV), 0.4% Triton X-100 and 3% bovine serum albumin in Tris buffer (Tris buffer: 8.5 mM Na $_2$ HPO $_4$, 3.5 mM KH $_2$ PO $_4$, 120 mM NaCl, 41 mM tris(hydroxymethyl)aminomethane, pH 7.6) and then incubated for ~ 48 h at 4 $^{\circ}\text{C}$. Slides were then rinsed with PB and incubated for 2-3 h at room temperature with a biotin-SP-conjugated affiniPure donkey anti-rabbit IgG H+L

secondary antibody (1:500; Jackson ImmunoResearch, Philadelphia, PA) and finished with streptavidin-Alexa Fluor 594 (1:2500; Jackson ImmunoResearch, Philadelphia, PA). Following a final rinse overnight with PB, slides were cover slipped with gelvatol containing the anti-fading agent, 1,4-diazabicyclo(2,2)octane (DABCO; Sigma-Aldrich; Grachev et al., 2016).

Visualized whole-cell patch recordings

Coronal brain slices (240 μm) containing the ARH from gonadectomized, or intact mice were made in an ice-cold sucrose-cutting solution (see recipe below) and stored in a bubbled chamber containing aCSF (see recipe below). Whole-cell patch recordings were performed in voltage clamp and current clamp using an Olympus BX51W1 upright microscope equipped with video-enhanced, infrared-differential interference contrast (IR-DIC) and an X-Cite 120 Series fluorescent light source (Excelitas Technologies Corp.). Electrodes were fabricated from borosilicate glass (1.5 mm OD; World Precision Instruments) and filled with a normal internal solution: 128 potassium gluconate, 10 NaCl, 1 MgCl₂, 11 EGTA, 10 HEPES, 3 ATP, and 0.25 GTP (pH was adjusted to 7.3–7.4 with 1N KOH, 290–300 mOsm). High chloride internal solution consisted of 140 mM KCl, 5 mM MgCl₂·6H₂O, 1 mM MgCl₂, 0.1 mM EGTA, 10 mM HEPES, 5 mM K₂-ATP, and 0.35 mM Na₃-GTP (pH was adjusted to 7.3–7.4 with KOH; 290–2945 mOsm). Cesium chloride internal solution consisted of 125 mM CsCl, 5 mM MgCl₂, 1 mM BAPTA, 10 mM HEPES, 5 mM K₂-ATP, and 0.4 mM Na-GTP (pH was adjusted to 7.3–7.4 with CsOH). Pipette resistances ranged from 3–5 M Ω . In the whole-cell configuration, access resistance was <20 M Ω ; access resistance was 80% compensated. Electrophysiological signals were amplified using the Axopatch 200B amplifier (Molecular Devices) and digitized using the Digidata 1440A digitizer (Molecular Devices), and the data were analyzed using p-Clamp software (RRID: SCR_011323, v10.3, Molecular Devices). The liquid junction potential was corrected for all data analysis.

Solutions/drugs

A sucrose solution was used during Vibratome slicing: 2 mM KCl, 1 mM MgCl₂·6H₂O, 1.4 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, 208 mM sucrose, 26 mM NaHCO₃, 2 mM MgSO₄·7H₂O, and 1 mM CaCl₂. Standard artificial cerebrospinal fluid was used: 124 mM NaCl, 5 mM KCl, 1.4 mM NaH₂PO₄, 5 mM HEPES, 10 mM glucose, 26 mM NaHCO₃, 2 mM MgSO₄·7H₂O, and 2 mM CaCl₂. All drugs were purchased from Tocris Bioscience unless otherwise specified. DAMGO (D-Ala², N-MePhe⁴, Gly-ol]-enkephalin) was purchased from Peninsula Laboratories (Bachem).

Data Analysis & Imaging

For electrophysiology, ClampFit 10.3 (Molecular Devices) and Prism (GraphPad Software) were used for analysis. Comparisons between different treatments were

performed using t-tests, where appropriate. Differences were considered statistically significant if $p < 0.05$. All data are expressed as mean \pm SEM.

Samples were imaged on an Axioscan 7 using a Plan-apo 10x0.45NA objective. Images were illuminated by a Colibri5/7 LED light source (385nm, 567nm) with excitation: emission filters 390/40:450/40 and 550/25:605/70 and captured on a Hamamatsu Orca Flash v3.0 camera. Tiled images were stitched using ZenBlue software.

ICC labeling of VGLUT2 was quantified using Zen Blue software. For each section, an ROI was drawn around the MB. Expression was determined by contrast, and size of spots in aged positive specimens and used for all sections. The percent area of expression was determined and compared to the control for each group. Data was analyzed compared to controls in each group as well as the experimental group being compared by age groups. Significance was determined using a two-way ANOVA.

Results

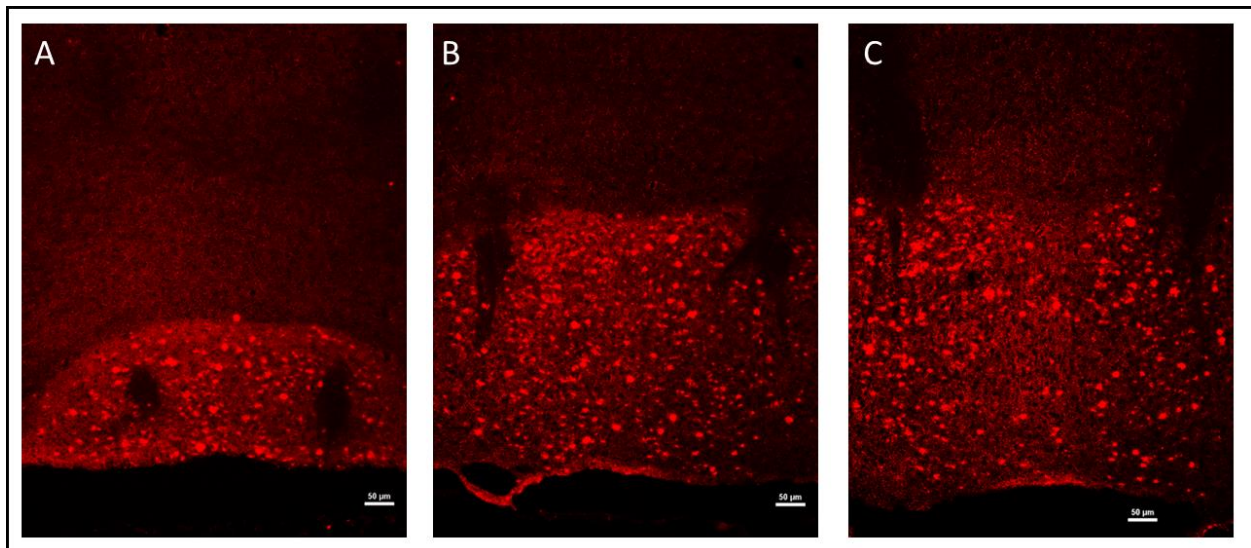


Figure 3: The accumulation of Aβ in a 6 month old positive 5xFAD specimen from caudal (A), medial (B) and then rostral(C)

Our examination of the mamillary bodies as an early target of Alzheimer's Disease first began with confirming the early expression of Aβ in comparison to other regions where plaque develops. It was observed that Aβ begins to accumulate around 2 months of age. Figure 3 shows the accumulation of Aβ through ICC staining. The mammillary bodies appear to have high expression of Aβ in the early stages of the disease. This progression was expected and points to the MB as an early target of the disease.

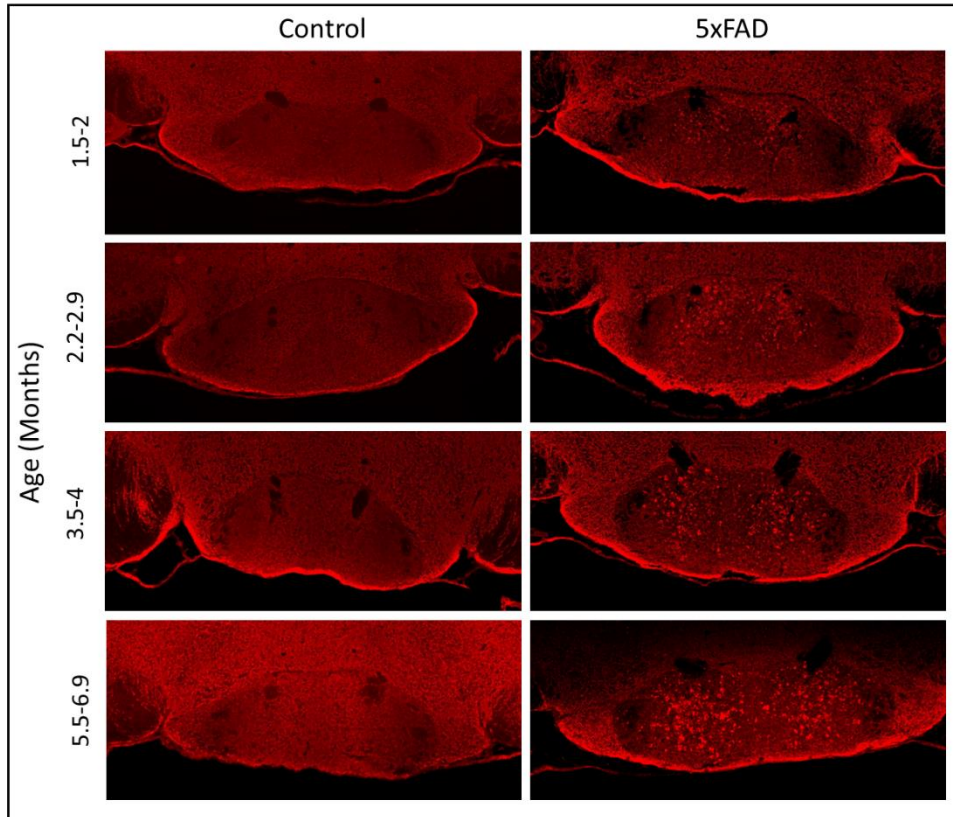


Figure 4: Vesicular Glutamate Transporter 2 labeling showing difference from control to 5xFAD positive mice within the mammillary bodies. The expression is shown to increase with age.

With the expression of A β documented in the MB, it was of interest to interrogate the mammillary bodies for other proteins that may be overexpressed as a result of Alzheimer's Disease.

To assess possible effects on neurotransmitter activity VGLUT2 expression was measured in the 5xFAD model. As shown in Figure 4, the expression

of VGLUT2 compared to controls was significant. Similarly, to beta-amyloid, initial observation of the ICC labeling of VGLUT2 showed early accumulation in the mamillary bodies. Additional staining was conducted to observe the progression of VGLUT2 expression in mice ranging from 1.5-6.9 months.

Over-expression of VGLUT2 in the mamillary bodies appeared around 2 months and drastically increases until 4 months when the expression plateaus (Figure 4). Quantification of the expression by percent area of expression was conducted showing significant differences between control and AD mice starting at 2 months and widening until 6 months of age. It should be noted that the amount of expression within the areas considered "positive" within the percent area calculation could show continued increased expression. This was not quantified as the fluorescence of these areas often becomes saturated.

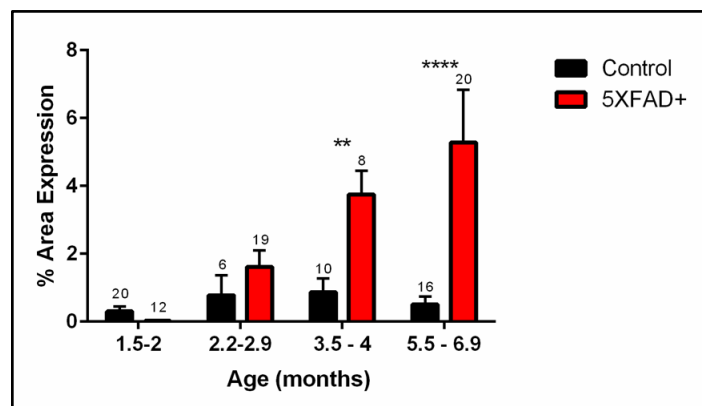


Figure 5: Quantification of VGLUT2 labeling by percent area of expression within the MB.

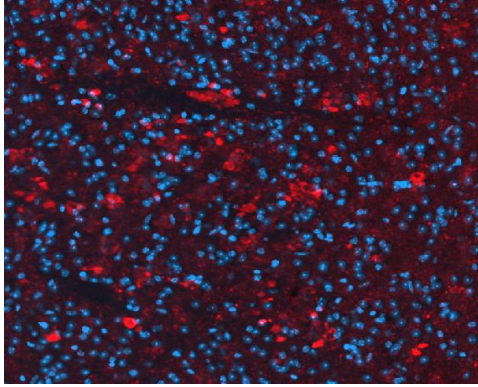


Figure 6: DAPI and VGLUT2 labeling of MB sections of positive 6.9 month old 5xFAD mice.

As with the accumulation of beta-amyloid, we found that VGLUT2 expression was first increased in the MB before spreading to other sections of the limbic system. Expression begins to appear at 2 months. A significant difference from the control was evident at 3.5-4 months. Expression increased drastically from that point until 6 months when the quantification of VGLUT2 reached a maximum.

Cross-staining 5xFAD mice for VGLUT2 and 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI), which stains for nuclear DNA, revealed interesting results. It was expected that VGLUT2 would be mainly expressed in the cellular bodies as this is where the protein normally functions. As shown in Figure 6, VGLUT2 was found around the nuclei of the cells within the cell bodies. This effect was accentuated with age with a greater distinction between cell body labeling and VGLUT2 labeling.

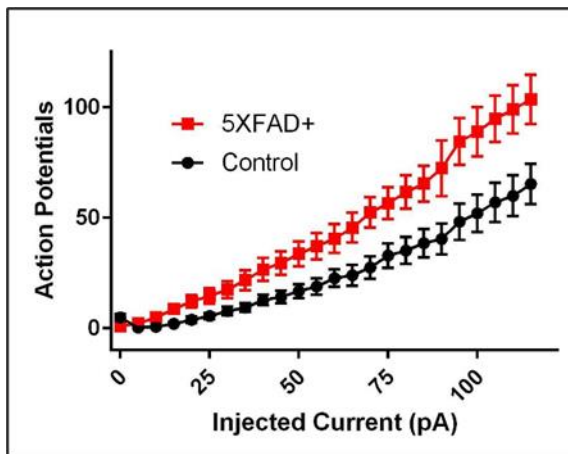


Figure 7: Action potentials fired upon injection of current comparing 5xFAD to controls.

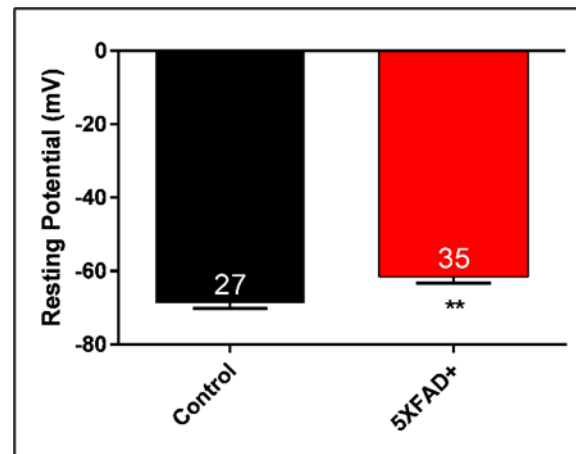


Figure 8: Measured resting membrane potential of 5xFAD compared to control.

To develop an understanding of the possible mechanisms involved with the pathology of AD as well as the impact the disease was having from neuron to neuron, basic patch clamp electrophysiology was used to monitor the excitability of the MB neurons. Injection of current found a significant difference in action potential firing as Figure 7 shows. Analysis of the resting membrane potential (RMP) of MB neurons found a significant depolarization of 7mV (Figure 8). The increase in the F/I current would translate into a greater response in firing action potentials from an increase in the excitatory input that the cell receives. Additionally, the cell sitting at a lower RMP (i.e., depolarized membrane potential) would allow for less excitatory input to generate an action potential. Therefore, both show that the cell is in a hyperexcited state. Changes in ion conductance, such as for Ca^{2+} and K^+ , likely play a role in these changes.

Discussion

Glutamate release is essential for the normal functioning of the central nervous system (Zhou & Danbolt, 2014). However, when glutamatergic overexcitation occurs there is increased activation of glutamatergic NMDA receptors (S. Chen & Diamond, 2002). VGLUT2 is overexpressed within the cell so more glutamate is released into the synapse. Initially, this will lead to the post-synaptic cell being overactivated as well. When coupled with a depolarized resting membrane potential and increased frequency of action potentials the effects of glutamate release are exacerbated. At high enough levels, not only are the synaptic NMDA receptors being activated, but glutamate can also diffuse out of the synapse and activate extracellular NMDA receptors. This leads to a large uncontrolled influx of Ca^{2+} that becomes concentrated in the mitochondria of the cell and leads to cell death (S. Chen & Diamond, 2002; Pivovarova et al., 2004; White & Reynolds, 1996).

It has been shown that Alzheimer's progresses through the brain as a cascade rather than sporadically developing throughout. This can be seen in the progression of $A\beta$ plaques. This would make it appear that the overexcitation of neurons affected by the disease is "spreading" the disease through a mechanism of overactivation. The MB acts as an important relay in the Limbic system that is still not fully understood. Important direct and indirect connections also show the accumulation of $A\beta$ after the MB (Gail Canter et al., 2019). It should be noted that the MB are a small region where expression could seem greater than others due to the increased density of synaptic connections or a greater homogeneity of functions (Dillingham et al., 2021; Hou et al., 2021).

Our analysis found that the expression of VGLUT2 increased congruently with $A\beta$ expression. Both VGLUT2 labeling and $A\beta$ appear to start within the mamillary bodies at a similar time. It is difficult to determine if one appears antecedent to the other with this technique as antigens may have different binding affinities and light intensities than one another. Future research can focus on this to be able to better understand the initiation of the disease.

$A\beta$ has been shown to bind to many synaptic receptors including NMDA receptors. The activation of these receptors is not completely understood. NMDA receptors are ionotropic glutamate receptors composed of two GluN1 subunits and two GluN2 or GluN3 subunits (Lee et al., 2014). Activation of the receptor has been proposed to stem from an interaction between the GluN2 or GluN1 subunit and the $A\beta$ oligomers. This was tested by blocking these subunits with targeted antibodies (G.-F. Chen et al., 2017; Olajide & Chapman, 2021). When treated with $A\beta$, cells showed an increase in VGLUT2 and a decrease in NMDA receptors (Texidó et al., 2011). This is likely neural adaptation to $A\beta$ activating NMDA receptors as there was increased glutamate release. In the disease state, the cell would be experiencing both $A\beta$ driven NMDA receptor activation as well as general activation from the increased glutamate release. This is a significant effect that should be investigated further. VGLUT2 may increase in expression simply due to the increased calcium influx since any calcium channel activator can have similar

effects (Doyle 2010). Understanding the possible pathways that lead to A β overproduction is also important. A β is formed from the decomposition of amyloid precursor protein (APP) from A β -secretase and gamma-secretase (G.-F. Chen et al., 2017). Understanding the possible activation of these enzymes within the context of the disease could help better determine the cause of the disease.

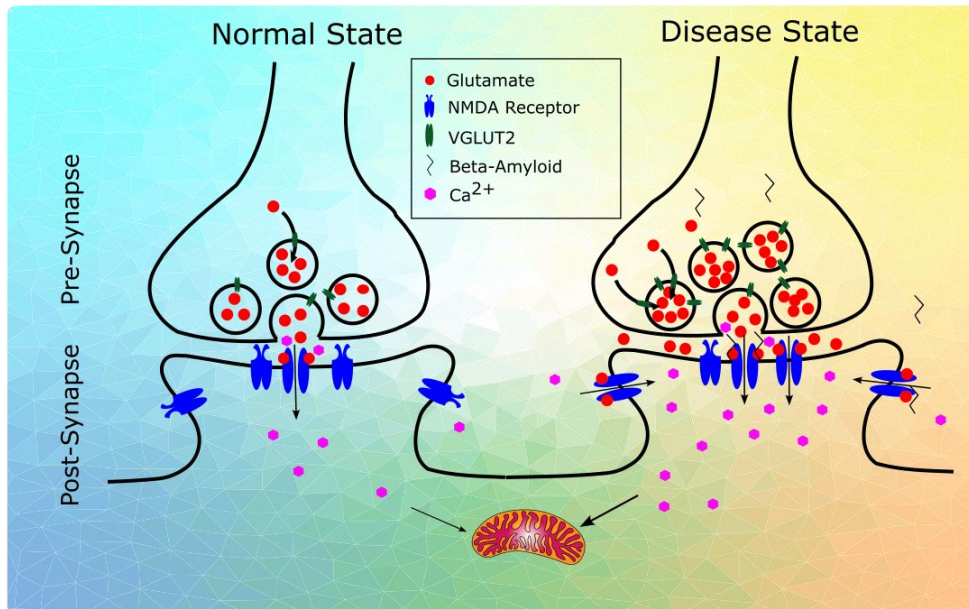


Figure 9: Diagram of Alzheimer's Disease effects on synaptic activity. Adapted from (Day-Cooney et al., 2023)

The pathology of the disease is difficult to characterize as spontaneous within the cell but rather stems from intervention from other neurons. Within the classical limbic system, this is well characterized following the A β plaques. At the start we see the mamillary bodies

are first affected by the increase of both VGLUT2 and A β . As far as our understanding, the MB are purely projection neurons. This characterization is why the MB are usually overlooked as the origination point for AD. Other factors such as cytokines dysregulation could lead to an explanation of MB origination as they play a large role in the homeostasis of the CNS (Marisa et al., 2018) and have been shown to possibly be affected by AD (Su et al., 2016; Swardfager et al., 2010). Also, an area that is often overlooked is the tegmental nuclei of the midbrain. This area of the brain both sends and receives direct impulses from the MB. In the last few years studies have shown that tegmental nuclei appear to be an early target of the disease (La Barbera et al., 2022; Nobili et al., 2017). Both in human and animal models neural degeneration is found with A β plaques being found early in the

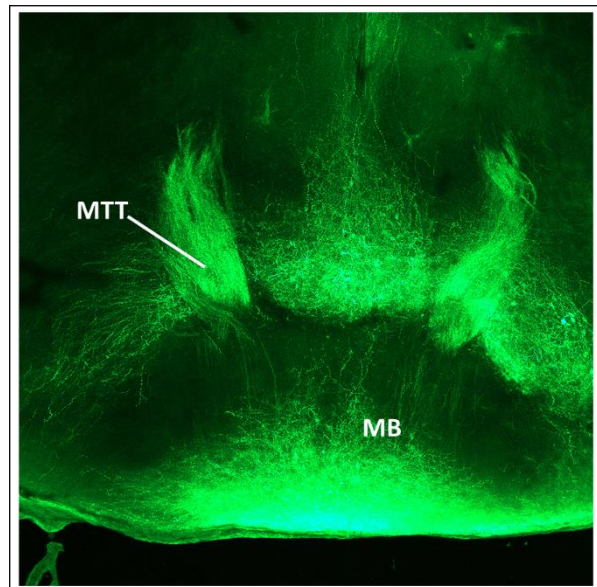


Figure 10: Vglut2-Cre mice injected with AAV1-YFP-ChR2 to fluorescently identify glutamatergic neurons in the mammillary bodies. Confocal imaging was used. MTT - mammillothalamic tract

tegmental nuclei of the midbrain. Interestingly we did not find any indication of this in our analysis. However, neurons in this area are mainly dopaminergic and GABAergic so investigation through the expression of VGLUT2 would not yield meaningful results (Cai & Tong, 2022; Tritsch et al., 2012). Moreover, if the cause of the disease is a spontaneous activation of neurons that leads to a cascade, it could point to a different source. Detailed investigation of this area and possible overexcitation is warranted.

In addition to continuing research on the initiation of AD, it is also important to investigate the progression of the disease. Possible therapeutic targets could be found to help treat AD. To understand the downstream effects of increased electrical activity as well as increased VGLUT2 expression in the MB the tracts of glutamatergic neurons are explored using VGLUT2-Cre mice that were injected with AAV1-YFP-ChR2 virus (with limited success) to visualize the glutamatergic tracts from the MB. As Figure 10 shows, the MTT tract consists of a dense collection of glutamatergic tracts.

In summary, using immunocytochemical staining, the accumulation of A β within the MB was confirmed at an early stage of the disease. This highlighted that the MB are an early target of the disease progression. The overexpression of VGLUT2 within the mammillary bodies was discovered. The progression of VGLUT2 was quantified and found to be significantly different from the control group at 3.5-4 months. However, visibly there is notable accumulation of VGLUT2 in some sections of the 1.5-2 month group and more within the 2.2-2.9 month group. This increase in glutamate activity was substantiated by electrophysiology findings showing a decreased RMP and an increase in the F/I current. Together these findings document that the mammillary bodies are in a hyperexcited state, releasing more glutamate to downstream targets.

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