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Abstract PP2A is protein phosphatase-2A. Inhibition of PP2A (by PP2A inhibitors 1 and 2) results in hyperphosphorylation and accumulation of tau protein. Hyperphosphorylated tau is a major component of neurofibrillary tangles – a pathological hallmark of Alzheimer's Disease (AD). Hence PP2A inhibition represents a putative new approach for a rodent model of AD.

The goal of this study was to evaluate the effects of PP2A inhibition on dendritic branching in cortical pyramids in rats that wereinjected ICV with an adeno- associated virus for expression of:

(1) Inhibitor 1 full length (I1 FL)
(2) Inhibitor 2 full length (I2 FL)
(3) Inhibitor 2 amino terminal fragment (I2 NTF)
(4) GFP (Green Fluorescent Protein), Control

Rat brains were subsequently Golgi stained and from coded slides randomly selected layer V pyramids were evaluated for the amount and distribution of their basilar dendritic arbor using Sholl analysis. Results showed that administration of both I2 groups (full length and amino terminal fragment) resulted in more dendritic branching than either the GFP control or the I1 full length inhibitor. Tau hyperphosphoryation by PP2A-inhibition did not result in a diminution of cortical branching. Additional studies are ongoing to further characterize the validity of this model.

Background Alzheimer's disease, also known as AD, is the most common form of dementia in the elderly population; with the elderly population being defined as those individuals 65 years old or greater. By 2010, AD has been known to effect 5 million people throughout the United States and 30 million people throughout the world (10). It is predicted that by the year 2050 approximately 1 person out of every 85 persons will suffer from AD (1). The cause and course of AD is still not fully understood, though some basic facts about AD are a consensus between researchers. AD has two major hallmarks in the brain: extracellular deposits of β-amyloid in neuritic plaques and intracellular neurofibrillary tangles (NFT) (8). The NFTs and β-amyloid in neuritic plaques are most often found in the hippocampus, fundus of the forebrain, and the cerebral cortex of the brain (10).

Both β-amyloid in neuritic plaques as well as NFTs are easily visible using basic microscopy techniques in brains suffering from AD. β-amyloid in neuritic plaques, often referred to as just amyloid plaques, are dense mostly extracellular insoluble deposits of amyloid-beta peptide deposited outside and around neurons (8). NFTs are aggregates of hyperphosphorylated tau, a microtubule-associated protein. The presence of the protein tau is also found in a great deal of other diseases known as tauopathies. NFTs are formed

by hyperphosphorylation of a microtubule-associated protein (MAP) known as tau, causing it to aggregate, or group, in an insoluble form (4). The clusters or aggregates of hyperphosphorylated tau are also referred to as paired helical fragments (PHF). Tau in PHFs is rid of its normal function as it is abnormally hyperphosphorylated. Tau proteins are proteins that stabilize microtubules. When tau protein is hyperphosphorylated evidence from several previous studies have shown that tau loses its biological ability to stabilize microtubules effectively and becomes quite resistant to proteolytic degradation (2, 3, 5, and 10). Since hyperphosphorylated tau results in it becoming quite resistant to degradation in comparison to normal tau, there is an accumulation of tau in tauopathic disease models, such as AD. Tau proteins are quite abundant in the central nervous system (CNS) and especially in neurons.

Naturally the regulation of the phosphorylation of the tau protein would be of primary concern when understanding AD and why NFTs develop. It is thus essential then to understand why and how tau is abnormally hyperphosphorylated. Evidence from previous studies show that all of the phosphorylation sites on tau are on two amino acid residues: serine and threonine (2). Previous studies have thus suggested that protein phosphatases (PP) are involved in the regulation of the phosphorylation of tau (2). There are four classes of PP: PP1, PP2A, PP2B, and PP2C (9). More recent studies have shown that the specific activity of PP2A is the major regulator of phosphorylation of tau (2). Successful and proper inhibition of PP2A has been shown to cause hyperphosphoryaltion of tau, and as a result the accumulation of tau and the development of PHFs and NFTs; and thus a novel way to develop a tauopathy rat model of AD (3).

In the present study, adeno-associated viruses serotype 1 (AAV1) vectors were used to express the three different inhibitors used in the experiment: full length (I1 FL), inhibitor 2 full length (I2 FL), inhibitor 2 amino terminal fragment (I2 NTF), and GFP (Green Fluorescent Protein) which was used as the control in this experiment. The AAV1 was injected into the female Wistar rats within their first 24 hours of life intracerebroventricularly (ICV). Three different cell populations were measured for assessment using sholl analysis after Golgi stained: granule cells of the dendate gyrus, CA1 pyramids of the hippocampus, and layer V pyramidal cells of the parietal cortex.

In the present study our laboratory focused predominantly on the morphologhy and the alterations in the dendritic arbor of three different neuronal populations, and not on the molecular and mechanistic aspect involving the adeno-associated virus and how the inhibitors were expressed. To view these different neuronal populations and evaluate them a drawing tube attached to a Zeiss brightfield microscope was used. The rat brains were required to be stained using a technique known as Golgi staining or the Golgi method. Golgi staining is a nervous tissue staining technique discovered by the physician and scientist Camillo Golgi in 1873. Golgi staining is often the method of choice when examing the morphology of neurons as this particular study does. Cells in the nervous system are densely packed and thus sometimes hard to view and distinguish from one another. The uniqueness of the Golgi method stems from them fact that the staining technique only stains a small percentage of the neuronal elements, allowing the ability to discern different cells and structures, and their interconnections (7). The Golgi method itself is capricious and stains a limited number of cells at random in a portion of brain tissue. The Golgi method randomly stains approximately 8% of all the neurons in the brain, staining the soma, entire dendriric arbor (95% of the volume of the neuron is made up of the

dendritic arbor), and dendritic spines of the neurons (7). Many times when employing the Golgi method, one section of brain tissue can be successfully stained while a neighboring portion of brain tissue might not be stained at all (7). The mechanism by which the Golgi method stains at random is still largely unknown. The main strength of using the Golgi method is that in regions it does stain, virtually all of these components are visible, giving a view that captures the entirety of the neural element. In the present study it is important to note that the morphology of the neuronal populations examined were a snapshot of what their morphology appeared to be when the female Wistar rats were between 11 to 13 months of age, with the average being 12 months in age.

Materials/Methods Formalin fixed brains of four groups of rat brains were sent to Neurostructural Research Labs. Coronal blocks of tissue incorporating the hippocampus and overlying parietal cortex were stained using the Rapid Golgi method. Briefly, the tissue blocks are immersed in a solution of potassijm dichromate and osmium tetroxide for 5-7 days. The tissue is rinsed in a freshly prepared solution of silver nitrate and placed in more silver nitrate for 39-42 hours. The tissue blocks are dehydrated through ascending concentrations of alcohol, then into ether-alcohol. The blocks are then embedded in low viscosity nitrocellulose which is hardened by exposure to choloroform vapors. The hardened blocks are affixed to wooden blocks and sectioned on an AO sliding microtome at 120um. The sections are cleared in alpha-terpineol, throroughly rinsed in xylene, placed on coded slides, and coverslipped under Permount.

CA1 Branching: Sholl Analysis

CA1 pyramids were randomly selected from coded slides. Camera lucida drawings were prepared from the basilar tree of the selected CA1s. Neurons had to be well impregnated, not obscured by other neurons, glia, blood vessels, or non-descript precipitate. Neurons had to be located in the middle third of the thickness of the slide. The camera lucida drawings were quantified using Sholl analysis. The Sholl analysis, also known as the Method of Concentric Circles, defines the amount and distribution of the dendritic tree. In the Sholl analysis, a template composed of concentric circles of increasing diameters equivalent to 10 microns apart is superimposed on the camera lucida drawing and the number of intersections of the dendritic branches with each of the shells is recorded.

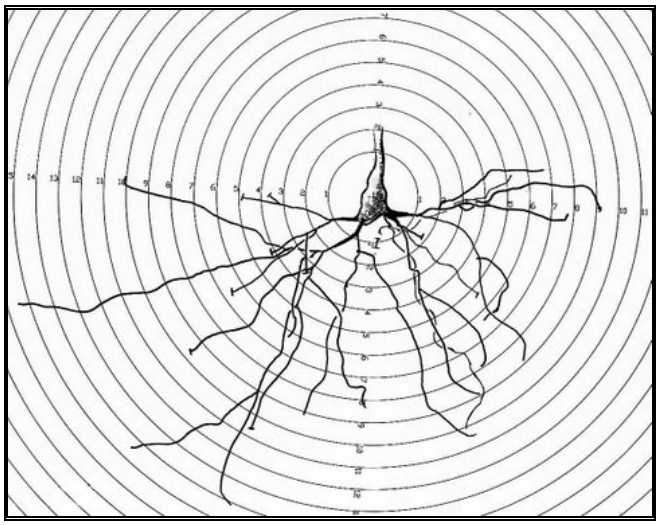

Figure 1. Example of a camera lucida drawing with the overlying Sholl template

The data that is generated from this Sholl analysis (or "Method of Concentric Circles") provides a profile of the amount and distribution of the dendritic arbor at increasing distances from the cell body. In addition to the analysis of the CA1s previously described, we also evaluated the dendritic branching of the granule cells of the dendrite gyrus as well as the layer V pyramid cells of the parietal cortex. The identical protocol was followed for analyzing this cell population as we did with the CA1s …e.g., all slides were coded and granule cells and layer V pyramids were selected at random for preparation and analysis of camera lucida drawings of the granule cells.

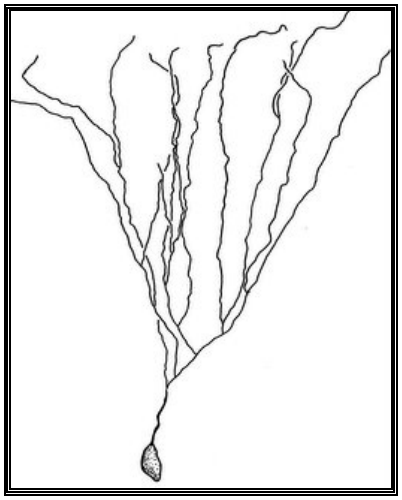

Figure 2. Example of a camera lucida drawing of a granule cell that was prepared for subsequent Sholl analysis

Statistical Analysis

Statistical analyses were performed using the one-way analysis of variance (ANOVA) test, and post-hoc analysis with the Bonferroni multiple comparison test. Statistical significance was accepted at the 95% confidence level($P<0.05$).

Results and Conclusions This study endeavors to characterize a novel disease-relevant non-transgenic model of Alzheimer's disease based on the adeno- associated viral inhibition of protein phosphatase 2A (PP2A). This inhibition, by inhibitors 1 and 2, results in hyperphosphorylation and accumulation of tau protein (as neurofibrillary tangles), which is a pathological hallmark of AD. Three different inhibitors were evaluated by examining their effects on dendritic branching from both cortical and hippocampal populations.

For the first cell population studied, the granule cells of the dendate gyrus were injected with AAVexpressing the three inhibitors, as well as the GFP control. The dendrite gyrus region, part of the hippocampal formation expressing inhibitor 1 – Full Length (I1 FL) had granule cells with the largest dendritic arbor at 23% greater than the GFP which was significant.

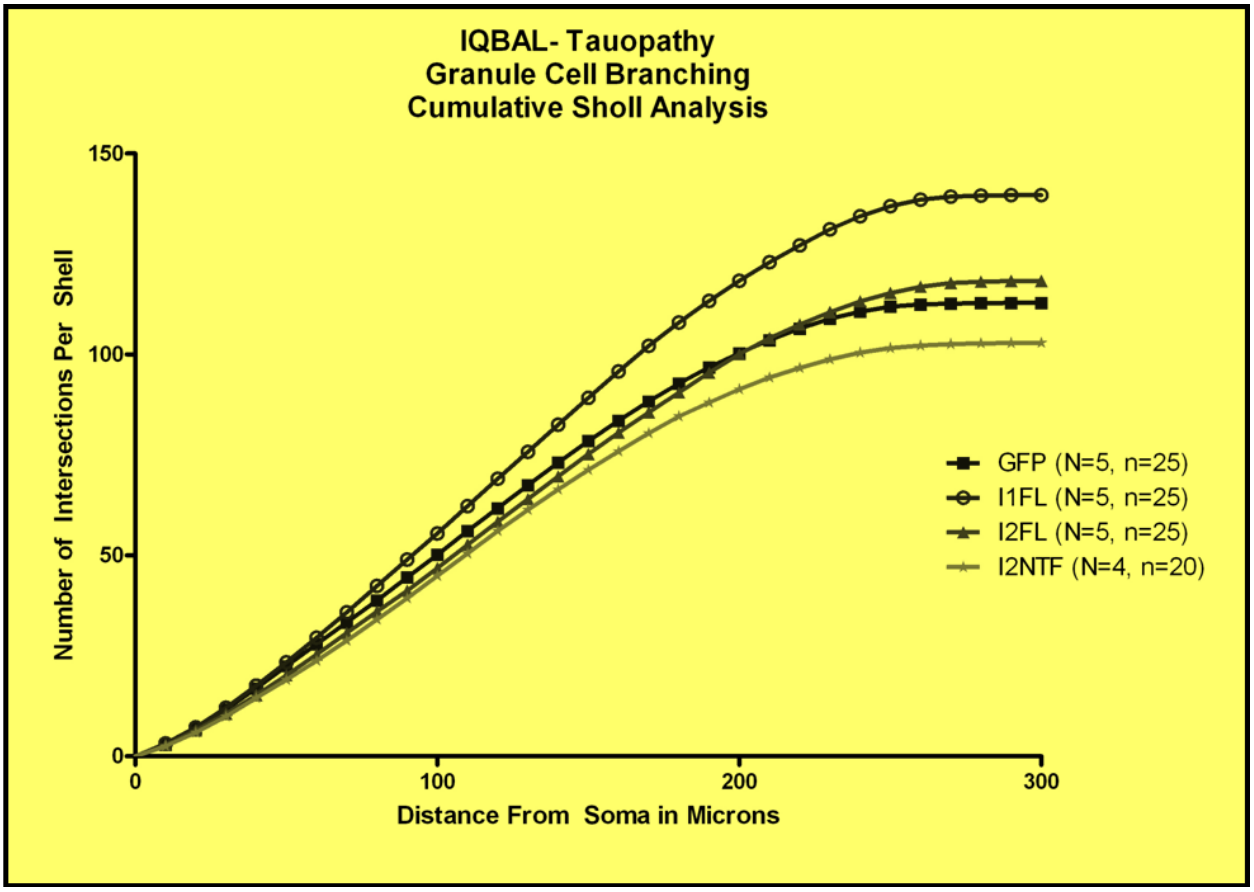

Figure 3. Granule cells of the dendrite gyrus were injected ICV with AAV expressing: Inhibitor 1 full length (I1 FL), Inhibitor 2 full length (I2 FL), Inhibitor 2 amino terminal

fragment (I2 NTF), and GFP (Green Fluorescent Protein) Control. GFP control is always set as 100% for number of intersections per sholl as a standard.

Expression of Inhibition and Total Amount of Dendritic Arbor Length of Granule Cells of the Dendreate Gyrus

Expression	**Total Amount of Dendritic Length**
GFP	100%
I1 FL	123%
I2 FL	104%
I2 TNF	91%

Table 1. The total amount of dendritic length was calculated using the distance from the soma in microns of the dendritic branching as well as the number of intersections per sholl for the branches.

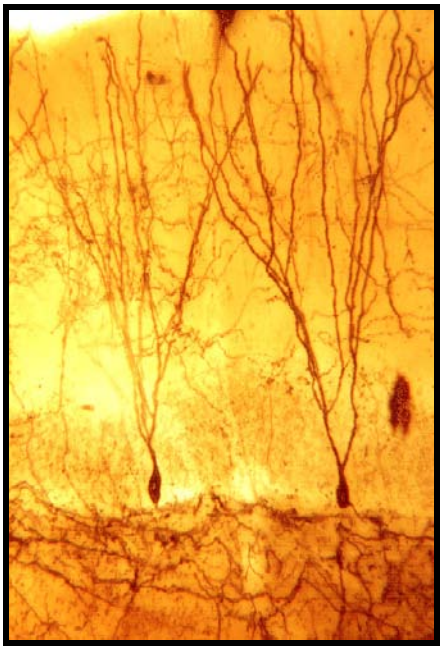

Figure 4. A photomicrograph of granule cells from the rat dendrite gyrus, as examined in this study.

For the second neuronal population studied in this experiment, the CA1 pyramids of the hippocampus, inhibition of PP2A with AAV resulted in a different pattern that presented itself when analyzing the dendrate gyrus neuronal population. In this neuronal population AAV I2 FL was the only PP2A inhibitor which resulted in significant dendritic atrophy (86% of total amount dendritic length). No other AAV resulted in either a significant increase or decrease in dendritic arbor.

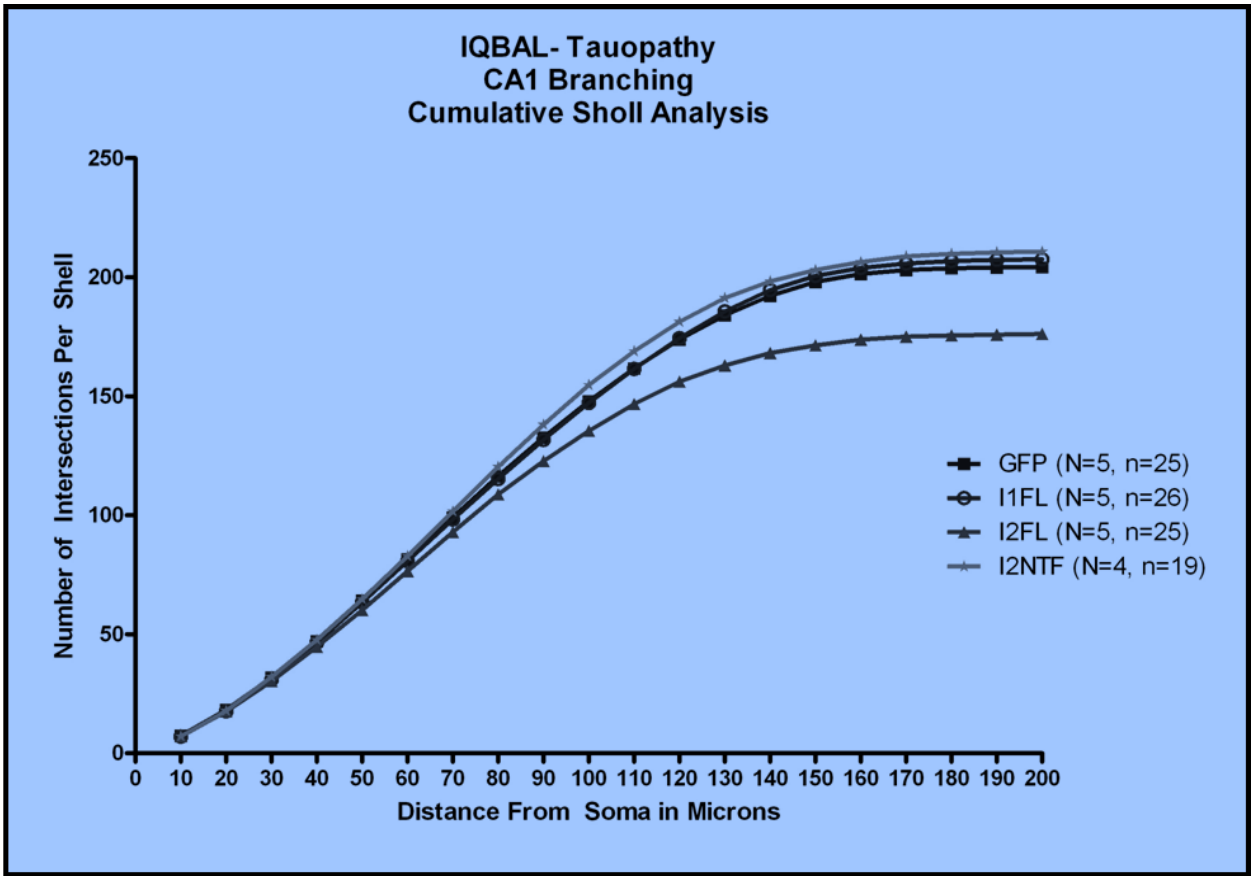

Figure 5. CA1 pyramids of the hippocampus(basilar tree) were injected ICV with an AAV expressing: Inhibitor 1 full length (I1 FL), Inhibitor 2 full length (I2 FL), Inhibitor 2 amino terminal fragment (I2 NTF), and GFP (Green Fluorescent Protein) Control. The GFP is set at 100% as the standard.

Expression of Inhibition and Total Amount of Dendritic Arbor Length of Granule Cells of CA1 pyramids of the hippocampus (basilar tree)

Expression	**Total Amount Dendritic Length**
GFP	100%
I1 FL	102%
I2 FL	86%
I2 TNF	103%

Table 2. The total amount of dendritic length was calculated in CA1 pyramids of the hippocampus (basilar tree) after an AAV was injected ICV expressing: Inhibitor 1 full length (I1 FL), Inhibitor 2 full length (I2 FL), Inhibitor 2 amino terminal fragment (I2 NTF), and GFP (Green Fluorescent Protein) Control

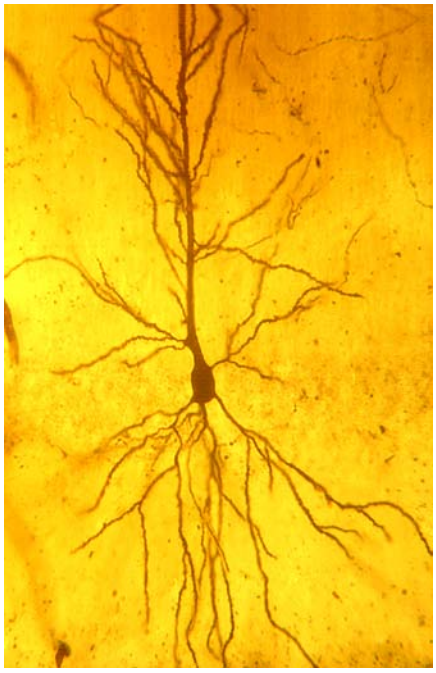

Figure 6. A photomicrograph of a CA1 pyramid of a rat hippocampus, as examined in the study.

As can be seen in comparing the two neuronal populations thus far, the CA1 pyramids of the hippocampus (basilar tree) and the granule cells of the dendrite gyrus, these two neuronal populations gave quite different patterns of dendritic branching alterations. Thus far, only one of which, AAV I2 FL in CA1s pyramids, is associated with significant dendritic atrophy relative to the control GFP group.

The third neuronal population examined in this study was the layer V pyramid cells of the parietal cortex (basilar tree). In this neuronal population, the effect of PP2A inhibitors 1 and 2 in the AAV treated rat yielded another pattern in cortical pyramids. Here, the GFP and I1 FL groups have neurons with similar dendritic arbors at 100% and 98% respectively. However, both I2 groups (I2FL and I2NTF) had larger dendritic arbors at 110% and 108% respectively.

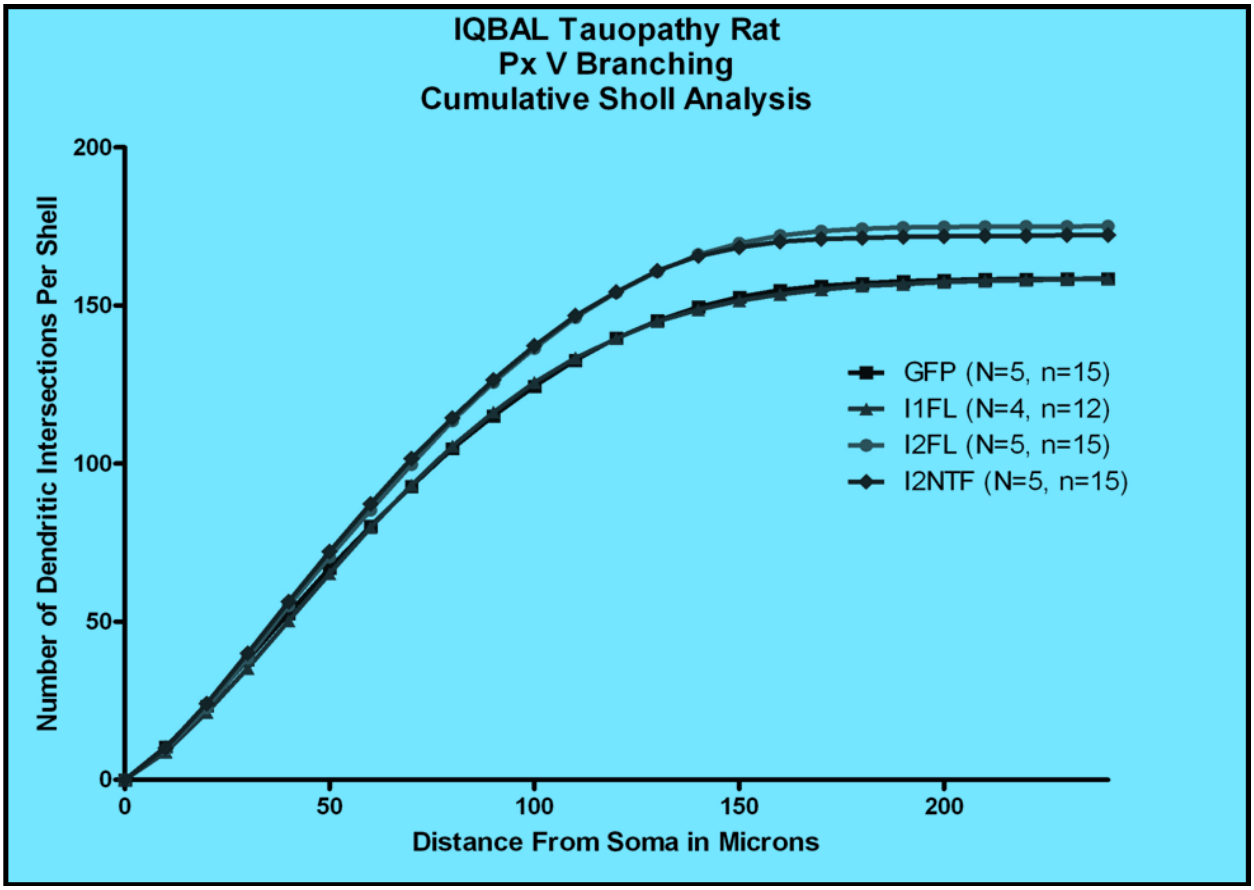

Figure 7. Granule cells of the dendrite gyrus were injected ICV with AAV expressing: Inhibitor 1 full length (I1 FL), Inhibitor 2 full length (I2 FL), Inhibitor 2 amino terminal fragment (I2 NTF), and GFP (Green Fluorescent Protein) Control. GFP control is always set as 100% for number of intersections per sholl as a standard.

Expression of Inhibition and Total Amount of Dendritic Arbor Length of Granule Cells of the Layer V pyramids of the parietal cortex (basilar tree)

Expression	**Total Amount Dendritic Length**
GFP	100%
I1 FL	98%
I2 FL	110%
I2 NTF	108%

Table 3. The total amount of dendritic length was calculated in layer V pyramids of the parietal cortex (basilar tree) after an AAV was injected ICV expressing: Inhibitor 1 full length (I1 FL), Inhibitor 2 full length (I2 FL), Inhibitor 2 amino terminal fragment (I2 NTF), and GFP (Green Fluorescent Protein) Control.

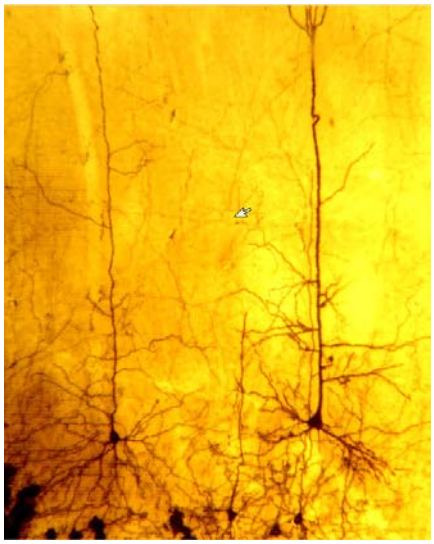

Figure 8. A photomicrograph of layer v pyramids of a rat parietal cortex, as examined in the study.

All three neuronal populations examined in this study show three different patterns of dendritic branching alterations. As with the comparison made between the first two neuronal populations, and after incorporating the third neuronal population of the layer V pyramids of the parietal cortex, there is only one expression in one cell type (AAV I2 FL of the CA1s) that showed significant decrease in total amount of dendritic length relative to the GFP control group. Nevertheless, these results represent an initial important assessment which may help us to understand potentially alternative etiopathological mechanisms associated with AD. Other ongoing investigations are currently also evaluating changes in dendritic spines in these same neuronal populations. Taken together, they may serve to better characterize and/or validate this novel animal model of AD as well as suggest novel therapeutic strategies to treat this devastating disease.

Discussion AD is major health concern, as more and more individuals continue to be afflicted by this devastating disease. By 2050 the number of people suffering from AD is approximated to be around 100 million, more than 3 times greater than the population currently suffering from AD (1). The staggering potential of AD patients to increase by more than 3 fold over the next 40 years could possibly be curbed if new drugs become available that can prevent or cure the disease. A significant step in the development of possible drugs to curb the increase in individuals afflicted with AD throughout the world, is illumination of mechanisms causing AD and developments of new tauopathy animal models.

An agreed upon key in the development and mechanism of AD is the hyper-phosphorylation of tau, thus depressing its activity and causing it to accumulate and aggregate into clumps of PHF and eventually playing a significant role in causing NFT, a pathological hallmark of AD (2). Several previous studies show that out of the 4 different classes of PP, PP2A is involved with regulating the phosphorylation of tau. Thus this study aims at a relatively novel technique in using AAV inhibitors injected ICV to cause downregualtion of PP2A and as a result hyperphosphoryaltion of tau. The hyperphosphoryaltion of tau eventually plays a crucial role in

the development of NFTs; therefore developing a quite new tauopathic animal model of AD that can be further studied for mechanism and drug design regarding AD.

The present study aims to quantify and compare the total amount of dendritic length of the basilar tree in three different neuronal populations using GFP at 100% as the control group in all three populations. The results were a bit atypical from what was hypothesized. The inhibition of PP2A by the AAV inhibitors causes an increase in hyerphosphorylation of tau, and eventually NFTs as explained throughout this paper. Thus, due to the development of NFTs and the tangling of the dendritic arbor, it was hypothesized that this would cause the dendritic arbor of the neuronal populations to be significantly smaller than the GFP control group of each neuronal population. Since the GFP control group is set at 100% for the total amount of dendritic length as a means of standard, it was hypothesized that the inhibitors: I1 FL, I2 FL, and I2 NTF would all show a significant decrease in total amount of dendritic length in all three neuronal populations.

Thus far in all 3 neuronal populations examined in this study, three different patterns were identified in each of the neuronal populations for the three different inhibitors expressed. This is an initial development in a novel way of using an AAV method for expression of inhibition in an animal model, as opposed to using a transgenic or knockout animal method to study AD. It is also very important to note that the morphology and dendritic branching of the three neuronal populations were taken and examined as a snapshot when the animals were between 11-13 months old, with the average age being 12 months old. This is significant, as it is still currently unclear when NFTs first start to develop in AD patients and AD animal models, though it is traditionally believed to be quite early in the AD process (6). Future beneficial studies may involve examining the animal not at one snapshot in time, but as a longitudinal study and over a greater period of time when the neuronal populations can be examined at multiple different times after being injected with the AAV.

AD is known to cause neuronal death and neuronal loss in individuals afflicted with AD. It is known that as NFTs increase that neuronal death also increases, though it has been shown that there is significant neuron loss before NFTs develop as well. Thus it is believed that some other factor is primarily responsible for the bulk of neuron loss and previous studies show that NFTs account for a small percentage (around 8.1%) of the neuron loss due to AD (6). Novel research has brought to light the possibility of a phenomenon called compensatory hypertrophy that may occur in the brain with neurons. In this phenomenon, as more and more neurons die and neuron loss increases due to AD, the dendritic arbor of the surrounding neurons actually increase in length to fill the void left by the surrounding dying neurons. Future studies are being done on compensatory hypertrophy involved with neuronal loss and could be significant in explanation of these results. Studies on compensatory hypertrophy and that phenomenon were not incorporated in the present study, though it is of great interest for future endeavors. Multiple future studies are required to validate and better understand these results and other results that have been gathered from studies involving PP2A inhibition and hyperphosphorlation of tau in rat models of AD. Using this new technique of using AAV for expression of inhibition of PP2A, in a tauopathy rat model, provides a beginning to a feasible approach for understanding the complexity of AD.

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