The Expression of Calm1, Calm3, adcy1, and Camk2n1 in Astrocytes may change with aging in male mice

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Thesis

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Introduction

Aging is the main contributing factor when it comes to the development of neurological diseases that lead to senile dementia and reduced cognitive functions[1]. The brain's ability to withstand the different environmental stresses that could end up damaging nervous tissue as well as its ability to compensate for age-related deterioration vary greatly between different subjects[2][3]. This ability is determined by both the brain's ability to withstand and compensate for damage and stress prior to the onset of the damage or disease as well as the resistance and regenerative properties of the specific nervous tissue being affected[2]. The neuroglia play a huge part in these areas, and the neuroglia's ability to protect and sustain the neural system, its various nerve tissue, and the central nervous system as a whole is an important factor in the progression from normal to pathological function during brain aging[4]. Of the neuroglia, one of the most important types that play a large role in maintaining molecular homeostasis are the astrocytes, which also have a wide variety of functions depending on where they are in the brain[1]. They can help transport ions which support neuronal excitability, they accumulate and catabolize neurotransmitters and supply neurons with neurotransmitter precursors which helps in sustaining synaptic transmission, they support neuronal metabolism and detoxify ammonium and reactive oxygen species, and they secrete numerous trophic factors, neuroactive agents, and hormones[5][6][7][8][9][10][11][12]. Due to taking part in such a wide variety of functions, it is unsurprising that the whole brain would suffer from age-related astrocyte changes, especially since astrocytes help the brain handle the aging process better[1]. These morphological changes in the astroglia associated with aging are multifaceted and most likely brain-region dependent[13][14]. Little is known about the physiology of aging astrocytes, however one of the most prevalent theories is the "calcium hypothesis of aging"[15]. The theory states that aging neurons experience increased Ca2+ influx during depolarization, which elevates resting Ca2+ concentration, thus triggering excitotoxicity. Although astrocytes are not neurons, they may suffer from a similar condition. In fact, the astroglial

Ca2+ signaling toolkits undergo modification in multiple neurodegenerative diseases including Alzheimer's Disease(AD) which can result in deficient neuroprotection and failure in glial homeostatic support[1].

Ca2+ is a key secondary messenger that is involved in the regulation of many different pathways, including synaptic transmission, neuronal survival, cellular bioenergetics, and gene expression over the course of minutes, hours, or even days [16][17]. It is postulated that the mechanisms controlling intracellular Ca2+ homeostasis play a fundamental role in normal cellular function and that variations in this maintenance may explain several changes in the neural functions related to aging and AD[15]. Maintaining proper Ca2+ concentrations within the cell is very important, since even modest prolonged elevations of Ca2+ concentration can be neurotoxic [18]. One of the most important structures through which cells maintain these concentration levels is through voltage gated calcium channels (VGCCs). As organisms age it is inevitable that the processes used to control the expression of these channels will deteriorate, which can negatively affect the homeostasis of neural cells by deregulating calcium concentration. In fact, the aging in mammals is characterized by an increased abundance of L-type VGCCs which can contribute to the neurotoxicity associated with intracellular calcium concentrations exiting their optimal range[19].

My work is based off the work a previous member of the Kreiling Lab, Amy Elias, who created a reference list of primary micro-RNAs by performing single nucleus sequencing [20]. The purpose of her study was to see how primi-RNA expression levels changed in the nucleus of the Subventricular zone (SVZ) cells as mice aged, however while doing so, due to the fact that the single nucleus gene expression collected data for all expressed genes and not just primi-RNA expression, she noticed that there was a difference in the change in expression of some genes between young males to old males and young females to old females. The Lab decided that this change in expression was worth looking into, and that the study would begin by checking genes related to calcium channel expression. Single nucleus

sequencing would not be used in this case since we want to check the change in expression level of these genes, which requires checking the cytoplasmic RNA as well. This project was then assigned to me along with a list of candidate genes to be checked. Of these genes, we were able to find 4 that could be reliably be checked with the primers we would order. These 4 are: Calm1, Calm3, Adcy1, and Camk2n1.

Calm1 and Calm3 are Calmodulin genes. Calmodulin is an indispensable component of signal transduction pathways that target a large variety of structural proteins, receptors, enzymes, and ion channels[21][22]. It is a ubiquitously expressed Ca^{+} sensor molecule that is found in all eukaryotic cells[23], playing a key role in enhancing the calcium sensitivity of certain enzymes[24][25]

Adcy1 is an adenylyl cyclase gene. Adenylyl cyclases (ADCYs) catalyze the production of cAMP from ATP [26][27]. cAMP plays a pivotal role in a variety of fundamental cellular processes [28], so it needs to be tightly regulated to ensure proper function of the cell. ADCYs also play a variety of important functions in the CNS and can affect learning, memory, and movement. Abnormal ADCY expression is found in and associated with many neurological disorders, including AD and depressive disorders [29]. For Adcy1 specifically, loss of ADCY1 leads to impaired synaptic plasticity and deficits in spatial learning [30] while overexpression of ADCY1 in the forebrain enhances recognition and memory [31]. ADCY1 is also Ca2+/calmodulin-sensitive, and as result exerts important functions in neuronal development [32].

Camk2n1 is Calcium/calmodulin dependent protein kinase II inhibitor 1 and as the name implies works by inhibiting Calcium/calmodulin dependent protein kinase II(CaMKII). CaMKII is a key molecule for memory formation [33]. It is activated by Ca2+ and calmodulin after which it gains the ability to auto-phosphorylate itself [34] until it is inhibited.

By studying the change in expression of these genes between young to aged males and young to aged females, we hope to show that there is a difference in the change of expression level of these genes with aging between sexes. From the experiment performed by Amy, which dealt with all cells from the

SVZ, we found that changes in these important genes occurred mainly in Astrocytes, so it was decided that the difference in the change of expression of these genes would be studied in Astrocytes isolated from the SVZ.

Methods

Mouse tissue

Male mice of strain C57BL/6N were obtained from the National Institute on Aging (NIA) Aged Rodent Colonies (www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook). The mice were split into 2 groups, Young males aged 4 months old, and Old males aged 23-24months old. SVZ tissue would be obtained from mice of all age groups, with 3 mice being combined into 1 sample due to low RNA yield from only a single SVZ sample. All procedures were approved by the Brown University IACUC.

Quantitative real-time PCR

In order to quantify the amount of RNA being expressed for the candidate genes, we used quantitative polymerase chain reaction (qPCR), using the comparative CT method as described in the paper by Dorak [35]. GapDH was used as the normalizer, since its expressions remains relatively unchanged over the course of aging.

For quantification of mRNA expression 200 ng of total RNA was transcribed into cDNA in 50 µL reactions using random hexamers and the TaqMan kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Biosystems, Foster City, CA, USA), 5.5 mM MgCl2 in 1x RT buffer in a final volume of 50 µl. qPCR was performed using the SYBR Green system (Applied Biosystems, Foster City, CA, USA) on an ABI ViiA 7 Real Time System instrument (Applied Biosystems, Foster City, CA, USA) and later

a QuantStudio™ 6 Pro Real-Time PCR System after the core facilities equipment was replaced, according to the manufacturer's specifications. Primers for mRNAs were designed using the Primer-BLAST software.

Primer selection

Our experiments began with testing candidate primers: Cacna1c, calm1, adcy1, camk2a, camk2n1, and calm3. Candidate primers were created using primerBlast, testing at least 2 variants of each primer each round. The cDNA used for the serial dilution tests was derived from a reverse transcriptase (RT) reaction involving whole brain mouse RNA isolated using our Lab's Trizol protocol and the RNeasey Qiagen kit. The primers would be run alongside verified GapDH primers and the CT value vs the log of the dilution of the sample's would be graphed. If slopes of the candidate primers were close enough to the GapDH value, which itself must have been between -3.1 and -3.6, then the primers would be accepted for use in the future qPCR experiment. The finalized primer sequences are shown below in Table 1.

Target	Forward Primer	Reverse Primer
Calm1_V2 (Chosen)	CCGAGTGTTTGACAAGGATGG	TAGTTGACTTGTCCGTCGCC
Calm1_Is2	GCTCGCACCATGGCTGAT	CTCGGCTTCTGTTGGGTTCT
adcy1	ATGGTCCAATGACGTGACCC	TGTGATGTGGACCTTCCCTG
camk2n1_F1	GGAGCAAGCGCGTTGTTATT	CAGCCCGCCACTCTTCTTAT
Calm3_V2 (Chosen)	TGGCCAGGTCAATTATGAAGAGTT	GTACGCAGGGGAGTGTTGAA
calm3_F2	AGTAACCTCGATCCCCGAGC	GGCTTCCTTGAACTCTGCAATC

Table 1. Finalized Primers for testing the RNA extracted from the SVZ tissue.

Astrocyte RNA Isolation

After this I could move on to gathering SVZ RNA for the next stage of the experiment. For this experiment we would dissect the SVZ from young and old mice and isolate their astrocytes using a combination of the miltenyi Neural Tissue Dissociation Kit protocol and the Anti-ACSA-2 Microbead Kit. This involved dissociating the tissue using the provided Miltenyi dissociation buffers and their octomax heating and mixing program. The resulting solution then needed to be filtered using a 40 µm filter and washed using a DPBS solution with $Ca^{+,}$ Mg^{+}, Glucose, and sodium pyruvate. Debris would then be removed using the miltenyi debris removal solution protocol. The astrocytes in solution would then need to be labeled and isolated using the methods in the ACSA-2 protocol, however instead of HBSS we combined BSA with DMEM F12 with 10% FBS to help improve cell viability.

Astrocyte RNA was isolated using the Trizol reagent protocol. The cells isolated in the Astrocyte isolation protocol were thoroughly lysed in 1 ml Trizol and extracted with 200µl of chloroform. Following centrifugation at 12,000 x g, 4°C for 15 min. the RNA in the aqueous phase was precipitated by adding 500 µl isopropanol and 5-6ul glycoblue (glycogen with a blue color that was easier to see visually). This would then be left in the freezer overnight after which it would be spun down at 12,000 x g, 4°C for 10 min and the resulting RNA pellet identified using the blue color marker. After aspirating solution, the pellet would then be washed with 1 ml RNase free 75% ethanol, which would then be removed after another centrifugation at 7,500 x g, 4°C for 10 min. The tube would then be left open to let all the ethanol evaporate, usually taking around 5 min, after which the pellet would be dissolved in 30ul of RNase and DNase free water. The concentration of this solution would then be measured using the Qubit HS RNA kit and the integrity checked using an Agilent screentape bioanalyzer.

After getting the Astrocyte RNA protocol working, we began to collect the RNA from the mice, convert them to cDNA via RT, and then testing the primers through qPCR. The same sample was run in triplicate

to provide better data. Over the course designing the protocol, it was decided that 3 mice of the same sex and age group would be used per sample, with all mouse coming from the C57BL/6N strain.

Astrocyte Enrichment markers

To ensure that our samples have been properly enriched for astrocytes. We collected the cells unlabeled by the ACSA-2 microbeads, and extracted the RNA using the same procedure as the labeled cells. The fold change for both samples would be calculated from the Cq/Ct values obtained from qPCR. The average of the labeled values for each marker would then be normalized to that of the unlabeled cells, and the fold change calculated using the 2^(- Δ ΔCT) calculation. This calculation would be used as the enrichment value. Samples that had a higher enrichment value would generally be held in higher confidence.

Before this could be done, markers that were more highly expressed in astrocytes than in normal cells would have to be selected. The candidates were: GFAP, GLAST, and BLBP[37][38][39][40][41]. RNA isolated from a whole brain sample would be used to test the primers for the astrocyte enrichment test. Each gene would have 2 primer pair variants prepared to test it initially, which were designed in primer BLAST. Afterwards, the normalized cq/ct mean values would be used to calculate the fold change in expression between cells that had been labeled by the microbead kit and the unlabeled samples that would have been washed away on the column. The primers tested are shown below in Table 2.

Target	Forward Primer	Reverse Primer
GFAP_#1	CAATGCTGGCTTCAAGGAGACACG	TCAGTTCAGCTGCCAGCGCCT
GFAP_#2	CACCTACAGGAAATTGCTGGAGG	CCACGATGTTCCTCTTGAGGTG
GLAST_#2	AATGCCTTCGTTCTGCTCAC	ATCCTCATGAGAAGCTCCCC
BLBP_#1	AGACCCGAGTTCCTCCAGTT	ATCACCACTTTGCCACCTTC
BLBP_#2	ATTTAGGTGACACTATAGAAGTGTGAGTACATGAAAGCTCTGG	GCGTAATACGACTCACTATAGGGAGACTACCTCCACACCGAAGAC

Table 2. Astrocyte marker primers

Results

Sadly, due to the limitations on how many mice our lab could receive it was quickly deemed infeasible to get enough data from male and female mice, so for my project we decided to focus on the change in the expression of the target genes between young and old males only. Future work with female mice could be done by a future lab member at a future date.

Primer Testing

After many rounds of primer testing, the candidates that passed were primers for: Calm1, adcy1, camk2n1, and Calm3 as they were able to produce linearization slopes closest to the -3.0 to -3.6 range which indicates quantitative amplification. The other candidate primers did not amplify in a quantitative manner. The graphs in Figure 1 show the linear fit of the tested primers as well as the date each primer test was done, the ones marked as "good" were the ones.

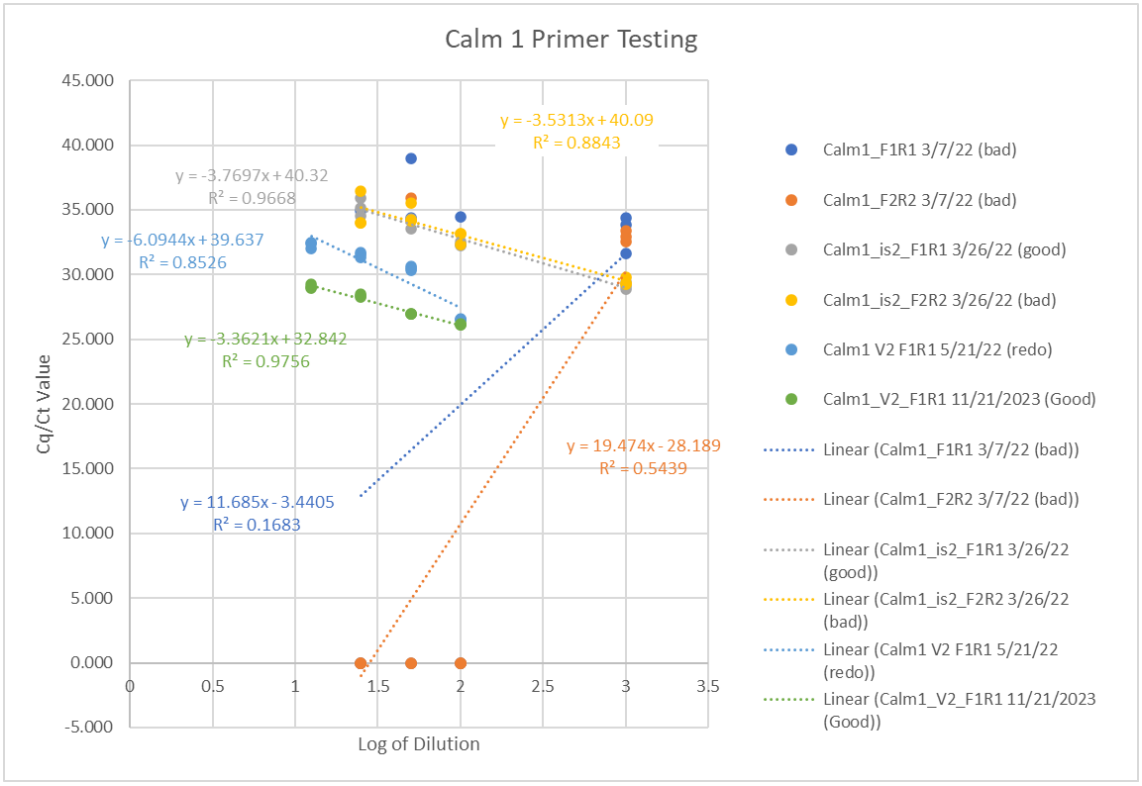

Figure 1a Calm1 Primer Testing

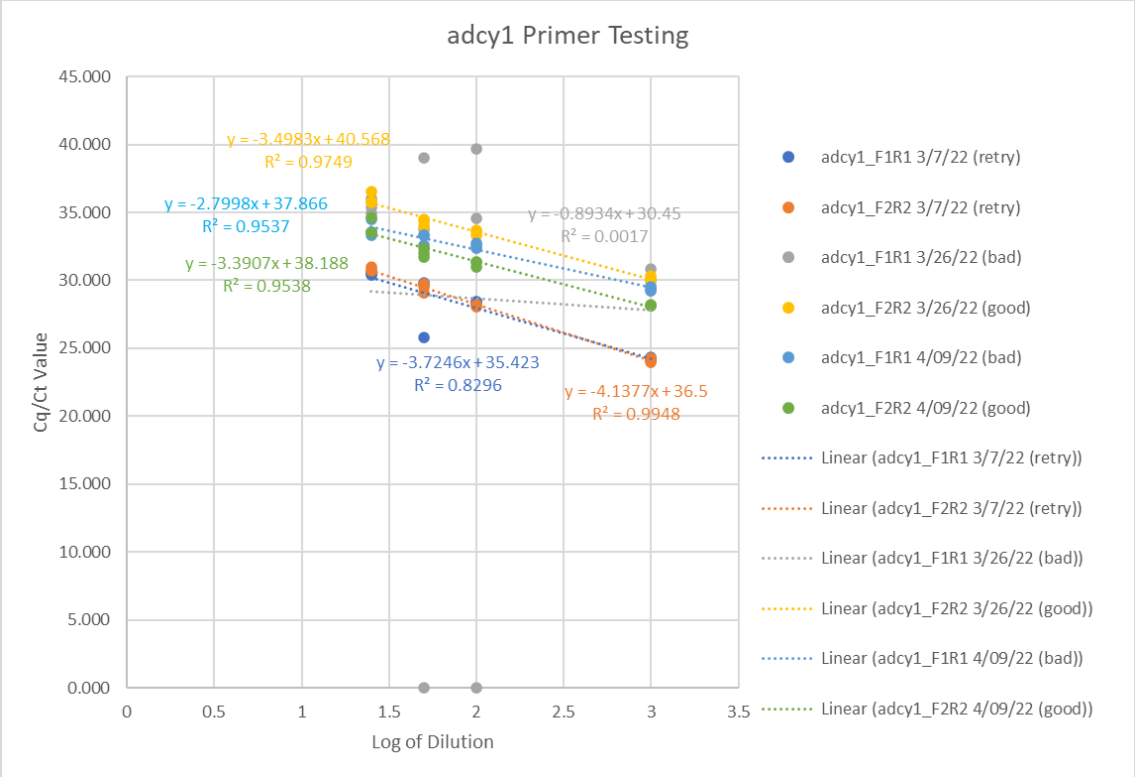

Figure 1b adcy1 Primer Testing

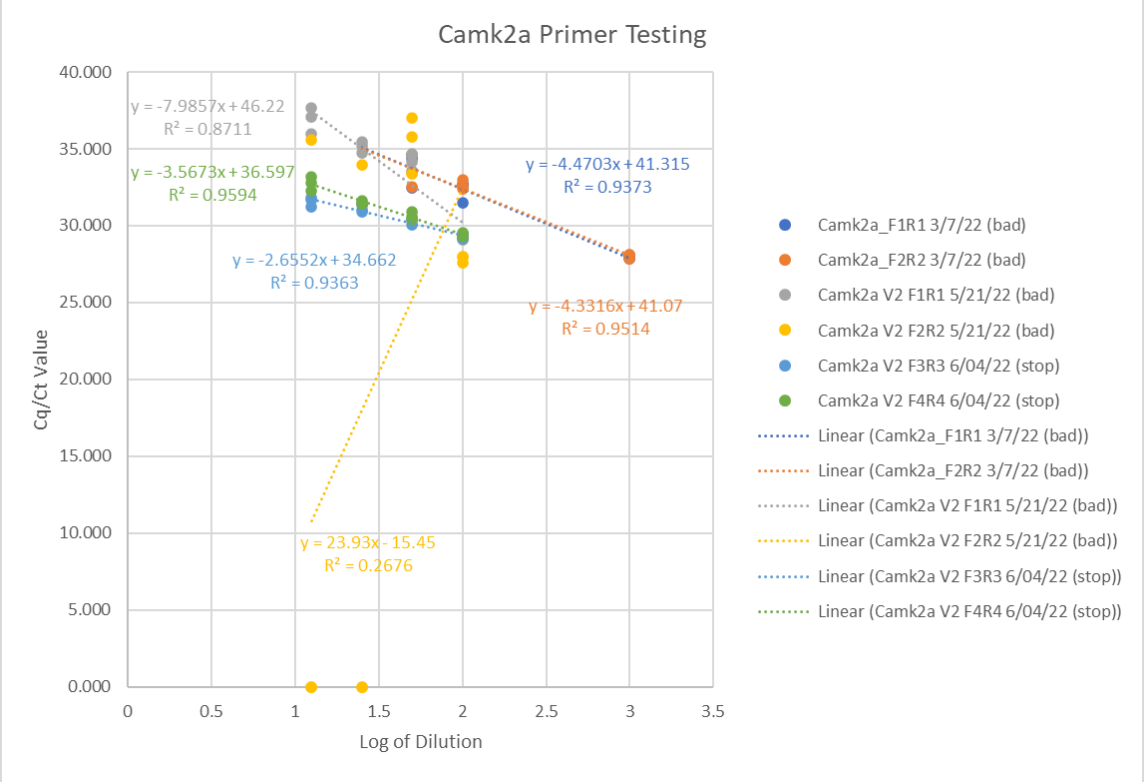

Figure 1c Camk2a Primer Testing

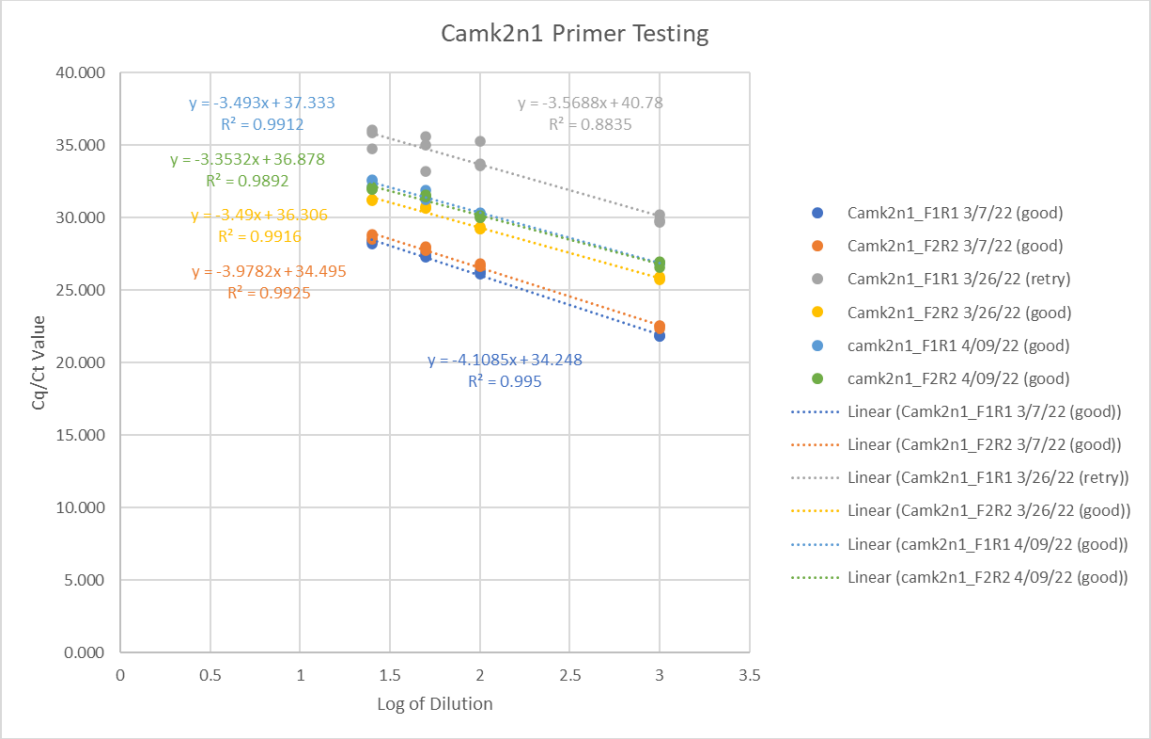

Figure 1d Camk2n1 Primer Testing

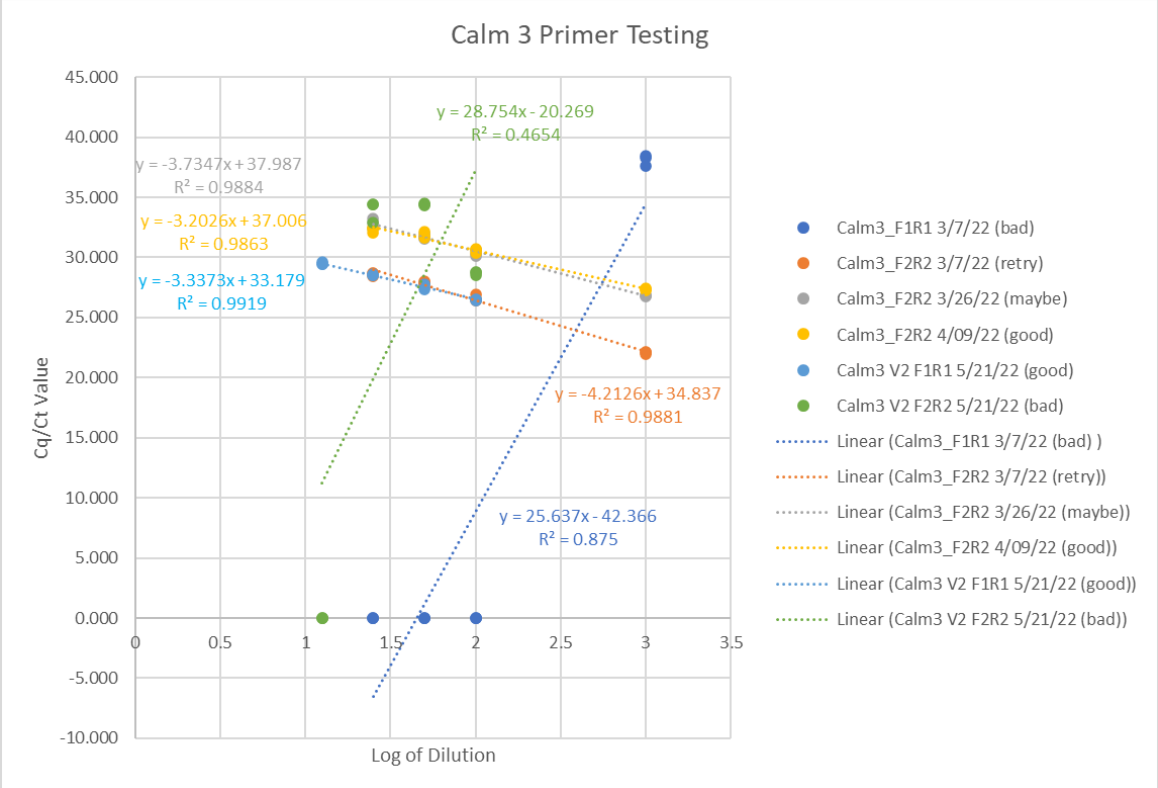

Figure 1e Calm3 Primer Testing

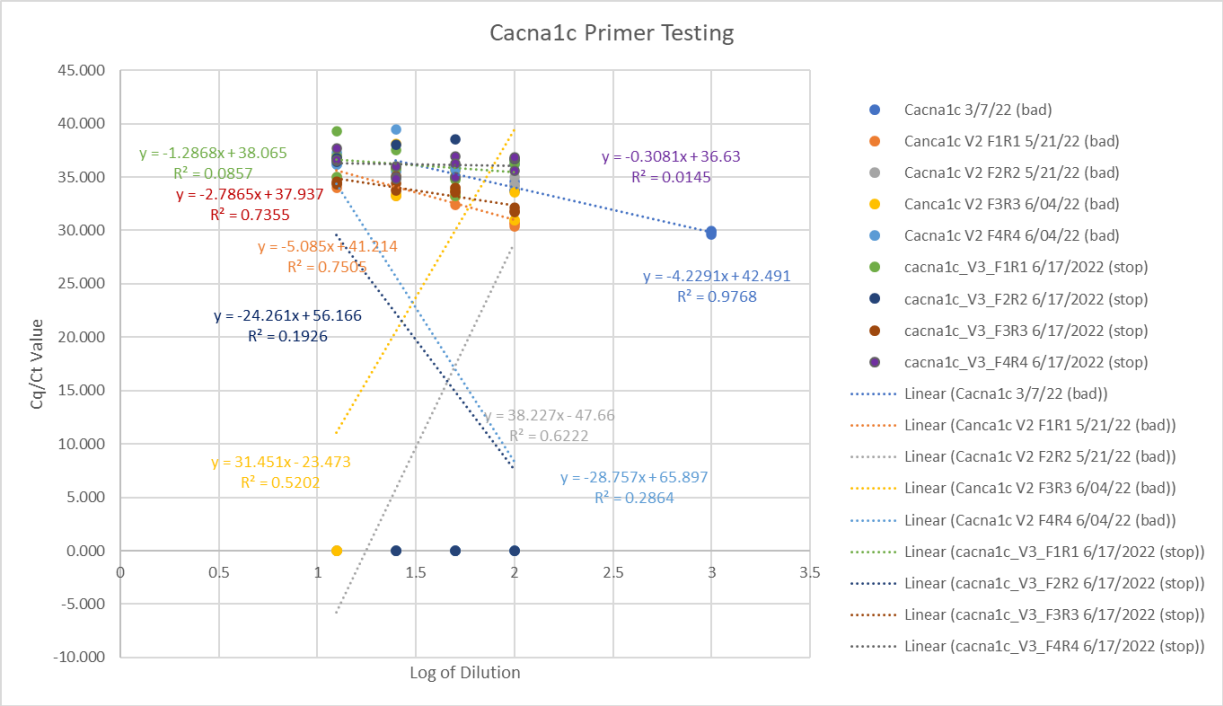

Figure 1f Cacna1c Primer Testing

Figure 1: Cq/Ct values vs Log scale graphs of all primers tested for these experiments.

Astrocyte Isolation Protocol Improvement

Getting the Astrocyte isolation protocol working properly was a long multi-month endeavor for our lab, the details summarized below in Table 3.

Date	live SVZ cells	dead SVZ cells	live forebrain cells	dead forebrain cells	SVZ percent vialbility	forebrain percent vialbility	RNA conc	Integrity	sidenotes
7/15/2022	3.10E+05	N/A	N/A	N/A	N/A	N/A	12.7ng/ul	N/A	3 mice
7/26/2022	1.15E+05	N/A	N/A	N/A	N/A	N/A	7.2ng/ul, 2nd elu 3.3ng/ul	N/A	1 mouse
7/28/2022	3.50E+04	N/A	N/A	N/A	N/A	N/A	31.2 ng/ul, 2nd elu 8.5 ng/ul	N/A	1 mouse
8/1/2022	1.25E+05	N/A	N/A	N/A	N/A	N/A	can't read	N/A	3 from then on
8/3/2022	2.60E+05	N/A	N/A	N/A	N/A	N/A	0.5420 ng/ul	6.9	Quarter forebrain would be control from this point on, also swapped to Trizol RNA isolation protocol
8/8/2022	2.20E+05	2.50E+06	1.00E+07	2.98E+08	8.00%	3.25%	not measured due to low viability	N/A	Debris removal with percoll protocol may be problomatic, neonatal stem cell

									isolation protocol swapped with Isolation and cultiavtion of adult neural stem cells from adult mouse brain protocol
8/17/2022	7.00E+05	5.15E+05	1.47E+06	1.13E+06	57.60%	56.56%	not measured due to low cell count	N/A	Rnase inhibitor used during trizol step, won't be used again due to waste
8/19/2022	5.50E+04	1.35E+05	1.50E+04	1.30E+05	28.90%	10.30%	Too low to read	N/A	pbs bsa suggest switch to DMEM/F12 BSA by miltenyi
8/26/2022	1.20E+05	4.60E+05	2.50E+05	1.12E+06	20.69%	18.20%	SVZ:172pg/ul, fore 738pg/ul	SVZ too low to check, forebrain:8.6	1 mouse today
9/9/2022	1.10E+05	4.00E+05	1.30E+05	1.99E+06	19.61%	6.13%	SVZ:8.02ng/ul, for:50.6ng/ul	SVZ:6.7, Fore: 5.9	2mice, 12.5% percoll final half the original, Enzyme to mix added to 80ul PBS bsa during blocking process. Took longer this day
9/14/2022	9.50E+04	1.16E+06	3.20E+05	3.59E+06	7.57%	8.17%	SVZ:11.9ng/ul, fore:13.5ng/ul	SVZ: 5.4. Fore: 4.8	
9/16/2022	1.20E+06	5.00E+05	4.65E+06	1.10E+06	71%	80.87%	SVZ: 4.4ng/ul, fore: 12ng/ul	not measured	Miltenyi stopped by this time, 2 yong female mice. Showed debris removal solution effictiveness. Stopped using Percoll. Didn't add penstrep or glutamate to DPBS, flick resuspension. Reduce bead incubation back to 15 min, remove chunks don't dissolve with enzyme 2. 70um strainer only
10/3/2022	1.24E+04	8.65E+05	2.35E+05	1.07E+06	1.37%	18.08%	SVZ:9.74ng/ul,Fore:4.6ng/ul	SVZ:4.0, fore:not measurable	2 young female mice, attempted to get more cells through column but knocked magnet loose causing marked astrocytes to flow through
10/14/2022	6.40E+04	3.20E+05	N/A	N/A	15.60%	N/A	SVZ: 6.98 ng/ul, unlabeled: 34.8 ng/ul	SVZ:7.2 integ, unabeled: 6.9 integ	Uncertain if the debris chunk is the problem or not. Stopped cell counting after this since it looked like we got the viability as high as we could. We also began isolating the unlabeled portion of cells to use for Astrocyte enrichment testing later. After this experiment is where we found that glycoblue should have been added before the overnight precipitation instead of after like we had previously been taught. (It was also decided that this would be the first sample used for the qPCR experiments)
10/17/2022	N/A	N/A	N/A	N/A	N/A	N/A	SVZ: 27.4 ng/ul	SVZ: 7.3 Integ	The addition of Glycoblue greatly increased the yield of RNA. At this point it was also

									decided that optimization would end and this would be the first experimental sample used.
10/25/2022	N/A	N/A	N/A	N/A	N/A	N/A	SVZ: 32.6ng/ul Unlabeled: 32.6ng/ul	SVZ: 7.7 Unlab: 8.5	This sample, along with the samples on 10_14 and 10_17 were combined and split among the three individuals who worked on the Astrocyte isolation optimization as the first sample for their own experiments. Due to small yield and lack of a good unlabeled cell comparison. The first sample would not undergo triplicates or astrocyte enrichment testing

Table 3. Overview of Astrocyte isolation protocol optimization

This protocol initially had very low yields of RNA which wouldn't be enough for the future stages of the experiment. To increase the yields our lab began testing ways to both increase cell viability and RNA yield during the experiment. We attempted many methods and were increasingly careful with samples, such as pipetting more gently and using clipped pipet tips to reduce the chance of damaging the cells we wished to isolate. We also began counting the viable cells after certain key steps, such as debris removal and the column process, to see if the changes we made had any noticeable affect. It was at this point we also decided that we would combine 3 mice per sample to help bring up the yield of each experiment. Initial changes that we made were treating any reagents we could with DEPC(Diethyl pyrocarbonate) and autoclaving to remove any trace RNases, replacing the HBSS with DMEM 10% FBS to increase cell viability, and changing the DPBS used to one that had Mg+, Ca+, Glucose and Sodium Pyruvate. The RNA isolation method was also changed from the Qiagen RNeasy kit to the RNA isolation steps of the Trizol Protocol.

We also changed the method by which we measured the RNA from nanodrop to RNA high sensitivity Qubit in order to get more accurate results, as the Glycoblue, a colored glycogen substance which was used as an RNA coprecipitant that could be visibly identified after centrifugation, that we had been using was not compatible with the nanodrop and confounded results. We also got in contact with miltenyi

representatives who helped us better understand how to use the debris removal protocol, and change from the 37C_NTDK_1 octomax program to the 37C_ABDK_02 program.

However, we only finally got yields to significantly increase after changing when the glycoblue was added to the RNA-isopropanol mixture after reading the cold spring harbor RNA Isolation protocol in more detail [36]. It seemed as though in our initial training with the RNA isolation from the Trizol procedure we were either incorrectly taught or misinterpreted the methods by which small tissue samples were handled, so we initially added in the glycoblue the morning after the RNA spent the night precipitating in isopropanol. The correct method was to add the glycoblue and vortex the solution before the overnight precipitation so it could help the small amount of RNA properly precipitate.

Astrocyte Marker primer testing

For the astrocyte marker testing it was decided by the lab that during the initial testing that all primer pairs would be acceptable except for GLAST#1, due to it having an incorrect slope. The slopes for the initial primer testing can be found below in Figure 2a. A whole brain sample was then processed using the Astrocyte Isolation protocol and tested using qPCR, where the cells isolated through labeling with the ACSA-2 marker and the unlabeled cells were compared with each other. The results in Figure 2b show that all the markers used showed an increase in expression for the labeled cells. GLAST#2 was shown to have the highest fold change increase between labeled and unlabeled cells, so I chose it as the main astrocyte enrichment marker for my future experiments.

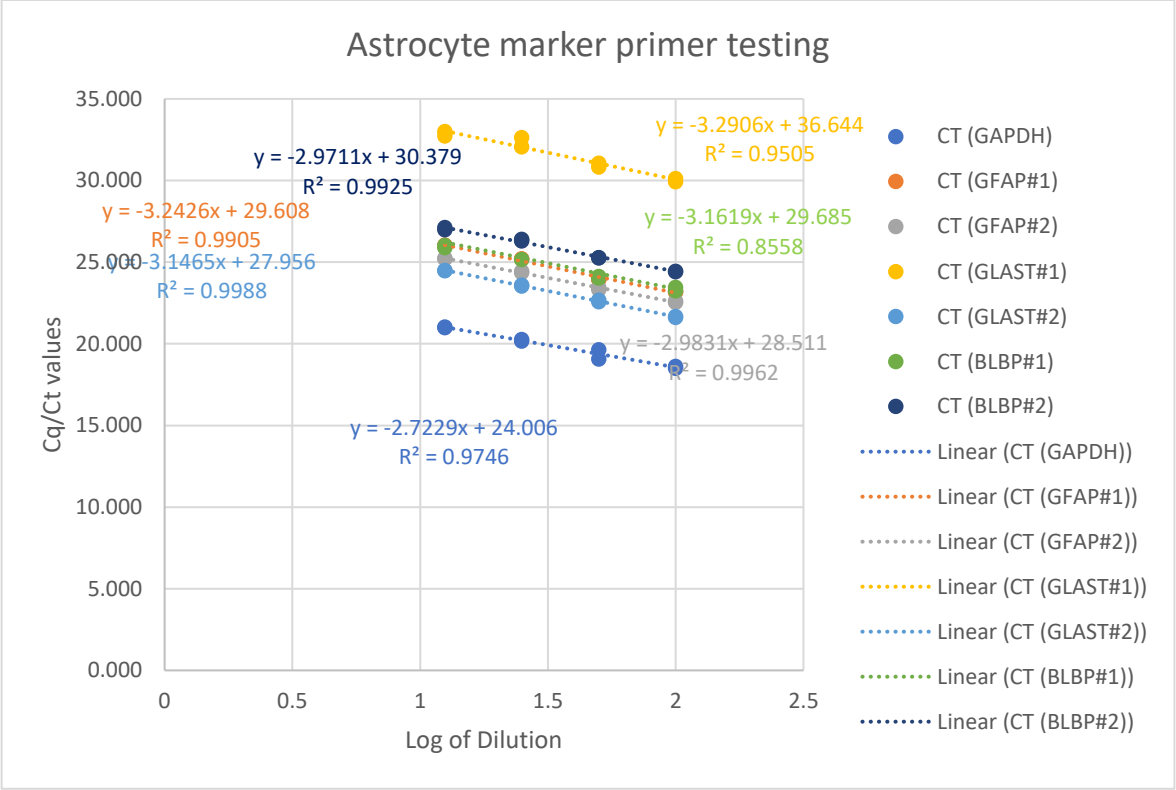

Figure 2a Testing Data for Potential Astrocyte Primers

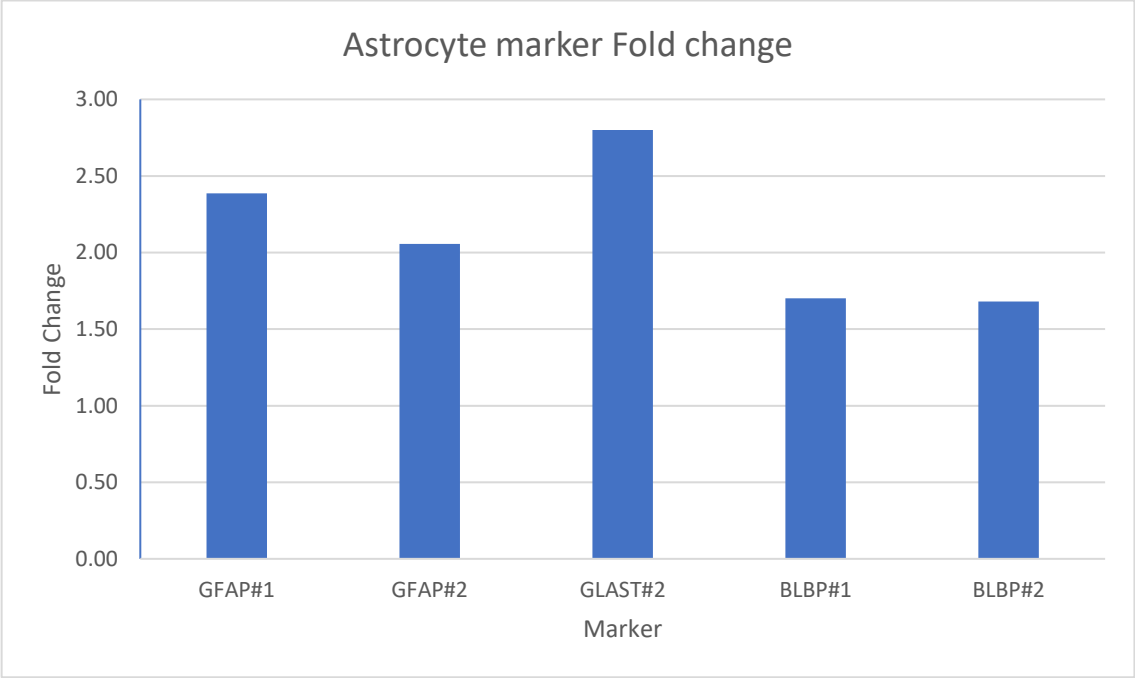

Figure 2b Fold change of successful candidate primers when tested with a whole brain sample

Figure 2 Astrocyte Marker testing graphs

Old vs Young Male gene expression data

As of the end of this project we have collected the Astrocyte RNA of 6 samples of 3 Young Male C57BL/6N mice aged 4 months each and 6 samples of 3 Old Male C57BL/6N mice aged 23-24 months old. The astrocyte enrichment of all of the samples appears to be excellent, with all samples having a more than 4-fold increase in astrocyte marker expression except for the last sample which was processed on 3_12 (Figure 3a). When looking at the normalized average gene expression for each gene group we can see slight changes in the adcy1, Calm1, and Camk2n1 groups, however due to the standard error size the old and young groups are not statistically significant. This is bolstered by the fact that none of the p-values generated when comparing the expression levels of the genes between young males and old males were able to meet the 0.05 cutoff (Figure3b). At best it could be said that there may be a trend that shows gene expression adcy1, Calm1, and Camk2n1 may change with age, however it will require a greater number of samples to prove statistical significance.

Of the samples collected the last 3 Young males samples began having Astrocyte isolation issues, giving gene expression results very different from the first three (Figure 3c). This may be due to the reagents we'd been using starting to lose effectiveness with age, or perhaps due to the fact that the increased viability of the young cells, due to the improvement of my methods, causing the solutions during the astrocyte isolation to become stickier. The stickiness of the cells likely played the biggest role in the last two young male samples, causing a sticky clump to form that could have trapped cells during the debris removal process. The loss of reagent potency may have had the greatest effect on the last young male sample due to the values being closer to that of earlier samples, but only halfway there. This idea is compounded by the fact that the last astrocyte isolation had the lowest enrichment (Figure3a). When comparing the first three young male samples to the old male sample (Figure 3d) or the last 3 young male samples to the old male samples (Figure 3e) the results were very different than that of all the

mice samples. In both cases the some of the p-values when comparing the expression of genes between old and young managed to break the 0.05 benchmark.

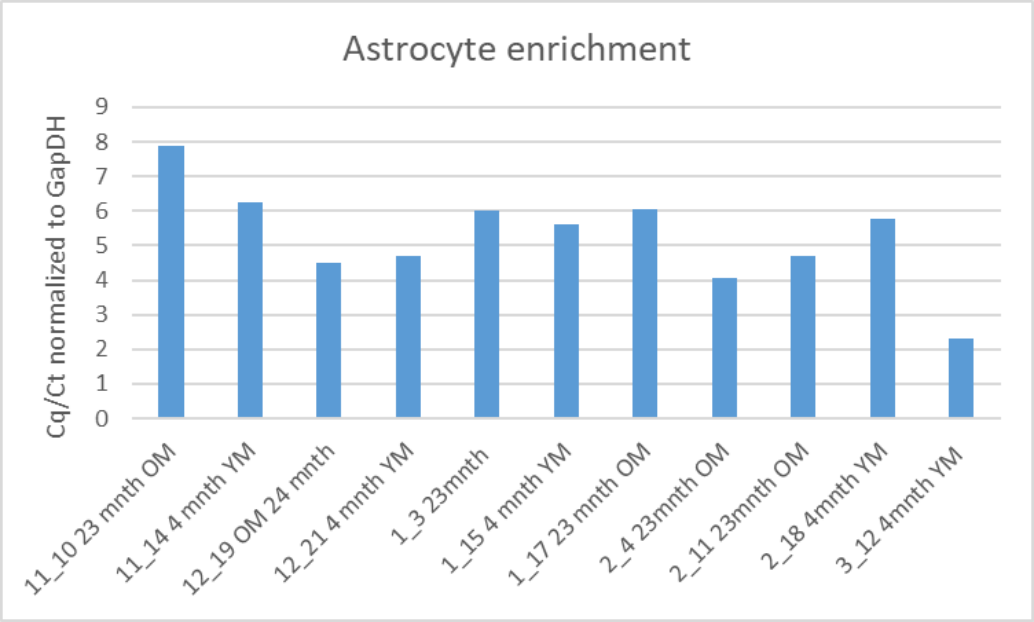

Figure 3a Astrocyte enrichment for each dissected sample.

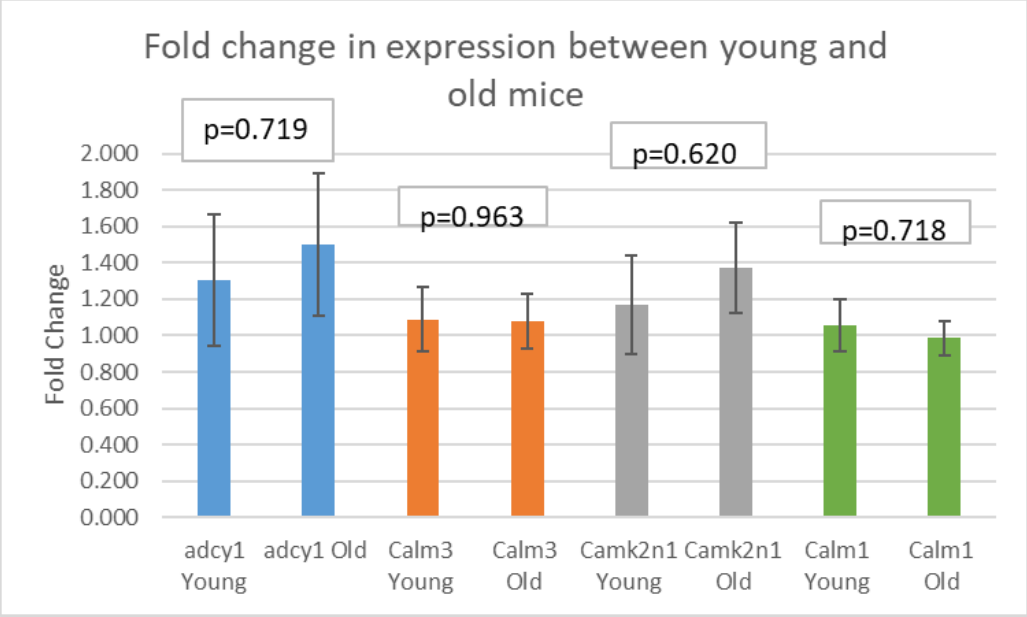

Figure 3b Change in gene expression between Young Males and Old males

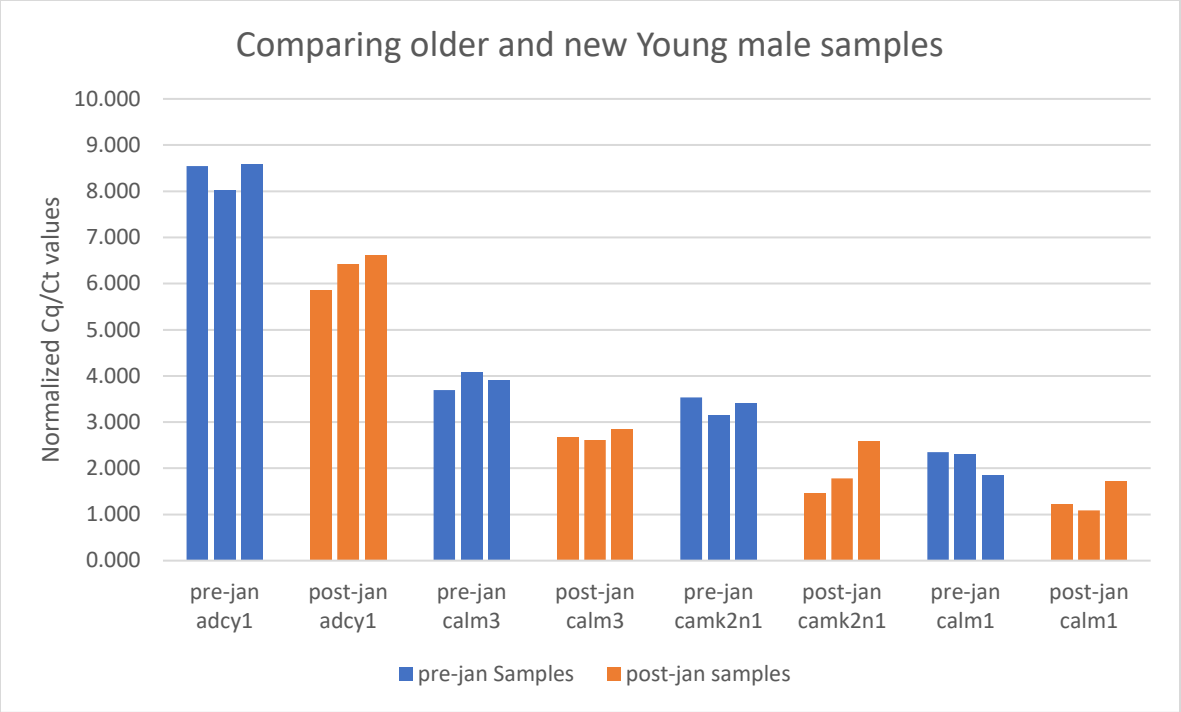

Figure 3c Comparing the Young male samples from before and after January.

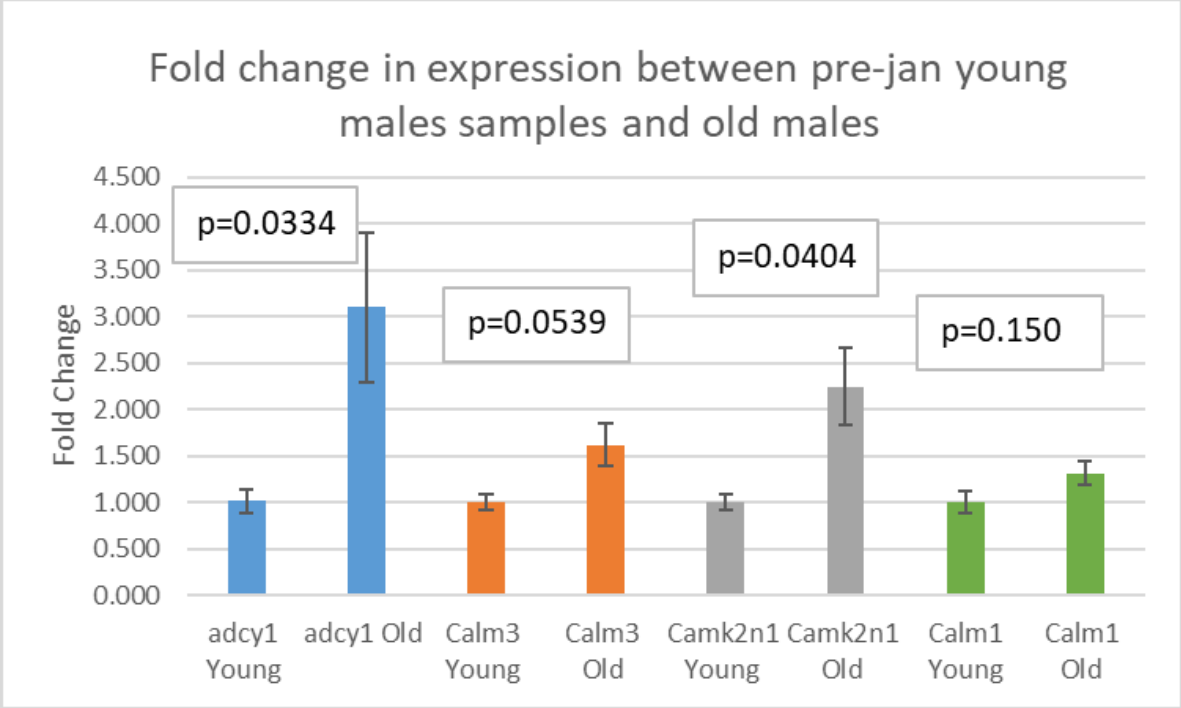

Figure 3d Change in gene expression between pre-jan Young Male samples and Old male samples

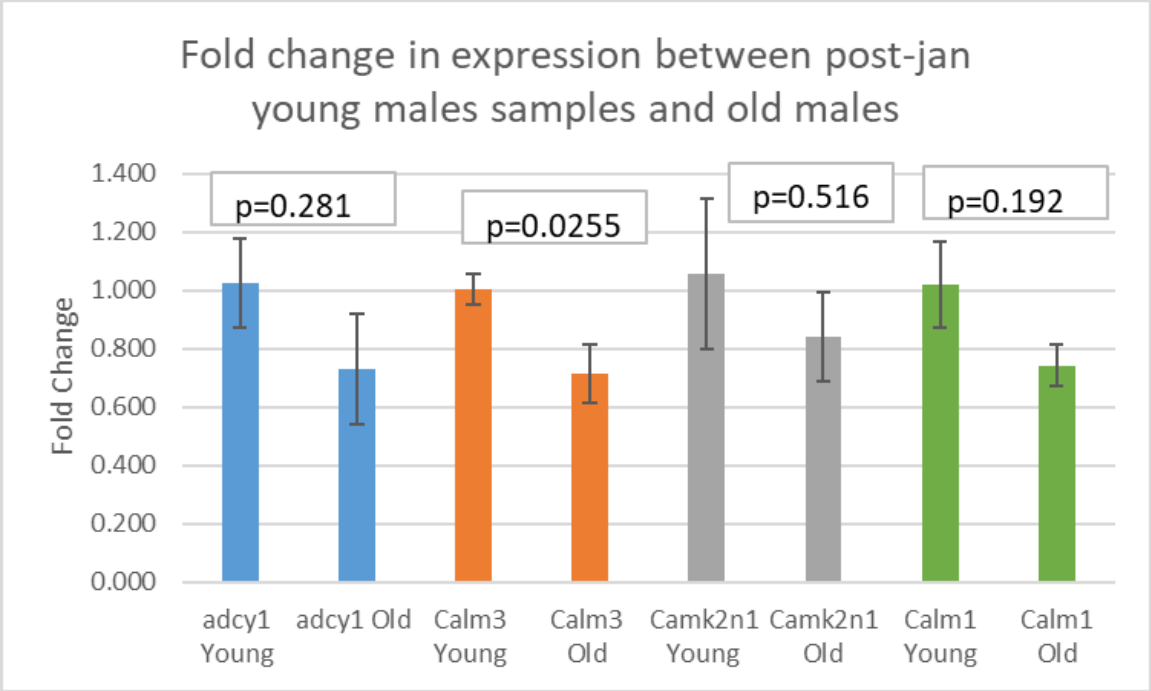

Figure 3e Change in gene expression between post-jan Young Male samples and Old males samples

Figure 3 Overall data for the Study: The enrichment of Astrocytes for each sample, and the overall comparisons between the Young Male mouse samples and Old Male mouse samples.

Discussion

This study has shown that there might be a trend in the change of expression of the genes adcy1, Calm1, Calm3, and Camk2n1 in male mice as they age. However, due to the lack of appropriate sample size and issues with the later young male samples it is hard to say at this point whether the expression of any of these genes changes over the course of male mouse aging. More samples would need to be gathered and processed with fresh Miltenyi reagents both to increase the statistical power as well as help identify which group of young mice have the better data and should be used in future experiments. However, due to the 4th and 5th young male sample having an issue where a sticky clump of debris may have trapped a portion of the astrocytes before being taken out which likely caused a smaller portion of astrocytes to be isolated in the end, I am inclined to believe that the true data would be closer to that of the older young male samples.

When comparing the p-values of the for all 3 fold change comparisons between the young and old groups (Figure 3b,d,e) and taking into account that the results of Figure 3d are likely to be closer to the true data, Camk2n1 and adcy1 stand out as being the closest to statistical significance, with Calm3 being another likely candidate. Which means they may be the most likely to change in expression with age in male mice with the data currently available.

Camk2n1, adcy1, and Calm3 are all very important when it comes to brain function. Camk2n1 is one of the endogenous inhibitors of CaMKII[42], which is a key molecule for memory formation[43]. It is also dependent on Calmodulin to function, so its expression is almost certainly linked with that of Calm1 and Calm3. Adcy1 is an Adenylyl cyclase(ADCY) gene that is expressed in a variety of Central nervous system tissue. ADCYs catalyze the production of cAMP from ATP, while phosphodiesterases (PDEs) degrade cAMP to 5'-AMP [44][45]. cAMP is a ubiquitous second messenger in various cell types[46], and must be highly regulated due to playing a pivotal role in a variety of fundamental cellular processes[47]. In the brain, Adcy1 is expressed in greater variety in the developmental stages but in more confined areas as the individual reaches adulthood[48][49]. Calm3 is one of the 3 genes that encodes calmodulin [23]. These enzymes regulate neural development, memory, and learning. The classic example being long-term potentiation in hippocampal CA1 neurons, which involves phosphorylation of AMPA and NMDA receptors by CaMKII[50].

Due to this, changes in the expression of these genes can have great impact on the brain function. There does not appear to be much research on how Camk2n1 expression changes with age, however as an inhibitor of CamKII, which blocks CaMKII signalling either by acting on the kinase activity or by preventing the CaMKII-NMDA receptor association[34]. Both over and under expression of this gene can lead to CamKII related issues in memory formation and induction of synaptic potential if it exits the ideal range. Adcy1 has been shown to be decreased during aging and increased during the acquisition of spatial learning[51][52]. Conversely overexpression of ADCY1 in forebrain can lead to elevated LTP,

improved memory and decreased social ability via increased extracellular signal-related kinase (ERK1/2)[53]. Due to Calmodulin being an indispensable component in the calcium signaling process, that targets a large array of structural proteins, receptors, enzymes, and ion channels. A change in expression in any of the 3 genes that code for it (Calm1, Calm2, and Calm3), can have a great effect on a variety of different cellular pathways, one of them being previously mentioned CamKII and Camk2n1 interaction.

If important genes such as these have differences in the change of expression with age based on the sex, it could provide valuable data in the personalization of neurological therapies for aging patients based on their sex. In order to find statistical significance for this data, not only will this study require more young male mouse samples in order to establish statistical significance for the change of expression with aging in male mice. A similar study will also need to be done with aging female mice before the change in expression with aging can properly be compared between sexes. This will likely be a project for future members of the Kreiling lab, and hopefully it can provide a baseline for new model system for neurological disease to be developed that better takes into account the differences in gene expression with aging between males and females.

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