Declaration Page

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This thesis is presented for the degree of Bachelor of Science Honours (Biomedical Science), College of Science, Medical, Molecular and Forensic Science, Murdoch University, 2021.

I declare this Scientific Manuscript is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institution.

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Investigating the expression and role of chloride ion channels in diffuse intrinsic pontine glioma

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Abstract

Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive type of glial brain tumour found in the pons region of the brainstem. DIPG accounts for about 10% of childhood central nervous system tumours and the prognosis for these children is poor. Resistance to radiation, the only current available therapy for DIPG, is one of the biggest challenges. This resistance could be due to the plasticity of DIPG cells, allowing them to rapidly adapt in response to different conditions. Preliminary RNA sequencing analyses of patient tumours identified the expression of ion channel genes including the GABA family. It is known that ion channels regulate tumour plasticity in other cancers and as such we aimed to investigate and characterise the role of ion channels in DIPG. This study aimed to validate the mRNA and protein expression of the GABA-A receptors associated with ligand-gated chloride channels, in DIPG patient-derived cell lines. The results of the study validated mRNA expression of the GABA-A receptor subunits using semi-quantitative and quantitative RT-PCR. Protein expression of three of the most highly expressed subunits (GABRA2, GABRA4, and GABRA5) was also demonstrated using western blotting and immunocytochemistry. Furthermore, a drug screen and titration showed that some GABA-A receptor modulators significantly inhibited proliferation of DIPG cells. This work confirmed that GABA-A subunits are expressed in DIPG cells and that blocking these ion channels inhibits DIPG cell proliferation. These findings form the foundation for future studies that will investigate GABA-A receptor drugs as potential treatments for DIPG using preclinical models.

Introduction

Diffuse intrinsic pontine glioma (DIPG) is an aggressive children's brain tumour that accounts for 10% of childhood central nervous system tumours and is diagnosed in children of five to ten years of age (1). DIPG is the most lethal type of paediatric brain cancer with a 5-year survival rate below 2% (1, 2). Surgical Resection of DIPG tumours is not possible, due to their location near the brainstem and so, despite standard radiation treatment, patients have a mean survival of only 12 months upon diagnosis (1,2). This prognosis has not improved in decades and this is could be the result of the cancer's resistance to therapies including radiation, which has long been explored in other cancers

but is a novel concept in DIPG (3). We hypothesise that ion channels drive this resistance due to their role in cellular plasticity of healthy brain cells, and several other types of cancer (4).

Ion channels are proteins that allow ion passage through the cell membrane. Ion channels at rest are tightly closed and impermeable, but they are opened by different changes occurring within and around the membrane (5). Instead of normal cell communication, the channels could facilitate oncogenic signalling and drive proliferation in DIPG cancer cells. Ligand-gated ion channels are neurotransmitter receptors such as GABA-A receptors (5). The GABA-A receptors and chloride ion channels could cause this resistance to therapy due to their ability to influence proliferation (6).

The GABA-A receptor is one of the major inhibitory neurotransmitter receptors in the brain (5, 7). It is comprised of a number of possible subunits including alpha 1 (α1) - α6 and beta 1 (ß1) – ß3 (7). The genes that encode these subunits are defined as, *GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRA6, GABRB1, GABRB2,* and *GABRB3* (7). To the best of our knowledge, there have been no published reports of identified GABA-A receptor subunits in DIPG cell lines at the time of writing.

The expression of chloride ion channels, particularly the GABA-A subunits in DIPG and their contribution to cancer progression and resistance is currently unknown. However, ion channels are important drug targets in other neurological conditions such as epilepsy (8). There are clinically approved drugs available to target GABA-A subtypes (8). Thus, identifying which GABA-A subtypes are expressed in DIPG could provide novel therapeutic targets that increase specificity and reduce the risk of off target effects, and can be rapidly progressed to clinical trials. We hypothesised that specific GABA-A subunits are functionally expressed in DIPG and act as drivers of cellular plasticity, thus blocking them with drugs will result in decreased DIPG cell proliferation.

Here, we aimed to identify the specific GABA-A subunits that are expressed in patient derived DIPG cell lines using semi-quantitative and quantitative RT-PCR to measure mRNA, and western blotting and immunocytochemistry to analyse protein. ViaLight assays were used to measure the anti-proliferative effects of seven GABA chloride channel-targeting drugs on DIPG cell lines.

Materials and Methods

Ethics Approval

Ethics for this project was obtained by the Oncogenic Signalling Laboratory at the Telethon Kids Institute through the relevant Human Research Ethics Committee (HREC), the Government of Western Australia Child and Adolescent Health Service HREC. The HREC approval number is 1769EP and expires on the 10/12/2021. These ethics were then reviewed by the Murdoch University HREC and reciprocal approval was obtained.

Antibodies

The following primary antibodies were all from Alomone Labs: Anti-GABA(A) α2 Receptor Antibody (AGA-002), Anti-GABA(A) α4 Receptor Antibody (AGA-008) and Anti-GABA(A) α5 Receptor Antibody (AGA-025). IRDye 680RD Goat anti-Mouse IgG secondary antibody (LCR-926-68070), IRDye 800CW Goat anti-Rabbit IgG secondary antibody (LCR-926-32211) and were supplied from Millennium Science.

Cell lines and Culture

The patient-derived DIPG cell lines DIPG36B, DIPG13, ICR194, DIPG38, ICR169 and the adult high-grade glioma cell line GBM6 were all initially established from surgically resected patient tumours (further detail on cell lines is provided in Supplementary Table 1). Tumour samples were collected after obtaining informed patient consent and with HREC approval from each site. DIPG36B, DIPG38, and DIPG13 were kindly gifted by Professor Michelle Monje from Stanford University (USA) and ICR169 and ICR194 were kindly donated by Professor Chris Jones from the Institute of Cancer Research (UK). Cell cultures were passaged once neurospheres had reached a suitable size or adherent cultures were ~90% confluent (typically after ~7days), and cultures were supplemented with fresh media every 3-4 days. All cell lines were maintained in 1× Working Tumour Stem Media (Supplementary Table 2) in a laminar flow Class II Biological Safety Cabinet. The suspension cultures were centrifuged at 300 g for 5 minutes to pellet the neurospheres prior to changing the medium.

Semi-quantitative RT-PCR

Total RNA was isolated from at least 5×10^6 cells from each cell line using a QIAGEN RNase Midi Kit (75142) according to the manufacturer's protocol. Following this, RNA concentration and quality was determined using NanoDrop spectrophotometer (Thermo Fisher) and 2µg of RNA was reverse transcribed using the Tetro cDNA Synthesis Kit (BIO-65042) following the recommended protocol. Custom primers were designed using NCBI Primer Blast and sourced from Integrated DNA Technologies and *GAPDH* was used as the reference gene due to its consistent expression throughout the human brain and different cell lines (primer details are provided in Supplementary Table 3). Semi-quantitative RT-PCR components included: 20 ng cDNA, 12.5 µL Promega GoTaq Master Mix, 1 µM forward and reverse primers and 8.5 uL Nuclease-Free water (In a total reaction volume of 25 µL). Samples underwent initial denaturation at 95 °C for 5 min;- 35 cycles of: 95 °C for 30 s (denaturation), 60˚C for 40 s (annealing) and 72 °C for 20 s (extension);- followed by a final elongation at 72 °C for 5 min. RT-PCR products were separated via gel electrophoresis for 45 minutes at 120 V on 2% agarose gels and visualised using SYBR Safe DNA Gel Stain (Thermo Fisher) on a Bio-Rad Gel Doc transilluminator. Healthy total brain (TB)-, and total cerebellum (TC) RNA samples were used as positive controls (controls sourced from Takara Bio USA). Non-template negative control (containing all reaction components except cDNA) and non-Reverse Transcriptase negative controls (containing all reaction components except reverse transcriptase) were also used.

Quantitative RT-PCR

For quantitative RT-PCR, total RNA was converted to cDNA using a QuantiTect cDNA Synthesis Kit according to the manufacturer's recommended protocol (Qiagen, 205311). qPCR reactions included 5 µL of Taq man (fast) master mix, 2µL of water, 0.5 µL of probe and 25 ng of the cDNA template and were prepared in optical 96 well reaction plates. Nuclease-free water was used for no template controls, instead of cDNA. Cycling was performed using a QuantStudio Flex 7 qPCR machine and the following conditions were used: UNG incubation, 50˚C for 2 minutes, polymerase activation 95˚C for 2 minutes, 40 cycles: denature 95˚C for 1 second, and anneal/extend 60˚C for 20 seconds.

Western Blotting

Cell pellets were washed with ice-cold phosphate-buffered saline (pH 7.2, Life Technologies, AM9624) and lysed using RIPA Buffer (Thermo Fisher, 89900) supplemented with 1× HALT Protease and Phosphatase Inhibitor Cocktail and 10 mM EDTA. Protein quantification was then performed using a Pierce BCA Protein Assay Kit (Pierce, 23225) as per the manufacturer's instructions. For visualisation of Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha2, Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha4, and Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha5 samples were prepared by addition of 1x NuPAGE LDS Sample Buffer and 1x NuPAGE Sample Reducing Agent (Thermo Fisher, NW04120BOX) and denatured at 95˚C. Total protein lysate was run on Bolt 4–12% Bis-Tris Plus Gels (Thermo Fisher) and transferred to PVDF membranes using the iBlot 2 system (Thermo Fisher).

Membranes were probed with primary antibodies; 4 µg/mL of Anti-GABA(A) α2 Receptor Antibody (AGA-002), 0.4 µg/mL of Anti-GABA(A) α4 Receptor Antibody (AGA-008) or 2.1 µg/mL Anti-GABA(A) α5 Receptor Antibody (AGA-025) diluted in Odyssey Blocking Buffer (Li-Cor, LCR-927-50003) and 0.1% Tween-20 overnight at 4 °C using a tube roller. Next day, membranes were washed vigorously using a plate shaker for 3 x 10 mins and then incubated in appropriate secondary antibodies for 1 hr at room temperature (~25 ˚C). A further 3 x 10 min vigorous washes were performed, and membranes were visualised on the Odyssey CLx (Li-Cor) using the 700 nm and 800 nm channels.

Immunocytochemistry

DIPG36B, DIPG38 and DIPG38 cells were seeded into separate wells of a 96 well flat-bottomed cell culture plates coated with Cultrex (Invitro technologies, RDS343201001) to promote adhesion and cultured until near confluent. Cells were fixed using 4% paraformaldehyde for 30 min at room temperature and washed with PBS 3×10 mins. To minimise non-specific staining, cells were blocked using 10% donkey serum in PBS at room temperature. Cells were then incubated with primary antibodies; 4 µ/mL of Anti-GABA(A) α2 Receptor Antibody (AGA-002), 16 µg/mL of Anti-GABA(A) α4

Receptor Antibody (AGA-008) and 8.5 µg/mL of Anti-GABA(A) α5 Receptor Antibody (AGA-025) at 4˚C overnight. The next day, the cells were washed again, this time in 1% goat serum/PBS for 10 minutes before being incubated in the fluorophore-conjugated secondary antibodies (AF488) diluted 1:5000 in 1% goat serum/PBS for 2 hours at room temperature in the dark. The cells were rinsed 3×10 minutes in the dark with 1% goat serum/PBS and then incubated in DAPI (Assay Matrix Pty Ltd, 0100-20) for 10 minutes away from light. Cells were imaged under a fluorescent microscope (Nikon Ts2R Fluorescent Microscope) at 40X magnification. Unstained cells, and cells incubated with secondary antibody only, were used as negative controls to account for any autofluorescence or non-specific binding.

Anti-Proliferation Assay

Drugs were provided as lyophilised powders and reconstituted in either DMSO (6-(4-Methoxyphenyl)-3-pyridazinamine, and GABA(A)-Compound 1b), water (Tiagabine hydrochloride, Isoguvacine hydrochloride and Muscimol hydrobromide) or PBS (Thiomuscimol), according to the recommendation on the product data sheets. Dilutions were performed to make a range of concentrations between 1 and 100 µM. 2000 cells were seeded per well into 96 well flat bottom (for adherent cell line) or ultra-low attachment (for neurospheres) plates and after 4 hours the drugs were added. The cells were then incubated for 7 days. After this period, anti-proliferation assays were performed using the ViaLight Plus BioAssay Kit (Lonza, LT07-121) as per the manufacturer's instructions. The ViaLight BioAssay measures ATP bioluminescence after drug treatment and incubation thereby allowing for comparison between remaining viable cell numbers of treated vs. untreated groups. GraphPad Prism 9 was used to prepare dose-response plots and perform statistical analyses (Tukey corrected).

Results

Expression of GABA-A subunits genes in DIPG cell lines qualitatively assessed by semi-quantitative RT-PCR

We first wanted to determine whether we could detect expression of any of the GABA-A subunits in a small subset of DIPG cell lines. This was completed by characterising the mRNA expression of 9 previously identified subunits in these cell lines through RT-PCR. The subunits were chosen previously as bioinformatic analysis demonstrated these 9 genes to be highly expressed in unpublished RNA sequencing data (9). Specifically, these were *GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRA6, GABRB1, GABRB2,* and *GABRB3* (Figure 1). All GABA-A subunits except *GABRA6* were expressed in the total brain control. According to the literature, *GABRA6* is only found in the cerebellum and so the total cerebellum control was used as the positive control for that gene. However, there was also no apparent expression of *GABRA6* in this cerebellum control, or any of the DIPG cell lines, and so *GABRA6* was not included in subsequent analyses. We did not prove that the *GABRA6 primers* worked and therefore this could have been due to a primer issue.

Seven of the nine target genes across our two patient-derived DIPG cell lines were expressed. *GABRA2, GABRA4, GABRA5, GABRB1* and *GABRB3* all showed bands across both cell lines, and this was similar to that of the control total brain tissue. While some genes were not expressed in either cell line (*GABRA1),* there was also some evidence of differential expression between the two cell lines. For example, no RNA was present for *GABRA3* in DIPG 36B and only a trace of RNA in DIPG38, while there was no RNA for *GABRB2* in DIPG38 but a trace of RNA for DIPG 36B.

Although this data gives a good indication of mRNA expression for these genes in our DIPG cell lines, it cannot be extrapolated to protein expression due to differences in translation, mRNA degradation, and post-translational modification of different genes.

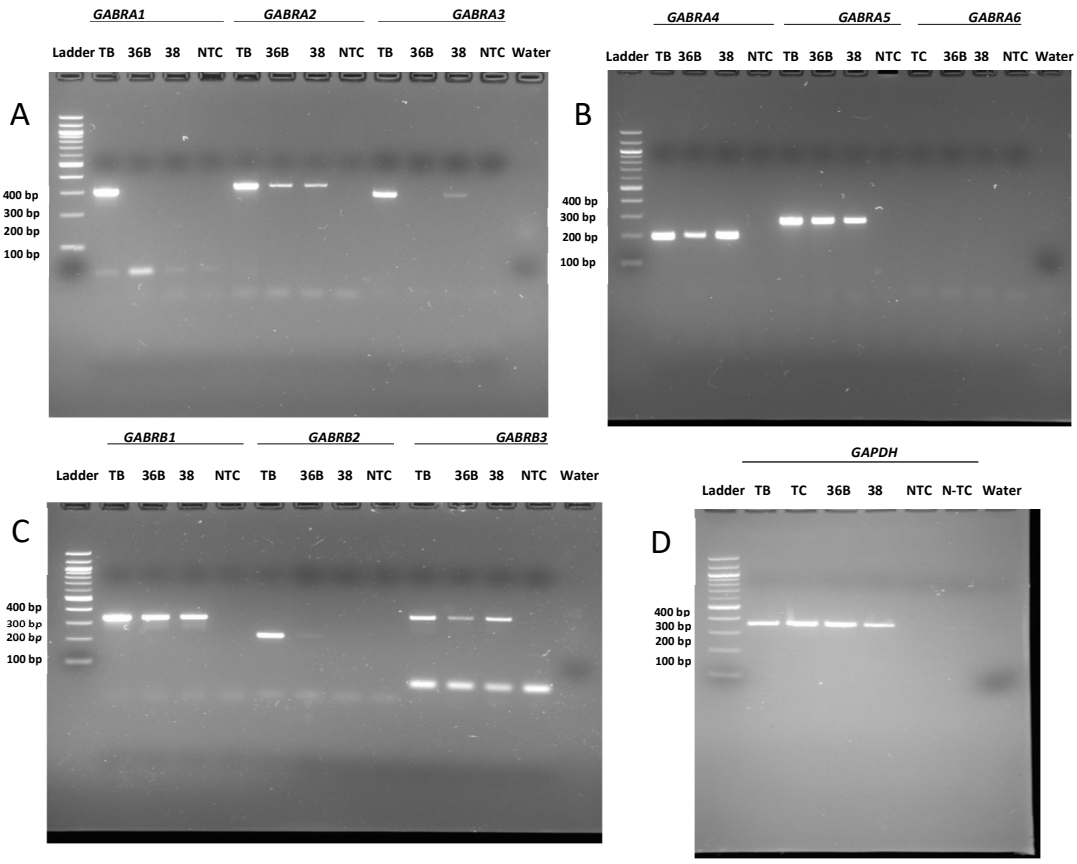

Figure 1: Qualitative analysis of GABA-A subunit gene expression in 2 DIPG cell lines. Gel images show A) *GABRA1, GABRA2* and *GABRA3*; B) *GABRA4, GABRA5* and GABRA6; C) *GABRB1, GABRB2* and *GABRB3*; D) the *GAPDH* controls. The ladder used in all gels was 100 bp. TB = Total Brain Control; TC = Total Cerebellum Control; 36B = DIPG36B; 38 = DIPG38; NTC = No Template Control; N-TC = No Transcriptase Control and all gels were repeated n=3.

Quantitative analysis of GABA-A subunit mRNA expression in DIPG cell lines

The next step in this project was the confirmation of the GABA-A subunit RNA expression in a larger and more diverse panel of DIPG cell lines, which included DIPG36B, DIPG38, DIPG13, ICR194, ICR 169. This was completed by further characterising the mRNA expression of the subunits through

qRT-PCR. This method was used to quantify the expression of the subunits in the DIPG cell lines and we compared them to an adult high-grade glioma cell line, GBM6, and the total brain control RNA.

GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRB1, GABRB2, and *GABRB3* were all expressed in each of the DIPG cell lines and highly expressed in the total brain control (Figure 2). Since the delta Ct in this study compares the expression of the target gene to that of the reference gene (GAPDH), a lower Ct value indicates higher expression of the target gene.

Notably, *GABRA5* was strongly expressed across the DIPG cell lines in a similar level to the total brain expression. Interestingly, *GABRA2* and *GABRA3* were also quite strongly expressed across most cell lines. Differential mRNA expression was observed for a number of genes across the patient derived DIPG cell lines. Validating the results from the semi-quantitative RT-PCR, *GABRA4*, *GABRB1* and *GABRA2* were strongly or moderately expressed in DIPG36B and DIPG38 cells.

Through ANOVA tests it was shown that there was a significant difference of subunit expression within the whole subunit data set ($p<0.05$). Specifically, this showed that each subunit was differentially expressed between cell lines and that the different subunits were differentially expressed within one cell line. Pairwise Tukey's tests identified significant differences in expression of the subunits between cell lines. Some important significances to note include *GABRA5* and *GABRB3* which were both significantly upregulated in all DIPG cell lines in comparison to GBM6 (high grade glioma cell line) ($p<0.0001$). Also, very interesting is the significant difference between the very low expression of *GABRA1* in all DIPG cell lines in comparison to the high expression in the total brain control ($p<0.0001$). Interestingly *GABRA4* and *GABRB1* both had quite high expression levels in DIPG36B, DIPG38 and DIPG13 but low expression in ICR cell lines.

The only subunits that were not significantly different were *GABRA3* in DIPG38 compared to total brain; *GABRA5* in DIPG36, DIPG38 and ICR169 in comparison to total brain, and *GABRB3* in DIPG38, ICR194 and ICR 169 in comparison to total brain.

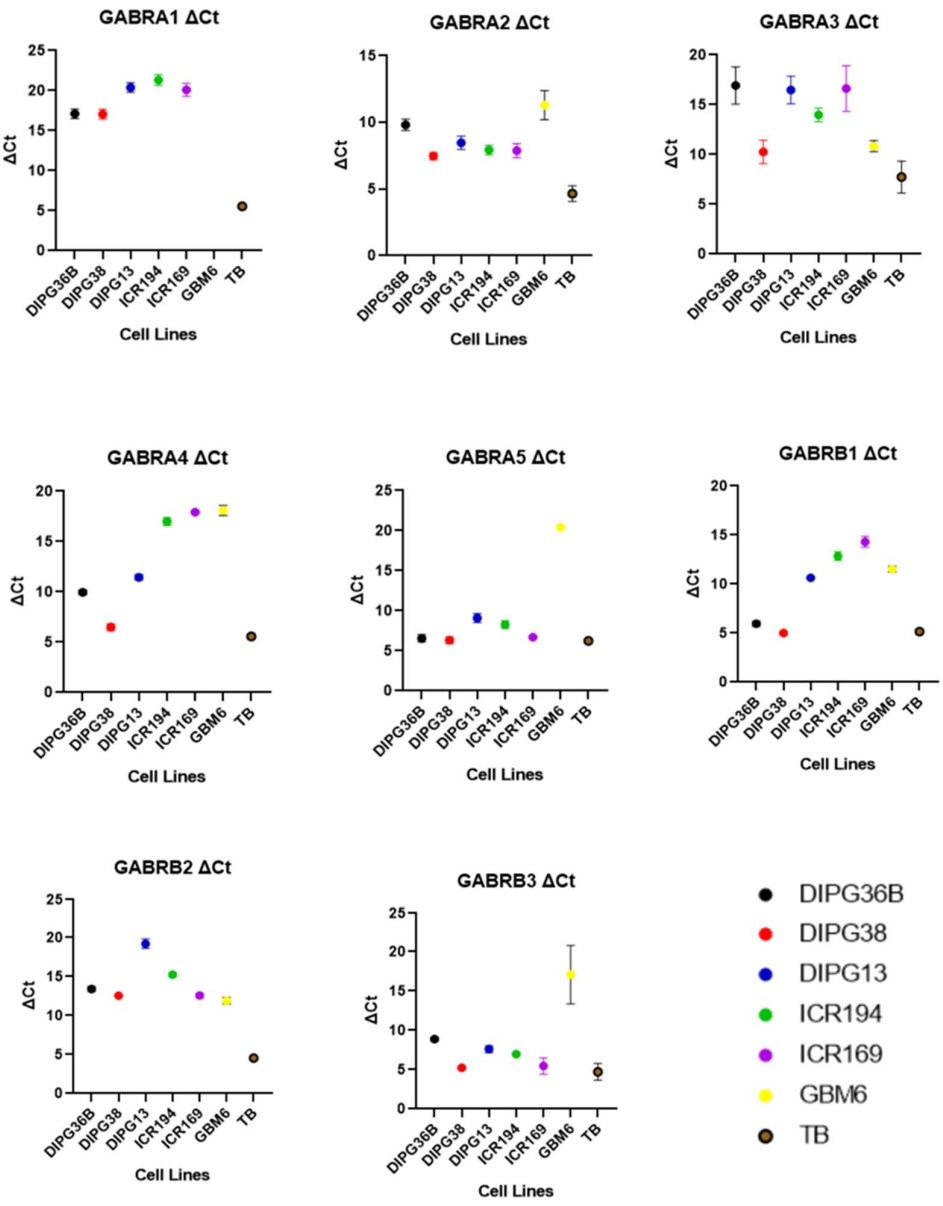

Figure 2: Quantitative analysis of GABA-A subunit genes in 5 DIPG cell lines and 1 GBM cell line. The graphs show the average delta Ct (ΔCt) of each subunit within the cell lines and total brain RNA (TB). The delta Ct is the Ct of a subunit in a cell line minus the Ct of *GAPDH* in each cell line. The graphs plot the mean of each subunit within a cell line and errors bars represent standard deviation. qRT-PCRs were repeated to n=6 for each subunit.

The log2 delta delta Ct compares the relative expression of each subunit within a cell line to the expression of that subunit in total brain. Almost all subunits in most cell lines have significantly lower expression in comparison to total brain. It is interesting to note that *GABRA3* is expressed less than -1-fold change in comparison to the total brain in DIPG38 (Figure 3). This is also similar to *GABRA4* expression in DIPG38. Expression of *GABRA5* was similar in total brain to most cell lines including DIPG36B, DIPG38 and ICR169. Significantly, *GABRB1* is the only subunit to be over expressed at all in comparison to total brain, and this was only evident in DIPG38 cells. *GABRB3* expression was also similar to that of total brain in DIPG38 and ICR169. It is important to note that although most of the subunits are under-expressed or downregulated in comparison to total brain, they still have very high levels of overall expression (Figure 2 and 3). This vitally shows that the subunits are not only expressed but could therefore also function.

When looking at qPCR as a whole, we can see that while some subunits are under-expressed in comparison to total normal brain, they still have very high levels of expression in DIPG. From these results, and the prior RNA-sequencing data analysis, we rationally selected our targets of interest for further validation of protein expression – Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha2 (*GABRA2),* Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha4 *(GABRA4),* and Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha5 *(GABRA5).*

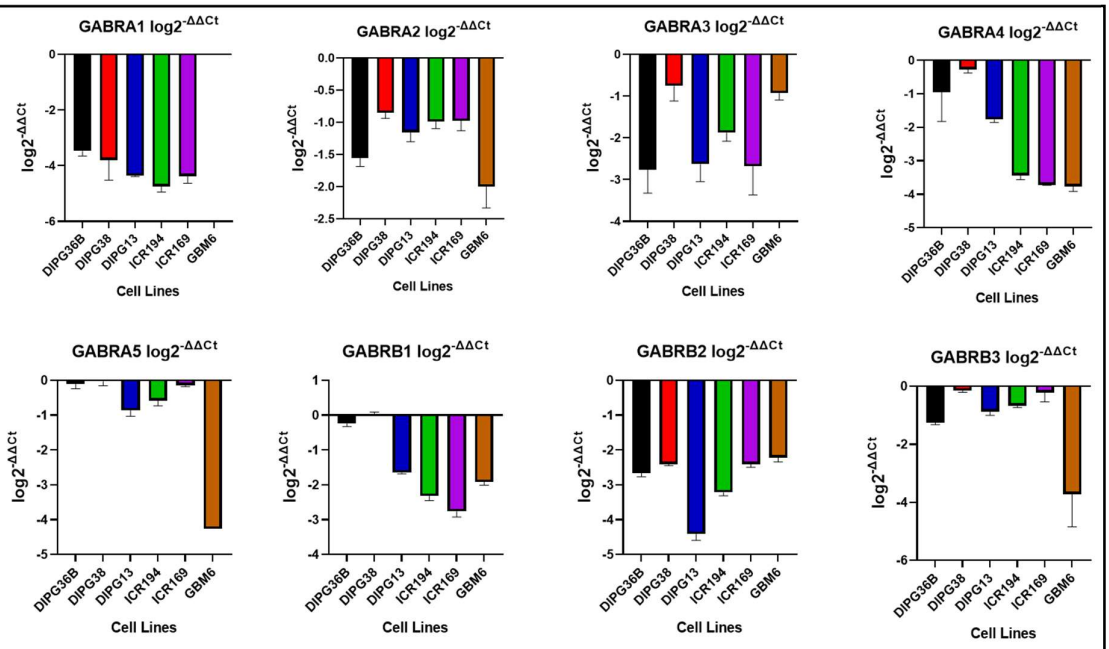

Figure 3: Quantitative analysis of GABA-A subunit genes in 5 DIPG cell lines and 1 GBM cell line. The graphs show the mean log delta delta Ct (ΔΔCt) of each subunit within the cell lines and total brain RNA and the error bars indicate the standard deviation. The log delta delta Ct is the ΔCt of subunits in each cell line minus the ΔCt of the subunit in total brain compared to GAPDH in each cell line. This result is then log2 transformed. Each subunit within each cell line had n=6.

Analysis of GABA-A subunit protein expression in DIPG cell lines

To assess expression of GABA-A subunit proteins, western blotting was performed using total protein lysates from DIPG36B, DIPG38 and DIPG13 cells (Figure 4). GABA-A Receptor Subunit α2 (GABRA2) protein was minimal in DIPG36B, and stronger, but still quite weak, in DIPG38 and DIPG13. GABA-A Receptor Subunit α4 (GABRA4) had moderate expression in all three cell lines, and a doublet was present in DIPG38. GABA-A Receptor Subunit α5 (*GABRA5)* was very strongly expressed in all three DIPG cell lines. All three GABA-A subunits were present at the expected sizes of 37 kDa, 62 kDa, and 50kDa for GABA-A α2, GABA-A α4, and GABA-A α5, respectively.

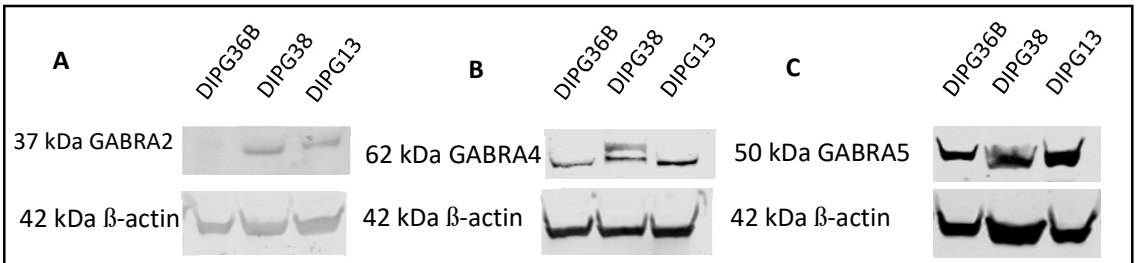

Figure 4: Protein analysis of GABA-A subunits in 3 DIPG cell lines. ß-actin was used as a positive control for all protein lysates. The three cell lines used were DIPG36B, DIPG38, DIPG13. A: shows *GABRA2* protein expression in the cell lines and *ß-actin*, B: shows *GABRA4* protein expression in the cell lines and *ß-actin* and C: shows *GABRA5* protein expression in the cell lines and *ß-actin*. Protein size of each subunit was estimated by comparing to a low molecular weight marker ladder and each subunit plot had n=3

Although mRNA expression and protein expression of the GABA-A subunits have been shown, it was also important to determine the location of these subunits within the cells as they will only be functionally active as ion channels if they are in the cell membrane. Therefore, immunocytochemistry was performed on three cell lines: DIPG36B, DIPG38 and DIPG13, using the same primary antibodies as the western blotting experiments. Each cell line also had a secondary antibody only control to rule out any autofluorescence and false positives.

The GABA-A Receptor Subunit α4, is clearly expressed in all cell lines. The small green fluorescent punctate areas show the individual GABA-A subunits. In the merged images of Figure 5 it appears that the punctate expression is present around the area of the cell membrane as would be expected. The expression of the other two subunits, GABA-A Receptor Subunit α2 and GABA-A Receptor Subunit α5 is inconclusive. The background fluorescence has shown to be quite high in order to image the subunits. There seemed to be weak levels of expression of the other two subunits when completing the fluorescent microscopy. At this size and resolution however, the results for GABA-A Receptor Subunit α2 and GABA-A Receptor Subunit α5 in all cell lines is best described as inconclusive.

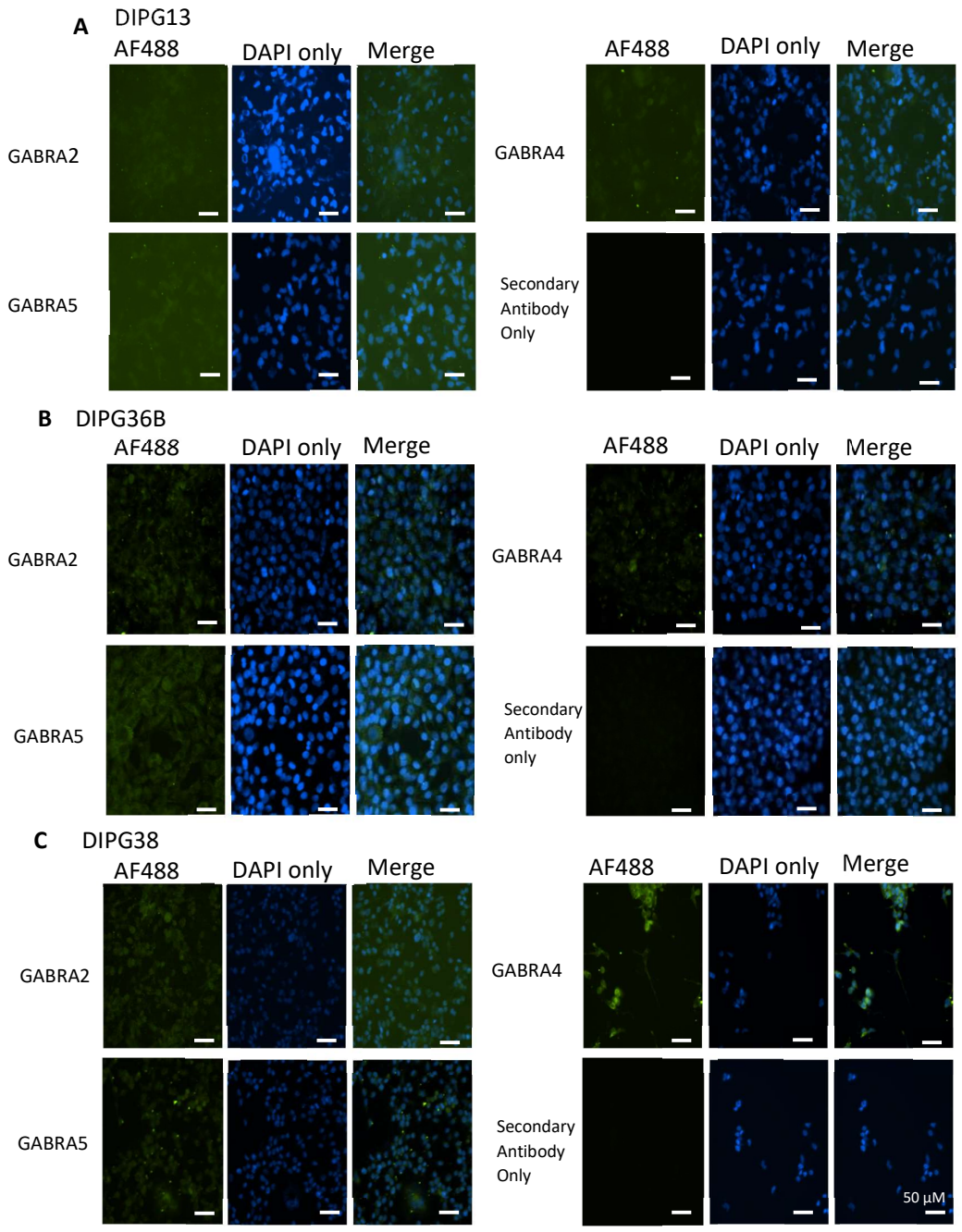

Figure 5: Immunocytochemistry images showing the cellular location of *GABRA2, GABRA4* and *GABRA5* proteins in three different DIPG cell lines: A) DIPG 13; B) DIPG36B and C) DIPG 38. Each of the GABA(A) receptor subunits were labelled with AF488 secondary antibody (green), and nuclei were counterstained with DAPI (blue). All cell lines also show images for the secondary antibody control. Images were taken at 40X Magnification and each experiment was performed n=3. Scale bar = 50 µM

Targeting GABA-A subunits with drug treatment

Drugs that specifically target the GABA_A receptor were tested for their anti-proliferative effects on DIPG36B cells, selected as a candidate cell line (Figure 6). An initial drug screen using 7 drugs was performed, specifically: GABA(A)-Compound 1B, U93631, Isoguvacine Hydrochloride, Muscimol Hydrobromide, 6-(4-Methoxyphenyl)-3-pyradazinamine, Tiagabine hydrochloride and Thiomuscimol.

Five of the seven drugs in the initial drug screen had no impact on cell proliferation at either 10 or 50 µM. Two drugs did stand out for having an adverse effect on DIPG36B cells. GABA(A)-Compound 1B and U93631 were both GABA-A receptor antagonists and expectedly caused a reduction in relative cell viability after 7-days of culture. They had significant reduction of 24.7% (± 4.57%) and 29.06% (± 8.45%) in viability at 50 µM respectively ($p<0.0001$). GABA(A)-Compound 1B further investigated in a drug titration cell proliferation assay since it also had an effect at 10 µM, with a reduction of 17.1% (± 11.8).

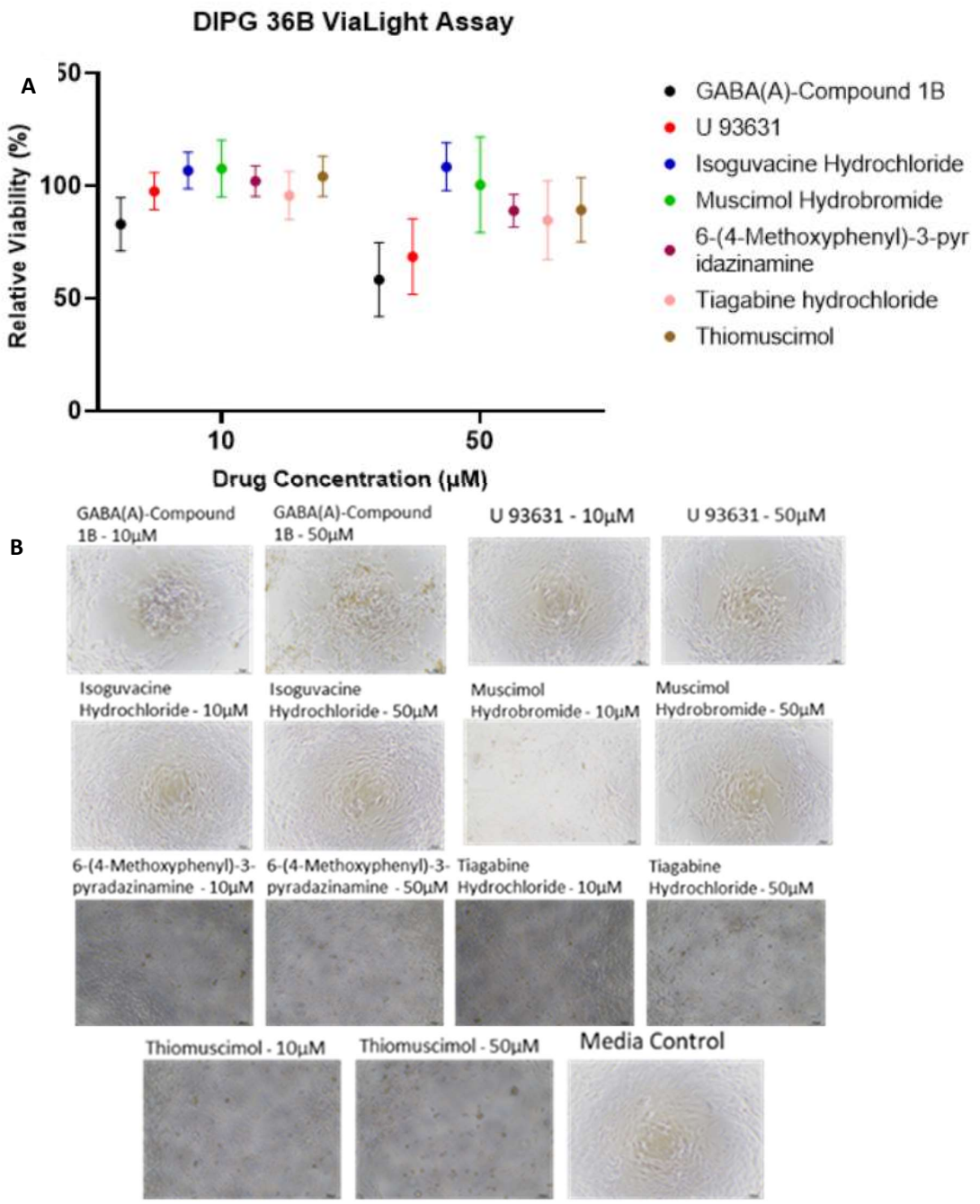

Figure 6: A) ViaLight assay drug screen results after a 7-day incubation period for *DIPG*36B treated by 10 and 50 µM of 7 different drugs: GABA(A)-Compound 1B, U93631, Isoguvacine Hydrochloride, Muscimol Hydrobromide, 6-(4-Methoxyphenyl)-3-pyradazinamine, Tiagabine hydrochloride and Thiomuscimol. 'Relative Viability %' refers to the reduction in cell viability as compared to the control group (untreated cells cultured in complete media). Error bars indicate standard deviation. B) Microscope *images (20x magnification) of untreated and treated cells. n=3*

Based on the drug screen results, a titration ViaLight assay was performed for GABA(A)-Compound 1B, using a range of concentrations from 1 to 100 µM (Figure 7). There was no significant effect on viability following treatment with 1 to 10 µM drug. However, treatment with 20 to 100 µM caused a significant reduction in viability to a maximum decrease of 47.79% (± 12%) ($p<0.0001$) for 100 µM GABA(A)-Compound 1B. The effective response of the drug on the GABA-A receptor indicated that the GABA-A subunits that are expressed in this cell line are more than likely functional.

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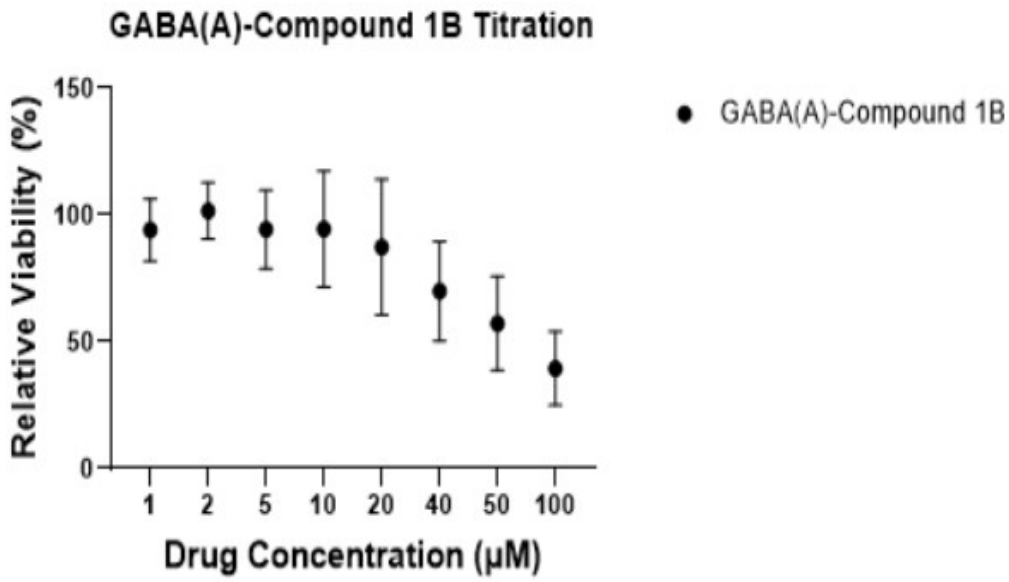

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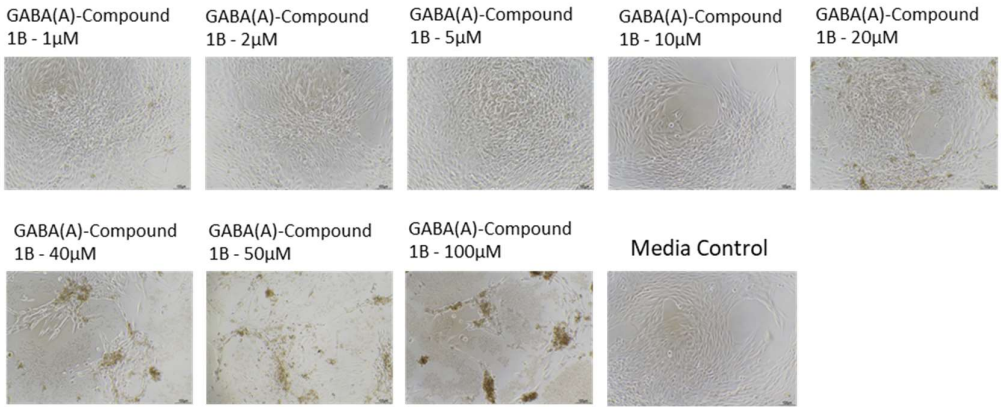

Figure 7: A) ViaLight assay results after a 7-day incubation period for DIPG36B. Cells were treated with 1 to 100 µM of GABA(A)-Compound 1B. 'Relative Viability' refers to the reduction in cell viability as compared to the control group (untreated cells). Error bars indicate standard deviation. B) Microscope images (20x magnification) of untreated and treated cells. Results are representative of n=3 independent experiments.

Discussion

This study aimed to identify the expression pattern of the different GABA-A subunits in DIPG, a rare but severe type of childhood brain tumour, for the first time. Preliminary RT-PCR results identified differential mRNA expression of GABA-A subunit genes in two patient-derived DIPG cell lines. GABRA2, GABRA4, GABRA5, GABRB1 and GABRB3 showed uniformly high levels of mRNA production across both cell lines, similar to that of the control total brain RNA. Subsequent quantitative RT-PCR analysis in a larger panel of DIPG cell lines identified expression of all GABA-A subunits except *GABRA6* in all 6 cell lines. These findings validated recent RNA-sequencing data from DIPG patient tissues analysed by computational biologist Dr. Anya Jones (9, 10). This highlights the genetic heterogeneity of DIPG cells. Differential expression of the GABA-A pentamer between cell lines could be a factor that contributes to their resistance to targeted therapies.

Similarly, to the RT-PCR it was quantitively shown that *GABRA5* was strongly expressed across the DIPG cell lines in a similar level to the total brain expression. *GABRA2* and *GABRA3* were also strongly expressed in most cell line. *GABRA4, GABRB1* and *GABR2* had varying levels of expression across the cell lines. *GABRA5* and *GABRB3* were both significantly upregulated in all DIPG cell lines in comparison to GBM6. *GABRA1* was significantly down regulated in comparison to the brain control. *GABRA4* and *GABRB1* were strongly expressed in DIPG36B, DIPG38 and DIPG13 but were weakly expressed in ICR cell lines. Some subunits did not have significant differences such as *GABRA3* in DIPG38 compared to total brain; *GABRA5* in DIPG36, DIPG38 and ICR169 in comparison to total brain, and GABRB3 in DIPG38, ICR194 and ICR 169 in comparison to total brain.

Since, mRNA expression does not always correlate to functional protein expression due to mRNA degradation, post-translational modification, and other factors, protein expression of selected GABA-A subunits was also investigated. Three proteins were chosen to proceed to protein analysis, GABA-A α2 (*GABRA2),* GABA-A α4 *(GABRA4)* and GABA-A α5 *(GABRA5)*, due to time constraints and availability of verified antibodies and other materials. Western blots confirmed that DIPG cell lines

showed differential expression of GABA-A subunits, and this directly correlate to the RT-PCR and q-RT-PCR results. Immunocytochemistry then verified the predicted membrane localisation of the α4 subunits in the patient cell lines while the other subunits seemed to be inconclusive. Lastly, anti-proliferation assays showed that treatment of the cell lines with GABA-A antagonist drugs decreased cellular proliferation, indicating that the GABA-A receptor subunits are functional in the DIPG cell lines. However, further studies such as patch clamp analysis are required to characterise the function of these channels.

The GABA-A receptor expressed and upregulated in numerous other cancers, including breast, prostate and colon, and they have been shown to be present and functioning in rare forms of cancer such as human insulinoma (11-17). Upregulation of GABA and GABA-A receptors has been directly linked to cancer cell invasion, proliferation, migration, differentiation and metastasis (15, 18-21). A specific example is the upregulation of the π-subunit of the GABA-A receptor in breast cancer and pancreatic adenocarcinomas (16, 22). These examples show that there is a strong possibility that blocking GABA-A receptor activity could reduce tumor growth.

It is yet unknown whether this expression and functionality is similar in DIPG. The ability of DIPG to become rapidly resistant to treatment is a major difficulty impacting survival outcomes, and this resistance could be directly related to ion channel expression. Therefore, identifying whether GABA-A receptors are involved in these resistance processes in DIPG could lead to the development of new treatment strategies that target these ion channels. These results have managed to introduce novel findings that are very similar to the literature in other cancers. The finding of this research has shown several GABA-A subunits are present in DIPG cells and are strongly expressed. Most of the subunits were downregulate in DIPG in comparison to total brain with the exception of GABRB1. However, this is not unimportant because they are still very strongly expressed and therefore could be functional. It is yet and unclear are in DIPG whether we expect GABA-A receptors to be up-or down regulated. It is therefore still plausible that the GABA-A receptor in DIPG has similar functions

to that in the other cancers (e.g., invasion, proliferation, metastasis) and similar targeted therapies can be used.

Whilst we have compared the expression of GABA-A receptor subunits in DIPG to that of a human total brain control used in other studies (23, 24), this cannot be extrapolated into differences between DIPG tissue and healthy brain mainly because the control comes from a healthy adult brain, whereas DIPG is a children's cancer. We identified that total brain had a very high expression of all the subunits with the exception of α6. Unfortunately, we were unable to detect expression of the α6 subunit which is specific to the cerebellum, in a specific cerebellum control. This could be due to the generally low expression of the α6 subunit in the cerebellum of a healthy adult brain. It is important to note that at the time of writing the total brain control in the best control available. However, a more appropriate control would be sourced of a child in the same age-group as typical DIPG patients. All controls are currently taken from adult human and this specific example was taken from an adult male. This may be more specific and beneficial in comparison to a pooled sample from a large number of patients however as all our cell lines came from female donors that could be problematic. A range of children's total brain DNA sourced from males and females would ideal. In the future it would very beneficial for the brain control to be more informative and for a children's control to be available for study.

This research has provided novel results as at the time of writing no other literature had examined the expression of GABA-A subunits within DIPG. GABA-A subunits had been identified in other high-grade glioma and cancers but never in this fatal disease (25). These results have provided evidence that the GABA-A subunits α2, α4 and α5 are expressed in DIPG patient derived cell lines at an mRNA and protein level.

These subunits are also expected to be functionally active since some GABA-A receptor antagonistic drugs caused decreases in cell proliferation. U93631 and GABA(A)-Compound 1B are both antagonists of the GABA-A receptor And have been previously shown to reduce proliferation in

other cell types (26, 27). Specifically, GABA(A)-Compound 1B was the most effective antagonist trialled and it specifically targets the GABA-A α5 subunit which was highly expressed in the DIPG cell lines (27). This antiproliferative effect suggest that the α5 subunit is not only functional but also could prove important in DIPG treatment. This is very important as it is not only the first time this subunit has been identified in DIPG but it is also the first time that blocking the GABA signalling pathway has been shown to affect DIPG cell proliferation.

The results discussed in this paper will have functional and therapeutical implications in the future. While further research is required, the identification of these GABA-A receptor subunits in DIPG has high therapeutic potential due to their importance in other cancers. Identification is the first step in researching the function that these subunits play in DIPG proliferation and invasion. Once these paths are identified, they can be therapeutically targeted in combination therapy, to overcome disease resistance and proliferation.

Clinically, any progress that can be made in early detection and diagnosis of DIPG patients would be significant. A new treatment strategy using ion channel drugs to prevent resistance and targeted therapy in order to block cancer growth could successfully prolong the life of a patient. This would represent the most significant leap in this field over the last 30 years. While my results are still preliminary and are a long way from providing this treatment, they may prove helpful in initiating this pathway. The main problem we do have to be aware of clinically however, is the presence of off-target effects in any treatment going forward. GABA-A subunits are identified in different peripheral organs such as the colon, breast, and prostrate and therefore treatment targeting these subunits could have off-target effects in these other organs (28). This research has shown that the GABA-A subunits are also highly expressed in the healthy brain. Some GABA-A target drugs have been attributed to immunosuppression, some also affect other signalling molecules and some benzodiazepines such as GABA(A)-Compound 1B can have off-target effects on different cell types (29).

There are a number of key strengths of this study, particularly attributed to its planning and execution. All the protocols used are routine techniques and the specific experimental condition noted in the methods were thoroughly optimised by myself and other members of the lab. Therefore, these results will be easy to replicate by other researchers around the world (30-33). The study used appropriate controls, safety measures and variables that proved critical to demonstrate that no off-target RNA or proteins were selected in the experiments. The use of a panel of patient-derived lines that reflect the genetic and molecular heterogeneity of DIPG and the consistent findings across the panel of lines strongly suggests the results are a true reflection of DIPG itself and not just one particular patient. All of the experiments were replicated at least three times and the results of this study can therefore be interpreted to provide scientifically valuable conclusions.

There were some limitations for this project that should be acknowledged. Firstly, the immunocytochemistry samples were analysed using a fluorescent microscope since confocal microscopy was not available. Due to the small size of the subunits and the low levels of expression, this led to high background fluorescence in the images in order to visualise the subunits. Despite this, there was still clear specific staining of the subunits when comparing the images to those of the secondary only controls. Secondly, although some agonists were used in the drug screening experiments, it was not possible to determine whether they had a positive effect on proliferation because the untreated cells grew to confluent at the end of the 7-day time period. This problem was mitigated by trialling reduced cell counts however this did not greatly impact the results. In future studies we could try to reduce the cell number further and take measurements at shorter time points.

This experiment would ideally be repeated to validate this data but it would also be ideal to investigate more of the subunits in detail which was not possible due to time and delivery constraints. The project would also benefit from some immunohistochemistry investigation in direct tumour tissue samples such as the immunohistochemistry conducted in other disease research

because this would add *ex vivo* validation to the study. (34). The subunit proteins would be the same size in tumour tissue samples as they are in the cell, however depending on the quality of tissue samples they may be difficult to visualize.

It would be of interest to follow this work on with more drug screening and analysis of potential drug therapies similar to other conducted in DIPG (35). In particular, studies that could identify whether GABA-A-targeting drugs induce cell cycle arrest at some stage would be useful similar to some melanoma studies (36). Other future directions could include investigating the subunits in combination together. The composition of subunit in the GABA-A receptor are important to the functionality of the receptor. If it is possible to identify the combination of GABA-A receptor subunits in DIPG, then perhaps all subunits or the receptor as a whole could be targeted to provide a therapeutic response. Future work will most likely trial combination therapy between conventional radiation therapy and targeted ion channel drug therapy similar to other studies (37, 38). With the development of more bioinformatic analysis future studies should be able to study the pathways and connections relating to these subunits. Lastly, the ultimate goal for the future would be to development suitable *in vivo* animal models for drug testing, before moving to pre-clinical and clinical trials such as those conducted in similar research areas. (39, 40).

In summary, firstly we identified mRNA expression of eight GABA-A subunits in DIPG cells. Secondly, we demonstrated protein expression of three of these subunits. Finally, targeting GABA-A receptors with antagonists can reduce cellular proliferation of DIPG cells. Since we hypothesised that specific GABA-A subunits were functionally expressed in DIPG where they are drivers of cellular plasticity, thus targeting them with drugs will result in decreased DIPG cell proliferation, these results have supported our hypothesis.

Conclusions

This thesis, investigated the expression of GABA ion channels in DIPG, which is a particularly lethal and aggressive form of children's brain cancer that has not seen any significant improvement in

treatment for the past 30 years. This is mainly due to its highly invasive and diffuse growth pattern, metastasis, development of drug resistance and increased cellular plasticity. All of these variables have been associated with ion channels such as GABA-A in other cancers. In this study, we have confirmed the presence of functional GABA-A subunits in DIPG cell lines via anti-proliferation assays. RT-PCR was used to identify mRNA production for 9 GABA-A subunits in multiple patient derived DIPG cell lines. Our findings suggest that further studies should be undertaken surrounding the functional role played by GABA-A subunits in DIPG progression with a focus on proliferation, invasion, synaptic plasticity and ion channel interaction.

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Appendix

Supplementary Information

Supplementary Table 1. Patient-derived DIPG cell lines utilised in this project.

Cell Line	Age at diagnosis	Sex	Tissue type (autopsy vs biopsy)	Histone status	Other mutations
DIPG13	6	female	autopsy	H3.3K27M	P53
DIPG36B	3	female	autopsy	H3.1K27M	
DIPG38	4	female	autopsy	H3.1K27M	COR frameshift
ICR169	10.9	female	biopsy	H3.3K27M	
ICR194	9	female	biopsy	Wild-type	

Supplementary Table 2. Recipe 1: Tumour Stem Medium (TSM) Base and Recipe 2: 1× Working Tumour Stem Media Recipe.

RECIPE #1: Tumor Stem Medium (TSM) Base

Reagent	Vendor	Catalog no.	Volume
Neurobasal-A Medium (1X), liquid	Invitrogen	10888-022	250mL
D-MEM/F-12 (1X), liquid, 1:1	Invitrogen	11330-032	250mL
HEPES Buffer Solution (1M)	Invitrogen	15630-080	5mL
MEM Sodium Pyruvate Solution 100mM (100X), liquid	Invitrogen	11360-070	5mL
MEM Non-Essential Amino Acids Solution 10mM (100X), liquid	Invitrogen	11140-050	5mL
GlutaMAX-I Supplement	Invitrogen	35050-061	5mL
Antibiotic-Antimycotic (100X), liquid	Invitrogen	15240-096	5mL

RECIPE #2: Working TSM 1x (Base Medium + Growth Factors)

Reagent	Vendor	Catalog no.	Working conc.	Stock conc.	Volume
TSM Base	--	--	--	--	50mL
B-27 Supplement Minus Vitamin A (50X), liquid	Invitrogen	12587-010	--	--	1mL
H-EGF*	Shenandoah Biotech	100-26	20ng/mL	20ug/mL	50uL**
H-FGF-basic-154*	Shenandoah Biotech	100-146	20ng/mL	20ug/mL	50uL**
H-PDGF-AA*	Shenandoah Biotech	100-16	10ng/mL	20ug/mL	25uL**
H-PDGF-BB*	Shenandoah Biotech	100-18	10ng/mL	20ug/mL	25uL**
Heparin Solution, 0.2%*	StemCell Technologies, Inc.	07980	2ug/mL	2mg/mL	50uL**

Supplementary Table 3. GABA-A subunit genes and their designed forward and reverse primer sequences.

Gene	Direction	Primer Sequence	Product size	Melting Temperature (Tm)
GABRA1	FWD	GAGAGCGTGTAACCGAAGTGA	307	60.07
	REV	GCTCTCACTGTCAGCCTCATGG		62.93
GABRA2	FWD	AGACCAGGACTGGGAGACAG	335	60.25
	REV	TGCATTGGGCATTCAGCTTG		59.75
GABRA3	FWD	GACATTGGCGGGCTGTCTC	282	61.11
	REV	TGGAAGGATCTTCATGGGGC		59.45
GABRA4	FWD	TTGAAATTCGGGAGTTATGCCT	201	58.04
	REV	CCGTCTGAGGTGGAAGTAAACC		60.61
GABRA5	FWD	GCACCAGCACAGGCGAATAC	271	61.98
	REV	TAGCACACGGCTATGAACCA		59.10
GABRA6	FWD	TTTGGGCCCGTGTCAGATGT	265	62.06
	REV	CAGCATTGATGGTAAGCCTCATGG		62.05
GABRB1	FWD	GATATGACATTCGCTTGCGGC	337	60.40
	REV	GCTGTGGTTGTGATTCGGA		58.07
GABRB2	FWD	GCAAGGGTGGCATTAGGAAT	381	58.22
	REV	GTCCATCGTATACAGAGAGAAATCG		58.92
GABRB3	FWD	GGTTTCCGAAGTCAACATGGATT	388	59.30
	REV	CACTCCGGTAACAGCCTTGT		59.96
GAPDH	FWD	CGGGAAGCTTGTGATCAATGG	357	59.60
	REV	GGCAGTGATGGCATGGACTG		61.38

Supplementary Table 4. Table of Two-way ANOVA conducted between all cell lines and subunits. Shows significance level, % of total variation, P-value and significance.

Two-way ANOVA	Ordinary			
Alpha	0.05			
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**
Row Factor	33.50	<0.0001	****	Yes
Column Factor	43.00	<0.0001	****	Yes

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Investigating the expression and role of chloride ion channels in diffuse intrinsic pontine gliomas; A review of the existing literature

Introduction

Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive glial brain tumour that is found in the pons region of the brain stem. This type of brain cancer accounts for 10% of all childhood central nervous system tumours and is usually diagnosed in children of five to ten years of age (1). DIPG is commonly diagnosed using magnetic resonance imaging (MRI) due to its characteristic appearance and location in the pons (Figure 1) (2). This MRI is a good example of the location and extent of the tumour in a child's brain. It shows the tumour in the Pons extending superiorly into the midbrain and inferiorly into the Medulla Oblongata.

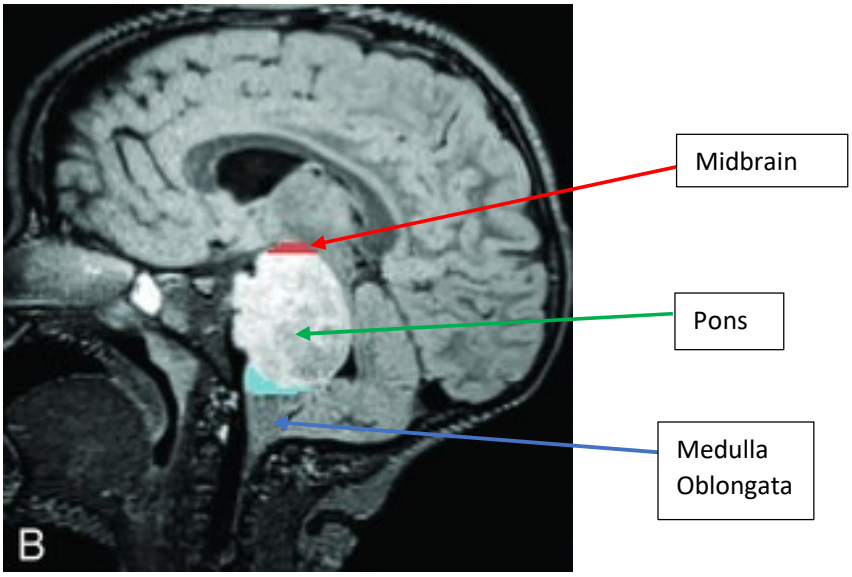

Figure 4: MRI showing the brain of a patient with a DIPG tumour extending into the midbrain and Medulla Oblongata (2).

The prognosis for many childhood brain cancer types is poor but worse in DIPG. The median survival for children with DIPG is <1 year and <2% of patients survive past 5 years (3). Comparatively, children with other central nervous system tumours have a five-year survival of approximately 70%

(1). This difference is due to significant advancements in the treatments of other central nervous system cancer in comparison to DIPG (4). Other central nervous system tumour survival rates have improved from between 40% and 60% in the 1970's depending on age group and tumour type, to over 70% in 2021 (4). However, in the case of DIPG, prognosis has not improved in 30 years (3). Whilst research has improved our understanding of the disease mechanisms and molecular characteristics of DIPG, this has failed to translate into improvements in patient outcomes.

This literature review relates to the hypothesis that expression of γ-aminobutyric acid (GABA) receptor subunits on DIPG cells may contribute to treatment resistance by exploiting cellular plasticity. This hypothesis will be tested by investigating the GABA family member subunits α1-α6 and ß1-ß3 defined as GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRA6, GABRB1, GABRB2 and GABRB3. This review describes the characteristics of DIPG and potential therapies, ion channels and plasticity in DIPG, cancer cell types in relation to DIPG, and therapeutic development using ion channels.

Diffuse Intrinsic Pontine Glioma

Diagnosis and Classification

A major difficulty with DIPG diagnosis in the past has been the ability to differentiate DIPG from other tumours on MRI imaging. Previously, diagnosis was based solely on MRI due to the location of DIPG, its relation to vital structures such as the brainstem and the diffuse nature of the tumour (5, 6). Up until 2016 biopsies were not performed due to the associated high risk of permanent disability or death (5, 6). However, in the last 5 years, improvements have been made to the brainstem biopsy protocols such that biopsies are now considered to be safe (7, 8). As such, biopsies are now used to confirm the diagnosis through histopathology or histone mutation analysis (9). Biopsies can be an important diagnostic tool and are used in other cancers such as gastrointestinal cancer (6, 7, 10). DIPG has a diverse histological spectrum, which can be similar to other types of

gliomas (7). Biopsies are important to confirm diagnosis but offer more help in identifying histone mutations and providing prognosis thereafter.

More than 60% of DIPG tumours contain a heterozygous mutation at genes that code for Histone H3 proteins, which can be identified in biopsy samples and aid in diagnosis (11). Specifically, these mutations replace the lysine at position 27 of histone H3 with a methionine (K27M), which affect the epigenetic regulation of gene expression (12-14). There are two relevant variants of this mutation: H3.3 (H3F3A) and H3.1 (Hist1H3B) (14). H3 K27M-mutant diffuse intrinsic pontine glioma is a type of DIPG identified quite recently (15). Diffuse intrinsic pontine glioma has now been reclassified as diffuse midline glioma (DMG), but DIPG still remains a commonly used and clinically relevant term. The presence of this mutation is associated with a much poorer prognosis (14). Additionally, Histone H3 wild-type tumours that overexpress EZHIP are less prevalent but also associated with a dismal prognosis (16). It has been found that a previously identified target mutation K27M-H3.1, would lead to increased rate in fatality as well (7).

Animal Models

Animal models, pre-clinical models, human cell lines and xenografts have improved the study of DIPG. Human primary tumour cells can be directly modelled and engrafted *in vitro* and engrafted into animal models to generate, *in vivo* patient-derived xenografts. These valuable tools can more accurately recapitulate DIPG as a whole and reflect the unique genetic and molecular make-up of each patient's DIPG tumours, in comparison MRI's and previous methods (8, 17). This has been made possible after the developments in biopsies (6-8, 10, 17).

The generation of mouse models for research has advanced. Traditionally genes were targeted by homologous recombination in embryonic stem cells (18). Current methods have progressed to enable allele-specific manipulation in zygotes (18). In the creation of DIPG models as well as others, *in utero* electroporation (IUE) has been used (19). Specifically, in DIPG the IUE method was used to transfect neural stem cells in the developing brainstem of mice. A combination of different growth

factors and DIPG histone mutations such as H3.3K27M are used to induce the DIPG tumour growth (19). More advanced and detailed DIPG mouse model generation has greatly improved DIPG research.

Treatments for DIPG

Current Therapy

Radiation Therapy

The only current treatment for DIPG is radiation therapy. While numerous chemotherapy strategies have been trialled over the years, they had little to no success (5). About 70–80% of patients respond to radiation therapy with improvement in their symptoms, and these improvements typically last three to eight months (1, 20). More than half will exhibit side effects from the steroidal treatment that is often administered alongside radiation therapy (20). Although this reduction of symptoms would seem positive, it is short-lived and there are detrimental effects on neighbouring healthy brain tissue and regression following treatment (20). After completion of radiation, in all most all cases there is progression of the cancer with evidence of local recurrence within 3–6 months (20). This shows that radiation treatment has a limited positive effect on patient outcome.

There are numerous variables contributing to the difficulty treating DIPG. The first is the cancers very close proximity to the brainstem which renders surgical removal close to impossible. The second is the frequency and speed with which DIPG tumours develop resistance to radiation (20). There are some DIPG treatment strategies being evaluated in early clinical trials and it is theorised that combination therapeutic approaches will be necessary for improving prognosis for DIPG in the future (21). This means that patients are still being treated with single radiation treatment regimens as this is still the only current standard (22). This suggests that a consensus of international guidelines for the treatment of DIPG is needed as well as more collaborative clinical trials globally in order to advance DIPG research and survival (22). The resistance to radiation and challenges surrounding the treatment shows the need for newer therapies.

Potential Therapy

At the time of writing (Aug 2021) a total of 95 clinical trials investigating DIPG have been registered (23). Of these, only a small number had study results available, and none reported an improvement in prognosis (23). There are a number of Australian clinical trials currently ongoing. However, with the rare and lethal nature of DIPG there are still more clinical trials needed and the necessity of combination therapy has also been bought to the forefront (23). Epigenetic and DNA modification therapy in DIPG has been showing promise (24). Combination therapy also applies to epigenetic therapies and is becoming extremely important, since regular single action therapies continue to fail. It is hoped that these therapies, in combination with expanding knowledge of epigenetic changes in DIPG (including histone mutations) will result in a better prognosis for DIPG (24). The results of clinical trials will determine the success of therapies such as ion channel therapy.

Targeted Therapies

Targeted therapies block the growth and spread of disease by interfering with specific cellular pathways. An example of targeted therapy is Herceptin that specifically blocks the human epidermal growth factor receptor 2 (HER2) on breast cancer cells, thereby inhibiting this growth signalling pathway and effectively treating HER2-positive breast cancers (25). For many other cancers there are successful targeted therapies available, however this approach has failed in DIPG.

Furthermore, targeted therapies, have failed most brain cancer clinical trials with none providing any significant improvement in disease-free survival (26). For example, kinase inhibitors targeting receptor tyrosine kinases such as EGFR has so far failed to prolong overall survival of patients with DIPG and other high grade gliomas (26).

One possible reason is the ability of brain cells to adapt to different stimuli and conditions by altering cellular pathways (27). This phenomenon is known as cellular plasticity (27). Neural cells such as glia have the ability to change their morphology or function in response to stimuli making them uniquely plastic (28). Overcoming this cellular plasticity is an important step to prevent treatment resistance

and improve survival. Other targets and treatments including histone mutations therapy, and chemotherapies have all failed (29). This could be due to the agents used, their inability to cross the blood brain barrier (BBB), or the presence of redundant pathways within the tumour (20). This has started investigation into other possibilities such as glial cell plasticity and the influence of ion channels in this process.

It is hypothesised that this plasticity is being driven by a special type of trans-membrane protein called ion channels. Ion channels are pore forming proteins that are found in the cell membrane and allow small, charged molecules (i.e., ions) to pass in and out of the cell. Neural cells possess more ion channels than other cells types, including voltage-gated sodium, calcium, and potassium channels, and ligand-gated ion channels such as chloride channels (30). The GABA (gamma aminobutyric acid) receptor family are a functionally important ligand-gated chloride channel family and are the focus of this research. These ion channels could be the key to unlocking the problem of treatment resistance that we currently face.

Mutation Specific Therapy

Genetic mutations such as the H3 K27M mutation currently represent a poor prognosis but they may also provide a novel target for therapy. Convection-enhanced delivery (CED), is a drug delivery technique that bypasses the blood-brain barrier (BBB). This occurs by locally applying a pressure differential to drive fluid flow throughout the tumor (31). The safety of this delivery technique in patients is supported, with a trial showing safe delivery of a radioimmunotherapy agent targeting the glioma-associated B7-H3 antigen (31). Further development of histone treatments is warranted and might offer hope to DIPG patients with Histone H3 mutations (31).

DIPG has been characterised as having a high prevalence of the H3K27M mutation (15, 32). Through a single agent and combination drug screen performed on a human DIPG cell line, a combination of panobinostat and marizomib treatment looks promising for future research and clinical trials (32). Even so, the *in vivo* efficacy in patient derived xenograft models is significant but only modest with

an increase in survival of ~20% (32). Prolongation of survival is important, and ultimately, curative therapy for DIPG will likely use combination therapy (32). This is important as our current research is aiming to develop a combination therapy that includes ion channel therapy.

ONC201, which is a small molecule selective antagonist of dopamine receptor D2/3 (DRD2/2), has been trialled as a treatment for DIPG tumours harbouring a K27M-H3.1 mutant type DIPG (15, 33). This is because this mutant type tumour expresses a dependency on DRD2 as a downstream epigenetic consequence of the mutation. ONC201 targets this vulnerability as a selective DRD2 antagonist can cross the blood-brain-barrier (15). Positive clinical outcomes in patients have been observed following the ONC201 treatment. These provided initial supportive evidence that targeting the H3 K27M-mutant with ONC201 could be a potential breakthrough and is robust for further testing (15). Whilst ONC201 has shown some promising early results, the trial only used single patient experiences in a heterogenous cohort, had limited follow up time of about 50 weeks and 28.6% of patients had undergone radiation previously which could confound the result (15). These variables could lead to a problem interpreting these results. Comparatively, another treatment investigated a mutation in the gene encoding protein phosphatase 1D (PPM1D) (33). The tumour mutation is found in only 9%–23% of DIPGs and is a reoccurring truncating mutation (33). It was found that when PPM1D is inhibited it also became sensitive to PARP inhibitor (PARPi) treatment (33). Reduced cell viability and induced cell apoptosis was observed, clearly showing the PARPi sensitivity (33). This together showed that a treatment combination of PPM1D inhibitor and PARPi is shown to be effective in treating PPM1D-mutant DIPG (33). The PARPi study did not have the same limitations as the ONC201 study. In this case *in vitro* results are promising and clinical *in vivo* trials are ongoing (33).

DIPG treatment and therapy conclusion

Several different approaches have been undertaken to find an effective therapy for DIPG. Several clinical trials of various systemic therapies have been tested or are ongoing (23, 34, 35). However, at

the time of writing (Aug 2021) they have not increased the survival of children with DIPG (34). One of the key challenges facing DIPG treatment is the difficulty in getting treatments across the blood-brain barrier. However, convection-enhanced drug delivery systems have shown some promise and may be able to help overcome this challenge.(36). A limitation to all therapies seems to be the availability of biological samples including generating cell lines for *in vitro and in vivo* testing as well as getting sufficient patient numbers for clinical trials. However, some progress with targets has been made. The majority of progress has been made in histone mutation treatment although there is still a lot of future research needed. Overall, there have not been any improvements in DIPG treatment success overall despite many trails over several decades, although recent studies have shown some promising results.

Ion Channels in DIPG

Ion channels are pore forming proteins that let ions pass across the cell membrane. An important feature of ion channels is that at rest they are tightly closed and impermeable, but they are opened by different changes occurring in the cell membrane (37-39). In terms of structure, they have two main components: a selective filter, and a gate (37-39). One major type of gated ion channel is the ligand-gated ion channel (37, 38). Ligand-gated ion channels include neurotransmitter receptors such as the GABA-A receptor (37-39). When the correct ligand binds to the ligand-binding site it triggers the gating of the channel (37, 38). Ligand-gated ion channels are the focus of this research.

DIPG cells are derived from glial cells

In the healthy brain, glial cells play an important role in regulating neural plasticity and keeping the central nervous system (CNS) functioning. The human CNS is normally made up of approximately 86 billion neurons and a similar number of glial cells (40). These cells communicate with one another through connections called synapses and the human brain typically possesses several trillion to quadrillion synapses (40). Two important types of glial cells in the CNS are: astrocytes, and oligodendrocytes. Oligodendrocytes form the insulating myelin sheath around axons and astrocytes

provide nutrients and support (41). Myelin is important since it enables nerve cells to transmit information faster and allows for more complex brain processes (41). These properties could make oligodendrocytes very important in glial plasticity and could contribute to cancer resistance by aiding cellular communication.

DIPG tumours originate in the pons and midbrain, where they continue to proliferate and invade surrounding healthy brain tissue. In DIPG cell lines, single cell sequencing of patient derived DIPG cell lines has suggested that tumours primarily contain oligodendrocyte precursor cells (42). Although the glial cell types that DIPG originate from are not known, this research would suggest it is a type of glial cell with oligodendrocyte lineage (43, 44). Regardless of the specific lineage, single glial cells are known to acetylcholine receptors and chloride ion channels.

GABA-A receptor and chloride ion channel structure and mechanism

GABA-A receptors are present on a class of ligand-gated ion channels (38, 45). The GABA-A receptor is one of the major inhibitory neurotransmitter receptors in the human brain (46). The GABA-A receptor is a pentamer and belongs to the Cys-loop receptor superfamily. It is comprised of several possible subunits including alpha 1 (α1) - α6 and beta 1 (ß1) – ß3 (39, 47). The genes that encode these subunits are defined as, *GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRA6, GABRB1, GABRB2,* and *GABRB3* (47).

Every permutation of this receptor consists of five subunits, usually two alpha, two beta and one other, surrounding a central chloride ion-selective channel, which is gated by the inhibitory neurotransmitter GABA (Figure2) (46, 48). In the brain specifically, these receptors have a very diverse subunit composition (45). A wide range of clinical therapeutic drugs for other conditions are known to bind to sites found on GABA-A receptors, which could be useful for the current study approach (45). The specific subunit composition of GABA-A receptors in DIPG and their mechanism of action in DIPG cells will the choice of drugs used in a therapeutic approach targeting these ion

channels. The expression of these genes and the GABA-A subunit composition in DIPG is currently unknown.

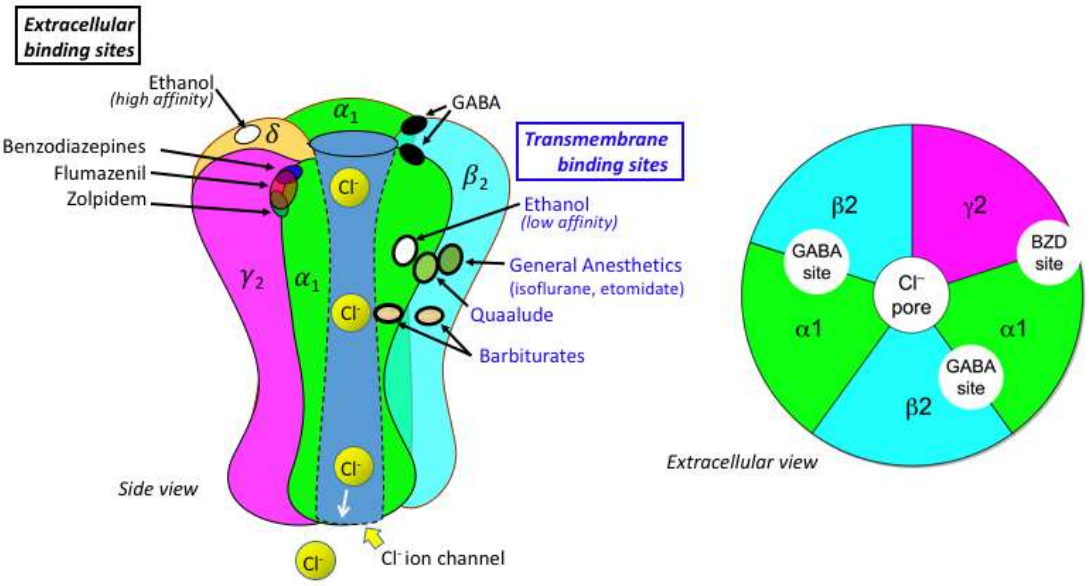

Figure 2: The side view shows the structure of the GABA-A receptor-chloride channel complex and indicates the positions of the binding sites for GABA and some drugs that inhibit this channel. The Extracellular view gives a "top" view of the GABA-A receptor, illustrating the most common pentameric combination of α1, β2 and γ2 subunits (48).

Although a number of different experimental approaches have been used over the years, treatment is challenging due to the complex pharmacology and mechanisms of these types of receptors (45). Firstly, the agonist GABA binds to its two binding sites (as shown in Figure 2, side view), which is then followed by a conformational change that keeps the GABA in its binding site (39, 46). After this, further conformational changes lead to an opening of the ion channel pore that allow the chloride ions to move through (39, 46). Genetic mutations and structural alterations to the subunits of the GABA-A receptor could trigger disease and may contribute to treatment resistance (39, 46). Targeting these channels could lead to therapeutic targets.

Ion channel subunit arrangement could pose an interesting and potentially impactful proposition. Currently, ion channel subunit arrangement is still thought to assembe in a fixed stoichiometric arrangement and this is due mostly to the functional responses to GABA (49, 50). With that in mind, this means that any combinations of subunits that do not possess the beta and alpha subunits would be scored as non-functional (49). These arrangements could still produce viable surface receptors for other ligands and have some kind of functional effect or act as a sort of sponge and prevent these ligands binding to functional receptors (49). This could lead to the possibility that other non-GABA binding configurations might also be functional (49). This is especially significant for ion channel identification and targeting in cancer therapy as all functional channels will likely need to be treated.

Since GABA is an inhibitory neurotransmitter, GABA-A receptors mediate inhibition in the adult and foetal brain (51, 52). This inhibits nerve transmission and thereby reduces neural excitability (53). Very little is known about the structure, mechanism and interaction that is occurring at various channel conformations (51). Problems with their function can lead to a variety of disorders, which are mainly treated with allosteric modulators (52). The GABA-A receptors are some of the biggest target receptors for major classes of drugs including benzodiazepines, ethanol and general anaesthetics (54). Existing modulators have lacked specificity, so the focus of more recent research has been to develop more specific drugs.

Ion channel expression in the healthy brain and DIPG

The expression and localisation of the different GABA-A receptor subunits is important since it could lead to a functional difference between receptors with different subunit composition. It is predicted that DIPG tumours originates from an oligodendrocyte lineage, and indeed the subunits of GABA-A receptors are predominantly expressed in the central nervous system (39, 46). It is important to note that the GABA-A receptor is also expressed throughout the rest of the body including the pancreas, gastrointestinal system, ovaries and breast for example. Some of the subunits have a very broad expression while others are more localised. GABA-A receptor subunit α6 for example is localised to

the cerebellum while the other alpha subunits are broadly expressed throughout the brain (46). The expression localisation as well as intensity of subunits could be very important as different drugs bind to the interfaces between different subunit arrangements.

My research project is focused on subunits α1-6 and ß1-3. This was based on the analysis of raw DIPG RNA-sequencing data, downloaded from the cBioPortal for Cancer Genomics (Accession: 10.24370/SD_BHJXBDQK) by computational biologist Dr. Anya Jones (55). A main finding of the analyses conducted on this bioinformatic data concluded that the α1 and α6 subunits were not strongly expressed (55). According to the literature the α6 subunit is only expressed in the cerebellum (46, 56). Since DIPG tumours have a diffuse nature and are located close to the cerebellum it is plausible that DIPG tumours located in the cerebellum express the α6 subunits. A study that conducted RT-PCR analyses did not identify mRNA expression of either the α1 or α6, subunit mRNAs in oligodendrocyte progenitor cells (57). This would align closely with cBioPortal data and could produce interesting research in whether location of tumour or type of glial cell holds a higher priority of subunit expression.

Expression of these subunits can also vary during brain development. Most research on the expression of these subunits in the human brain has been conducted in the adult brain. However, GABA-A subunit expression is known to be regulated dependent on development (58). The α2, α3, α5, and β3 subunits are predominately present during early development (58), while the α1, α4, and β2 subunits are seen in adult brain (58). These changes are due to GABA transitioning from being depolarizing to hyperpolarizing (58). Interestingly, the α5 subunit is found mainly in the hippocampus in the brain (59, 60). In mice the lack of the α5 subunit of the GABA-A receptor improves spatial learning and memory, indicating GABA-A activity modulates synaptic plasticity (59, 60). The expression of α5 is of particular interest due to this reported role in plasticity and because DIPG tumours occur in the developing childhood brain.

Preliminary Laboratory Analysis on GABA-A subunit expression in DIPG

Recent DIPG RNA-Sequencing data analysed by computational biologist Dr. Anya Jones has supported the value of ion channel research in DIPG (55). The analysis identified a large number of ion channels are expressed in DIPG tumours, and many of these are differentially expressed between DIPG and paired normal brain samples. Of particular note was the GABA chloride channel, with several GABA-A receptor genes being expressed in DIPG tumours. The findings showed that the subunits of the GABA-A receptor are differentially expressed in patient tumours. This is important to investigate as all of the alpha and beta subunits were expressed in both data sets. Figure 3 shows the preliminary RNA sequencing results of two data sets of GABA-A subunit expression in counts per million from which these results were drawn from (61). The figure also shows the averages, ranges and outliers of each subunit in counts per million Although this current research will study all subunits, the alpha 2,3,5 as well as beta 1 and 3 subunits were all strongly expressed and are of particular interest.

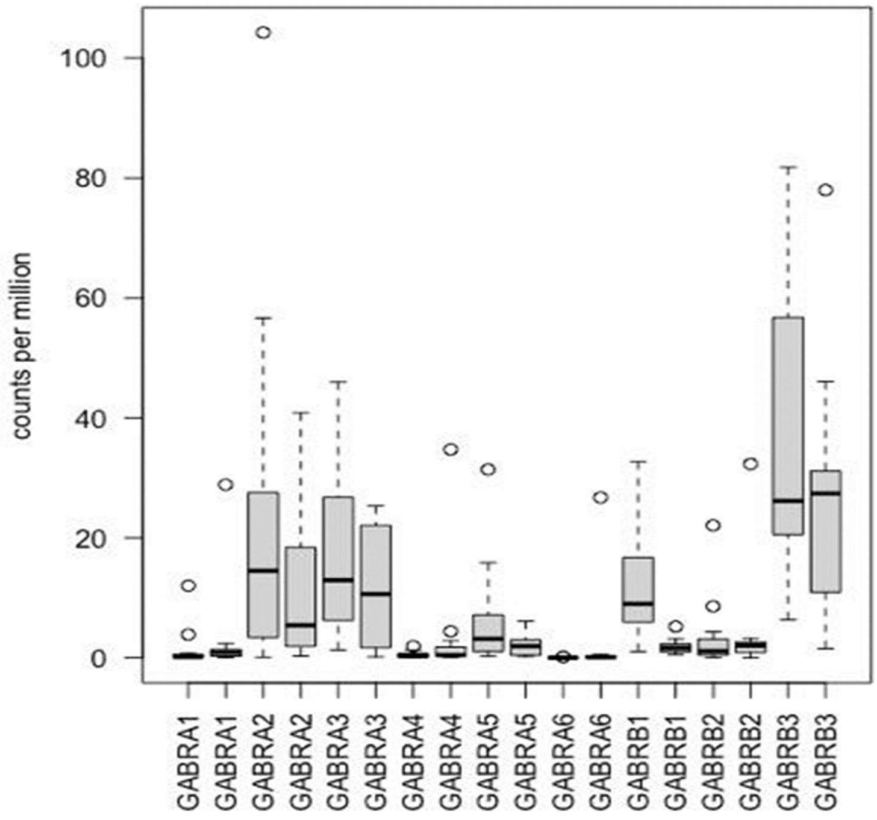

Figure 3: Preliminary RNA Sequencing Data in two data sets showing specific GABA subunit expression in counts per million. The figure shows the average count per million, ranges for each subunit and outliers (61).

Ion channels in Cancers

Research has been conducted on acid sensing ion channels ASIC1 and ASIC3 (62, 63). It identified these ion channels by detecting transcripts of the *ACCN2* and *ACCN3* genes which code for ASIC1 and ASIC3 (62, 63). Western blotting has been used confirm the presence of these ion channels in different brain areas including glioblastoma cell lines (62, 64). The identification of *ACCN2* and *ACCN3* expression could improve survival of patients suffering from gliomas (62). This is important as it shows that a similar method including Western Blotting could also identify GABA-A subunit expression. If this is the case there is a possibility that GABA-A subunit expression could improve patient survival in patients suffering from DIPG tumours.

The cellular processes which drive cancer severity are cell proliferation, metastasis and apoptosis, and treatments typically aim to halt these processes. However, these pathways can also play important roles in physiology in a healthy body. Cells can either be at a non-dividing quiescence stage or proliferating (growing and dividing) in accordance with the cell cycle (65). Humans need continuous cell proliferation throughout their lives in most cell types and for development, maintenance and repair (66). Cells proliferate when needed, but also there are key checkpoints in place to trigger apoptosis when required (66). Apoptosis is the process of programmed cell death (67). The failure of this process and cells proliferating uncontrollably leads to cancer (66). Stopping this abhorrent proliferation in cancer is a key treatment strategy, and ion channels could be important in this process. Apoptosis is vital in normal cell turnover and typical development of the human body (67). Irregular apoptosis (either too much or too little) can lead to many types of cancer including DIPG (67). The ability to control cell apoptosis could be an ideal therapeutic target and is directly related to proliferation and metastasis in DIPG (67).

There is a large existing body of evidence on the contribution of ion channels to the growth of cancer and the malignancy of tumours (68, 69). However, this research has not been specific to DIPG, most research has studied ion channels in cancer overall (68). Membrane ion channels are essential for cell proliferation and they appear to play a large role in the development of cancer (68-70). The role of ion channels in brain cancer cells is an ongoing topic of research, and it is important to complete more studies on the primary tumour. There is still a lack of specific blockers for many of the ion channels related to cancer (68). One of the biggest goals at the moment is to identify drugs and therapies that inhibit or activate ion channels in brain cancer. By increasing this research, it should be possible to treat cancer by manipulating ion channel activity and provide an avenue to treatment (68).

Although ion channels can contribute to tumour growth through promoting proliferation, they can also be involved in apoptosis and necroptosis (71, 72). However, the precise role of ion channels in apoptosis is not well understood (71). The same channels could support regulated death on one hand, but could cause cancer development on the other (71). Two of the major pathways of apoptosis are the extrinsic and intrinsic pathways which are known to have occurred in tumour metastasis and could be largely influenced by chloride ion channels (71, 73). Overcoming cancer cells' resistance to apoptosis that could be mediated by ion channels has been one focus of tumour therapy (73). A great example of this are studies that show that Apo2L/TRAIL can induce apoptosis in cancer cells while not harming normal cells (74, 75). Apo2L/TRAIL is part of the TNF gene superfamily and is a ligand that induces apoptosis through engagement of death receptors (74, 75). Unfortunately, Apo2L/TRAIL treatment has failed to induce apoptosis in DIPG cells, which highlights that further research into the biology of DIPG is required.

Another key process in cancer is metastasis, whereby the disease spreads from a primary site to a different part of the body and it is important to look what impact ion channels have in this process. Data suggests that metastatic cancer cells adapt to treatment after exposure to a therapy agent such

as radiation (76). Whilst there is no published data regarding the role of ion channels in brain cancer cell metastasis, they have been shown to play a role in the metastasis of other types of cancers. For example, GABA-A receptor signalling was shown to play a role in the metastasis of colon cancer cells, and blocking this pathway with a GABA antagonist, Nembutal, significantly inhibited metastasis in *in vitro* cell proliferation assays (77). Therefore, blocking these ion channels in DIPG cells could have a similar effect and inhibit cancer metastasis.

Lastly, it is important to note that chloride ion channels and GABA-A receptors have been identified in numerous different cancer types including gastrointestinal, ovarian, breast, and pancreatic cancers (10, 78-80). GABA-A receptors and chloride ion channels are seen as biomarkers in these cancers (81). GABA can be measured through a blood test but specific receptors will need to be investigated via immunostaining of a tissue biopsy or fluorescent antibody assay. GABA-A receptors are seen to aid proliferation of the various cancers around our body, aid in metastasis, cancer progression, and apoptosis. The role of ion channels in cancer is an area of active research, and the current project will specifically examine the expression and role of GABA-A receptors in DIPG cells.

Conclusion

This review summarised the existing research into DIPG, a highly aggressive glial brain tumour. The prognosis for most childhood brain cancers is poor but DIPG accounts for approximately 10% of all childhood central nervous system cancers and the median survival is less than one year. Despite the clinical trials, survival hasn't improved. This might be due to ion channels, which can be dysregulated in cancer and lead to proliferation, metastasis, and treatment resistance. With that in mind this review focused on a number of key points; the severity of the disease, its incredibly low overall survival rate, and the inability of research to improve this outcome over past decades showed the overarching significance of current research. The large impact biopsies and animal models have had on our diagnostic standards and protocols was also covered. Subsequently, the ineffectiveness of radiation has been the major issue facing DIPG research and while potential therapies such as

targeted therapy and histone modification therapies have been trialled, none have provided conclusive improvements in patient outcome. This led to the exploration of ion channel structure, function and the hypothesis that ion channels could be the source of the problem that research is currently facing. With the significance and usage of these channels in other cancer types and the similarities of previous research with unpublished ion channel data in DIPG there seems to be an area of potential research. GABA-A receptors in particular with their functions in normal brain physiology could hold potential answers for this line of research. GABA-A receptors have not been previously studied in DIPG and to identify their subunit expression in the disease would not only provide important novel research but could lead to the development of future therapeutic targets for DIPG.

More research is clearly needed to determine the expression and role of ion channels in DIPG/DMG, and whether they represent a novel therapeutic target. As such, my project aims to characterise GABA-A receptors in patient derived DIPG cell lines. The specific aims of this project are: 1) characterise the mRNA expression profile of the GABA family members *GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRA6, GABRB1, GABRB2* and *GABRB3* in DIPG patient derived cell lines; 2), investigate the protein expression of GABA ion channels in DIPG patient cells and tumour tissue samples;, and 3) determine the effects of targeting these channels on cellular characteristics such as viability and proliferation. I hypothesize that DIPG cells will express GABA receptor subunits and these may be contributing to their resistance to current therapies.

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