5-HT2A receptors and their role in neuronal development

209005657

Dr Volko Straub

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Contents

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1.0 Abstract

Introduction

The study of serotonin has been ongoing for more than 50 years. Its dysregulation has been liked to multiple disorders ranging from Alzheimer's to Autism (Jauhar, Cowen and Browning, 2023). This monoamine has many roles within the body acting as a neuromodulator or transmitter as well has a hormone. It has may properties that make it a desirable molecule to study but its neurotrophic properties are some of the most interesting and highly researched areas. While some of its receptors have been studied in quite some depth the 5-HT2A receptor has not been at the forefront of research until recently. This study was conducted in an attempt to bridge the gap in knowledge surrounding this particular serotonin receptor.

Objectives

This study aimed to identify effects of activation of the 5-HT2A receptor. This was done through monitoring of growth cone mobility and total neurone length when treated with varying concentration of 5-HT2A agonist.

Results

There was found to be a significant difference in the growth rate and neurone length in lower concentrations of TCB-2 (5-HT2A receptor agonist). However, there was no significant effect on growth or length when looking at all the concentrations.

Conclusion

This study shows promising results that suggest a possible link between the activation of 5-HT2A and the growth of neurones. It also provides logical evidence to suggest a potential role of the 5-HT2A receptors involvement with neural development.

2.0 Introduction

2.1 Serotonin

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine that has played an important role in many physiological processes. It acts as a hormone, neuromodulator and neurotransmitter. The role of serotonin stretches from the regulation of hunger and reward systems to cognition, memory and learning (Zhang and Stackman, 2015). Most commonly serotonin has been understood as a hormone that is active in the role of producing the feelings of happiness. The dysregulation of serotonin has been connected to the development of disorders such as depression and anxiety and hence modern medicine uses

inhibitors of serotonin reabsorption (SSRI) in therapeutics to increase serotonin levels in individuals (Hyttel, 1994). However, more recent studies have shown that alone the levels of serotonin are not a comprehensive therapeutic and other avenues must be explored (Jauhar, Cowen and Browning, 2023).

Similarly, inhibitors of serotonin reuptake have been associated with the increase in grey matter in patients with depression concluding the theory that SSRIs help to rejuvenate neuroplasticity in these patients (Kraus et al., 2017). Though the size of grey matter is not directly correlate to the increase of neuroplasticity volume and density has been linked to influence of neuroplasticity (Mercadante and Tadi, 2023). Brains with higher volumes of grey matter have more neurons and synapses that provides a richer foundation to enable the formation and adaptions of neural networks (Albert, 2019). The correlation between 5-HT and neuroplasticity is essential to the understanding of the mechanisms of adaptation and development of the brain.

The significance of 5-HT in plasticity also underpins its role in learning and memory of humans. In 1997 Yan, Wilson and Haring showed the neurotrophic behaviours of 5-HT, that when 5-HT was depleted in the hippocampus shows a reduction in synaptic density. This identified a possible property of 5-HT was its neurotrophic effects in the brain. Alongside this, neonatal studies showed a reduction in the number of dendritic spines but did not affect the total length of the dendrites. This effect is not seen across all brain regions as for example studies of the olfactory bulbs concluded it was unlikely serotonin had a role in the cellular development of that region (McLean and Darby-King, 1994).

2.2 5-HT in brain development

It has been suggested that 5-HT has a fundamental role in neurodevelopmental processes. The development of the rodent brain is influenced by 5-HT that has been derived from the mother's placenta before the brain is able to produce its own. In rodent brains it has been identified that by the equivalent of the second trimester of a human pregnancy (up to 10th postnatal day in a rodent) the transcription of the necessary genes, such as the TPH2 (tryptophan hydroxylase 2) encoding gene, for 5-HT production is occurring in the raphe nuclei (in the hindbrain). Serotonergic neurons are then formed extending their projections throughout the brain to terminal regions such as the prefrontal cortex, hippocampus and amygdala. The growth stage of these neurones can last lasts until the birth of the rodents. For a short period of time during fetal development there is fleeting expression of 5-HT in sensory regions of the brain and subsequent thalamocortical radiations (Gaspar, Cases and Maroteaux, 2003). This is found to be between the 14th (E14) embryonic day and the 16th embryonic day (E16). This transient expression could further indicate the involvement of 5-HT in neural development. Considering the numerous developmental effects of 5-HT it is highly likely that neural development is affected during the disruption of the 5-HT transporters. Evidently, in the postnatal stage of rodent's

development manipulations of the 5-HT levels can result in the malformation of sensory regions affecting motor coordination and sensory processing (Esaki *et al.*, 2005). This stage is comparable to the third trimester of human pregnancies (Zeiss, 2021).

Later postnatal development (post-natal day 10, P10, onwards) in rodents (1-4 years old in humans) is where much of the mass growth, synaptogenesis and maturation of the monoaminergic systems are completed (Maciag, Coppinger and Paul, 2006). Environmental factors in this period have a large effect on the overall refinement of brain systems circulatory. Changes to the levels in 5-HT during both pre and postnatal stages have been seen to affect behavioural habits in later life. Mice that were observed to have a mutation in TPH2 presented with deficits in social interactions and compulsive behaviours that were comparable to known traits of autism (Kane *et al.*, 2012).

2.3 5-HT receptors

There are seven families within the 5-HT receptors these are 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6 and 5-HT7. They are all G-proteins apart from 5-HT3 which are ligand gated iron channels. Many of them have sub-groupings (Frazer and Hensler, 1999). The most heavily researched receptor is, 5-HT1 with its subgrouping 5-HT1A being one of the first discovered receptors and being linked with the regulation of serotonin. Other subtypes include: 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F. while this family has been mostly found in the CNS (central nervous system), they have been found in the gut too. They have been linked to behavioural traits and disorders such as aggression and anxiety while also being involved in pain perception. Its initial link to such behaviours lead it to be the highly studied. The other receptors including 5-HT2 have not had as much research conducted on their functions. They are all largely present in the CNS apart for 5-HT4 which is found in the GI tract and so has been on interest in the recent studies linking the brain to gut. The most poorly categorised receptors are 5-HT5, 5-HT6 and 5-HT7 though they are known to be involved in modulation of mood and circadian rhythms. 5-HT2 has subgroups, 5-HT2A, 5-HT2B and 5-HT2C and is widely found in the CNS as well as the peripheral nervous system. Currently it is known to regulate mood, appetite and vascular tone. One very interesting property is its hallucinogenic effect produced by psychedelics like LSD through the activation of the 5-HT2A receptor.

2.4 Distribution of the 5-HT 2A receptor

Previously, research has uncovered that hallucinogenic drugs to be stimulants of the 5-HT2A receptor; this is likely due to the similarities found between the chemical structure of tryptamines, one of the three psychedelic groups, and serotonin. The properties of the serotonergic psychedelics include the stimulation of dendrite growth and complexity in a paper by Ly *et al.*, in 2018. Not only this but that 5-HT2A agonist ligands enhance cognition

and propagate hallucinogenic effects. This discovery is a positive incentive to suggest the connection of 5-HT2A receptors' role in neural development.

There has been significant evidence to suggest the involvement of serotonin (5-HT) in neural development. Studies such as Faber and Haring in 1999 link the limitation of 5-HT to be negatively correlated to the growth of dendrite in rat pups. Papers such as Rojas et al., in 2017, also report on the dual effect of serotonin on the growth of dendrites, specifically, they outline the shortening of primary dendrites when levels of serotonin are raised. This effect was initiated by the 5-HT1A receptor as well as shortening the growth of secondary dendrites with the activation of 5-HT7. But both of these receptors were also found to be activate the ERK pathway that is responsible for the outgrowth of dendrites. Consequently, this study was started in an attempt to uncover other possible associations like the 5-HT 2A receptor for signalling and neuronal developments.

5-HT 2A is classed in the G protein-coupled receptor (GPCR) A family but there have been recent investigations into the novel mechanisms used by this receptor. It was initially cloned in humans (Julius et al., 1990) after its previous cloning in rats (Prichett et al., 1988). Initial associations began with the numerous disorders that are associated with the receptor along with psychoactive effects appearing due to hallucinogens acting as agonists on the 5-HT 2A receptor. Blocking 5-HT 2A has been seen to reduce psychotic behaviours as well as depressive and anxious symptoms in preclinical studies. Hence high affinity agonists have been used to effectively treat the symptoms of schizophrenia.

5-HT 2A receptors are widely distributed in the central nervous system (CNS) including the cerebral cortex of the brain, both in rats and humans (Cornea-Hébert *et al.*, 1999). While it is also found in other areas of the rat brain such as the basal ganglia, entorhinal cortex and olfactory bulbs however, it is absent from the cerebellum. Similarly, human brains show very little expression of 5-HT 2A receptors in the cerebellum, brainstem and spinal cord, but high-density expression in the hippocampus and Nucleus acumens (NAc). It is thought that the 5-HT 2A receptor is primarily expressed in layer 5 pyramidal neurons and putative interneurons. Due to its presence on these neurons and interneurons it has been hard to predict their core functions as they have the ability to not only regulate the inhibition circuitry but also excitation pathways. 5-HT 2A were also found to be distributed on glutaminergic neurons in the cortex, cholinergic neurons and glial cells. Previous studies focused on immunolabeling have shown 5-HT2A to be localised dendritic spines postsynaptic ally though there has been evidence of presynaptic expression.

2.5 Signalling

5-HT 2A has a plethora of signalling pathways ranging from ERK pathway to phospholipase signalling via G protein dependant, ligand dependant or independent signalling. Commonly Ca^{2+} levels are raised by Gα q -PLC-IP3 signalling within the cells. 5-HT 2A activation

modulates calcium currents as well as intracellular calcium levels. 5-HT 2A is also expressed on glial cells again helping to mediate calcium influx the and the secretion of vesicles. The ERK pathway is often also triggered by 5-HT 2A activation working as a regulatory mechanism. Other pathways such as the β-arrestin2 and tyrosine kinase pathway, the former used as modulator of the PI3K/Src/Akt cascade which is a critical signalling pathway that regulates multiple cellular functions essential for cell survival, metabolism, growth and function. The activation of the 5-HT2A receptor binds with β-arrestin2 triggering this downstream signalling pathway resulting in the ERK pathway that communicates the extracellular signals to the nucleus. Dysregulation of the β-arrestin2 pathway has been associated with diseases including cancer and various neurological disorders. The tyrosine kinase pathway is also indirectly initiated by the activation of the 5-HT 2A receptors due to the interactions with the ERK pathway which results in Rac1 activation which controls cytoskeletal dynamics, cell adhesion, migration, invasion and proliferation as well as many other cell behaviours(Quinn *et al.*, 2002). These pathways can be seen in figure 1. Dysregulation of Rac1 signalling has also been linked to diseases such as cancer, cardiovascular disorders and also neurological conditions. Such neurological conditions include intellectual disabilities; such as ASD which is classed as a neurodevelopmental disorder (Sun *et al.*, 2021). Other conditions affected by both pathways include links to anxiety, depression and neurodegenerative conditions like Alzheimer's and Parkinson.

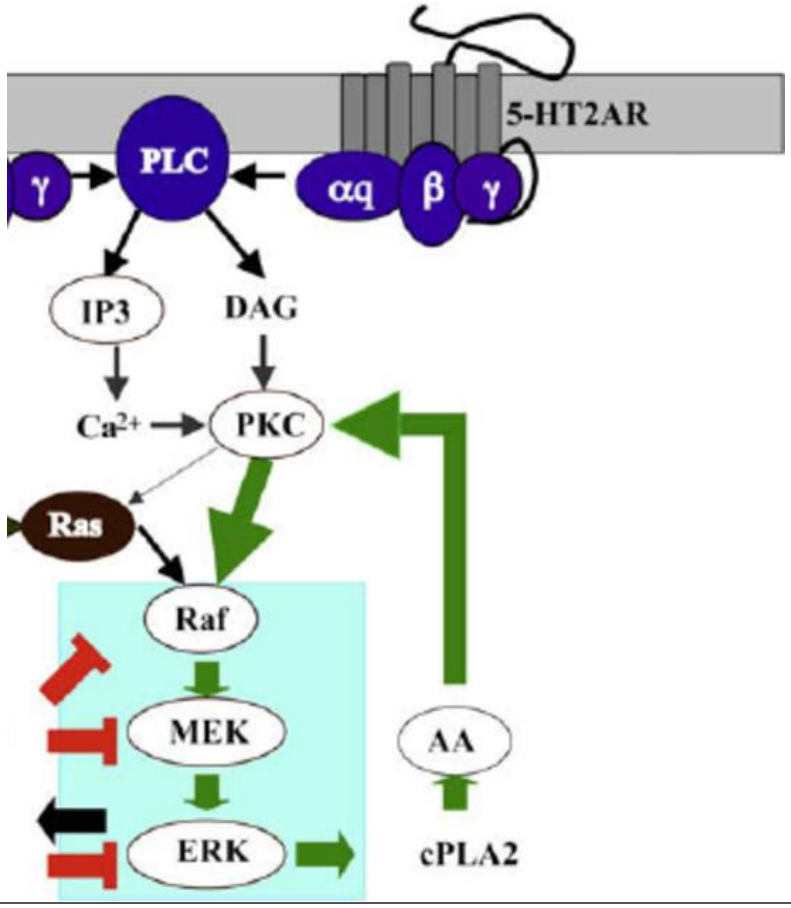

Figure 1 - 5-HT2A receptor signalling pathways - Adapted from Chang *et al.*, 2009. This figure shows β-arrestin2 binding to the receptor and setting off the cascaded reaction activating the signalling pathway of 5-HT2A receptor.

2.6 Importance

Understanding the role and of 5-HT 2A receptors is just a fraction of the total picture required to understand the mechanisms, functions and practicality serotonin has within species. 5-HTergic manipulations within key developmental stages could indicate links between behavioural differences and various development stages. Hence, knowing how each of serotonin receptors are involved in different aspects of serotonin function like its effects on neural development could help to demystify serotonin activity. Therefore, this study hypothesised the activation of the 5-HT2A receptor would have an effect on the growth of neurons using single cell cultures of mice cortex. Understanding any effect, the activation of 5-HT2A has on the growth of neurons could enlighten possible functions of the receptor on the development of neural pathways.

This study was done to identify the possible significance of the 5-HT 2A receptors' role in the neural development of mice. The study aims to look at the correlation between TCB-2, a 5-

HT2A receptor agonist, and the receptor observing the effects on the growth of neurons in the brains of mice. Specifically, the study looked at the effects of activation of the 5-HT2A receptor motility of neuronal growth cones and neurite growth in cultured cortical neurons.

3.0 Methods

3.1 Wet lab

Methods were adapted from protocols in Dr Volko Strabs' lab.

Plate preparation

For this project 24 well imaging and non-imaging plates were used. The non-imaging plates used 13 mm glass coverslips for cell adhesion.

Solutions

POLY-L/D-LYSINE - diluted stock solution to 0.05 mg/ml in cell culture grade water.
50 ml of culture medium - 38.8ml of neurobasal medium, 0.8ml of B27 50x, 400ul Pen/Strep 100x, L-Glutamine 200 mM 100ul.
20ml Horse serum solution - 18ml of neurobasal and 2ml horse serum. This was then swapped to BrainPhys in culture 2.

Method

The 24-well plate coating for cell adhesion was conducted in sterile fume hoods. When using coverslip plates the coverslips were washed in ethanol by dipping them into a tube of ethanol. They were then left on a tissue to dry. Using tweezers, the coverslips were placed into the wells of the plates ensuring all sit flat.

Both imaging and non- imaging plates had 400 µl of PLL (Poly-L-Lysine) (20x stock: 1 mg/ml stock solution in 0.15 M sodium borate buffer, pH 8.4) pipetted onto every coverslip or well completely covering the surfaces. Coverslip plates were checked for floating coverslips and tapped down if floating to ensure coverage. Then plates were left for 1 hour and washed with cell culture grade water three times in 5-minute intervals. Again, coverslips were pushed to the bottom of the wells so they were fully coated. All liquid was removed and left to dry in fume hoods to dry fully.

Preparation of mice brains - method adapted from Beaudoin et al paper in 2012.

Conducted in a laminar hood two mice brains (one female and one male) were dissected individually from a litter. They are collected from P1 to P3 (P1 - postnatal day one). The dissection is undertaken in a Petri dish filled with refrigerated Hibernate. The dish is placed

on an aluminium plate that has been kept in a freezer (ideally at a temperature of -20 degrees).

Using a scalpel, tweezers and scissors the skin first and then the skull were cut open. This was done in a straight line following the midline of the brain; from brainstem to the middle of the eyes. Then cut over the top of the eyes. This allowed the brain to be gently scooped out. Olfactory bulbs and meninges were discarded leaving the cortex of the brain to be chopped into small pieces. These pieces were collected and placed into a centrifuge tube. Once the tissue had settled the supernatant was removed. The tissue was resuspended in pre-warmed papain solution (2 ml per brain; 1 mg/ml in Hibernate medium). The samples would then be incubated in a water bath between 29 degrees to 32 degrees for 10 minutes agitating every 3 minutes. After which the supernatant is removed and 10% horse serum is added (~500ul). Both samples were left in the hood at RT for at least 4 minutes occasionally swirled. Once again, the supernatant was removed, careful not to remove any of the tissue, and resuspended in 1 ml of hibernate. The solution was then aspirated to break the tissue into smaller pieces. Titration continued 5 times removing the supernatant and collecting it in a new centrifuge tube; collecting ~10 ml.

The solutions were then centrifuged at 800 rpm for 8 minutes at room temperature. Again, the supernatant was removed for the sample to be resuspended in 2 ml of cell culture medium. The solutions were then passed through a 100 µm cell strainer. A cell count chamber was then used to determine the amount of cell culture medium needed to create cell suspension of 60,000-100,000 cells/ml. Place the cell suspension in the incubator while counting the cells.

Plating

The cell suspensions are then pipetted onto each prepared coverslip and/or well, fully covering the surface of the well. (~400 µl for 24-well plate wells). Each of the columns were treated with different concentrations of TBC-2 (5-HT 2A receptors agonist) so each concentration had four repeats per plate. This treatment was done anonymously to the researcher to remove possible bias. To store and incubate the plates were placed in a CO2 incubator (5% CO_2) at 37°C.

Fixation

Undertaken inside a fume hood. Defrosted 4% Paraformaldehyde and added to the wells after the cell culture is removed. This is left for at least 20 minutes and then removed and washed in PBS at 5-minute intervals three times. The plates were left in PBS as not to dry the samples out and for storage in fridges (4°C).

Immunochemistry

Primary antibody

The cultures were kept in PBS if steps could not be carried out immediately. The dilutions of the antibodies varied due to the particular antibody used. It was assured as a consequence of staining with multiple antibodies that each antibody was raised in different hosts as not to overlap staining.

The PBS solution was removed from the wells and replaced with ~200 µl of blocking solution (Triton X-100 (0.3%), goat serum (4%) in PBS). After leaving for 20 mins at room temperature the blocking solution was removed and replaced with 200 ul of primary antibody solution (primary antibodies of variable dilutions in blocking solution). The plates were then placed in a cold room at 4°C on a shaker overnight. The primary antibody was removed the next day and replaced with 200 µl PBS to be washed and repeated 3 times at 5-minute intervals.

Secondary antibody

Secondary antibodies were anti to the hosts of the primary antibodies used. The primary antibodies consisted of: Chicken antibody MAP2 (AB5543 EMD, LOT: 3795262, dilution 1:2000) and rabbit antibody for 5-HT2 (A307137, 1:200 dilution) and Mouse GAD67 (188 211, C/ 126G12, 1:250 dilution). Conjugates of the secondary antibodies were made to be different for each primary target.

PBS was removed and 200 ul of secondary antibody solution (goat serum (2%), Hoescht 33342 (LOT: 1156367) (10 mg/ml at 1:10,000), secondary antibodies (1:1000) in PBS) was added. This was left on a shaker covered to keep the plates in darkness for 90 minutes. The secondary antibody solution was removed and the wells were washed 3 times with PBS in 5-minute increments. The plates were left in PBS for storage at 4°C. Cells were stained with DAPI for viewing and a sample of < 10 µm counting in a counting chamber under the microscope.

Slide preparation

Some cell cultures, grown on coverslips, were mounted onto coverslips for long term usage. Each slide was labelled before affixing coverslips. Each slide had two coverslips placed on the glass slide. Slides were set flat with as few air bubbles as possible.

10 ul of Prolong Gold was placed onto the glass slide. Using tweezers, the coverslip was carefully extracted from the relevant well and dunked into dH2O. The edge is then placed against a paper towel to blot off the excess. The coverslip was held at an angle and gently lowered onto the drop of Prolong Gold. These were left flat to dry at 4°C.

3.2 Dry lab

High precision microscopes were used to capture the images. A Phasefocus Livecyte microscope was used to live image the samples. It enables imaging every thirty minutes of each of the 24 well plates and can be set to capture up to three sections of each well plate. The plate can be left in the microscope overnight or for long periods of time as it is an incubated chamber with CO2 ventilation to sustain the growth of the samples enclosed. The images can then be analysed after stabilisation of the images. The stabilisation is necessary to get accurate measurement later on. This analysis was done using ImageJ/FIJI using various plugin applications. For the live images a point selection was used to measure movement over the time of imaging. As seen in Figure 2, each point was measured and then added to regions of interest manager (ROI). Then the x and y coordinates were used to formulate the distance moved between points. The first calculation done for comparisons between the cultures was the mean total movement of growth cones. The microscope would take images every 30 minutes enabling the analysis of the photos in a time lapse stop motion system. Growth cones were identified and then tracked throughout the images by placing a point on the growth cone. Due to limited access to animal samples and therefore relying on an unpredictable breeding schedule of the mice, the number of repeats were restricted. Along with imaging issues when the live imaging shut down mid imaging and constrained time to perform the experiments meant the total number was reduced. Three images of each well were taken initially in an attempt to boost n numbers.

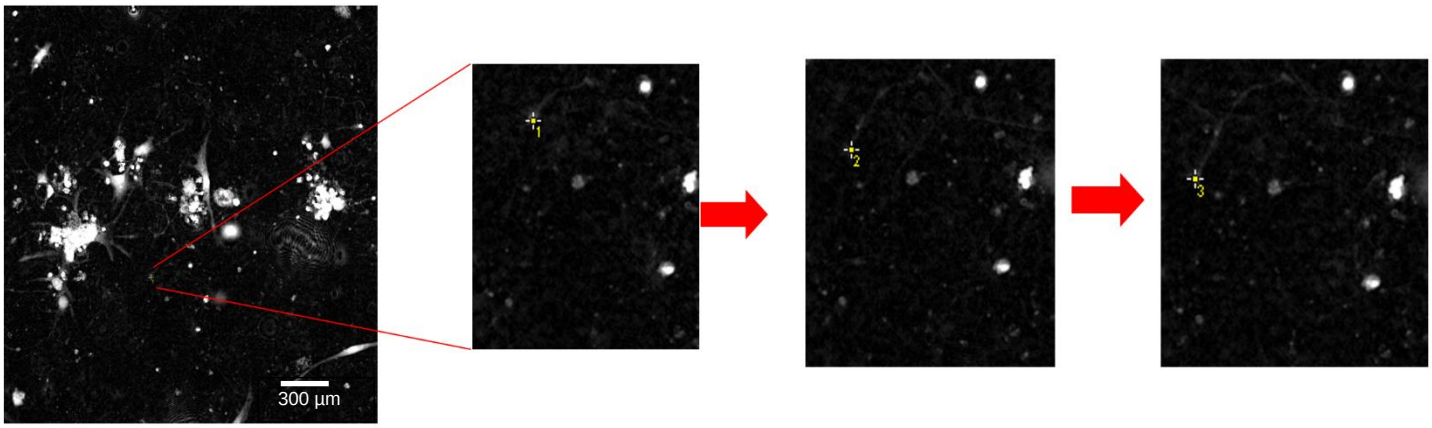

Figure 2 – Shows the analysis method used to track growth cones by using points of to indicate regions of interest to enable quantitative analysis of the images.

For the imaging of the samples that were fixed both the Livecyte microscope and Widefield system Nikon microscope were used. Some images were able to be imaged in the Livecyte for just one time frame. However, the focusing was out of range, due to being non-imaging plates or coverslips, so the Nikon was used. The Nikon allowed imaging of fluorescent dyes

and added the fluorescent colour onto the images after initial imaging. This allows for a more accurate image as it reduces the pixels used to colour the image creating a more precise image of just black and white preserving the accuracy and definition of the images. The colour was then overlayed onto the images later. In order to analyse these images ImageJ (FIJI) was used along with the plugin called SNT (simple neurite tracer). This function allows the prosses of a neurone to be traced over essentially mapping neurons. This is then converted into measurements and data that can be analysed. Neurons of similar nucleus sizes were compared with each other. Every process originating from the nucleus was traced. The data recorded consisted of things like: branch length, cable length, no of branch point ect. This can be seen in Figure 3.

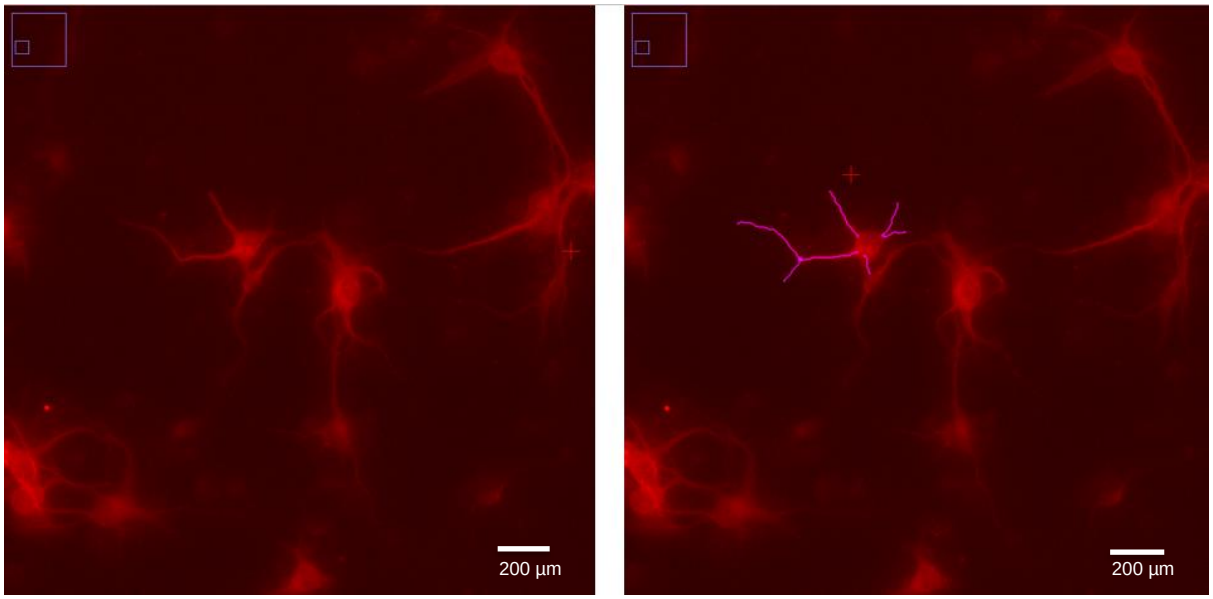

Figure 3 – Shows images taken of neurons stained with MAP2. The first panel shows the image before analysis and the second image shows the image after SNT analysis has been conducted. The SNT mapping is indicated in pink,

To check the data being collected the MAP2 stain was checked by not using primary antibody on two wells. These wells were used as negative controls and showed no staining. These results can be seen in Figure 4.

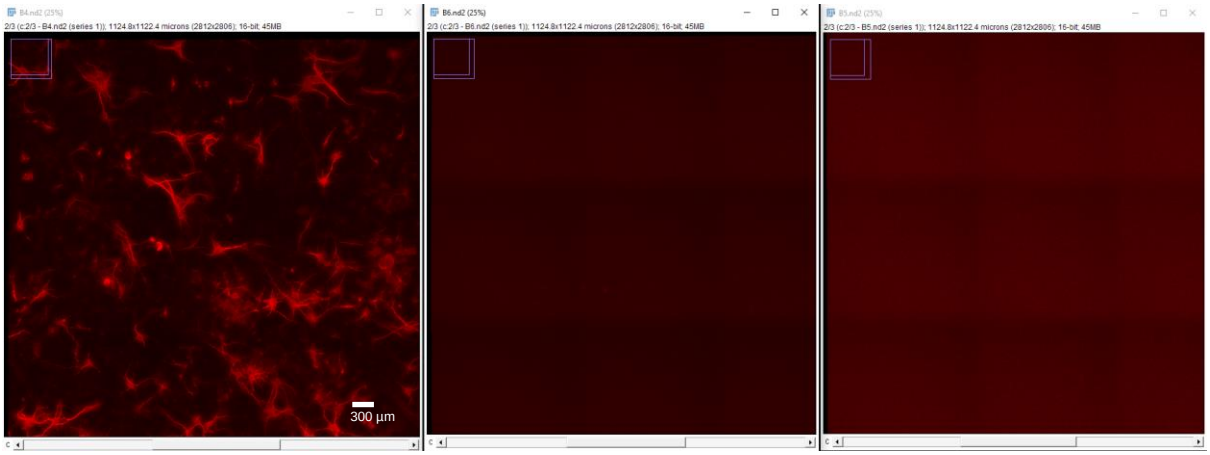

Figure 4 – The first panel shows an image taken of neurons stained with MAP2. The next two panels show negative controls of the immunostaining.

4.0 Results

4.1 Growth cones

The first round of data collected was the growth cone movement. This was conducted to observe any changes to the growth rate of the neurones. Growth cones are used for the growth of neurones as well as axon pathfinding and effective synaptogenesis which make them very clear features of neurodevelopment. By tracking their movement and would create viable indications of TCB-2 concentrations effect on neurodevelopment. Therefore, if the activation of 5-HT2A receptors have a role in neural development.

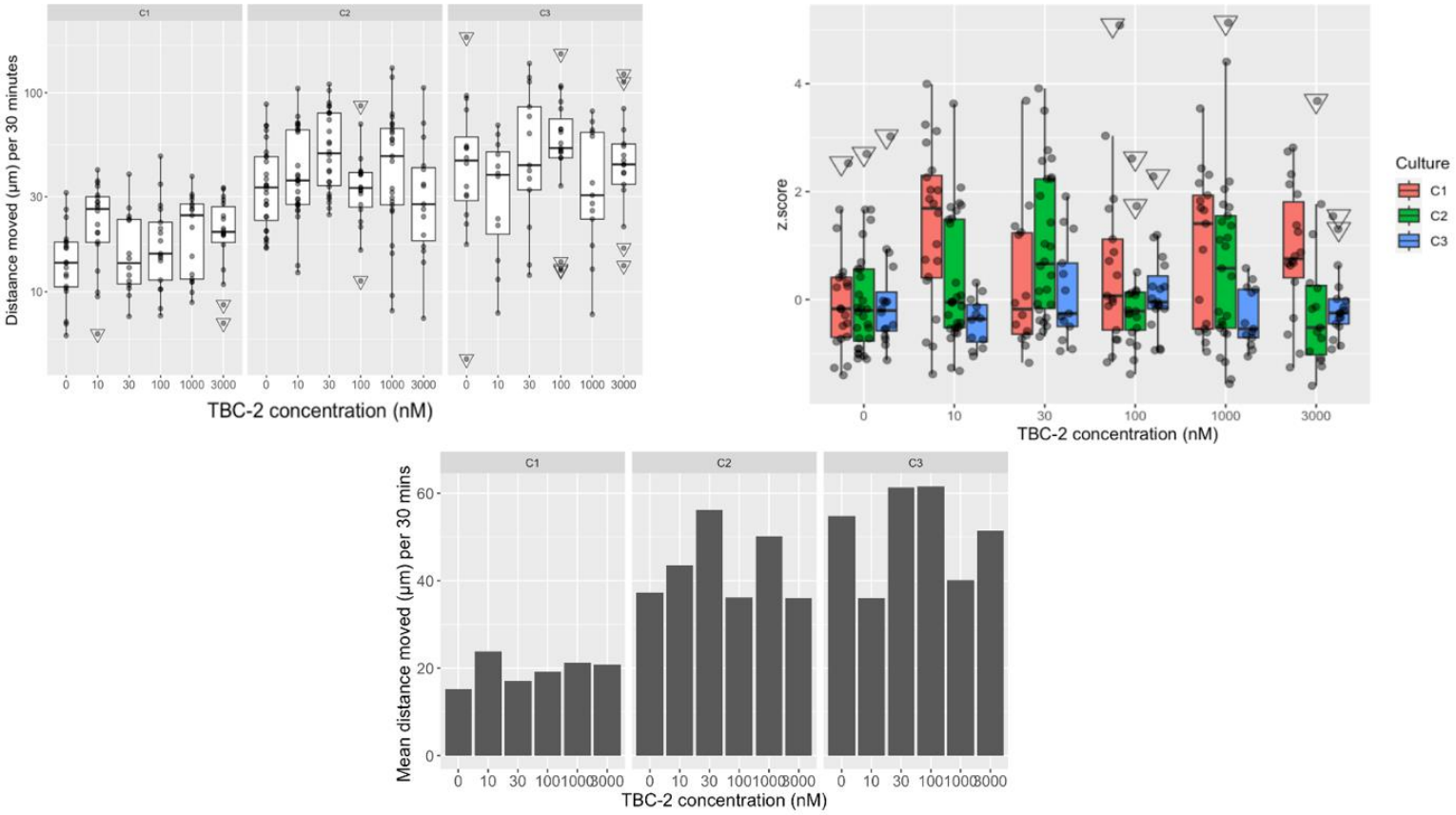

Figure 4 – This figure shows three graphs, the first shows a box lot of the distance moved by growth cones per 30 minutes at ascending concentration of TCB-2 (5-HT2A receptor agonist) which is split into the three cultures that were used. The second panel shows the corresponding z scores. The last graph illustrates the mean of the distance moved by growth cones per 30 minutes at the corresponding TCB-2 concentrations

The first element that was measured for was growth cone tracking. Using live imaging it was possible to identify moving growth cones. As seen in Figure 1 both cultures 2 and 3 show a curve shape initially increasing to then descend back to near the control concentration. The third panel shows the means of the culture at each treatment level and this also echo the curved shapes seen in culture 2 and 3 while culture 1 shows a gradual increase but a spike at 10nM. Cultures 1 and 3 showed a general increase in movement from the growth cones.

Culture 1 has the most obvious increase over all but the most distance coved was found in the second lowest concentration of 10 nM. However, in culture 2 there is a decrease in movement. From control to the highest TCB-2 concentration. As the concentration of TCB-2 treatment is increased it initially spikes to the most movement seen at 30 nM. Interestingly there is a spike in movement at 30 nM for both culture 2 and 3.

The z scores were calculated to normalise the data presented by expressing the data changes as multiples of the standard deviations from the reference data. This enables the analysis the data across the multiple experiments conducted even with data that had different baselines. This showed Culture 1 had the least deviation in comparison to culture 2 and 3 which seem to be relatively similar to each other. The largest difference between culture 2 and 3 is at the final concentration.

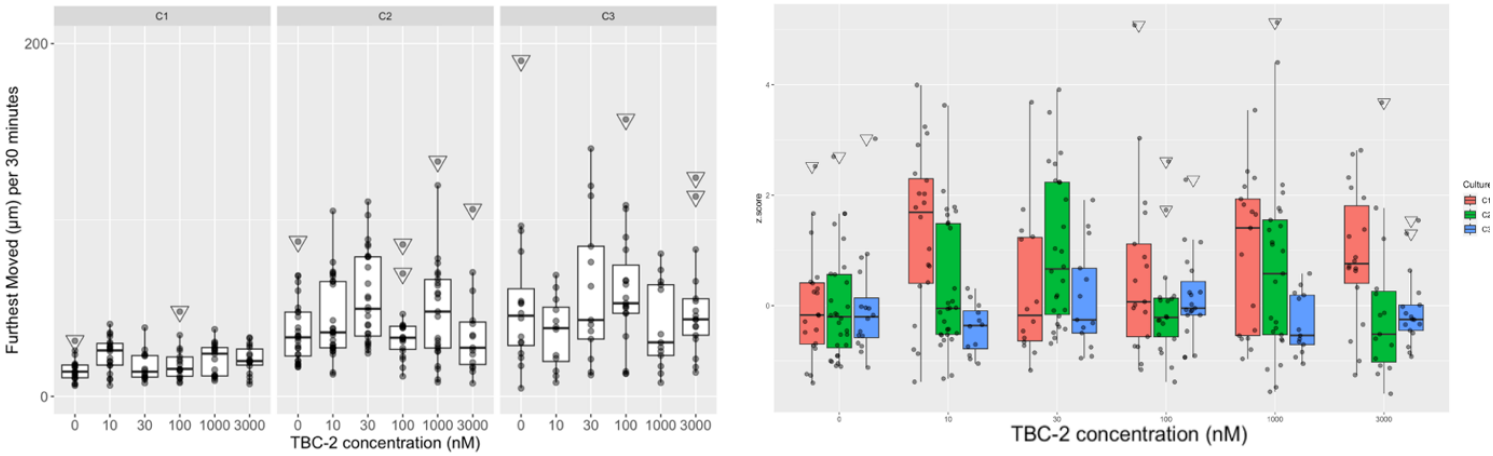

Figure 5 - This figure shows two graphs, the first shows a box lot of the furthest distance moved by growth cones per 30 minutes at ascending concentration of TCB-2 (5-HT2A receptor agonist) while the second panel shows a boxplot of the z scores of the data shown in the first panel.

When looking at the maximum movement of the neuron's growth cones at each concentration culture 1 had minimal differences between concentrations, but still followed the slight increasing trend seen in the distance moved. Both cultures 2 and 3 seem to experience an initial increase in maximum movement that then decreases. Culture 2 has the most movement at 30 nM but the least amount of movement at the next concentration of 100 nM. Culture 3 has the highest maximum movement at 30 nM where the means begin to drop. At the highest concentration there is an increase in length. Both of the cultures still follow the trend seen in the all the growth cone movement. Looking at this extreme helped to understand the effect the extreme may have had on the overall data, since the data seems to hold the same shapes there is a high change the overall data were impacted by the extremes.

Undergoing statistical analysis, a mixed linear model on the z scores showed significant changes seen at 10 nM and 30 nM form the control data. When an ANOVA was run it revealed no significant effect of treatment on growth cone motility at the 5% significance level. When using the emmeans function was used it produced pairwise comparisons for all treatment conditions to the control groups. This showed significant changes at 30 nM (p=0.0289) but did not find any significance in changes for the other concentrations.

4.2 SNT

Secondly, in an effort to study whether possible changes in the growth cone motility had any effect on the overall growth of the cultured neurons, neurites were labelled with MAP2 and their total length quantified by tracing individual neurons using SNT (Image J).

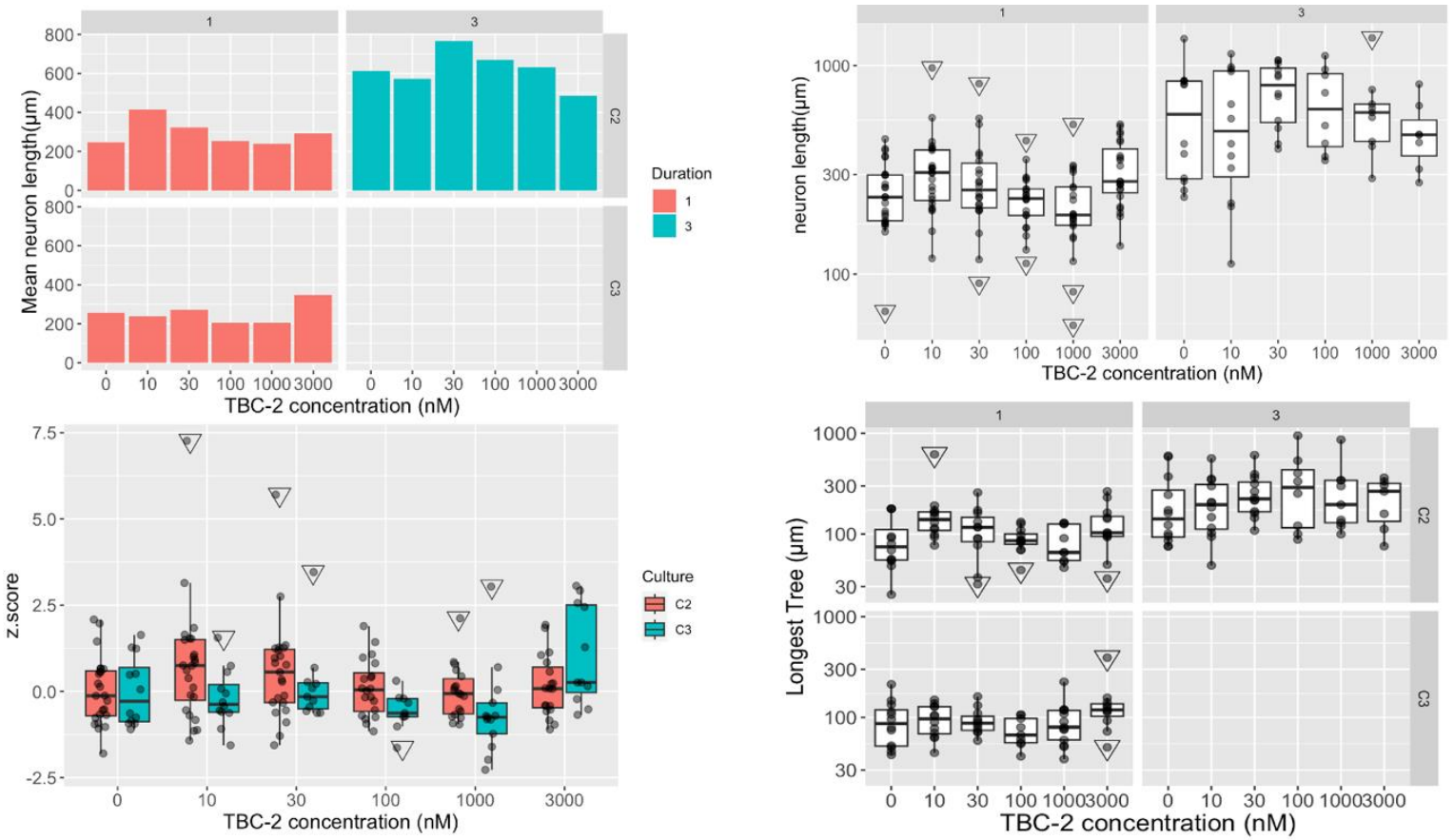

Figure 6 - This figure shows four boxplot graphs, the top left panel shows a box lot of the mean neurone cable length at different concentration of TCB-2 (5-HT2A receptor agonist), while the top right panel shows the total neurone cable lengths The bottom left shows the corresponding z scores for the data of total neurone cable length. The bottom right panel shows the longest cable lengths recorded per treatment group. The top right left and bottom right that have been split by duration of growth (1 day =1 or 3 days =3). The top left and bottom right have also been split by culture.

This type of analysis was only done for cultures 2 and 3. Culture 2 once again showed bell curve at a duration of 3 days as the TBC-2 concentration was increased. The mean length of the neurones decreased overall from control to 3000nM but both plates do show an increase before 30 nM. Culture 3 has little variation in the mean movement, though, it does mimic the same shape seem in Culture 2 when grown for 1 day. There is an initial increase that dips and then rises for the longest lengths at 3000 nM. Interestingly both high point in the day 1 cultures is at 3000 nM. When looking at the cultures combined and only viewed for their durations the trends seen in the means is also visible here. The 1-day cultures sine wave trend is amplified when combined.

The longest neurone also mirrors the trends in the mean lengths showing a general decrease in the plate that was cultured for 3 days. Overall, the plate cultured for 3 days shows both higher means and total neuron length compared to the plates only cultured for a day. When looking at the z scores for the neurone length the biggest difference the cultures are between the highest concentration.

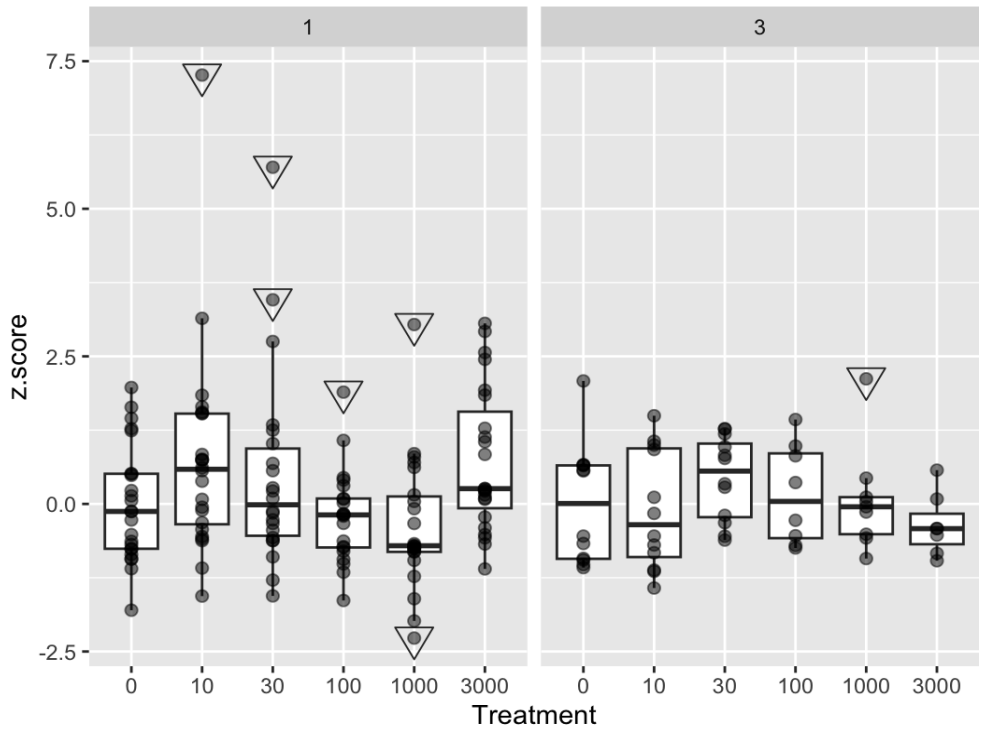

Figure 7 – This figure shows a boxplot of the longest cable lengths z scores.

The z scores from the neurone lengths in respect to the duration rather than culture does show that the highest concentration provides the most change. It also showed both durations are fairly contained to the median though this is much more apparent in the 3-day cultures. Some of the outliers in the 1-day cultures deviate significantly from the mean. This can be seen at the concentrations of 10 and 30 nM. These concentrations also have the largest deviations from the mean of the entire data set. This is not seen in the 3-day cultures with even outliers remaining within 2.5 SD. The general decline is seen in both durations of cultures.

A quantile-quantile plot was made to check for normal distribution. As seen, most of the data points hold close to normal distribution with only slight deviations at each extreme. When running statistical analysis on both the neural length and the total tree length a mixed linear model was used. This model then undertook an anova followed by a pairwise comparison and then configured into residual plots. Both models found no significant effects from the treatment of the length of the neurons at a 5% significance level. When looking at neuron length, duration was found to have a significant positive effect on the lengths of the neurons ($p=0.014$) while treatment was not significant at $p=0.081$. Similarly, significant effects were found between the treatment and duration suggesting the effects of the treatment were dependent on the time the plates had to culture. This was also seen when looking at the longest trees. The pairwise comparison did show that at certain durations and treatment levels there were significant changes. When looking at the longest tree the duration was significant again showing a positive relationship ($\beta = 0.313$, $p < 0.001$). The main effect of the treatment was not significant ($p=0.146$). Plots for both the neuron length and longest trees showed normal distribution and a fairly good spread of point when looking at pearson residuals. Though there was slight clustering seen in both models which could suggest heteroscedasticity. Some points did show a higher proportion of deviation which suggest the outliers may have skewed the data.

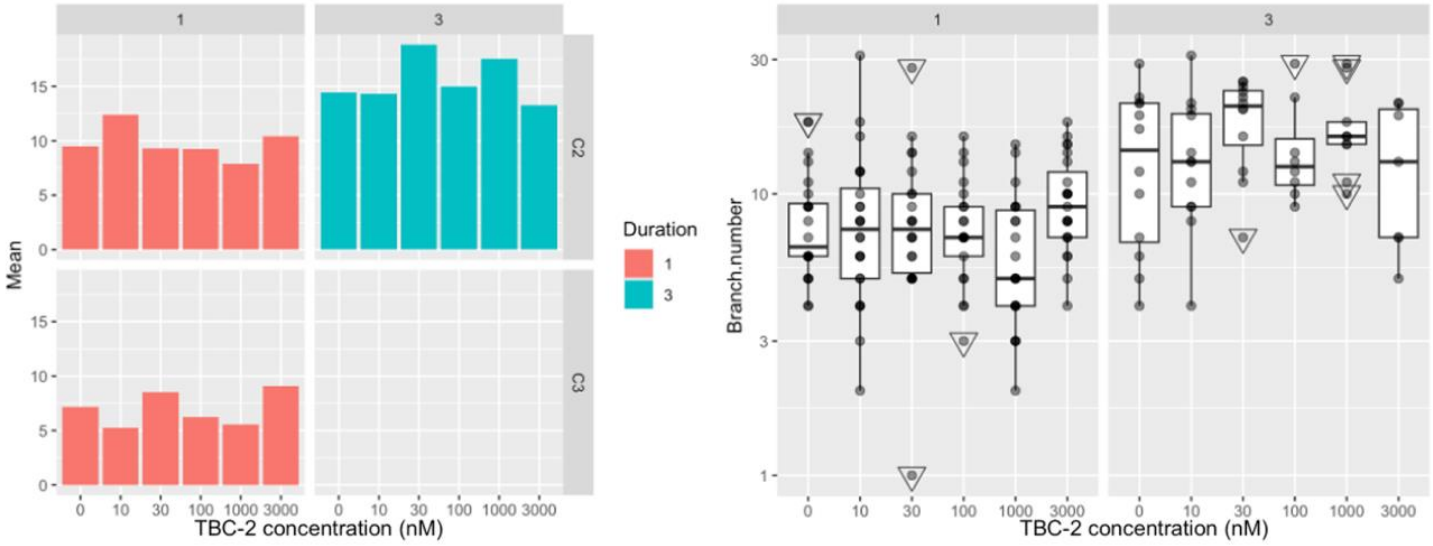

Figure 8 - This figure shows two boxplot graphs, the first panel is the mean branch number per neurone and the second which has been split by that have been split by duration of growth (1 day =1 or 3 days =3) shows the total branch number seen at each treatment level.

The number of branches per neuron were also considered during analysis. Similarly, there is less branching occurring at shorter durations. Decreasing trends can be seen in the plates especially prominent in the 1-day cultures through an increase can be seen at 3000 nM. The 3-day culture has a smaller data range.

The statistical analysis conducted was also using a mixed linear regression model. There was no significance found from the following ANOVA (p=0.388 (3sf)) and the pairwise test showed only a very small number of treatments. Once again there was a significant trend in treatment and duration but only just at a 5% significance level (p= 0.05). The difference between cultures was also significant in relation to duration (p=0.150 x10^-7).

5.0 Discussion

The neural pathways within the brain are incredibly intricate and as such the development of these pathways can be even more complex. A multitude of molecular signalling and other cellular interactions play a role in the development of neural pathways and each interaction can trigger various pathways. The 5-HT and its receptors are known to be involved in signalling pathways that affect the regulation of neuronal differentiation and synaptic plasticity along with other cellular activities. Of the receptors the 5-HT2A receptor is of

particular interest when studying the development of neural pathways due to its abundant expression in developing brains. The expression of the 5-HT1A receptors is not just localised to the brain they are also found in the muscle layer of the gastrointestinal tract (myenteric plexus) as stated by Pithadia and Jain, in 2009. The myenteric plexus is part of the enteric nervous system colloquy referred to as the 'brain of the gut' (Geng et al., 2022). This, along with other reasons like its links to disorders, has made the pursuit of knowledge of the 5-HT1A receptor very extensive. However, the absence of expression of 5-HT2A does not diminish the interest in studying this receptor. While this G-protein coupled receptor has been well studied and has established involvement with neural development it is not understood how the mechanisms of the receptor impact neural development nor the extent to which the development is impacted. Receptors such as the 5-HT1A have had more extensive research conducted on them due to the initial link to anxiety and depression. This led to the use of SSRIs to regulate mood through these receptors due to their ability to raise the levels of serotonin. But as research continued there has been further evidence to suggest there is a weak correlation and more effective treatments are needed. Hence, the move towards further understanding the other receptors within the 5-HT family. 5-HT2A receptors are of particular interest due to hallucinogenic effects of manipulating 5-HT2A receptors. 'Magic mushrooms'/psilocybin is currently been studied quite extensively as treatment for hard-to-treat cases of depression and so far, has proved very effective being 3x more effective than a placebo (Metaxa and Clarke, 2024). Consequently, the aim of this study was to address the gap in knowledge surrounding the 5-HT2A receptor and investigate the effect of TCB-2 on the growth of neural pathways. The model spices used were mice due to the highly organisational and functionally similar properties to not only other mammals but specifically to humans' brains. TCB-2 is a selective agonist for the 5-HT2A receptor and has a high affinity to the receptor hence is a useful molecule for study of the effects of activation of the 5-HT2A receptor. Leveraging these properties for this study allowed further investigation of dose dependent effects on neuronal morphologies. Explicitly, the individual neuron process lengths, total cable lengths and movement of growth cone were studied and analysed. Understanding the possible impact that TCB-2 has on neurons could help to elucidate underlying mechanisms of the processes involved in neural development. This knowledge could also lead to breakthroughs within neurodevelopmental disorders and new more effective therapeutics in this area. Through analysis of the effects of TCB-2 interactions with 5-HT2A receptors *in vitro* this study aims to contribute to a more in-depth knowledge of 5-HT2As role in neurodevelopment supporting a foundation for future research to build upon for more targeted therapeutic possibilities and understanding neural developmental disorders.

5.1 Findings

Initially this study aimed to look at the growth rate of the neurons. The data collected from the growth cones showed various patterns in terms of the movement seen in each individual culture. The results from culture 1 show overall much shorter growths than the

other two cultures. This is likely due to the enzyme (papain) that was used being old and not particularly effective. The cells clumped and therefore the growth cones were much harder to find as they were sparse in comparison to the other cultures. The less efficient dissociation may have contributed to lower density of healthy neurons but interestingly, in some ways the effect on growth cone motility appeared to be the most pronounced with the clearest positive trend in this culture. There was also a change in preferred culture medium after reading about the improved benefits for Brainphys in neurological studies (Bardy *et al.*, 2015) and was swapped in for Neurobasal.

When looking at any statistical significance a mixed linear regression model was used. While there was no overall significance, although it was approaching significance, there were treatment values found to be significant. The model was found to be a good fit. This type of model was used for its flexibility and ability to account for data that was missing along with the allowance of unbalanced experimental design. Nevertheless, when considering the data size, the initial trends seen hold promise that if repeated with larger sample sizes the data would hold significant trends. Interestingly, very similar positive effects on dendrite growth cones were seen in the study done by Zhang et al. in 2013.

The other technique used during this research was SNT. The reason for analysing the total neuronal growth, rather than just the growth rates, was to identify whether changes in the growth cone motility translated into any morphological changes. A change in the rate of growth may not automatically result in a change in neuron structure as the neurons may still all grow to the same size, but at different rates. Hence, analysing the growth cone motility alongside the total neurite growth provides a better overview with different information. This extra analysis gives a more comprehensive understanding of the effects of 5-HT2A activation. These analyses enabled full mapping of neurons identifying branches, cable lengths and much more. The focus of the analysis conducted was on individual neuron lengths and the maximum cable length of individual neurons as these were more likely to give indications of neural development.

Initially when looking at the mean neuron lengths there seems to be a very similar trend to the growth rates with an initial increase that then decreases. There is an obvious increase in length between durations of the cultures. These same patterns were also observed when considering the longest lengths. This analysis was also conducted on the number of branches each neuron had little correlation but did seem to be generally positive. When undergoing statistical analysis, it was not found to be significant. Like the neuron length data, the only significant correlation was found between the treatment and duration of the plate culture. The reflection of the trends in both the growth rates and the over all lengths of the neurons suggest relative validity at the replication of data trends seen. This gives very promising foundations for future research to confirm.

While the treatments remained anonymous during the analysis of the SNT imaging it is much harder to rule out unconscious bias. Not every neuron was measured and judgement calls had to be made on whether processes extended from the nucleus being recorded or

possibly a neighbouring cell. Naturally the researcher undertaking the analysis will be drawn to bigger or more visible neurons. Even attempts to counteract this would bias the data. However, using this blind analysis approach should help to limit the effects of this natural biased as it will affect all images equally. Each treatment had n=12 per plate in an attempt to keep the analysis fair and consistent as some wells had less visible neurons forcing a limited sample size. These small sizes again enable the results to be easily skewed. Culture 3 also used the primary antibody that was made prior, during culture 2, this may also have affected the data pool as the neurons may have been harder to see if the staining was less effective especially when considering the scale of the neuron processes being measured.

Consequently, the data collected suggests a possible positive correlation between the increased concentration of TCB-2 and the development of neural pathways. This was particularly evident in the lower concentrations. At higher concentrations it is possible that the receptors may have been affected by cross-reactivity with other receptors. This is where TBC-2 may have been able to bind with other receptors and effect the activity of these receptors. At higher concentration the 5-HT2A receptors may have been saturated and therefore increase the competition for other receptor activation if TBC-2 had a high enough affinity with other receptors (Frank, 2002). It is known to have an affinity to the 5-HT2C and B receptors as they are very structurally similar (Di Giovanni and De Deurwaerdère, 2018). These two receptors are less known and researched but they may have inhibitory effects in relation to the pathways activated by 5-HT2A receptors. For confirmation of these positive trends much larger data sizes would be needed to conclusively identify this trend. Further suggestions would include various durations up to a week of growth to see if the correlation is stronger when cultured for longer. It is possible that due to the weak trends seen in the data and fairly plateaued spread that the activation of 5-HT2A modulates the growth neurons. Instead of stimulating or inhibiting growth solely the 5-HT2A receptor may have the ability to signal for either pathway.

5.2 Current research

Contextualising the finding is crucial to fully explore the role of 5-HT-2A receptors in neuronal growth and development. The current research surrounding the understanding of the 5-HT2A receptor is largely divided. Though the consensus from recent studies suggests that 5-HT2A has a role in modulating the growth and development of synaptic connectivity, both in vitro and vivo, many studies differ in the modulating effect. This is reflected in the data collected within this study. Papers such as Boothman *et al.* in 2003 and Martín-Ruiz *et al.* in 2001 found that when 5-HT2A receptors were treated with an antagonist the effects of phenylethylamine were prevented. Phenylethylamine is known for its ability to interact with serotonin receptors and is thought to have a modulating role in the pathways associated with mood and energy. Both studies showed that with the addition of phenylethylamine

(agonist) cell firing was reduced and the inhibitory mechanism stimulated. The studies used different antagonists (MDL 100907 and ritanserin) for 5-HT2A receptor but both concluded that when used these antagonists reversed the effects of phenylethylamine. Hence it was presumed that 5-HT2A had a function in the inhibitory control of 5-HT activity. 5-HT is known for its stimulatory role in neuronal growth therefore implying an inhibitory role in the growth of neurons for 5-HT2A receptor.

Much of the visual data produced in this study showed a return back to control levels in length or growth in by the final concentration of 3000 nM. This produced either a bell curve effect seen in both the growth rates and total lengths. Wave like patterns were also seen with higher concentration often returning to closer values to the control. When looked at in conjunction with recent studies there is a possibility that the receptors would have become desensitised to the TCB-2. Tsybko *et al.* in 2020 noted a desensitisation of receptors after chronic exposure to TCB-2. Though this was over a 14-day period which is much longer than this study. Other studies like Berg *et al.,* (2001) also noted that when 5-HT2A receptors reached a 99% or more occupancy the receptors returned to control levels of responsiveness even if the agonist was still present. This could indicate why the positive trends initially seen in the figures then declined under increased concentrations of TCB-2 as well as seeing the slight wave pattern at these high concentrations when returning to control levels. The 2020 paper by Tsybko *et al.,* also indicated oligodendrocytes (OL) death may be regulated by 5-HT2A receptors. Oligodendrocytes are types of glial cells, which are known for expressing 5-HT2A receptors, and in the study 5-HT1A and 2A were expressed and therefore involved in the signalling of serotonin in relation to this cell. When exposed to serotonin the development of the OL cells were disrupted often causing process outgrowth, reduction in myelin protein expression and in high doses cell death. The result of cell death was replicated when DOI (5-HT2A agonist) was added indicating the role of 5-HT2A receptors in this pathway. OLs are responsible for the production of myelin which is vital for the formation of axons and the structural support of neurons therefore a vital component for the growth and neural development of organisms. Interestingly, the cell death occurred more frequently in immature OLs which might suggest a stronger link to 5-HT2A receptors being involved in inhibiting development of neural pathways rather than maintaining neural pathways. However, the other effect seen is the increased growth of processes. This suggests a stimulatory effect on neural development when 5-HT2A receptors are activated.

The role of 5-HT2A receptors have also been linked to the improvement of learning and memory in rodents (Zhang and Stackman, 2015). When mice were treated with TCB-2 there was a significant enhancement in the time the mice took exploring a new object when presented rather than objects that were not novel (Zhang *et al.*, 2013). Highlighting the link between 5-HT2A receptors and memory and learning. This was further proven by the reversal of this effect when treated with MDL 11, 939, a 5-HT2A antagonism, which saw a

reduction in the mices' object memory. Though this is only referencing to short term memory experiments it lays possible foundations for the role of 5-HT2A receptors in long term memory. This development is also facilitated by the findings of Zhang and Stackman in 2015 as the activation of the 5-HT2A receptors by TCB-2 reduced the conditioned fear responses of the mice after non reinforced exposure to the conditioned fear stimulus also known as fear memory extinction (Myers, Ressler and Davis, 2006). Due to long term memory being partially enabled by neuron growth and new connections once again the 5-HT2A receptor could be linked to the modulation of neuronal growth (Deng, Aimone and Gage, 2010). Though all that is known currently is 5-HT2A receptors are definitely linked to memory and learning but the mechanisms must be explored further. These links have made the receptor a very exciting area of research for future therapeutics for memory.

5.3 Future research

The possibilities that the 5-HT2A receptor holds are largely untapped have huge potential to change the multiple fields within the scientific community. Though receptors like the 5-HT1A receptor have had far more extensive research conducted, the other receptor families are not very well understood. The need for more in depth understanding of the other receptor families is integral as the pathways and mechanisms that have been linked to 5-HT are extensive and complex. Some of the key pathways include serotonin signalling, neurotransmitter release, neuroplasticity, psychedelic effects and mood disorders.

From this study, though the overall trend seems to suggest the stimulation of 5-HT2A receptor reduces the growth of neurons there was no significant evidence and so similar studies should produce large amounts of data that can be analysed fully. Additionally, as previously suggested longer culture durations alongside the shorter ones used in this study would be beneficial to identify patterns that may be exclusive to certain developmental stages.

Mood disorders such as depression have long been linked to the lack of serotonin and therefore have been treated with SSRIs to black the reuptake of serotonin to maintain higher levels of serotonin in patients' brains. Yet, recent paper such as Moncrieff *et al.,* in 2023, express a huge oversight in this therapeutic that while evidence suggests the link of serotonin to depression there has never been evidence for serotonin levels to be the cause of depression. As a result, patients rarely recover and often present with new symptoms of apathy or indifference, which may be less extreme but is not an effective therapeutic (Sansone and Sansone, 2010). If the mechanisms for the low serotonin levels were better understood, like understanding all receptor families, like the 5-HT2 family, the possibility of finding better more targeted therapeutics are much higher.

Another avenue of future study for therapeutic benefits would be the enhancement of memory efficiency. Within current studies it was discussed that significant evidence was found to suggest the stimulation of 5-HT2A receptor promoted the learning and memory in mice (Zhang *et al.*, 2013). It was suggested that further research identifying the extent of involvement 5-HT2A receptors have would need further investigation. However, when considering disorders such as ADHD that suffer from poor memory function, 5-HT2A receptors may hold the key to future therapeutics for these individuals. While 5-HT2A receptors have been identified as targets for memory disorders such as Alzheimer's (Tang *et al.*, 2017) there has been little literature to suggest deeper understandings of possible links to ADHD.

An interesting avenue of future studies would be the analysis of the possible connections of the psychedelic's effects on neuroplasticity and whether this is linked to the activation of 5-HT2A receptors. Considering the 5-HT2A receptor has been linked to neuron growth and some psychedelic drugs have been found to promote the growth of new neuron processes, the link could be through the 5-HT2A receptor. Though psychedelics have been identified for therapies to repair atrophied neuronal connection in the brain the mechanisms are not fully understood and the correlation with 5-HT2A receptor has yet to be fully explored (Ly *et al.*, 2018).

6.0 Conclusions

This study did not show a significant link between the growth or length of neurons when treated with TCB-2 there were significant differences between culture duration and the lengths produced at each treatment level. This significant effect is to be expected but does highlight a possible need for longer culturing to see clearer trends within the data. With longer time for experiments and analysis it would have been beneficial to record more neurons per treatment to garner more conclusive results. However, the initial findings of this study suggest a strong foundation and need for more extensive research into the mechanisms of the 5-HT receptor families. This report highlights just some of the future areas of study within neural development like memory.

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8.0 Reference

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