Tracking DAergic Neuron Ablation and Regeneration in the Brain of Adult Zebrafish

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Thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science in Biology

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Abstract

As the prevalence of Parkinson's disease is expected to increase gradually over the years based on recent scientific predictions, developing a treatment plan to mitigate the development of this disease is essential. Previous research tried to tackle the motor and non-motor symptoms associated with the disease. That said, some symptoms seem to persist, and the quality of life of PD patients continues to decline. Zebrafish have emerged as a strong model to study the regeneration of DAergic neurons as they have the ability to show robust adult neurogenesis. Here, we used the Tg(*dat:CFP-NTR*) zebrafish line to investigate DAergic neuron regeneration following ablation in various brain regions. In addition, we tested the efficacy of Nifurpirinol, an alternative substrate to MTZ, in ablating DAergic neurons in the adult zebrafish brain. Lastly, we tracked how the ablation of DAergic neurons influences the motor activity of adult zebrafish and how they tend to recover over time. Results showed a significant reduction in DAergic neurons at 7 days following the MTZ treatment in the olfactory bulb, telencephalon, and the periventricular pretectal nucleus. NFP also caused similar changes, albeit they were less statistically significant. In response to ablated DAergic neurons, MTZ-treated fish showed a significant increase in the number of neural stem cells undergoing proliferation at 1 dpt. However, the highest spike in proliferative cells, especially neural stem cells, was found at 7 dpt. This time point corresponded with the greatest decrease in DAergic neurons following ablation. These cellular changes were observed in the olfactory bulb and the telencephalon. That said, more drastic changes were noticed in the rostral and medial telencephalon. Results also showed that the adult zebrafish brain was not able to significantly replenish the number of DAergic neurons as early as 15 dpt. Based on previous observations, it seems that adult zebrafish need at least 45 days to regenerate their DAergic neurons to levels comparable to the DMSO control. Lastly, behaviour analysis showed

that NFP has the most significant impact on motor activity across three different parameters at 0 hpt. MTZ also had similar effects on motor activity; however, it was less pronounced. The impact on the behaviour level seems more transient as some recovery was observed at 7 dpt. Overall, this transgenic zebrafish line allowed us to explore how and when the adult zebrafish brain was able to efficiently recover following the specific ablation of DAergic neurons. In addition, it expanded our understanding of adult neurogenesis which will hopefully allow us to better approach patients with Parkinson’s disease.

Acknowledgements

I want to start first by thanking my wonderful supervisor, Dr. Marc Ekker, for giving me this great opportunity to work in his lab. Thank you so much for your continued support, guidance, and mentorship throughout the years. Your mentorship allowed me to grow and develop into a better and more curious researcher. I will always remember the discussions we had during our meetings and will try my best to carry over your advice in my future endeavors. Thank you dearly for everything!

Secondly, I would like to thank Khang Hua for showing me how to master various laboratory techniques and how to analyze my experimental results properly. I truly appreciate your constant feedback, input, and suggestions. I would also like to thank Herman Mbesha for the manual cell counts and Vishal Saxena for his assistance and recommendations throughout my project.

I would also like to express my great appreciation to Dr. Bruce McKay and Dr. Marie-Andrée Akimenko, my Advisory Committee Members, for their great feedback and helpful comments. Your remarks helped me adjust my approach to some aspects of my project, and it truly allowed me to steer in the right direction.

Lastly, I would like to thank my amazing family for their patience and continued encouragement over the past two years. Their support was paramount to my success, and I am really grateful for them.

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List of abbreviations

CFP	Cyan fluorescent protein
DAergic	Dopaminergic
DAPI	4′,6-diamidino-2-phenylindole
DAT	Dopamine Transporter
DMSO	Dimethyl Sulfoxide
dpf	days post-fertilization
dpt	days post-treatment
dpi	Days post-lesion
IHC	Immunohistochemistry
MTZ	Metronidazole
NTR	Nitroreductase
OB	Olfactory Bulb
PCNA	Proliferating Cell Nuclear Antigen
PD	Parkinson's Disease
Sox2	Sex determining region Y-box 2
Tg	Transgenic
WT	Wild type

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1. Introduction

1.1 Parkinson's Disease

Parkinson's disease (PD) is a complex neurodegenerative disease that is clinically characterized by cognitive, motor, and an array of non-motor psychiatric defects (Kalia, 2015; Kouli, 2018; Han, 2018). PD is regarded globally as the second most prevalent neurodegenerative disease following Alzheimer's disease (Tanner, 1996; Kalia, 2015). The prevalence of PD has been proven to become more aggressive with increasing age, and it accounts for 0.5% – 5% of the population above the age of 65 years (Nussbaum, 2003; Han, 2018). Based on scientific predictions, it is expected that the incidence and prevalence of PD will both increase by > 30% by the year 2030 (Chen 2001; Kouli, 2018). Since it was first discovered and described by James Parkinson in 1817, some noticeable symptoms have been observed (Parkinson, 1969; Jost, 2017).

The hallmark motor symptoms of PD include muscle rigidity (during early stages), slow rhythmic tremors, bradykinesia, gait distortions, postural instability, and other balance-related dysfunctions (Rinalduzzi, 2015; Kouli, 2018; Han, 2018). Recently, more studies are realizing the importance of considering the non-motor symptoms caused by PD. Examples of non-motor symptoms include rapid eye movement (REM) sleep behaviour disorder, hyposmia, constipation, and hallucinations (Poewe, 2008; Pfeiffer, 2016). Psychiatric disturbances are often overlooked and are incredibly hard to manage in PD patients as they are often sporadic and can vary from one patient to another. Some of these psychiatric symptoms that tend to manifest in PD patients include psychosis, depression, impulse control disorders (ICDs), delusion, and dementia (Bronnick, 2005; Poewe, 2008; Aarsland, 2014; Han, 2018). These symptoms have gained much more attention recently as they significantly impair the day-to-day activities of patients suffering from PD. **Figure 1** outlines some of the common psychiatric symptoms that tend to manifest in PD patients.

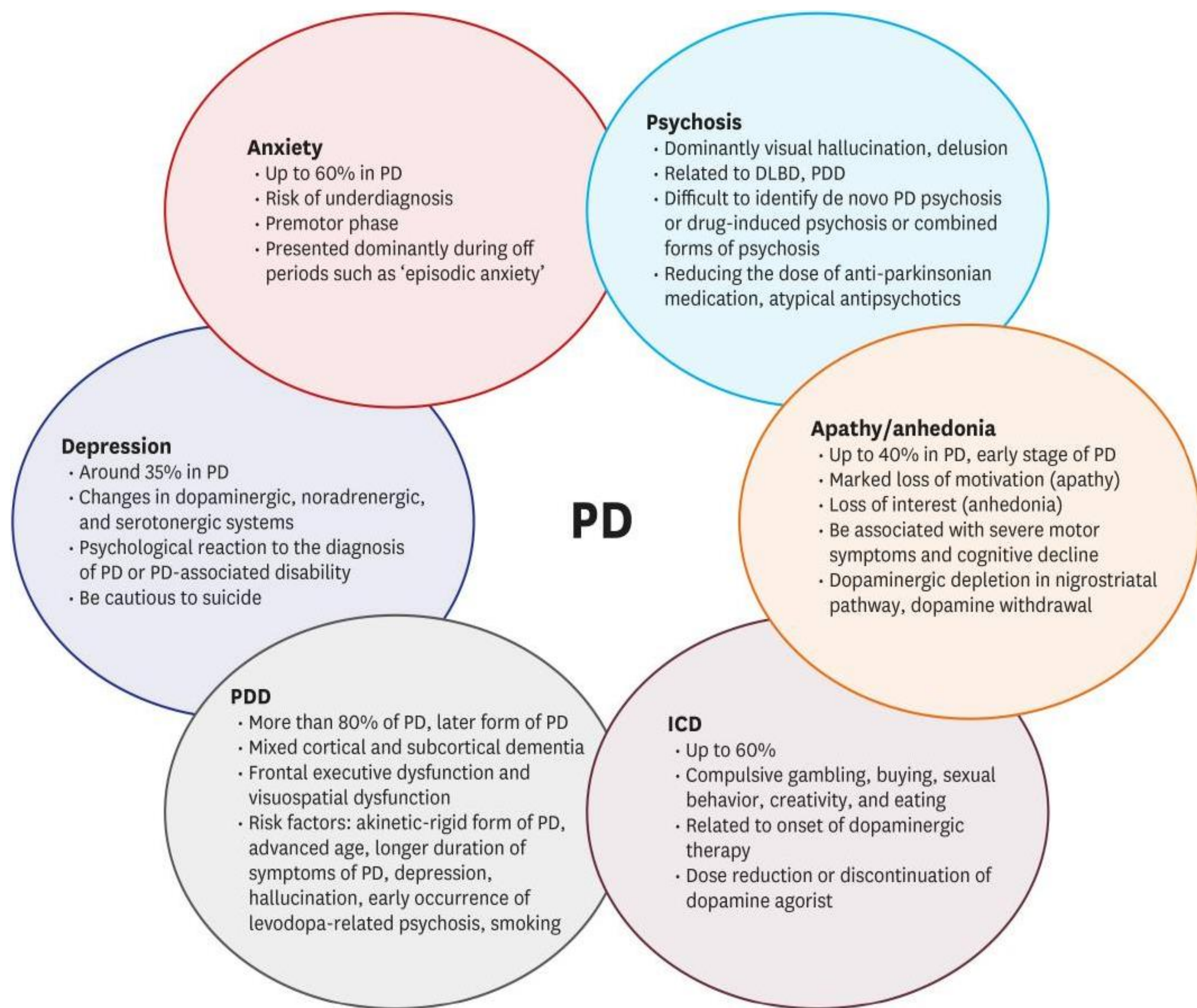


Figure 1. Summary of the common psychiatric symptoms that tend to manifest in patients with Parkinson's disease.

Anxiety, psychosis, ICDs, depression, and apathy are some of the familiar non-motor symptoms observed in PD patients. Some of these symptoms can be alleviated in the short term by taking dopamine agonists or other atypical antipsychotics. Most of these symptoms tend to progress gradually and worsen over time, drastically affecting the patient's quality of life. This diagram was obtained from Han et al. (2018).

As Parkinson's disease slowly develops over time, both motor and non-motor symptoms associated with this disease tend to worsen progressively, so early detection is essential to help treat the symptoms as they appear and help control pain levels (DeMaagd, 2015; Han, 2018). In later stages of the disease, treatment can become futile as these symptoms may become more resistant to current medications and treatment plans (Kouli, 2018). A lot of factors contribute to the formation and progression of Parkinson's disease; however, the consensus among scientists is that the interplay between genetic and environmental factors is the main reason (Benmoyal-Segal, 2006; Han, 2018; Kouli, 2018; Zafar, 2020). Despite PD being an idiopathic disease, 10% – 15% of patients report having some cases of PD in their families and 5% – 10% report having a monogenic type of the disease with Mendelian inheritance (Deng, 2018, Kouli, 2018).

More research lately has been trying to unravel the role of polygenic risk factors in increasing the risk of developing PD (Escott-Price, 2015; Gialluisi, 2021). Genes that are now known to potentially cause PD are referred to as *PARK* genes and are ranged from *PARK*1-*PARK*23, where the number indicates the order by which they have been discovered (Schulte, 2011; Klein, 2012). Patients with mutations in the *SNCA* gene tend to have the early-onset symptoms of PD, while patients with mutations in the *LRRK2* gene usually report experiencing the more late-onset symptoms of PD (Klein, 2012; Blauwendraat, 2019).

1.2 The role of Dopaminergic neurons

The unique morphological alterations in the brain of patients suffering from PD include the abnormal presence of Lewy bodies and the absence of dopaminergic (DAergic) neurons in the darkly pigmented area in the substantia nigra pars compacta (SNpc) (Alexander, 2004; Dickson, 2012; Kouli, 2018) (**Figure 2A**). It is the gradual progressive degeneration of DAergic neurons that leads to the deficit in the number of dopamine molecules present in the SNpc (Alexander,

2004; DeMaagd, 2015; Emamzadeh, 2018). As a result of lower levels of dopamine available due to the degeneration of DAergic neurons in the SNpc, reduced dopamine molecules will subsequently be transmitted to the basal ganglia, eventually affecting its function (**Figure 2B**). This is what leads to the range of motor and non-motor symptoms seen in PD patients (Alexander, 2004; Kouli, 2018). It is important to note that the SNpc is connected to the striatum by the nigrostriatal pathway (DeMaagd, 2015). The gradual degeneration of these DAergic neurons in the substantia nigra (SN) promotes more inhibitory signals to be sent from the globus pallidus interna (GPi) to the thalamus (**Figure 2B**). This, in turn, leads to a drastic decrease in functional motor activity in the brain suffering from PD. In the healthy brain, the signal from the GPi to the thalamus is less inhibitory and thus stimulates regular motor activity (**Figure 2B**) (Subramaniam, 2017).

Recent research focused on developing pharmacological solutions to tackle PD; however, the effective alleviation of the symptoms remains a challenge (Smith, 2011; Stoker, 2018). Some treatments focus on managing the symptoms caused by PD by administering DAergic drugs. However, they are by no means considered disease-altering treatments. One treatment, in particular, gives patients a mixture of levodopa with dopa-decarboxylase inhibitor, which helps prevent nausea caused by PD (Katzenschlager, 2014; Stoker, 2018). Levodopa is a precursor of dopamine and so it helps replenish some of the lost dopamine molecules (Knecht, 2004; Floel, 2008). Other dopamine agonists, such as Rotigotine, help treat some PD symptoms (Splinter, 2007). These drugs help restore some motor activity in PD patients by tackling the chemical imbalances happening in the striatum by re-establishing weakened DAergic activity (Stoker, 2018). The downside of these treatments is that they are futile when it comes to addressing the non-motor symptoms. In some situations, they can worsen these symptoms, making them

ineffective in the long run (Kujawa, 2000; Stoker, 2018). Ongoing research on PD examines how dopamine levels can be replenished by understanding how DAergic neurons can be restored in the SNpc (Harris, 2020; Godoy, 2020; Qian, 2020). These recent studies used a variety of animal models to study PD, including mice and zebrafish.

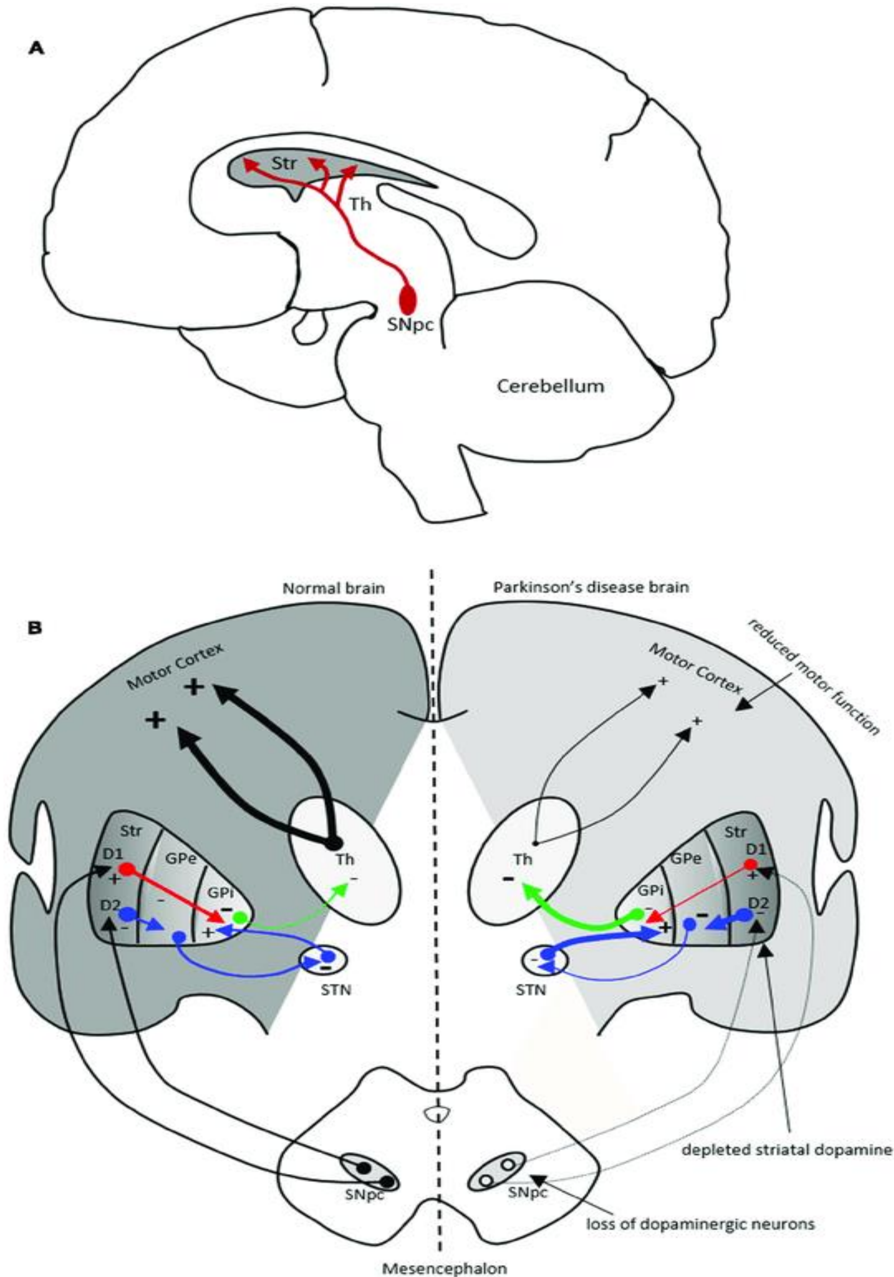


Figure 2. Schematic displaying the projections of the motor basal ganglia circuitry and the nigrostriatal pathway.

(A) Projections of DAergic neurons from the SNpc in the midbrain region to the striatum via the nigrostriatal pathway. **(B)** Abnormalities in the motor basal ganglia circuitry in the brain suffering from PD (right) and a healthy normal brain (left). The thickness of the projections describes the strength of the stimulus. A positive sign (+) indicates an excitatory signal while a negative sign (-) indicates an inhibitory signal. Striatum (Str), thalamus (Th). This schematic was obtained from Subramaniam et al. (2017).

1.3 Zebrafish as an in vivo model to study PD

The prevalent use of the teleost zebrafish (*Dania rerio*) in studying brain neurogenesis can be attributed primarily to the activation of neural stem cells from early development all the way to later stages, including adulthood (Kizil, 2012; Schmidt, 2013; Diotel, 2020). More studies are starting to utilize zebrafish models as they are advantageous at various stages of development, especially in their early embryonic and larval stages. For example, the external growth of embryonic zebrafish happens rapidly, and their embryos appear transparent when visualized under a fluorescence microscope (Berry, 2007; Gut, 2017). This allows them to be an excellent model to follow and study, especially for studies that focus on understanding the early stages of physical development. For example, the vascular morphogenesis of blood vessels in zebrafish embryos can be visualized using simple labelling methods (Gore, 2012; Gut, 2017). In addition, zebrafish are small in size, and their females can lay hundreds of eggs on a weekly basis, providing researchers with a great number of fish to analyze. Furthermore, these embryos will only take three months to

achieve sexual maturity and be considered adults (Kimmel, 1995). This is why a lot of researchers prefer to use them to investigate cellular and behaviour changes as opposed to mice or monkeys.

After the completion and publication of the complete zebrafish genome sequence in 2013, 26,206 protein-coding regions were identified (Howe, 2013). Furthermore, about 71.4% of human genes were found to contain at least one zebrafish orthologue while 69% of zebrafish genes contain a minimum of one human orthologue (Howe, 2013). These findings were interesting given the drastic anatomical differences between zebrafish and humans, allowing zebrafish to be an excellent tool for genetic manipulation experiments. Of significance to my thesis project are their markedly increased capacity of neuronal regeneration and ability of adult neurogenesis (Ghosh, 2016; Godoy, 2020; Zambusi, 2020). **Figure 3** shows the highly prolific nature of the adult zebrafish brain and the distinct progenitor niches spread along the rostro-caudal axis of the brain.

The zones where levels of cell proliferation seem to be elevated contain specific cells that are characterized with stem cell-like features. What makes these cells unique is that they are considered regenerative neural precursors (Kizil, 2012; Diotel, 2020). Some key regions to consider when observing neuroregeneration in the adult zebrafish brain can include the olfactory bulb, telencephalon, diencephalon, and the prectal area (Grandel, 2006; Kizil, 2012) (**Figure 3**).

Since neurogenesis is possible in the adult zebrafish brain, such a characteristic can provide us with a valuable insight as to how neurogenesis can be extrapolated and linked to adult mammalian brains with PD-like symptoms. In addition, identifying key adult stem cell niches in the adult zebrafish brain can potentially allow us to locate evolutionary similar regions that could be essential in adult neurogenesis in humans. Thus, the zebrafish brain has the potential to model complex diseases in humans like Parkinson’s disease.

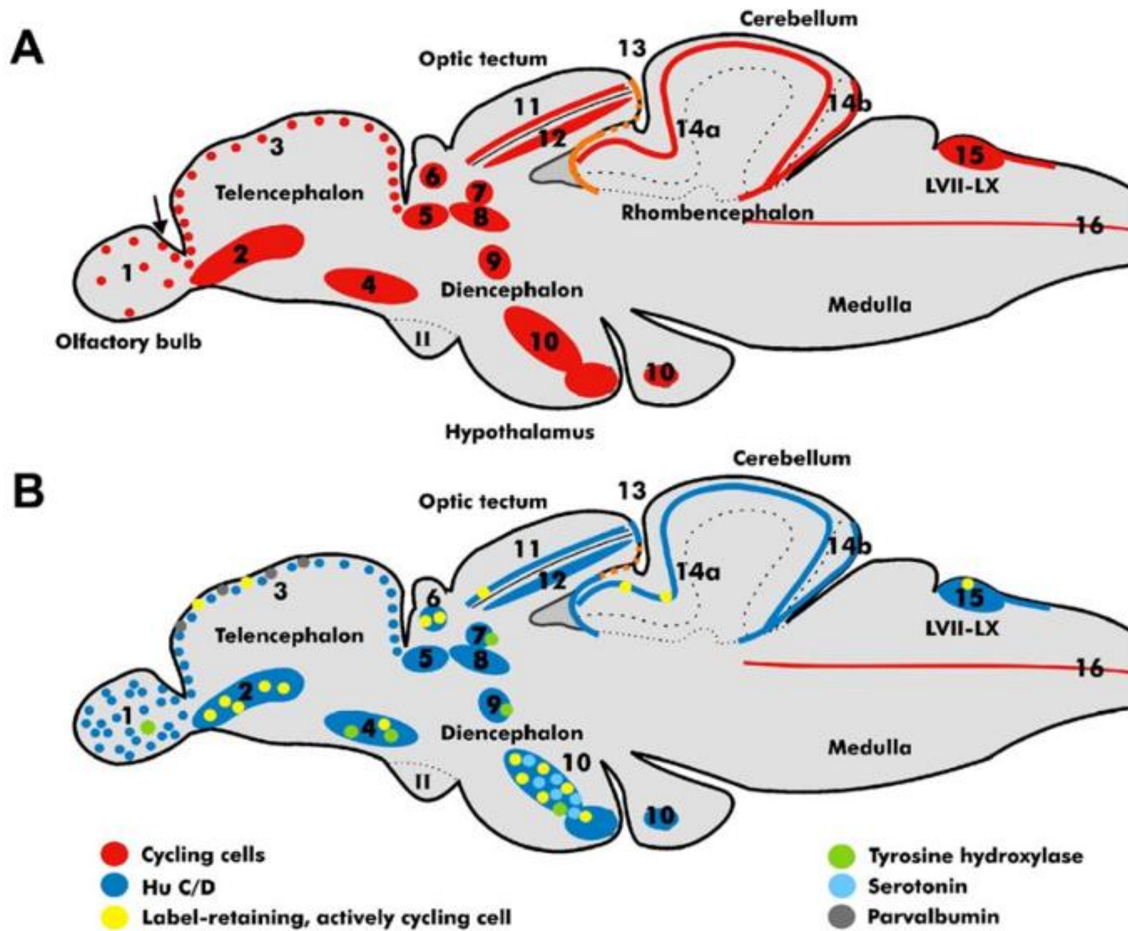


Figure 3. Distinct proliferation zones scattered along the rostro-caudal axis of the adult zebrafish brain.

(A) Red shaded areas indicate the localization of proliferative activity mostly in areas such as the olfactory bulb, ventral and dorsal parts of the telencephalon, caudal regions of the diencephalon, and in DAergic neuron clusters found in the pretectal area. **(B)** Blue shaded areas house specialized

neural cell types like Tyrosine hydroxylase, a known marker of DAergic neurons (green). This schematic was obtained from Grandel et al. (2006).

1.4 Dopaminergic neuron ablation

In order to study neurogenesis and the regenerative capabilities of adult zebrafish, different methods have been developed over the years that specifically target the ablation of DAergic neurons (Kroehne, 2011; Schmidt, 2014; Godoy, 2015). One common method to examine brain regeneration involves the use of mechanical stab lesions (Kishimoto, 2012; Schmidt, 2014; Weinschutz Mendes, 2020). This method is usually done by inserting a needle directly in a specific brain region to initiate a regenerative response. Researchers would euthanize fish at different days post-lesion (dpl) to study the impacted area and look at cellular markers for neurogenesis, including ongoing proliferation and differentiation (Schmidt, 2014; Diotel, 2020) . However, this approach is often considered invasive and non-specific, triggering pathways that might promote undesirable side effects (Schmidt, 2014).

Other studies rely on potent neurotoxins to destroy dopaminergic neurons (Tieu, 2011; Kalyn, 2019). Neurotoxins like 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, rotenone, and 1-methyl-4-phenyl-pyridinium (MPP+) have been used previously to target and ablate DAergic neurons (Alam, 2002; Peng, 2004; Tieu, 2011; Kalyn, 2019). However, the degree of variability in neuron ablation and the effectiveness of these neurotoxins remain varying. (Caldwell, 2019; Kalyn, 2019).

Chemogenetic ablation, a more novel approach, is regarded as one of the best tools to examine neuroregeneration in the adult zebrafish brain. It is considered advantageous as it allows the specific manipulation of genetically defined target cells (Godoy, 2015). This tissue-specific targeting can be visualized by the expression of an attached designer receptor that is stimulated

only upon the addition and binding of a unique ligand molecule (Curado, 2007; Godoy, 2015; Roth, 2016). To better understand this approach, it is important to consider the genetic and chemical aspects implicated in this method.

Godoy et al. designed the Tg(*dat:CFP-NTR*) zebrafish line as a way to specifically label and ablate DAergic neurons in larval and adult zebrafish (Godoy, 2015) (**Figure 4**). To understand the different elements of this transgenic line, it is important to note that DAergic neurons are uniquely identified by the presence of transmembrane dopamine transporter (*dat*) proteins (Holzschuh, 2001). These transmembrane proteins help translocate dopamine molecules into the cytosol of DAergic neurons from the extracellular space (Ciliax, 1995) (**Figure 5**). To drive the expression of downstream genes or reporters found exclusively in DAergic neurons, *dat* cis-regulatory elements were used (Godoy, 2015) (**Figure 4**).

Another key element of this transgene construct is the *Escherichia coli* (*E.coli*) bacterial nitroreductase (NTR) enzyme. The expression of the NTR enzyme is driven primarily by the activation of the DAT promoter. It has been previously established that the bacterial NTR enzyme has the capacity to catalyze the conversion of the non-toxic prodrug Metronidazole (MTZ) into a cytotoxic DNA-damaging compound (Curado, 2008). This concept is important as only cells that use the *dat* cis-regulatory elements to produce NTR will be impacted by MTZ administration. On the other hand, cells that lack *dat* cis-regulatory elements and consequently are unable to drive the expression of the NTR enzyme will not be capable of converting MTZ into a toxic metabolite and thus will not be impacted by the MTZ treatment (Curado, 2008; Godoy, 2015) (**Figure 5**). Thus, this chemogenetic method is used to selectively and conditionally ablate DAergic neurons without affecting neighbouring cells, preventing undesirable side effects (Curado, 2007; White, 2013; Godoy, 2015; Godoy, 2020).

The mechanism of action involving MTZ starts with the reduction of NTR by NADH or NADPH. This is followed by the attachment of MTZ to the reduced form of NTR, allowing MTZ to be reduced into a toxic cross-linking DNA damaging substance (**Figure 5**). That is why cell death only happens in cells expressing the *E.coli* NTR enzyme. Furthermore, it is believed that cell death following MTZ administration occurs via an apoptotic pathway (Curado, 2008; White, 2013; Godoy, 2015).

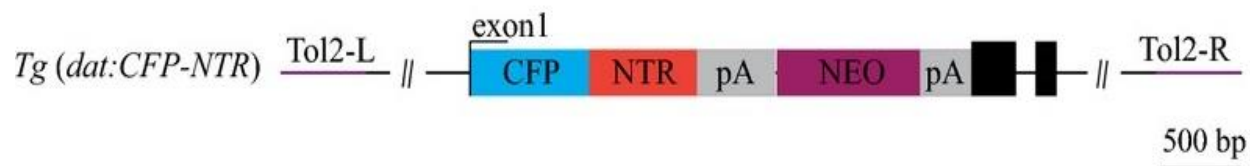


Figure 4. Schematic representation of the Tg(*dat:CFP-NTR*) transgene construct.

The construct consists of cyan fluorescent protein (CFP), nitroreductase enzyme (NTR), polyadenylation sequence (pA), and Neomycin (NEO). This schematic was obtained from Godoy (2015).

Recent studies are starting to use the nitroreductase-metronidazole (NTR-MTZ) approach to selectively ablate other unique types of cells in the body (Iervolino, 2020; Sharrock, 2020). Interestingly, cell-specific ablation tests are also being conducted in *in vitro* and *in vivo* systems. In addition, other variants of the bacterial NTR enzyme are being used as they seem to be more efficient (Sharrock, 2020). For example, the bacterial species *Vibrio vulnificus* was used to extract a variant referred to as NTR 2.0, which was reported to enhance NTR-MTZ cell ablation efficiency

by about 100-fold. In addition, this potent variant was able to destroy the remaining cell types that appear to be “resistant” (Sharrock, 2020).

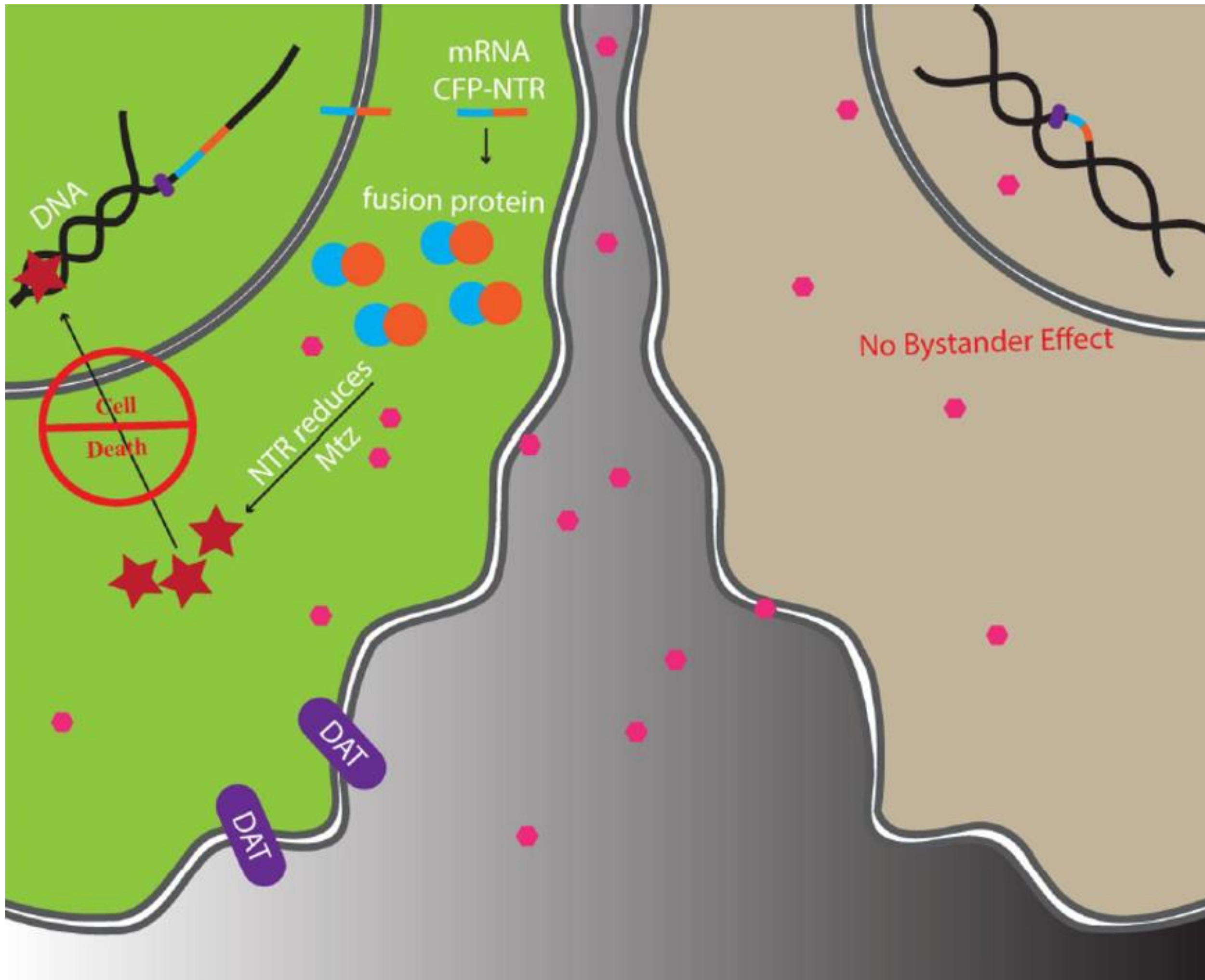


Figure 5. Schematic illustrating the conditional and selective ablation of DAergic neurons.

As the prodrug MTZ penetrates the blood-brain barrier, it begins to disperse across all types of cells. That said, the NTR enzyme can only be found in *dat*-expressing cells. Thus, only cells that have the CFP-NTR fusion protein will be able to successfully reduce the harmless MTZ substance into a toxic DNA damaging compound. This eventually leads to the death of DAergic neurons via an apoptotic pathway. Although MTZ is available to neighbouring cells, they will not be impacted

as they do not have the CFP-NTR fusion protein. MTZ molecules can be seen in pink, CFP in blue, and the NTR enzyme in orange. This schematic was obtained from Godoy (2015).

1.5 Nifurpirinol as an alternative substrate to MTZ

A recent study conducted by Bergemann et al. examined the efficacy of Nifurpirinol (NFP) as an alternative substrate to MTZ for the ablation of DAergic neurons in Tg(*dat:CFP-NTR*) zebrafish larvae (Bergemann, 2018). This study claimed that NFP caused rapid and reliable DAergic neuron ablations at a concentration 2,000-fold lower than MTZ. In addition, this study showed that neuronal ablations occurred at an NFP concentration that is three times lower than its own toxic concentration (Bergemann, 2018).

NFP toxicity was first assessed in non-transgenic wildtype (WT) zebrafish larvae. No morphological or direct adverse side effects were observed on target cells. Moreover, NFP did not show any signs of impacting innate regeneration mechanisms (Bergemann, 2018). Thus, similar to MTZ, the effect of NFP in NTR-mediated cell ablations was only specific to target cells in transgenic larvae and did not show a bystander effect on neighboring cells. Tg(*dat:CFP-NTR*) zebrafish larvae treated with 5 μM NFP at 3 days post fertilization (dpf) had less DAergic neurons in the diencephalon when visualized with CFP staining relative to MTZ and DMSO-treated fish two days following the treatment (Bergemann, 2018) (**Figure 6**). In addition, the remaining DAergic neurons observed in the diencephalon in the NFP-treated brain had irregularly shaped nuclei and a larger cytoplasm. Furthermore, larvae treated with MTZ still had more morphologically viable DAergic neurons and less cell debris relative to NFP-treated larvae (Bergemann, 2018) (**Figure 6**).

Finding an alternative substrate to MTZ for nitroreductase-mediated cell ablations can be beneficial as there are some limitations to the use of the prodrug MTZ. For example, using the established 10 mM MTZ concentration for extended periods (more than 24h) was shown to be toxic and MTZ seems to be toxic at a concentration that is only 1.5 times higher than the concentration that is generally used (Mathias, 2014, Bergemann, 2018). Furthermore, the use of MTZ as a substrate for nitroreductase-mediated cell ablations still produces inconsistent ablations and, in some cases, no ablations in some regions of the brain (Mathias, 2014; Godoy, 2020).

Given that MTZ can be administered at a maximum concentration of 10 mM for a 24h period, this limits the possibilities available to experiment with MTZ due to toxicity issues (Curado, 2007; Mathias, 2014; Godoy, 2015; Godoy, 2020). It would be useful to consider administrating MTZ directly into the brain via cerebroventricular microinjection (CVMI) to improve its delivery and make it more specific (Kizil, 2011; Bhattarai, 2017) (**Figure 7**).

This approach could help us examine whether MTZ can penetrate and affect caudal regions in the brain and cause reliable ablations without increasing its concentration or prolonging the treatment period. However, potential studies will have to focus on finding the optimal concentration that will induce efficient DAergic neuron ablations without causing lethality.

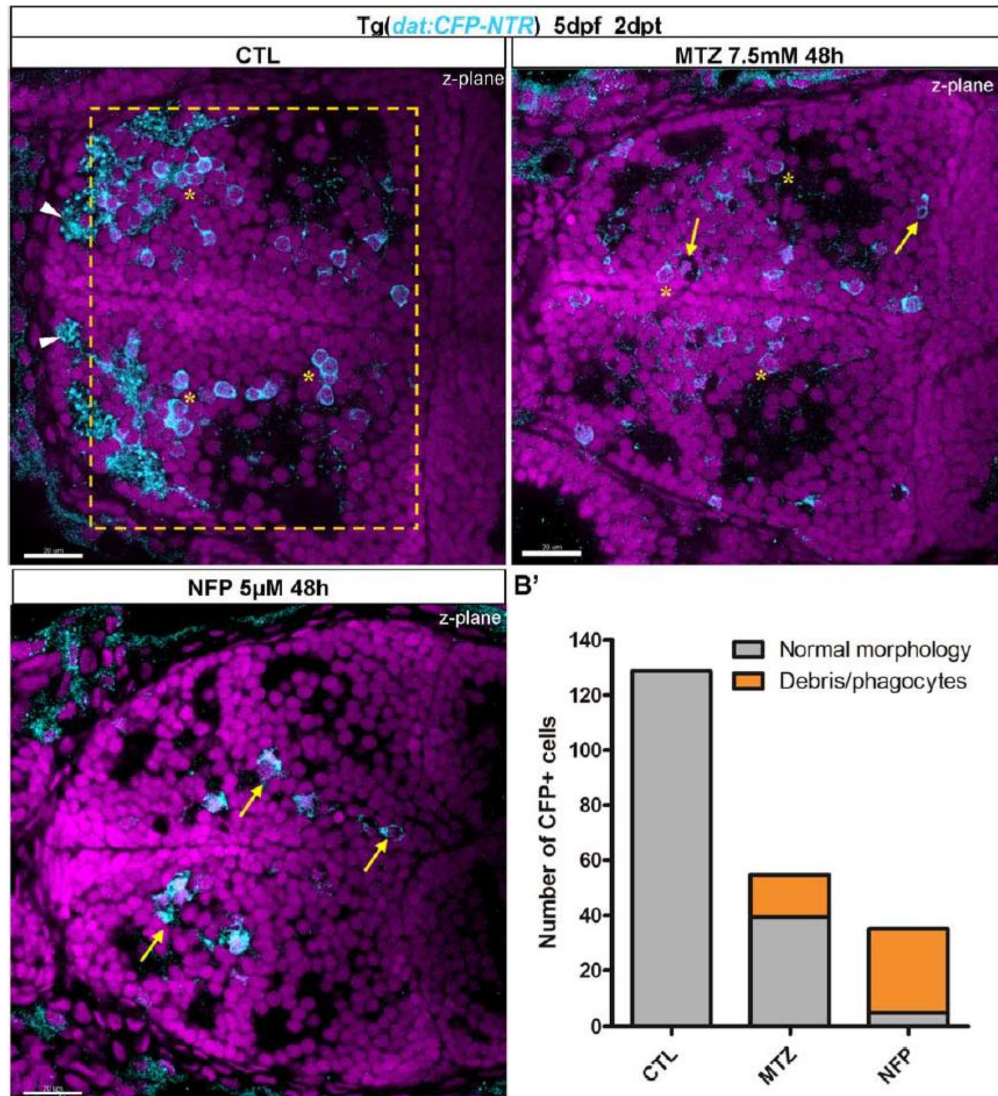


Figure 6. DAergic neuron ablation efficiency in the diencephalon of Tg(*dat:CFP-NTR*) zebrafish larvae under different treatments.

Zebrafish larvae were treated with 0.2% DMSO, 7.5 mM MTZ, or 5 μM NFP for 48 hours from 3 to 5 dpf. Z-plane images of representative cryosections are shown here. DAergic neurons (cyan) contain cell nuclei, seen with DAPI staining (magenta). Debris of DAergic neurons can be observed as marked by yellow arrows. **(B')** quantification of the number of CFP^{+} cells in the different treatment groups, with grey representing cells with normal morphology and orange indicating debris/phagocytosis. This figure was obtained from Bergemann et al. (2018).

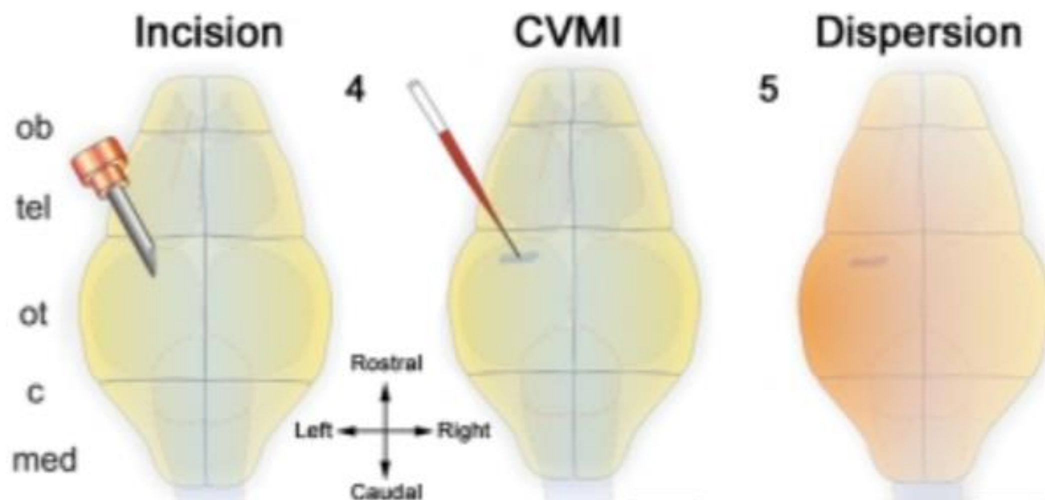


Figure 7. Schematic showing the insertion of a given substance via cerebroventricular microinjection (CVMI).

An incision is first created in the optic tectum region. The drug/substance can then be inserted directly through that open slit allowing it to spread throughout the brain. This method can also be applied to other brain regions like the telencephalon and the olfactory bulb. This schematic was obtained from Kizil et al. (2011).

Even though immersion treatments seem to be less efficient relative to other more invasive methods, it would be valuable to test the potency of NFP just like MTZ by administering it through immersion on adult Tg(*dat:CFP-NTR*) zebrafish. Determining if NFP is indeed a more powerful substrate than MTZ for nitroreductase-mediated cell ablations could unravel more potential for the already valuable chemogenetic ablation approach.

1.6 Dopaminergic neuron regeneration in adult zebrafish

It has been previously reported that the MTZ treatment effectively ablates DAergic neurons in juvenile Tg(*dat:CFP-NTR*) zebrafish (Lambert, 2012; Godoy, 2015). To build on previous findings, Dr. Godoy examined whether MTZ can still promote similar levels of DAergic neuron ablations in adult zebrafish at different periods following treatment to study adult neurogenesis (Godoy, 2020). The greatest degree of DAergic neuron ablation in adult zebrafish was observed 7 days post-treatment (dpt) in the rostral OB with an 83% reduction in the number of CFP^+ cells. This was validated with tyrosine hydroxylase (TH), an alternative marker for DAergic neurons (Grandel, 2006). Results showed an 86% reduction in the number of TH^+ cells in the rostral OB (Godoy, 2020). Ablation of DAergic neurons was also noticed in other parts of the OB, like the medial and the caudal regions. Adult zebrafish treated with MTZ were compared with ones treated with DMSO (control) at 7 dpt in this study (Godoy, 2020).

Godoy et al. also reported a 61% reduction in dopamine levels within the OB and the telencephalon (Godoy, 2020). When other neurotransmitters like serotonin and norepinephrine were analyzed in the olfactory bulb and the telencephalon at 7 dpt, results showed that their levels were comparable to the DMSO control (Godoy, 2020). This further confirms the selective ablation of DAergic neurons following the MTZ treatment in Tg(*dat:CFP-NTR*) adult zebrafish.

1.7 Impact of DAergic neuron loss and regeneration on olfactory sensation

To understand how the loss of DAergic neurons on the cellular level translates to behavioural impairments, a repulsive stimulus test was performed (Godoy, 2020). Results showed that MTZ-treated Tg(*dat:CFP-NTR*) zebrafish at 7 dpt spent more time in the area containing the repulsive scent of cadaverine (Godoy, 2020). This observation suggests that MTZ-treated fish at 7 dpt are unable to differentiate between the scents present in two different areas and cannot properly sense the repulsive scent of cadaverine. Furthermore, this hints at the impairment of the olfactory bulb region, as MTZ-treated fish are unable to steer away from the region with the repulsive scent. Interestingly, results showed that MTZ-treated fish were able to regain their olfactory senses and recover as they regained their ability to steer away from the repulsive scent of cadaverine at 45 dpt, which coincides with the restoration of DAergic neurons on the cellular level in the OB region (Godoy, 2020).

Furthermore, a recent study by Merhi et al. reported that adult zebrafish that are homozygous mutant for the *parla* gene showed a significant loss in olfactory function relative to Wild type adult zebrafish (Merhi, 2021). This study demonstrated that the loss-of-function *parla* mutant zebrafish suffer from a remarkable loss of DAergic neurons in the olfactory bulb and the telencephalon region (Merhi, 2021). In addition, their results validated the previous observation of Godoy et al. in which fish with decreased levels of DAergic neurons showed severe defects in olfactory function (Godoy 2020, Merhi, 2021). Merhi et al. also observed that adult zebrafish that are homozygous mutant for the *parla* gene spent more time in the stimulus arm containing the repulsive stimulant relative to the WT control (Merhi, 2021). Moreover, *parla* heterozygous mutant fish spent less time in the stimulus arm relative to the homozygous mutant; however, they

spent more time than WT fish. These results clearly demonstrate the importance of DAergic neurons in assisting zebrafish in distinguishing between different scents (Merhi, 2021).

1.8 Link between DAergic neuron loss and abnormal locomotor activity in zebrafish

In addition to DAergic neurons' role on olfactory function, motor activity also seems to be disturbed by the loss of DAergic neurons. It has been previously reported that DAergic neurons present within the diencephalospinal tract are directly implicated in motor behaviour (Jay, 2015). Jay et al. demonstrated that the specific ablation of the DAergic diencephalospinal neurons (DDNs) in zebrafish larvae resulted in impaired locomotor behaviour post-ablation (Jay, 2015). Fish larvae with ablated DDNs travelled on average 30.37 ± 9.71 cm, while control larvae travelled 72.95 ± 9.92 cm over 10 minutes (Jay, 2015). Average time spent swimming was another parameter that was tested. Fish larvae with ablated DDNs spent only 5.81% of the 10-minute testing period in an active swimming state, while the control larvae spent 15.84% (Jay, 2015). Surprisingly, there was not a significant difference in mean peak velocity between the two groups. This study provided a unique insight as they used a precise laser ablation technique to ablate distinct DAergic neuron populations (Jay, 2015). It is interesting to explore how this ablation method applies to adult zebrafish and how it might differ from the chemogenetic approach.

Since PD patients experience various motor symptoms as the disease progresses, it is critical to investigate the link between the gradual loss of DAergic neurons and changes in motor behaviour. Examining the unique regions where DAergic neuron regeneration happens and the specific time points following ablation can provide us with an understanding as to how adult zebrafish can respond to such disruptions in the brain.

1.9 Aim of the study

The overall goal of this thesis project was to extend on the previous work from our laboratory to better understand DAergic neuron ablation and regeneration in adult zebrafish using a non-invasive and cell-specific chemogenetic approach (Godoy, 2020). Using the Tg(*dat:CFP-NTR*) zebrafish line, the first aim revolved around investigating DAergic neuron ablations at additional time points following the MTZ treatment and in previously uncovered brain regions. For example, this included examining the rostral and medial telencephalon in greater depth in addition to the pretectal area. Uncovering the effects of MTZ on the more caudal regions of the brain will broaden our understanding of the efficacy and reliability of MTZ. Extensive analysis was done on time points shortly after the termination of the MTZ treatment. This included immediately following the treatment at 0 hours post-treatment (hpt), 1 dpt, 2 dpt, 7 dpt, and 15 dpt. This allowed us to identify the timepoints when extensive regeneration happens and how long the entire process takes. In addition, markers for proliferation and neural stem cells were used to precisely determine when and where regeneration initiates. It was hypothesized that if MTZ selectively ablates DAergic neurons, then the number of CFP^+ cells in the brain of adult zebrafish would change and consequently lead to more neural stem cells undergoing proliferation.

For the second aim, I tested the efficacy of the novel prodrug NFP in ablating DAergic neurons in adult zebrafish. Due to the limitations reported previously about MTZ, having a potent alternative substrate for nitroreductase-mediated cell ablations could allow researchers to achieve more effective and specific ablations of DAergic neurons. The available literature on the effects of Nifurpirinol is still very limited. Thus, I tried to optimize the conditions for using this prodrug to achieve reliable DAergic neuron ablations without causing lethality. It was hypothesized that if

NFP is a more potent substrate than MTZ for nitroreductase-mediated cell ablations, then it will cause a greater reduction in the number of DAergic neurons.

The last aim was focused on understanding the link between the ablation of DAergic neurons and the resulting changes in swimming behaviour. This allowed us to determine if the motor impairments were transient or more long-term. It was hypothesized that after the MTZ treatment, treated fish would gradually regain motor activity if they were able to regenerate their ablated DAergic neurons.

2. Materials and Methods

2.1 Animal care, husbandry, and transgenic zebrafish line

All experiments conducted in this thesis were in accordance with procedures approved by the Animal Care Committee at the University of Ottawa. Zebrafish were housed in tanks at 28.5 °C and maintained under a supervised light/dark (14h/10h) cycle. The main transgenic zebrafish line used in all experiments in this thesis is the Tg(*dat:CFP-NTR*) line. Only adult (4-6 months) fish were used for experiments. A combination of fluorescence microscopy and PCR was used for the screening of transgenic zebrafish. The majority of the protocols followed in this thesis were adapted from Godoy et al. (2015).

2.2 Chemogenetic ablation of DAergic neurons

Adult Tg(*dat:CFP-NTR*) fish were placed in 5 L glass tanks for two days prior to any treatment. Following this adjustment period, adult zebrafish were immersed in a tank containing 1L of either 0.2% DMSO (Sigma-Aldrich) or 10 mM MTZ (Sigma-Aldrich). This drug exposure immersion method is well established and has been adapted from multiple protocols (Agetsuma, 2010; Godoy, 2015; Godoy, 2020). Only three fish were placed in the breeding tanks containing either solution,

and the solution immersion exposure lasted only 24 hours. During the exposure period, all tanks were covered and were kept in the dark as DMSO and MTZ are light sensitive. After the 24-hour period, the exposure treatment was terminated by doing three continuous washes. Fish were transferred into breeding tanks containing fresh system water. The water was changed every hour for a total of three times. After the washes were done, fish were returned to their previously designated 5 L glass tanks. Fish did not receive any food the morning of the treatment nor during the exposure period. The fish were fed the next morning after the last wash. For some of the experiments, Nifurpirinol (Dr Ehrenstorfer GmbH, Augsburg, Germany) was used as an alternative to MTZ. The concentrations used for Nifurpirinol solution exposure treatments include 5 µM, 6 µM, 8.5 µM, 10 µM, 20 µM, and 30 µM. Nifurpirinol was also dissolved in 0.2% DMSO.

2.3 Histology and cryosectioning

Adult zebrafish were euthanized by placing them in water containing ice at specific days post-treatment (dpt). This was done mostly at 0, 1, 2, 7, and 15 dpt. Fish heads were removed and submerged directly in 4% Paraformaldehyde (PFA) for at least 24 h at 4 °C for fixation. Brains were then dissected and placed for 15 minutes in PFA. Brains were then washed three times with 1X Phosphate Buffered Saline (PBS) for 1 hour. After the last wash with 1X PBS, brains were immersed in a 30% sucrose/PBS solution for at least 24 h at 4°C. Brains were then oriented in cryomolds and fully submerged with Tissue-Tek OCT compound and frozen in liquid nitrogen for at least 1 minute before being stored at - 20°C. Frozen brains were then sectioned using the cryostat machine in order to obtain cryosections with a thickness of 16 µm. Tissue sections were kept at room temperature (RT) for at least 20 minutes before they were stored at -20 °C for future experiments.

2.4 Immunohistochemistry

Brain sections were left at RT for at least 20 minutes before being washed with PBST (PBS with 0.5% Triton X-100) for 20 minutes. Antigen retrieval was performed before the blocking step for all experiments. Washed slides were placed for 15-20 minutes at 85°C in a solution containing 10 mM sodium citrate (pH 6.0) with 0.05% Tween-20. After the incubation period, the slides were then washed three times (10 minutes each) in PBST. The slides were then placed in 5 % fetal bovine serum (FBS) in PBST, blocking solution, for 2 hours at RT. About 225µL of the primary antibody solution was added to each slide, and the slides were incubated overnight at 4°C. All primary antibodies were diluted in the blocking solution. A list of all the primary antibodies used in this thesis can be found in **Table 1**. Slides were then washed in PBST at least five times (15 min/wash) at RT. After the last wash, 225 µL of the secondary antibody was then added to each slide. The slides were kept in the dark overnight at 4 °C. Slides were then washed in PBST at least five times (15 min/wash) at RT. The slides were covered during this process as the secondary antibody is light sensitive. A list of all the secondary antibodies used can be found in **Table 1**. After the last wash, slides were allowed to fully dry for about 20 minutes before adding 65 µL of VECTASHIELD® Antifade Mounting Media with DAPI (4',6-diamidino-2-phenylindole) to each slide. Slides were eventually covered with a Microscope Cover Glass and stored at 4°C.

Table 1. Primary and secondary antibodies used in immunohistochemistry experiments

Primary antibody			
Host organism	**Target**	**Catalogue number**	**Antibody dilution**
Mouse	CFP	Clonetech	1:400
Mouse	PCNA	Ab29 (Abcam)	1:400
Rabbit	Sox2	Ab97959 (Abcam)	1:400
Secondary antibody			
Host organism	**Target**	**Supplier**	**Antibody dilution**
Goat Alexa 488	Mouse IgG (H+L)	Thermo Fisher Scientific	1:400
Goat Alexa 594	Rabbit IgG (H+L)	Thermo Fisher Scientific	1:400

2.5 Image analysis and cell counting

All images in this thesis were captured using the Olympus FV1000 BX61 LSM confocal microscope. To account for all the cells present at the different depths of a tissue, only the composite image of the various Z-stacks was acquired. Images were then processed and analyzed using the Fluoview 10-ASW 4.2 Olympus software. To count the number of cells in a specific region of the brain, three subsequent tissues with similar characteristics were chosen. Two independent researchers manually counted the number of cells in each tissue and the average number was used. Manual cell counts were done using Fluoview 10-ASW 4.2 Olympus software. Only one representative image of the specific tissue was included in this thesis.

2.6 Behavioural testing

Prior to the start of the behavioural test, fish were placed individually in tanks filled with 1.5 L of system water. Fish were allowed to acclimate by keeping them in the testing chamber for at least 10 minutes before the start of the test. If fish displayed signs of stress by swimming sporadically, the acclimatization period was prolonged by at least 15 minutes. Motor activity was tested at two key time points: 0 dpt and 7 dpt. Each trial lasted 10 minutes. Three different parameters were evaluated: total distance travelled, total time spent in a stationary state, and average velocity. Total distance was calculated by adding the inactive distance, small distance, and large distance. Distances were recorded in centimetres (cm). Total freezing time was determined by recording the inactive duration and was measured in seconds (s). Average velocity was calculated by dividing the total distance (cm) by the total trial time of 600 s. Tracking of the various parameters was done via Viewpoint ZebraLab tracking software.

2.7 Statistical analysis

Statistical analysis was done using GraphPad Prism 9 software. Quantification of cells between two independent groups was done using an unpaired student t-test. Behavioural analysis was assessed using a one-way ANOVA followed by Dunnett's multiple comparisons test. Statistical significance was determined when the p-value was found to be <0.05. Summary of symbols: not significant or ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), ***($p < 0.001$), **** ($p < 0.0001$). Error bars in this thesis represent the standard error of the mean (SEM).

3. Results

3.1 Ablation of DAergic neurons in MTZ-treated fish at 7 dpt

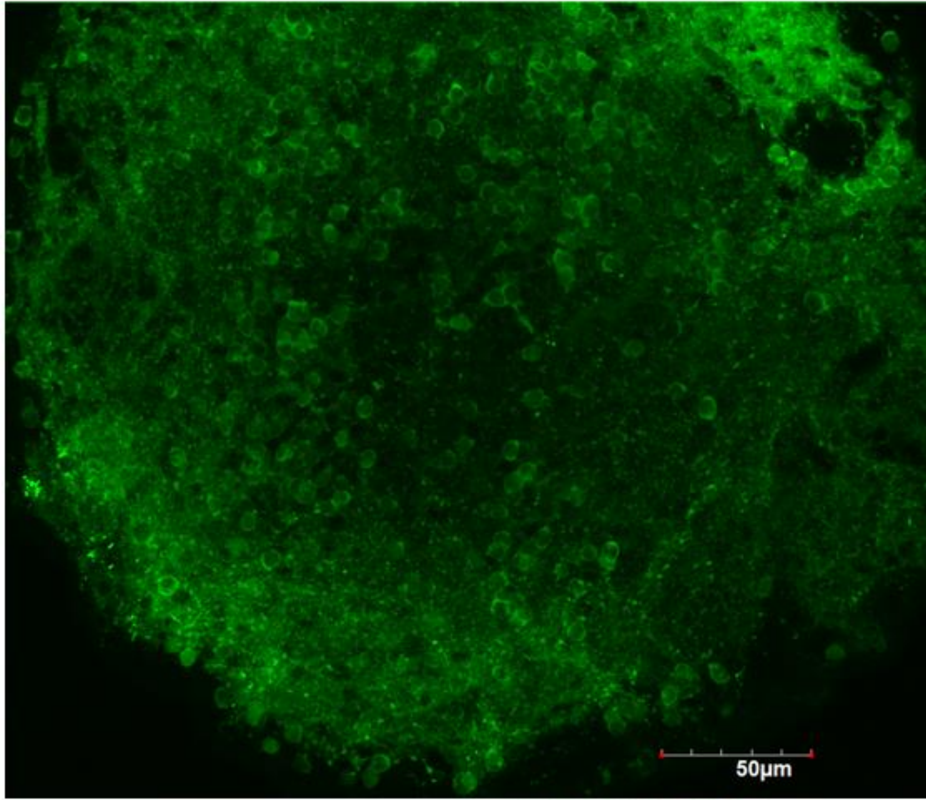
In order to examine and follow the regeneration of DAergic neurons in the adult zebrafish brain, it is essential first to confirm that they were successfully ablated. Another important thing that was considered in this analysis is the time point at which maximum ablation happens. Dr. Godoy successfully designed a transgenic line that made it possible to visualize DAergic neurons (Godoy, 2020). Adult Tg(*dat:CFP-NTR*) zebrafish were immersed in a solution containing MTZ for 24 hours. Fish were sacrificed at 7 dpt in order to examine various brain regions to determine the effect of this prodrug on DAergic neurons. Immunohistochemistry was performed on coronal brain sections obtained from MTZ and DMSO-treated fish using an anti-CFP antibody to label DAergic neurons.

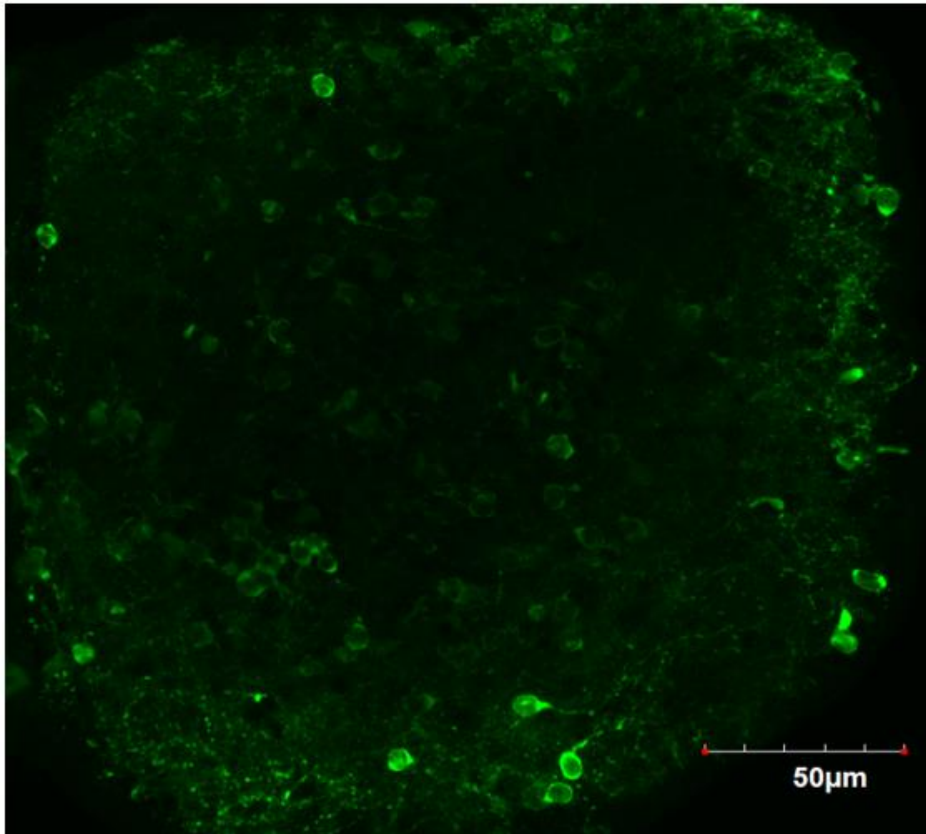
Results showed a drastic decrease in the number of CFP^+ cells in the OB of MTZ-treated fish relative to the DMSO control at 7 dpt (**Figure 8A**). Quantification of the number of CFP^+ cells in the OB showed a significant two-fold decrease in the MTZ treated group ($p=0.0037$, $n=3$, **Figure 8B**). This confirms the successful ablation of DAergic neurons in the OB region as previously observed by Godoy et al. (2020). Most CFP+ cells were localized in the central region, and some were along the periphery of the OB. In addition, it seems that CFP^+ cells tend to be packed together in close proximity in the control group. CFP^+ cells seem to be more dispersed in the MTZ-treated group (**Figure 8A**).

Another region of interest was the telencephalon, which has not been previously examined in great depth in adult zebrafish. Results showed a significant reduction in the number of CFP^+ cells present in the telencephalon in the MTZ-treated group relative to the control ($p=0.0157$, $n=3$, **Figure 9B**). CFP^+ cells were mostly localized near the ventricular region of the telencephalon; however, they were dispersed near the dorsal part (**Figure 9A**).

To determine if the effects of MTZ extend to the more caudal regions of the brain, the pretectal area was examined. Preliminary results showed a drastic reduction in the expression of CFP^+ cells within the DAergic clusters of the pretectal region in the MTZ-treated group relative to the DMSO control at 7 dpt (**Figure 12**).

A





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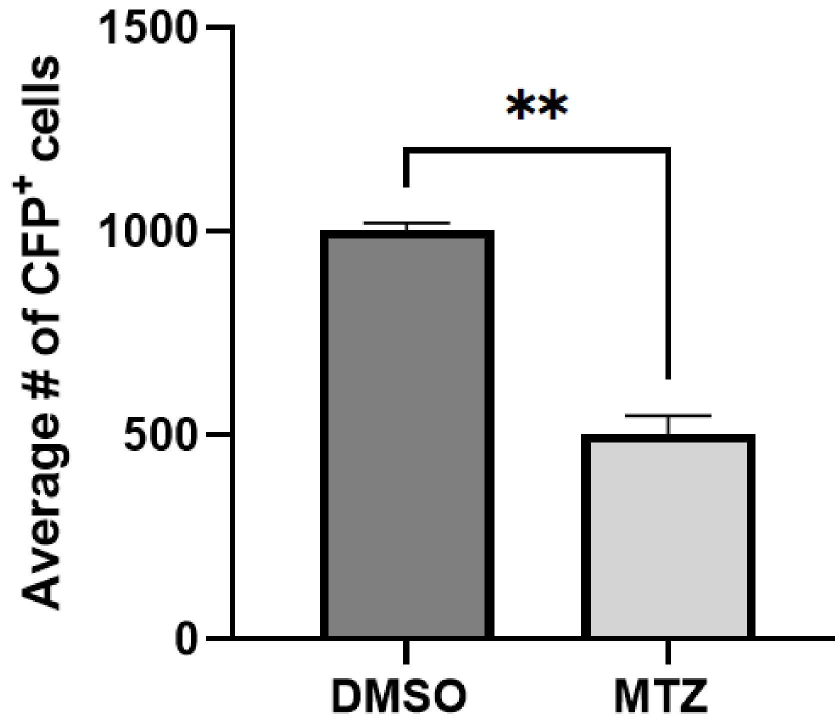
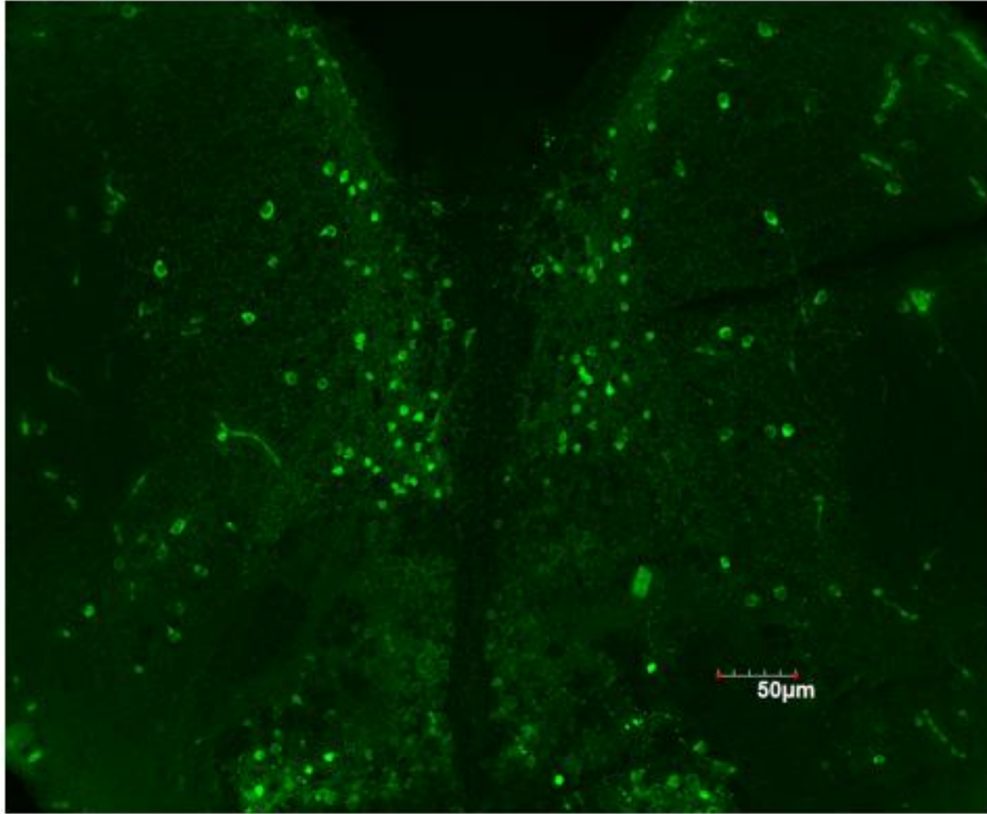


Figure 8. DAergic neuron ablation in the olfactory bulb of adult zebrafish at 7 dpt.

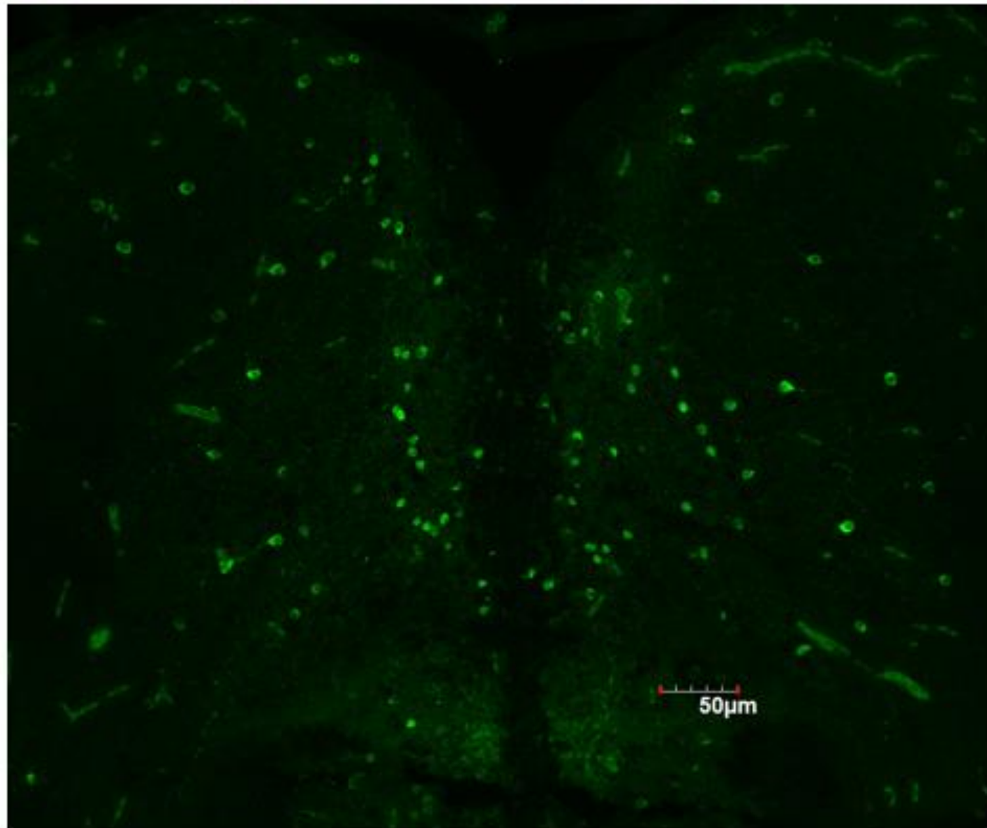
(A) Immunohistochemical staining of CFP^{+} cells (green) in DMSO and MTZ-treated fish. Coronal sections of tissue samples were taken at 16 µm. **(B)** Quantification of CFP^{+} cells present in the OB at 7 dpt (n=3). Scale bar: 50 µm. Error bars represent S.E.M. ** ($p < 0.01$)

A

DMSO



MTZ



B

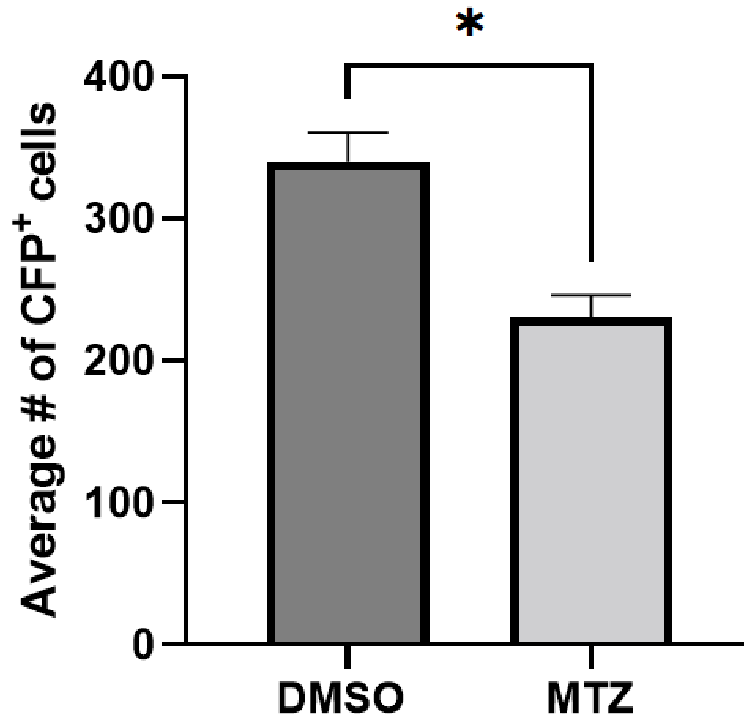


Figure 9. Number of CFP+ cells in the telencephalon of adult zebrafish at 7 dpt.

(A) Immunohistochemical staining of CFP^{+} cells (green) in DMSO and MTZ-treated fish. Coronal sections of tissue samples were taken at 16 µm. **(B)** Quantification of CFP^{+} cells present in the telencephalon at 7 dpt (n=3). Scale bar: 50 µm. Error bars represent S.E.M. * ($p < 0.05$).

3.2 NFP is efficient in ablating DAergic neurons in the OB and telencephalon at 7 dpt.

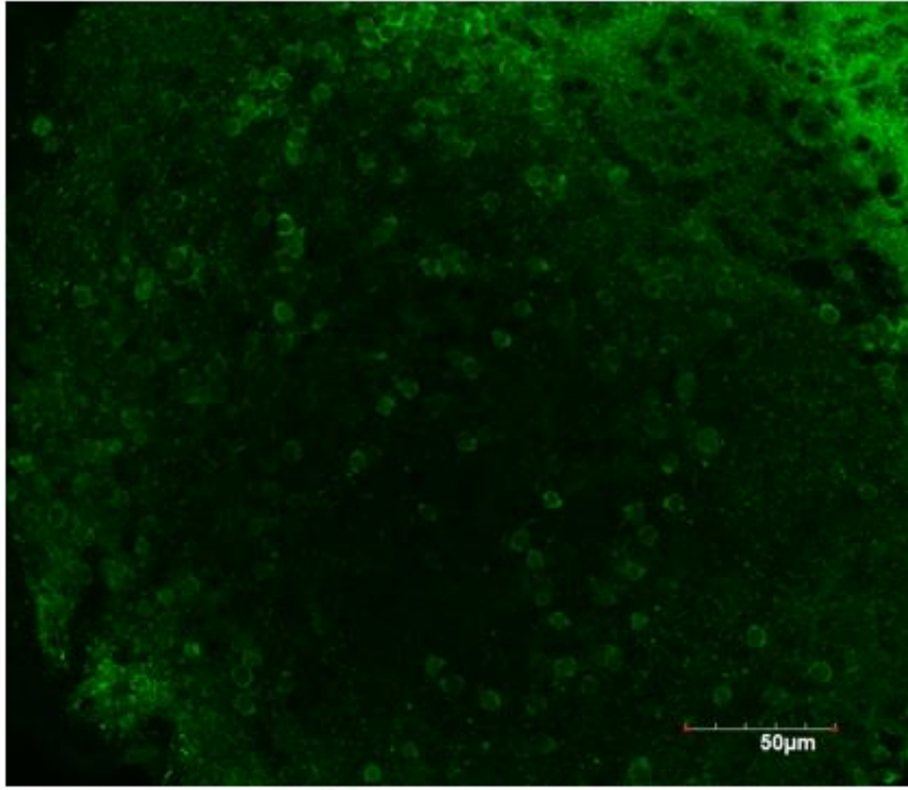
Given that NFP is still considered to be a novel drug in terms of its effectiveness in ablating DAergic neurons in the brain of adult zebrafish, various concentrations of the drug were initially tested. Adult Tg(*dat:CFP-NTR*) zebrafish were immersed in a solution containing 5 µM, 10 µM, 15 µM, 20 µM, or 30 µM NFP for 24 hours. Adult zebrafish did not survive for more than 8 hours when placed in an NFP solution with a concentration greater than 5 µM (n=4 fish/group). Next, fish were placed in a solution containing 5 µM NFP for 48 hours. None of the fish survived when checked after the 48h treatment period (n=6). Thus, it was determined that the best method to test this drug is by immersing adult Tg(*dat:CFP-NTR*) zebrafish in 5 µM for 24 hours. Results showed that NFP-treated fish had a significant decrease in the number of DAergic neurons in the OB at 7 dpt relative to the DMSO control (p=0.0112, n=3, **Figure 10B**). The telencephalon region was examined and results also showed a significant reduction in CFP^+ cells (p= 0.0123, n=3, **Figure 11B**).

When examining the DAergic clusters located in the pretectal area, similar CFP expression patterns were noticed relative to the DMSO control (**Figure 12**). MTZ seems to affect that region more than NFP and that could be due to several reasons explained in the discussion.

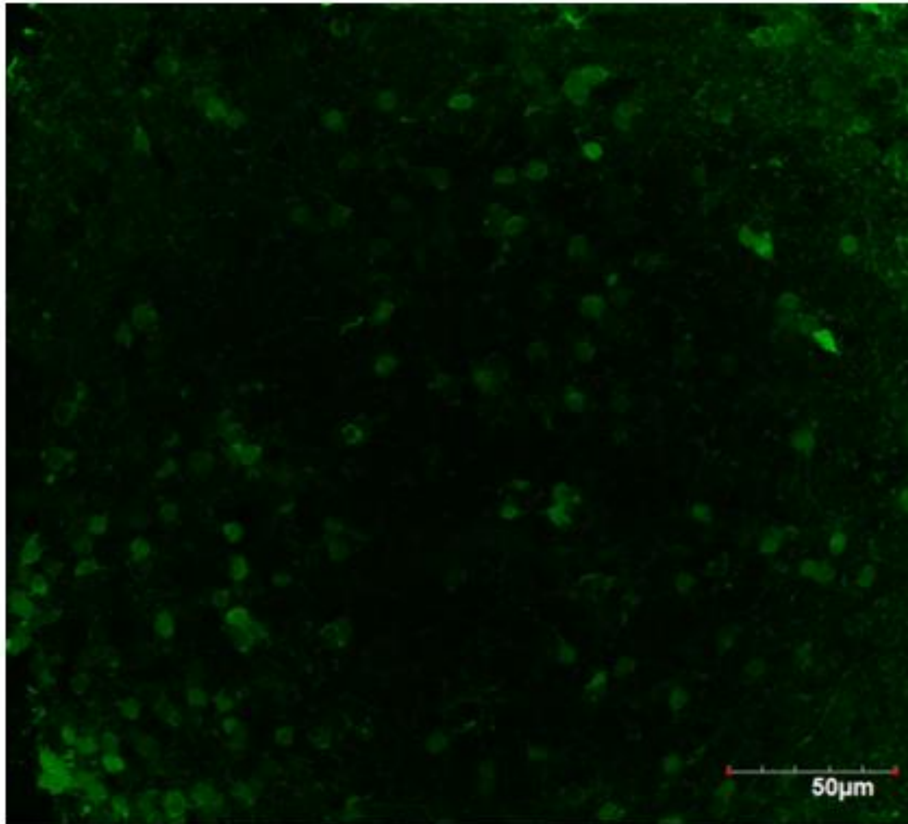
To determine if the effects of NFP are specific to Tg(*dat:CFP-NTR*) zebrafish, adult WT zebrafish were immersed in a solution containing 7.5 µM NFP for 24 hours. All WT fish survived the treatment period and had normal swimming behaviour directly after the treatment (n=6). It is important to note that when Tg(*dat:CFP-NTR*) zebrafish were placed in the solution containing 7.5 µM NFP for 24 hours, none of them survived (n=6).

A

DMSO



NFP



B

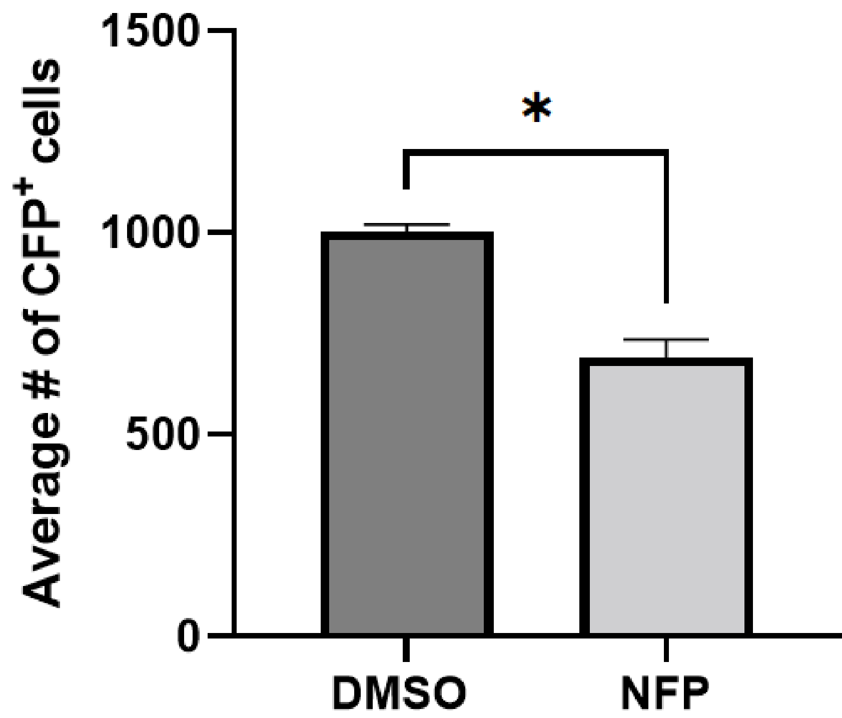
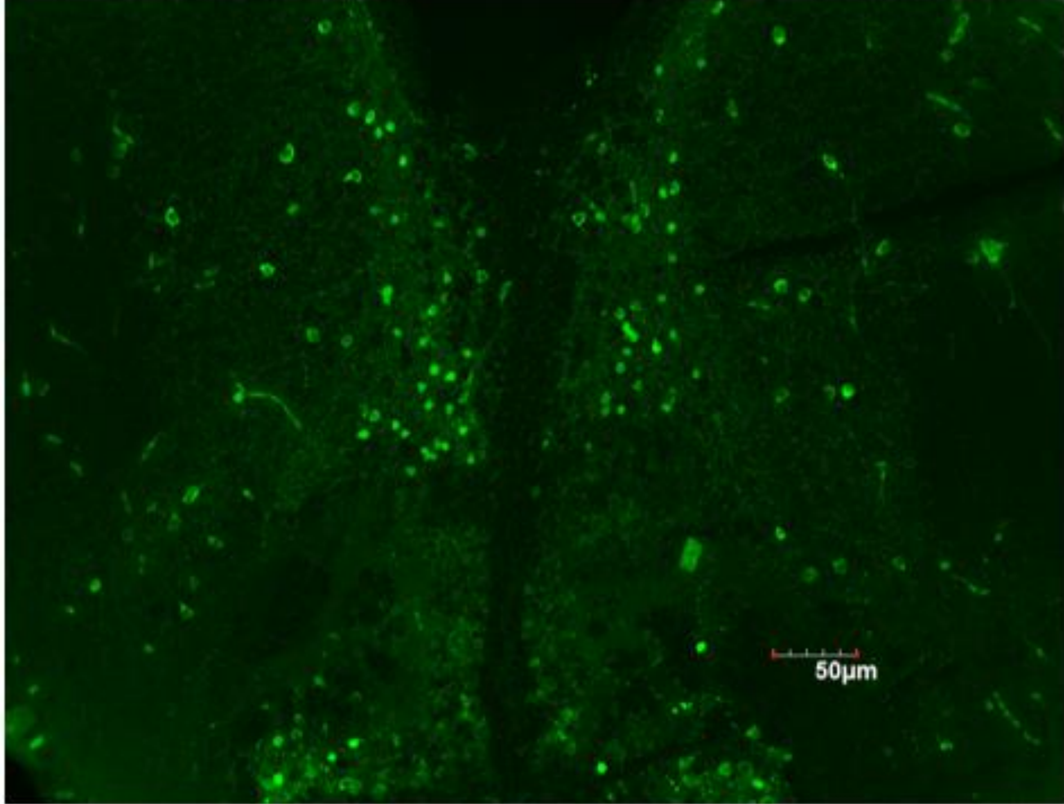


Figure 10. Reduction in the number of CFP+ cells in the OB of NFP-treated zebrafish at 7 dpt.

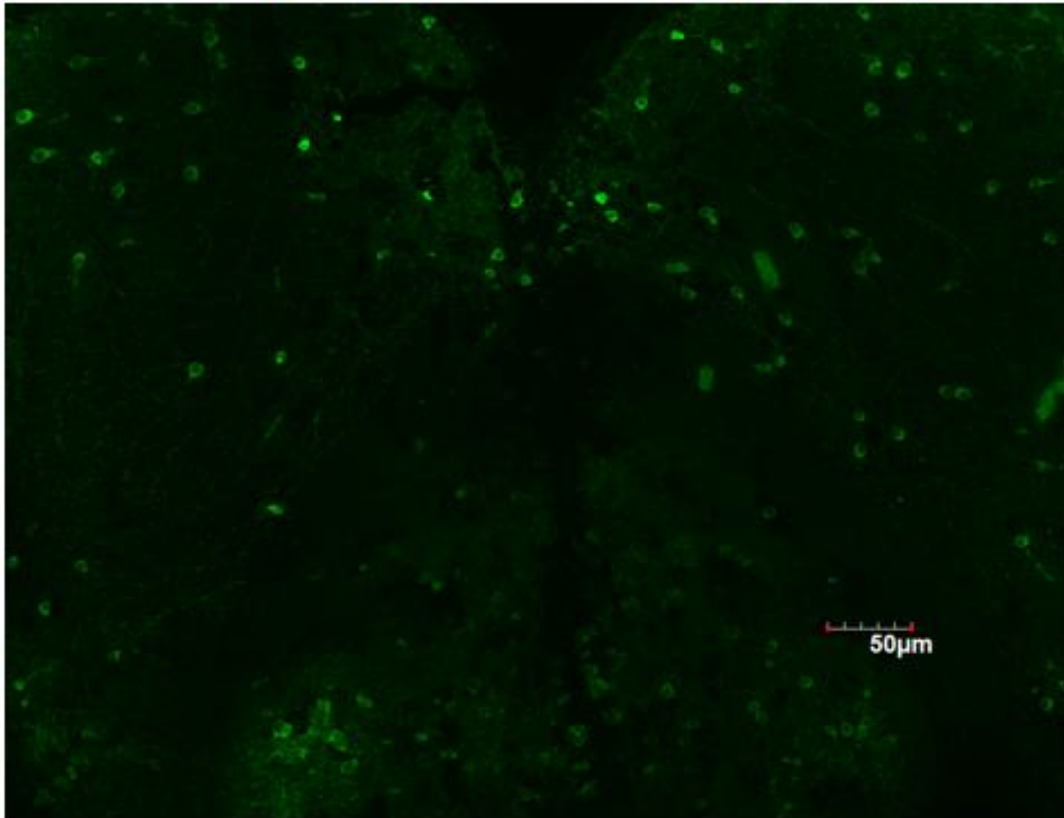
(A) Immunohistochemical staining of CFP^+ cells (green) in DMSO and NFP-treated fish. Coronal sections of tissue samples were taken at 16 µm. **(B)** Quantification of CFP^+ cells present in the OB at 7 dpt (n=3). Scale bar: 50 µm. Error bars represent S.E.M. * ($p < 0.05$)

A

DMSO



NFP



B

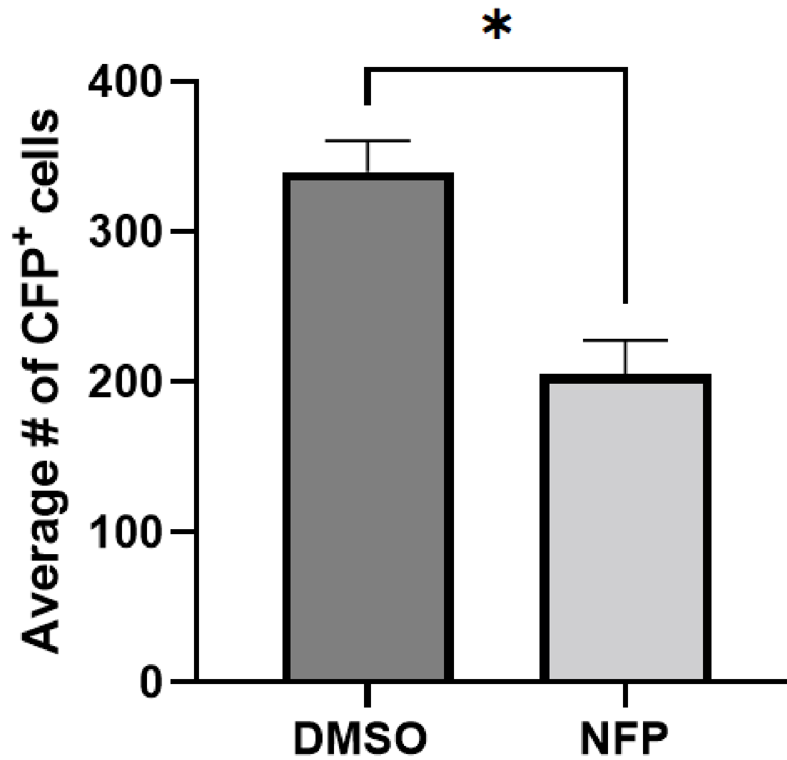


Figure 11. Ablation of DAergic neurons in the telencephalon of NFP-treated zebrafish at 7 dpt.

(A) Immunohistochemical staining of CFP^+ cells (green) in DMSO and NFP-treated fish. **(B)** Quantification of CFP^+ cells present in the telencephalon at 7 dpt (n=3). * ($p < 0.05$). Scale bar: 50 μm. Error bars represent S.E.M. * ($p < 0.05$).

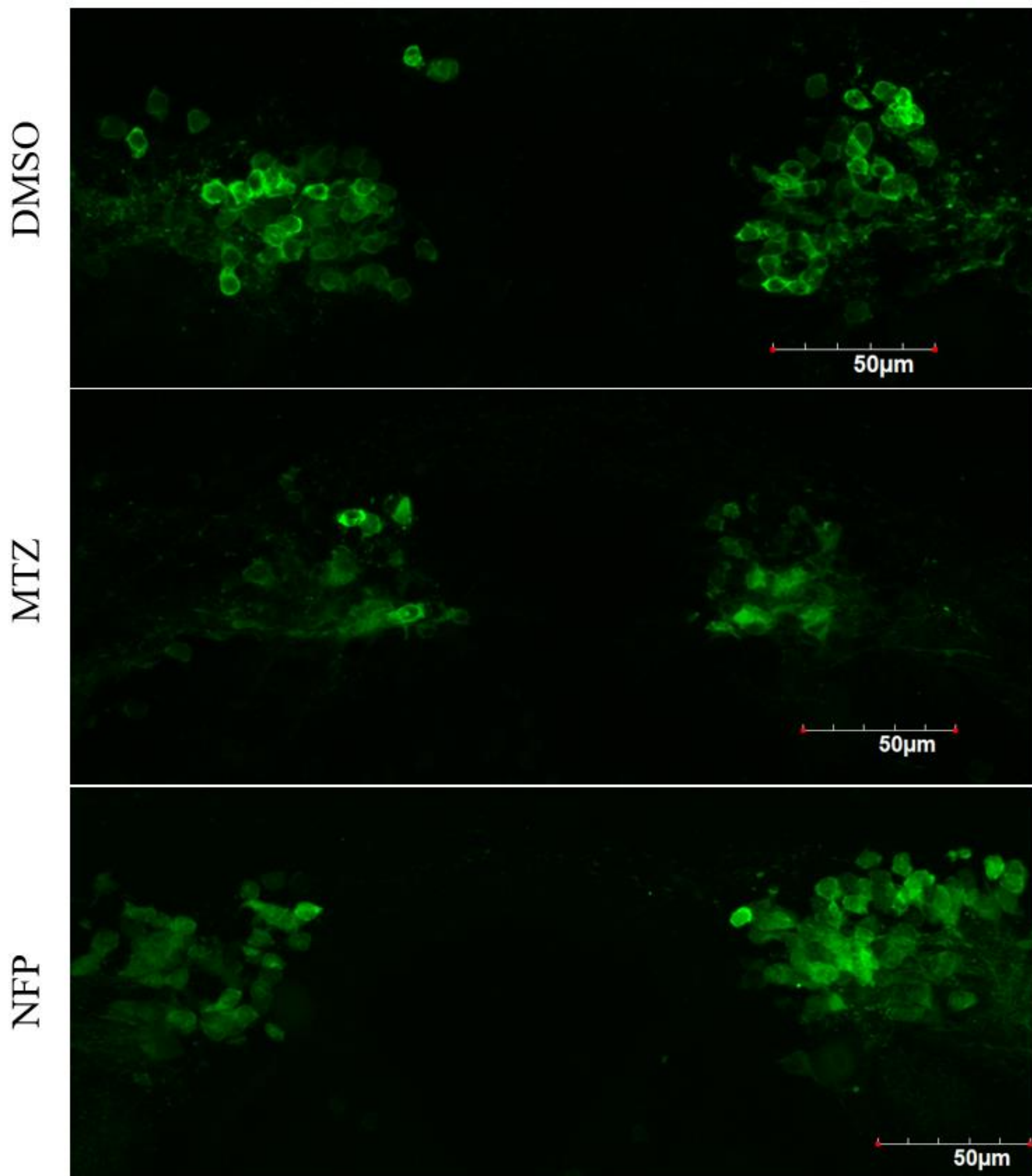


Figure 12. The effect of the various treatments on DAergic neuron clusters found within the pretectal region at 7 dpt.

Immunohistochemical staining of CFP^+ cells (green) in DMSO, MTZ, and NFP-treated fish. Coronal sections of tissue samples were taken at 16 µm. Scale bar: 50 µm.

3.3 Tracking DAergic neuron regeneration at later time points.

To understand how adult zebrafish tend to regenerate their DAergic neurons following ablation, different time points were examined following the MTZ treatment. Godoy et al. found that the differences in CFP^+ cells between MTZ treated fish and the DMSO control tend to diminish in the OB at around 45 dpt (n=6, **Figure 13B**) (Godoy, 2020). Since no intermediate time points were examined after 7 dpt, DAergic neurons were observed at the 15 dpt mark. The reason 15 dpt was chosen was to allow enough time for possible regeneration to happen. Results showed a slight increase in the number of CFP^+ cells in the OB region for MTZ-treated fish at 15 dpt relative to MTZ-treated fish at 7 dpt; however, it was not statistically significant (n=3, p= 0.5868, **Figure 13C**). This hints that the adult zebrafish brain needs much more time in order to start regenerating the ablated neurons. This observation is further analyzed in the discussion section.

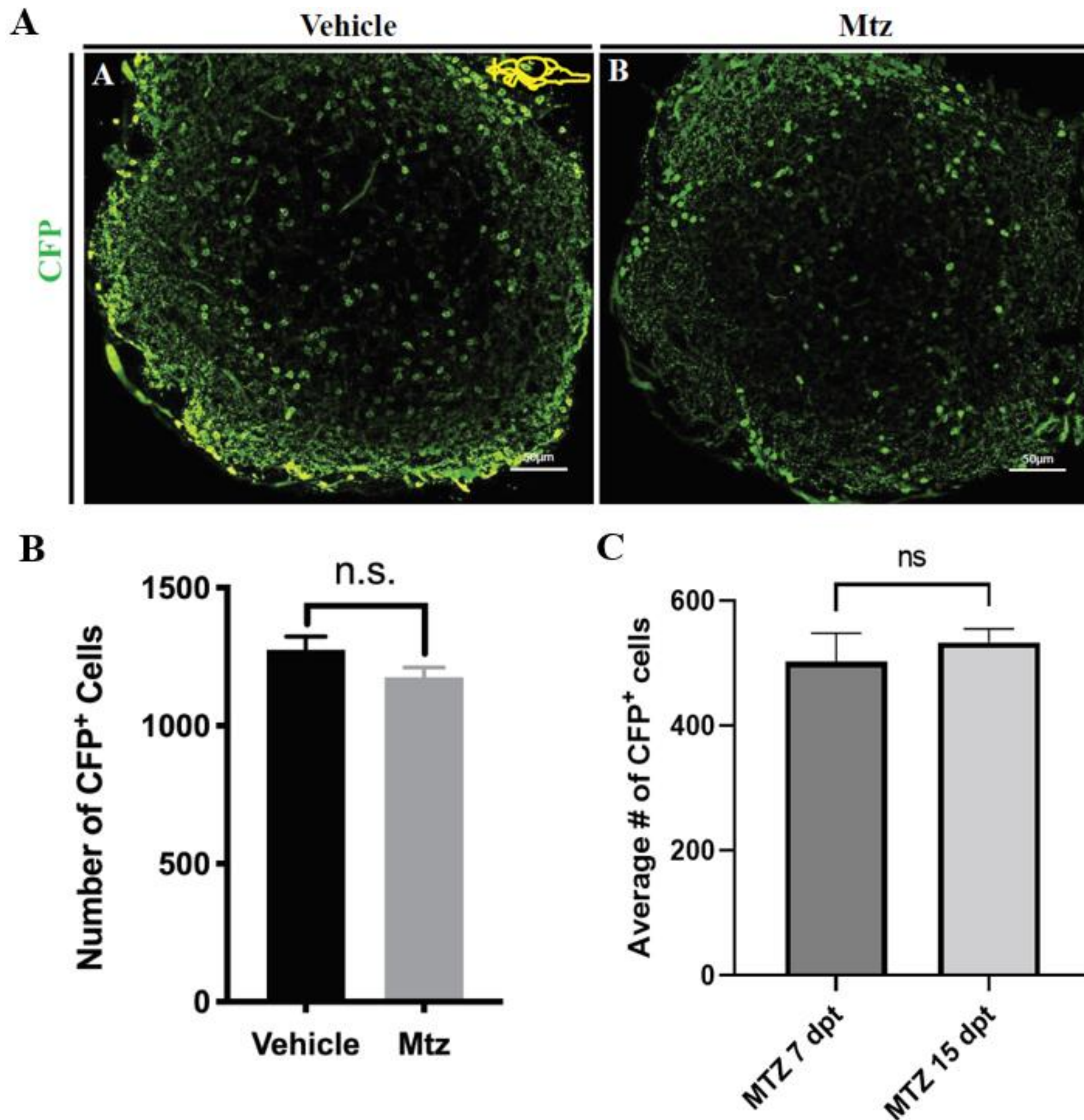


Figure 13. Comparable numbers of CFP+ cells in the OB of MTZ-treated zebrafish at 45 dpt.

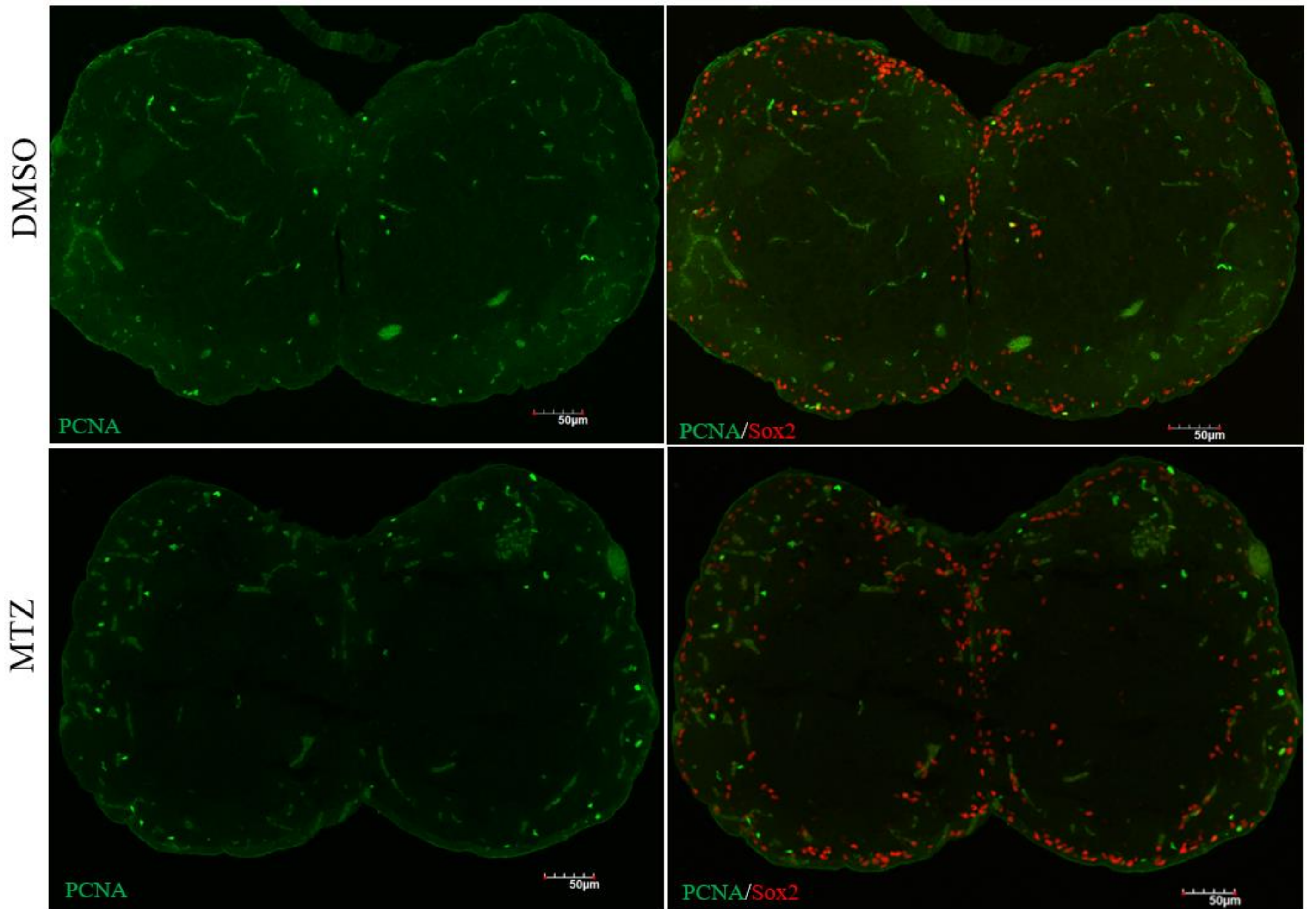
(A) Immunohistochemical staining of CFP^+ cells (green) in DMSO and MTZ-treated fish at 45 dpt. **(B)** Quantification of CFP^+ cells present in the OB at 45 dpt (n=6). (**C**) Quantification of CFP^+ cells in the OB of MTZ-treated fish at 15 dpt (n=2) relative to MTZ-treated fish at 7 dpt (n=3). ns ($p > 0.05$). Error bars represent S.E.M. Scale bar: 50 µm. Results in parts **(A)** and **(B)** were obtained from Godoy et al. (2020).

3.4 Changes in proliferative and neural stem cells in the OB and telencephalon following ablation

To understand how cells respond to nitroreductase-mediated cell ablations, it is essential to consider the factors involved in the neuroregenerative response. It is also important to determine when regeneration initiates and precisely where in the adult zebrafish brain. To tackle this, double immunohistochemistry was performed using Proliferating Cell Nuclear Antigen (PCNA) and Sex determining region Y-Box 2 (Sox2) antibodies. PCNA is a standard marker of cell proliferation, while Sox2 is a common marker of neural stem cells. Co-localization of both markers labels neural stem cells that are undergoing proliferation.

Results showed that fish treated with MTZ for 24 hours expressed a significantly higher number of $PCNA^+$ cells in the OB region at 1 dpt ($p=0.0348$, $n=3$, **Figure 14B**). No significant difference was found in the number of $PCNA^+$ $Sox2^+$ cells in the OB at 1 dpt ($p= 0.6797$, $n=3$, **Figure 14C**). The telencephalon region was also examined at 1 dpt in the MTZ-treated group and the control. I did not observe a significant difference in the number of $PCNA^+$ cells ($p= 0.3344$, $n=3$, **Figure 15B**), however there was a significant increase in the number of $PCNA^+$ $Sox2^+$ cells ($p= 0.0492$, $n=3$, **Figure 15C**). Most $PCNA^+$ cells were localized along the ventricular zone of the telencephalon. In addition, the expression of PCNA and Sox2 was strong along the dorso-lateral region of the telencephalon. The expression of PCNA and Sox2 was also examined in the OB and telencephalon of the MTZ-treated group at 2 dpt. Similar trends were observed to MTZ-treated fish at 1 dpt.

A



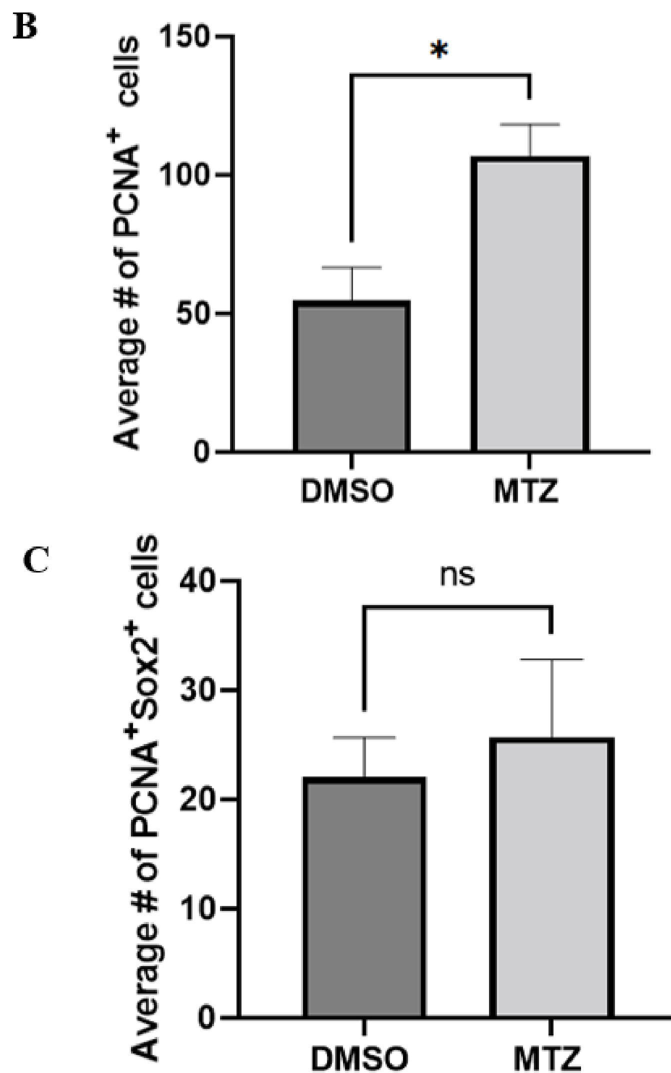
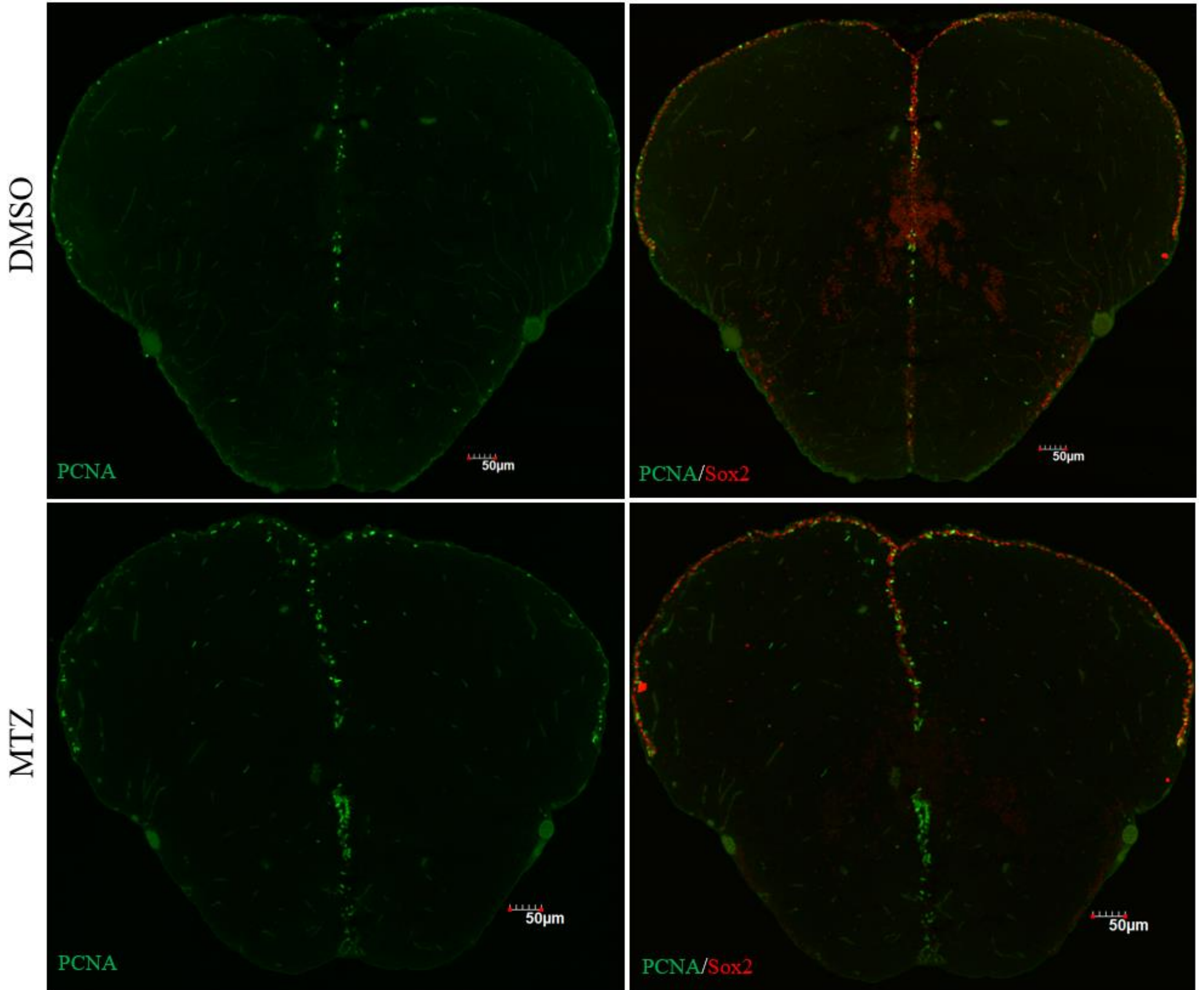


Figure 14. Effect of DAergic neuron ablation on proliferating and neural stem cells in the OB at 1 dpt.

(A) Coronal sections were immunostained with PCNA (green) and Sox2 (red) antibodies. **(B)** Quantification of $PCNA^+$ cells **(C)** Quantification of $PCNA^+$ $Sox2^+$ cells (n=3). Scale bar: 50 µm. Error bars represent S.E.M. ns ($p > 0.05$), * ($p < 0.05$).

A



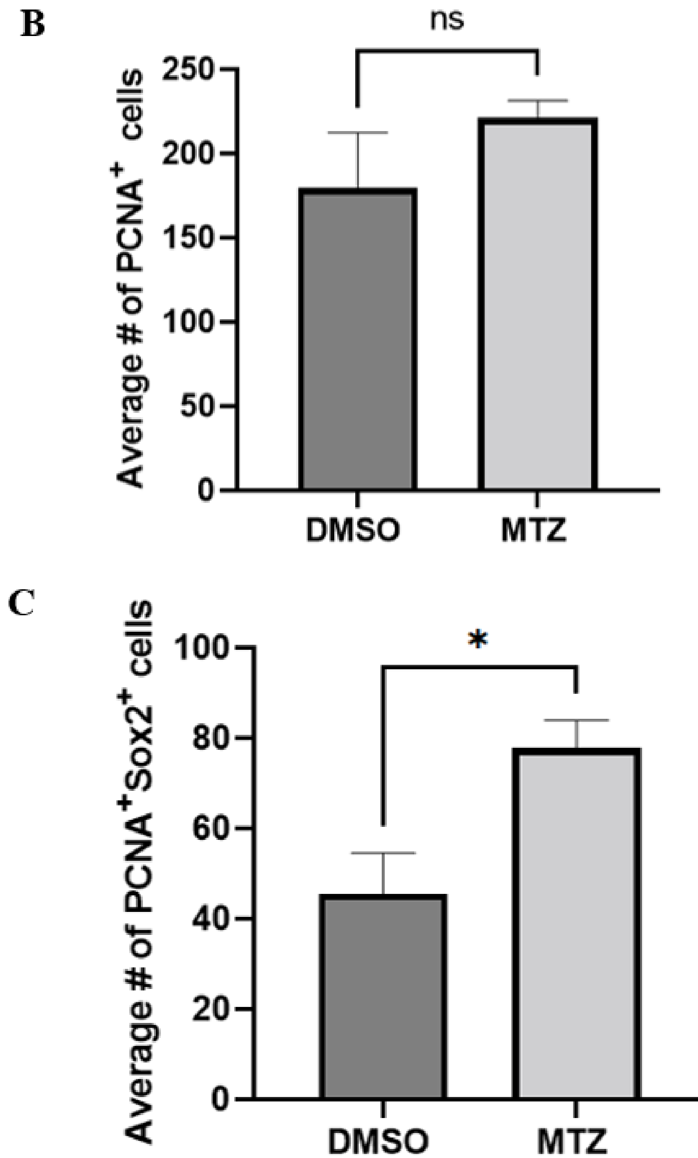


Figure 15. Effect of DAergic neuron ablation on proliferating and neural stem cells in the telencephalon at 1 dpt.

(A) Coronal sections were immunostained with PCNA (green) and Sox2 (red) antibodies. **(B)** Quantification of $PCNA^+$ cells **(C)** Quantification of $PCNA^+$ $Sox2^+$ cells (n=3). Scale bar: 50 µm. Error bars represent S.E.M. ns ($p > 0.05$), * ($p < 0.05$).

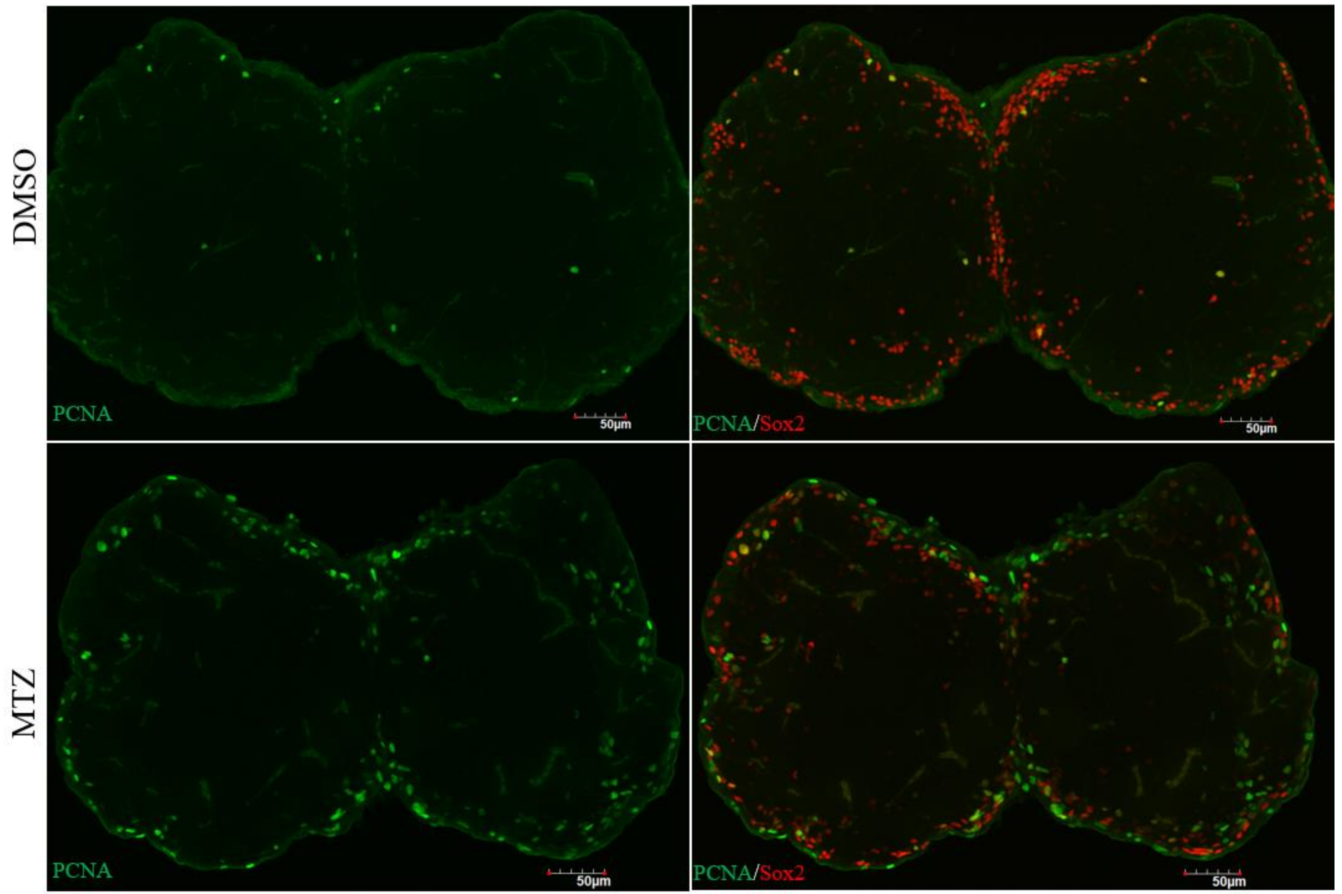
3.5 Cellular analysis in the OB and telencephalon at 7 days following treatment

To allow enough time for possible neuroregeneration to happen, further analysis was performed at 7 dpt. It was noticed that the number of $PCNA^+$ and $PCNA^+$ $Sox2^+$ cells drastically increased at this time point in the MTZ-treated group relative to 1 dpt and 2 dpt. Results showed a significant increase in $PCNA^+$ cells ($p= 0.0383$, $n=3$, **Figure 16B**) in the OB at 7 dpt. This was also the case for $PCNA^+$ $Sox2^+$ cells, as almost half of proliferating cells were neural stem cells ($p= 0.0473$, $n=3$, **Figure 16C**).

When observing the more rostral parts of the telencephalon, it was noticed that MTZ-treated fish had a drastic increase in the expression of PCNA near the ventral nucleus of the ventral telencephalon (**Figure 17B**'). This is relative to the DMSO control (**Figure 17A'**).

The highest spike in $PCNA^+$ cells was detected in the telencephalon region of MTZ-treated fish at 7 dpt ($p= 0.0054$, $n=3$, **Figure 18B**). The number of $PCNA^+$ $Sox2^+$ cells also spiked drastically in the telencephalon of MTZ-treated fish at this timepoint ($p= 0.0353$, $n=3$, **Figure 18C**). When comparing MTZ-treated fish at 7 dpt relative 1 dpt, results showed a significant increase in the number of $PCNA^+$ $Sox2^+$ cells in the OB at 7 dpt ($p= 0.0469$, $n=3$, **Figure 19A'**). That said, there was not a significant difference in the number of $PCNA^+$ cells between the MTZ-treated group at 1 dpt relative to 7 dpt ($p=0.0696$, $n=3$, **Figure 19A**). When comparing the same time points for the MTZ-treated group, a significant increase in $PCNA^+$ cells ($p=0.0177$, $n=3$, **Figure 19B**) and $PCNA^+$ $Sox2^+$ cells ($p=0.0495$, $n=3$, **Figure 19B'**) was found in the telencephalon region.

A



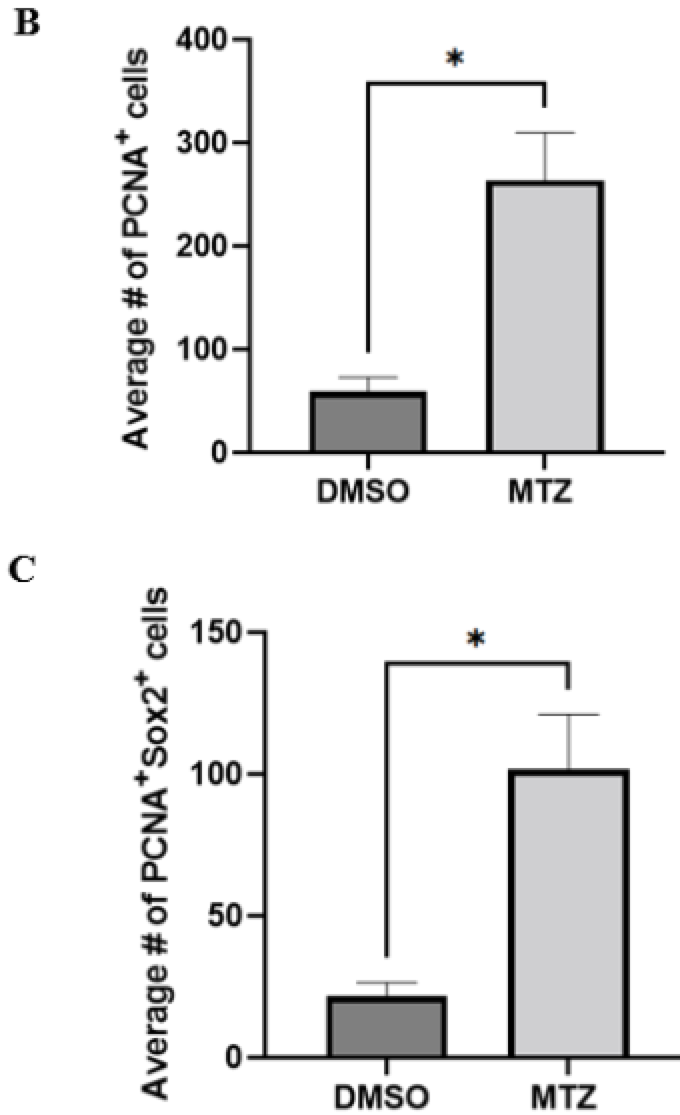


Figure 16. Changes in PCNA and Sox 2 levels in the OB at 7 dpt.

(A) Coronal sections were immunostained with PCNA (green) and Sox2 (red) antibodies. **(B)** Quantification of $PCNA^+$ cells **(C)** Quantification of $PCNA^+$ $Sox2^+$ cells (n=3). Scale bar: 50 µm. Error bars represent S.E.M. * ($p < 0.05$).

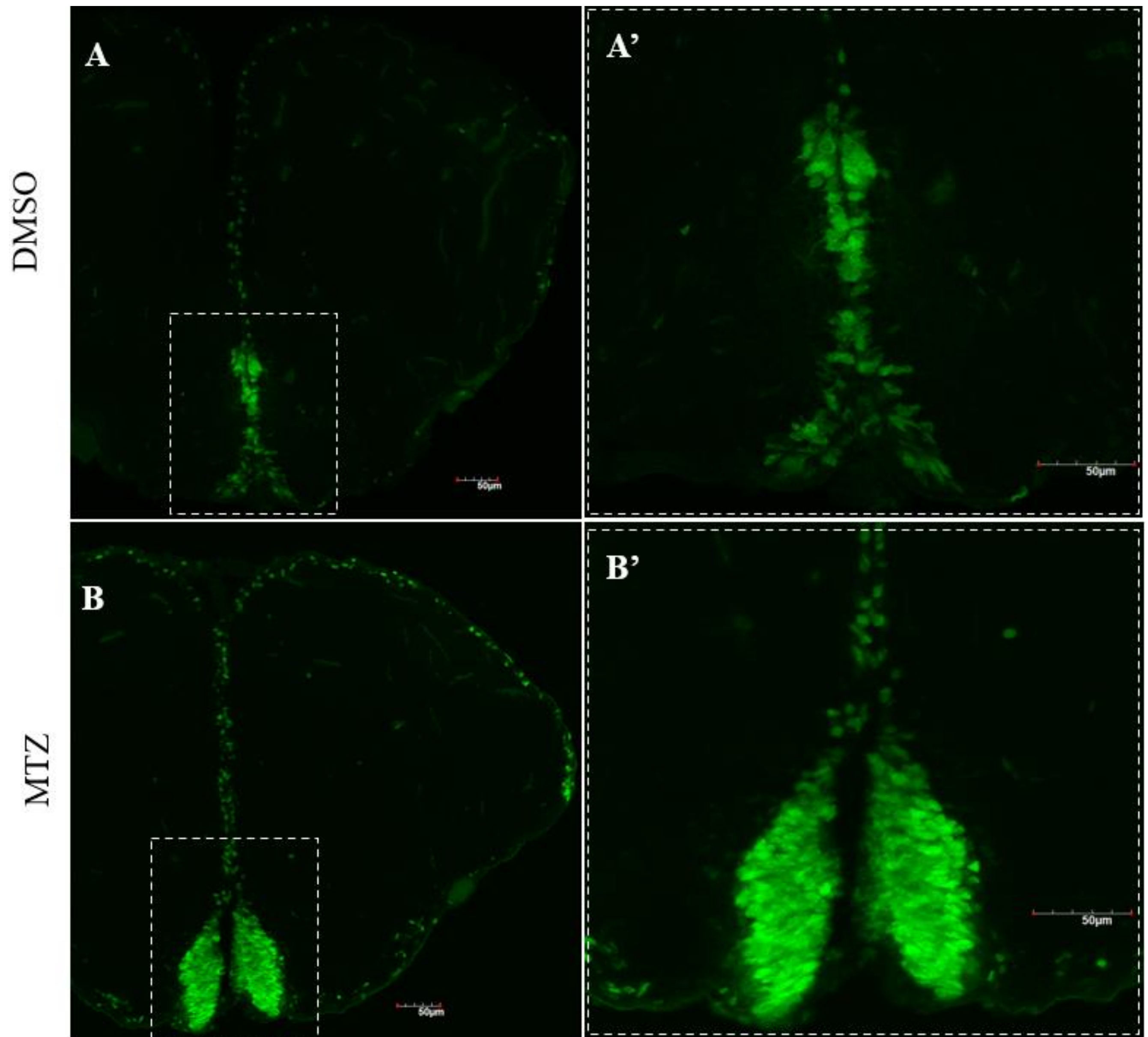
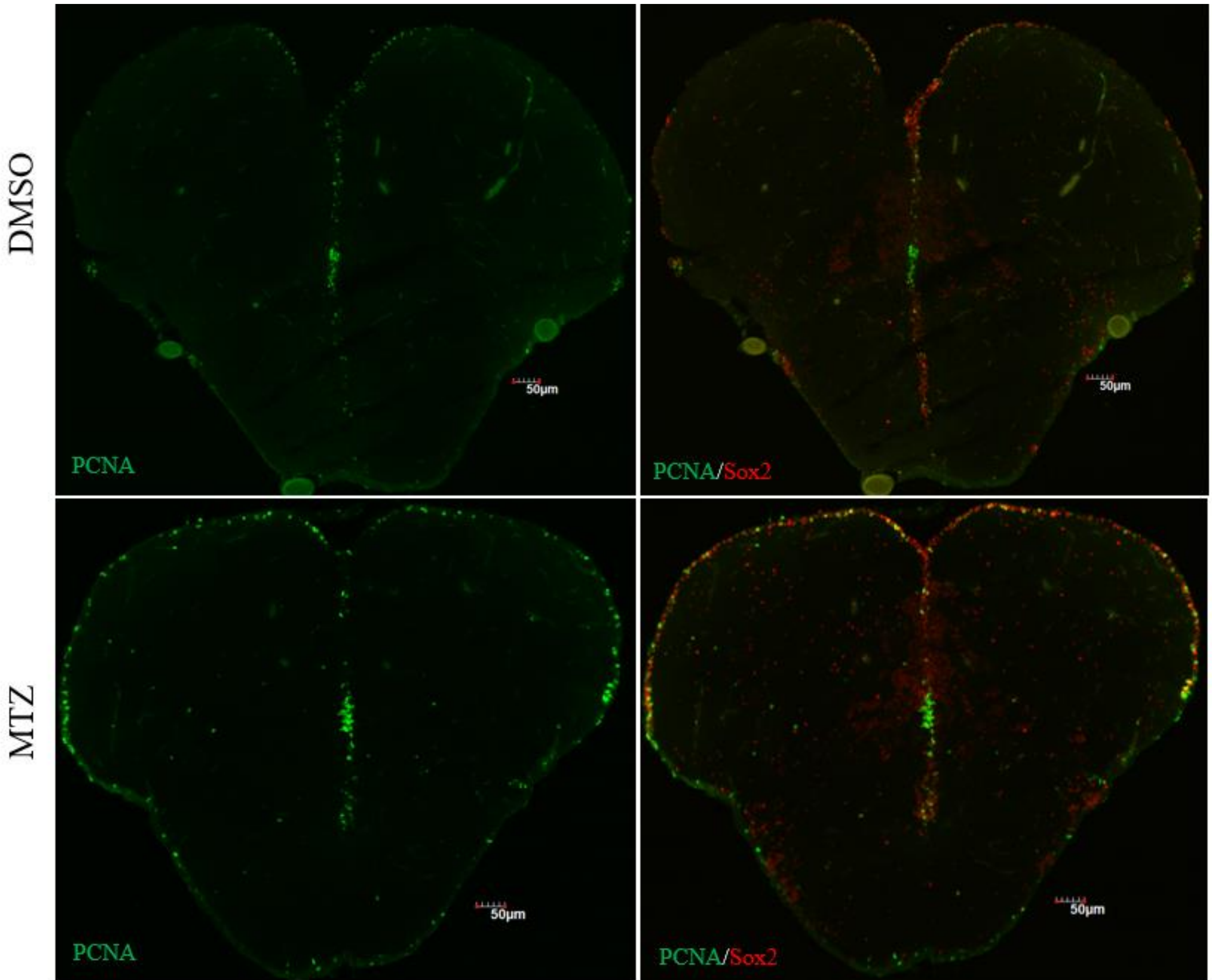


Figure 17. Increase in cell proliferation in the MTZ-treated group at 7 dpt in the telencephalic ventricular zone.

(A) Coronal sections of DMSO-treated fish were immunostained with PCNA (green) (**A'**) higher magnification of panel A showing the ventricular zone of the telencephalon. (**B**) Coronal sections of MTZ-treated fish were immunostained with PCNA (**B'**) higher magnification of panel B. Dotted box shows the magnified region. Scale bar: 50 µm.

A



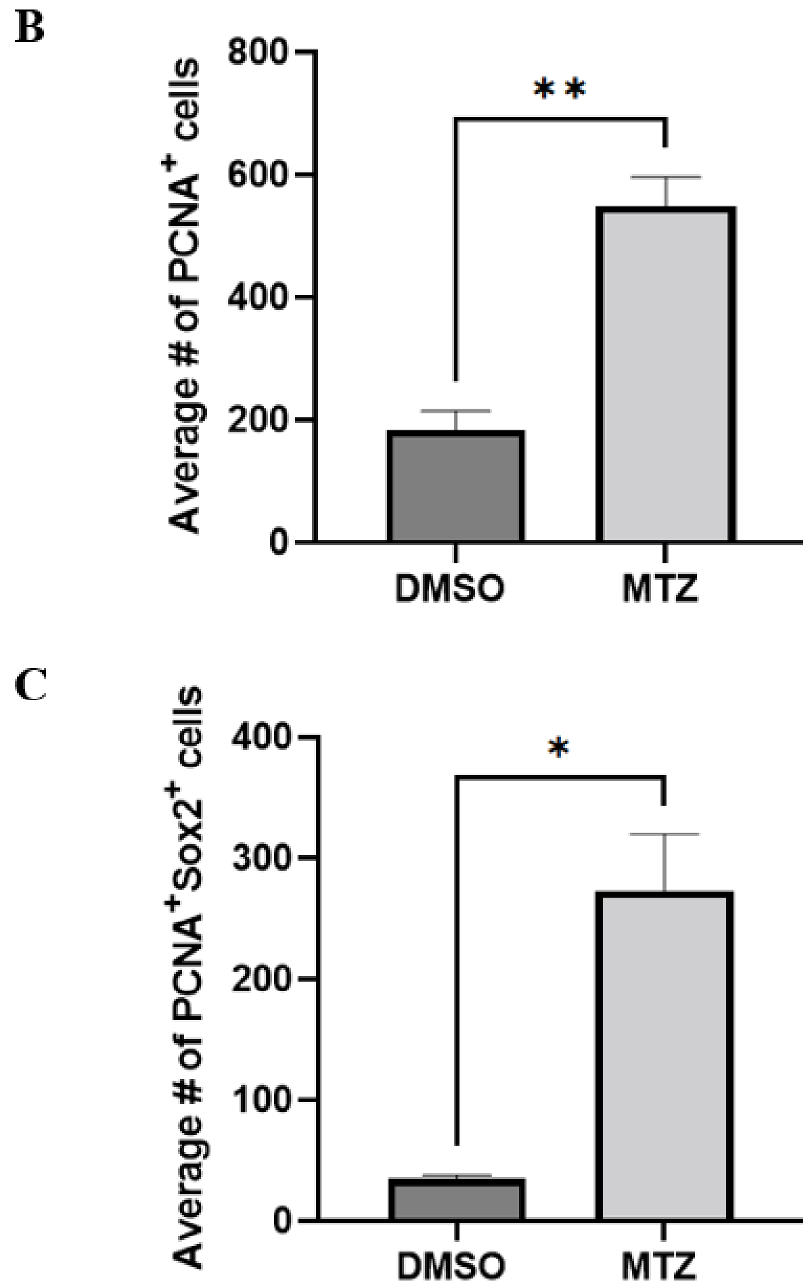


Figure 18. Changes in PCNA and Sox 2 levels in the telencephalon at 7 dpt.

(A) Coronal sections were immunostained with PCNA (green) and Sox2 (red) antibodies. **(B)** Quantification of $PCNA^+$ **(C)** Quantification of $PCNA^+ Sox2^+$ cells (n=3). Scale bar: 50 µm. Error bars represent S.E.M. * ($p < 0.05$), ** ($p < 0.01$).

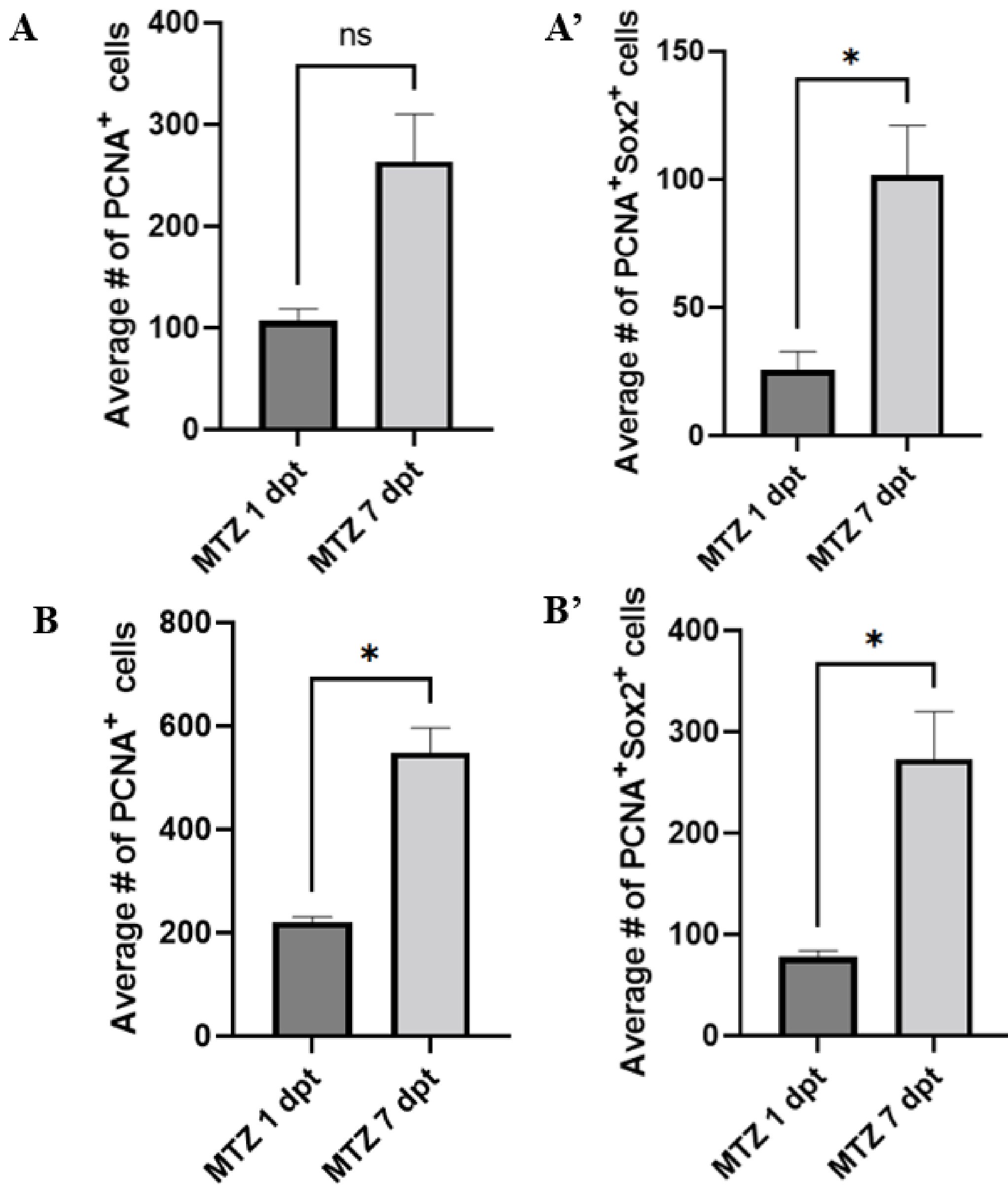


Figure 19. Summary of the changes in PCNA and Sox 2 levels at 1 dpt versus 7 dpt.

(A) Quantification of $PCNA^+$ cells in the OB **(A')** Quantification of $PCNA^+ Sox2^+$ cells in the OB (**B**) Quantification of $PCNA^+$ cells in the telencephalon **(B')** Quantification of $PCNA^+ Sox2^+$ cells in the telencephalon (n=3). Scale bar: 50 µm. Error bars represent S.E.M. ns ($p > 0.05$) * ($p < 0.05$).

3.6 Impairments in swimming behaviour following DAergic neuron ablation

Since PD patients suffer from motor defects due to the loss of striatal DAergic neurons, the next step was to determine how adult Tg(*dat:CFP-NTR*) zebrafish respond to DAergic neuron ablation. One important point to consider here is the time point of analysis, as this will give us an understanding as to how long adult zebrafish might need to show signs of possible recovery. To observe how zebrafish might respond to the sudden ablation of DAergic neurons, they were tested directly after the termination of the treatment (0 hpt). Another time point that was considered was 7 dpt. This time point corresponds to when almost half of the DAergic neurons were ablated in the OB of MTZ-treated fish (**Figure 8**). To mimic the behavioural symptoms displayed by PD patients, the three analyzed parameters included the time spent in a stationary state, the average velocity, and the total distance travelled by zebrafish.

Directly after the termination of the treatment, results showed that the total distance travelled by fish treated with either MTZ or NFP was significantly shorter relative to the DMSO control. NFP-treated fish, analyzed at 0 hpt, travelled the shortest average distance of 1839.6 ± 126.3 cm (n=10, **Figure 20A**). MTZ-treated fish travelled 3002.0 ± 145.0 cm, almost twice the distance travelled by the NFP treated fish (n=10, **Figure 20A)**. DMSO-treated fish travelled 3708.1 ± 49.4 cm, about 700.0 cm more on average relative to MTZ-treated fish (n=10, **Figure 20A)**. Analysis at 7 dpt shows that MTZ and NFP-treated fish displayed some motor recovery when compared to the DMSO control. NFP-treated fish travelled 3385.9 ± 366.6 cm while MTZ-treated fish travelled 3939.8 ± 295.9 cm (n=5, **Figure 20B**). DMSO-treated fish travelled 3521.6 ± 236.6 cm (n=5, **Figure 20B**). No significant differences were found between the three treatment groups in terms of distance travelled at 7 dpt (**Figure 20B**). This demonstrates that there was some sort of recovery in swimming mobility in MTZ and NFP-treated zebrafish relative to the control.

Further analysis showed that the velocity of NFP-treated fish was almost half of that of the DMSO-treated group directly after the termination of the treatment. The average velocity of NFP-treated fish was determined to be 3.1 ± 0.2 cm/s, MTZ-treated fish at 5.0 ± 0.2 cm/s, and DMSO-treated fish at 6.2 ± 0.08 cm/s at 0 hpt (n= 10, **Figure 21A**). The average velocity of MTZ-treated fish was found to be lower relative to the control at 0 hpt and was calculated to be statistically significant (n= 10, **Figure 21A**). Similar to the distances recorded at 7 dpt, there also seems to be some sort of recovery in the average velocity. At 7 dpt, the average velocity of NFP-treated fish was determined to be 5.6 ± 0.6 cm/s, MTZ-treated fish at 6.6 ± 0.5 cm/s, and DMSO-treated fish at 5.9 ± 0.4 cm/s (n= 5, **Figure 21B**).

The third parameter tested involved recording how long treated fish remain in a stationary state over the 10-minute testing period. At the 0 hpt timepoint, NFP-treated fish showed a significant spike in freezing duration. NFP-treated fish spent an average of 440.4 ± 22.2 seconds, MTZ-treated fish spent 227.0 ± 23 seconds, and DMSO-treated fish 136.1 ± 5.6 seconds in a stationary state during the 10-minute testing period (n=10, **Figure 22A**). In terms of percentages, NFP-treated fish spent almost 73.3% of the period in a stationary state. MTZ-treated fish spent 37.8% of the total time in that state while DMSO-treated fish spent only 22.6% (**Figure 22A**). After allowing 7 days for the fish to possibly recover, MTZ-treated fish spent only 164.4 ± 34.5 seconds in a stationary state which was similar to DMSO-treated fish as they spent 165.3 ± 21.9 seconds (n=5, **Figure 22B**). NFP-treated fish spent more time in a stationary state; however, the difference was not statistically significant relative to the DMSO control (n=5, **Figure 22B**).

To better visualize the swimming behaviour of the treated fish, a representative overhead image showing their complete swimming patterns was taken. At 0 dpt, it was noticeable that NFP-treated fish had impaired motor movements as most of the line paths were black colored (minimal activity) (**Figure 23**). Furthermore, NFP-treated fish tend to swim in small circular patterns instead of a straight linear path, as seen in the DMSO control at 0 hpt (**Figure 23**). MTZ-treated fish also had a remarkable degree of black path lines (minimal activity), however, more green path lines (normal activity) were present at 0 hpt (**Figure 23**). MTZ-treated fish also tend to swim in circular patterns; however, the radius of each circle seems to be bigger. At 7 dpt, both NFP and MTZ-treated fish had similar travel path lines compared to the DMSO control (**Figure 24**). Overall, this data suggests that NFP-treated fish show the most significant reduction in total distance travelled, lowest average velocity, and the greatest increase in total freezing duration at 0 hpt. That said, some recovery in motor behaviour seems to be evident at around 7 dpt.

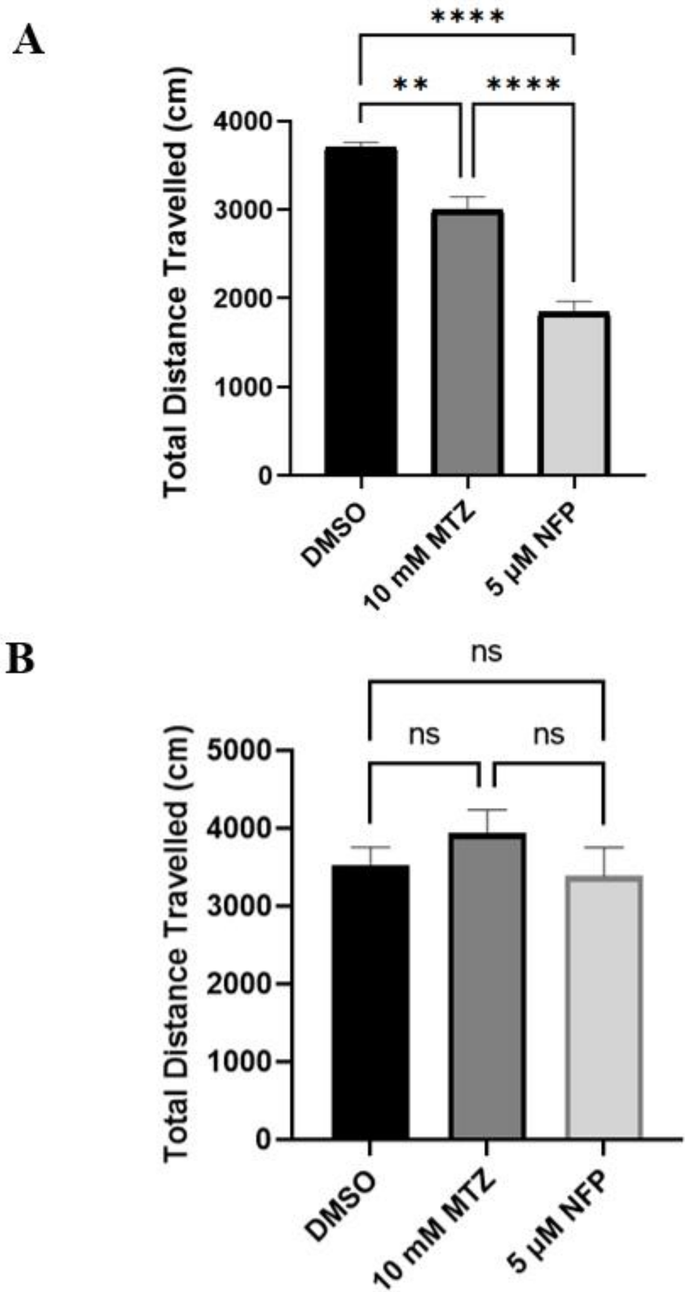


Figure 20. Total distance travelled by the treatment groups at 0 hpt and 7 dpt.

(A) Treated fish were allowed to swim immediately after the treatment for 10-minutes and the total distance travelled was recorded. **(B)** After 7 dpt, the distance travelled for the treated fish was recorded again (n=10 at 0 hpt, n=5 at 7 dpt). Statistical significance was calculated using one-way ANOVA. Error bars represent S.E.M. ns ($p > 0.05$), ** ($p < 0.01$), **** ($p < 0.0001$).

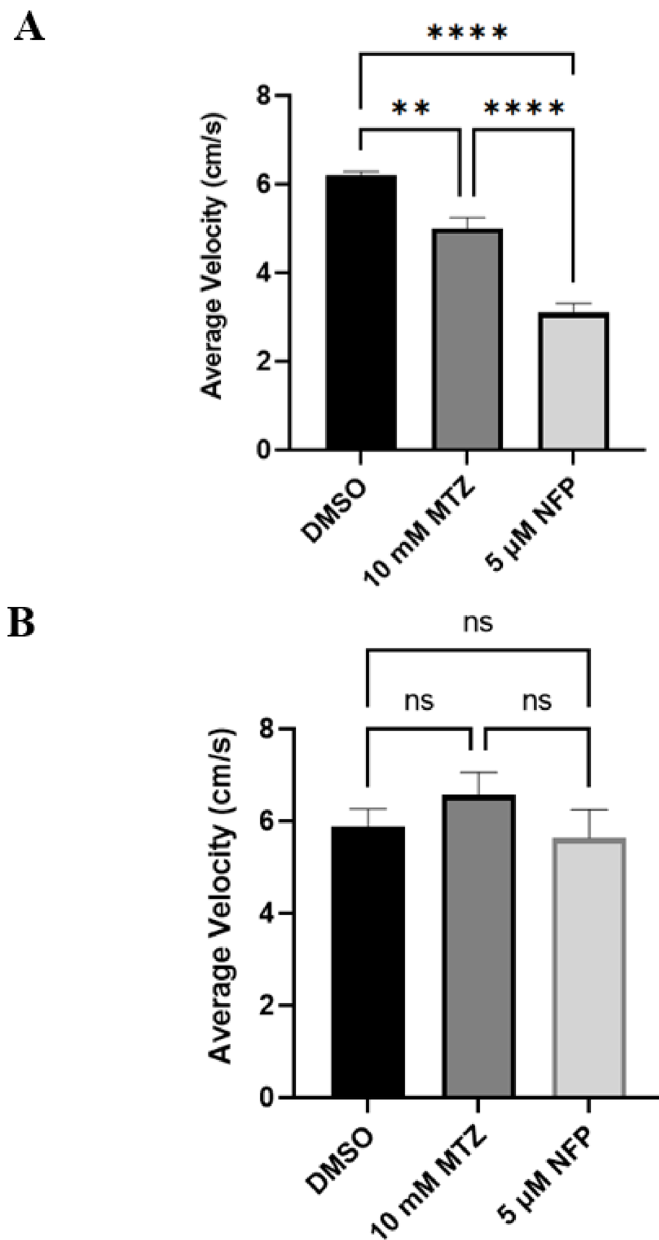


Figure 21. Average velocity of the various treatment groups at 0 hpt and 7 dpt.

(A) Treated fish were allowed to swim immediately after the treatment for 10-minutes, and the average velocity was measured. **(B)** After 7 dpt, the average velocity for the treated fish was tracked (n=10 at 0 hpt, n=5 at 7 dpt). Statistical significance was calculated using one-way ANOVA. Error bars represent S.E.M. ns ($p > 0.05$), ** ($p < 0.01$), **** ($p < 0.0001$).

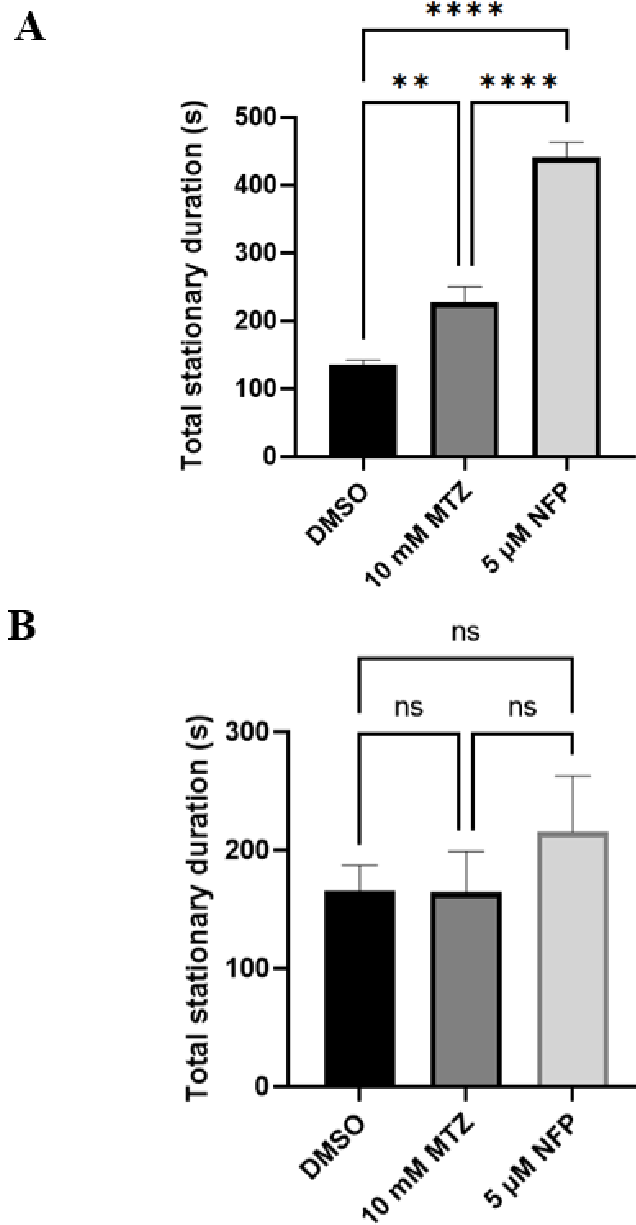


Figure 22. Total time spent in a stationary state by fish treated with either DMSO, MTZ, or NFP at 0 hpt and 7 dpt.

(A) The time spent by the treated fish in a stationary state was recorded immediately after the treatment **(B)** After 7 dpt, the total stationary duration for the treated fish was recorded (n=10 for 0 dpt, n=5 for 7 dpt). Statistical significance was calculated using one-way ANOVA. Error bars represent S.E.M. ns ($p > 0.05$), ** ($p < 0.01$), **** ($p < 0.0001$).

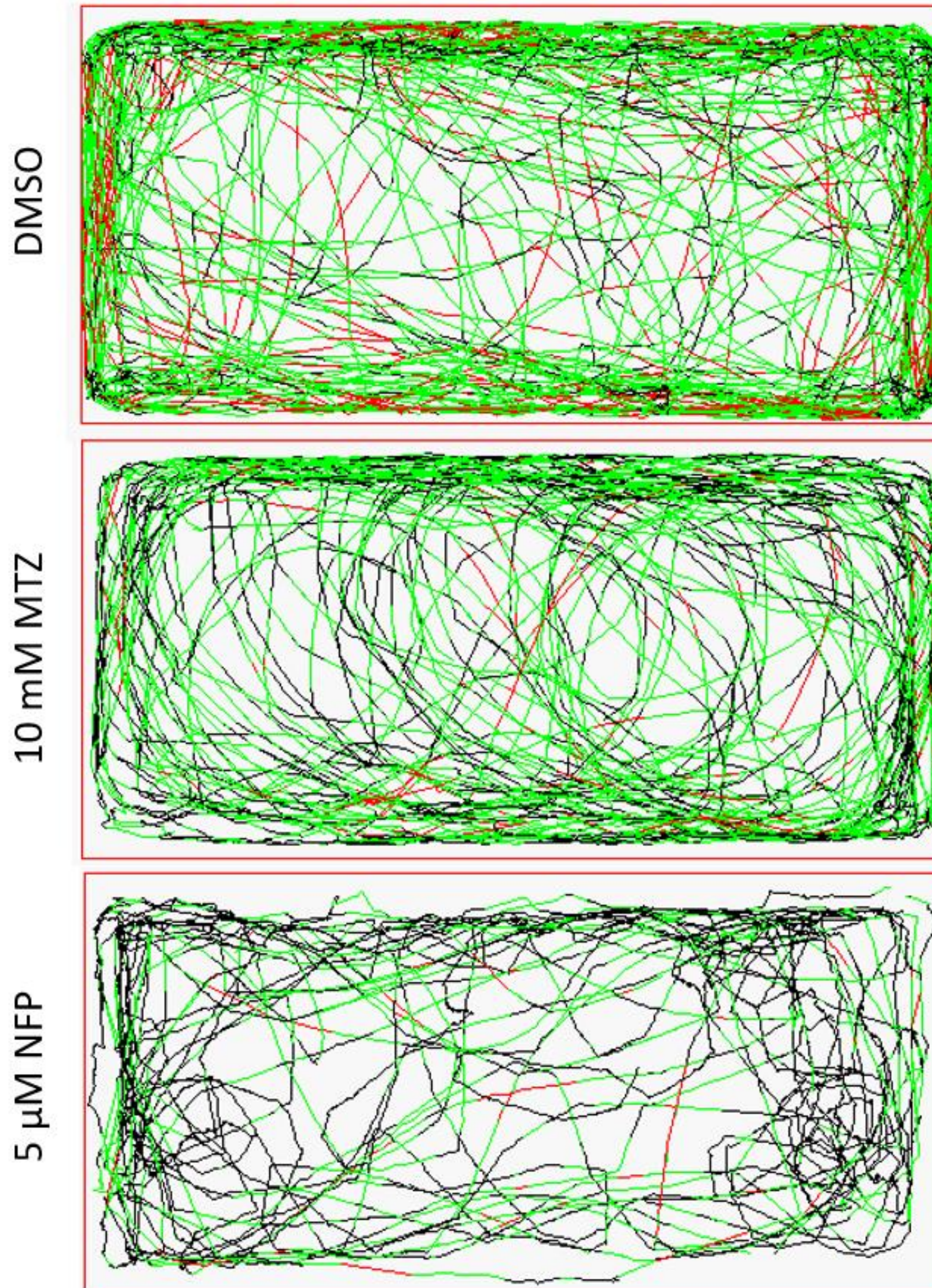


Figure 23. Representative path images over a 10-minute testing period for treated fish at 0 hpt.

The red color tracking line indicates fast movement, green indicates normal movement, and black indicates inactivity or minimal movement. Fish were acclimatized for at least 30 minutes before each trial. A sample size of 10 was used for each treatment group.

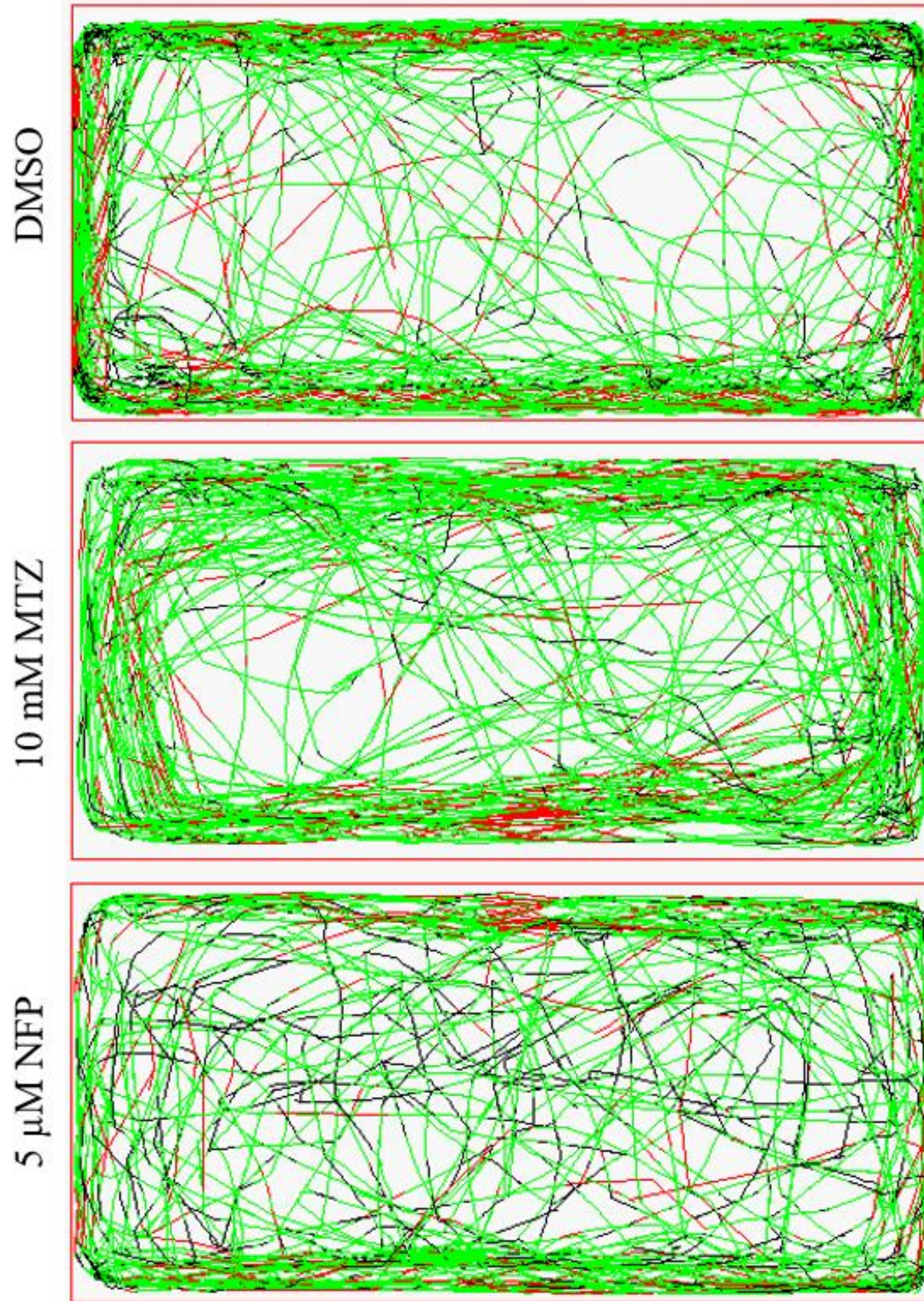


Figure 24. Representative path images over a 10-minute testing period for treated fish at 7 dpt.

The red color tracking line indicates fast movement, green indicates normal movement, and black indicates inactivity or minimal movement. Fish were acclimatized for at least 30 minutes before each trial. A sample size of 5 was used for each treatment group.

4. Discussion

Developing and utilizing a reliable approach to mimic PD-like symptoms in zebrafish is an ongoing project that is gaining more attention (Godoy, 2015; Wang, 2017; Vaz, 2018; Barnhill, 2020; Robea, 2020; Merhi, 2021). Using a chemogenetic approach, in particular, to induce such symptoms has been revolutionary as it is considered more specific and, in some cases, can be conditional upon the addition of specific agents (Bergemann, 2018; Godoy, 2021). In this project, prodrugs such as MTZ and NFP were used as substrates for nitroreductase-mediated cell ablations. The effectiveness of each prodrug in terms of DAergic neuron ablation was compared, and the target regions were analyzed. To examine how zebrafish regenerated their DAergic neurons after ablation, key markers were used. Using the PCNA antibody allowed us to label cells that are undergoing proliferation, while the Sox2 antibody allowed us to mark neural stem cells. The co-localization of both markers gave us insight into the degree of neural stem cells undergoing proliferation and potentially giving rise to new specialized DAergic neurons. Lastly, how the ablation of DAergic neurons translated to behaviour impairments was visualized by looking at three different swimming parameters.

4.1 Successful ablation of DAergic neurons using MTZ in three key regions

Extending on the work of former student R. Godoy, the ablation of DAergic neurons was examined at additional time points and in new brain regions. After confirming that almost half of DAergic neurons were ablated in the olfactory bulb region (most rostral part of the brain), the next objective was to dive into more caudal areas. The purpose of this was to see if MTZ can penetrate and cause effective DAergic neuron ablations. Results showed that MTZ was able to significantly decrease the number of DAergic neurons, as labelled by the CFP antibody, in the telencephalon at 7 dpt. The number of CFP^{+} cells was reduced by about 30% in the telencephalon in the MTZ-

treated groups relative to the control. It seems that the effects of MTZ are more pronounced in the more rostral parts of the brain as it reduced the number of CFP^+ cells by almost 50% in the olfactory bulb. This could be possibly explained by the route of drug administration. Since fish are immersed in a solution containing the drug for 24 hours, it could be that not enough MTZ molecules were able to penetrate and saturate the more caudal regions of the adult zebrafish brain, thus decreasing its effect on DAergic neurons. This could explain why the impact of MTZ in the telencephalon might be weaker relative to the olfactory bulb.

To further investigate the efficacy of MTZ in more caudal regions of the brain, the pretectal area was analyzed. This pretectal region contains a unique cluster of DAergic neurons called the periventricular pretectal nucleus. Interestingly, the MTZ treatment markedly reduced the number of CFP^+ cells present in the cluster at 7 dpt. This is critical as it suggests that MTZ is still able to effectively ablate DAergic neurons in brain regions posterior to the olfactory bulb and the telencephalon.

4.2 Optimizing NFP to promote effective ablation of DAergic neurons in Tg(*dat:CFP-NTR*) zebrafish

Even though MTZ can cause efficient ablation of DAergic neurons in various brain regions, finding a more potent alternative can potentially lead to more consistent and reliable cell-specific ablations. A recent paper published by Bergemann et al. suggested that another prodrug called NFP could potentially replace MTZ as a substrate for nitroreductase-mediated cell ablations (Bergemann, 2018). Given that they reported that NFP was able to promote reliable DAergic neuron ablations in zebrafish larvae at a concentration that is 2000 lower than MTZ, I wanted to test the efficacy of this novel drug in adult Tg(*dat:CFP-NTR*) zebrafish (Bergemann, 2018). Results showed that NFP was able to cause a significant ablation of DAergic neurons in the

olfactory bulb region at 7 dpt. When comparing the two prodrugs, MTZ was able to ablate about 27% more DAergic neurons in the olfactory bulb at 7 dpt relative to NFP. Similar to MTZ, NFP was also able to significantly decrease the number of CFP^+ cells in the telencephalon. Interestingly, NFP reduced the number of DAergic neurons in the telencephalon more than MTZ by 11%. Unlike MTZ, NFP was not able to drastically affect the DAergic neurons located in the periventricular pretectal nucleus.

One crucial aspect to consider when looking for alternative substrates is their level of specificity. To determine if the effects of NFP are only selective to NTR-expressing cells, adult WT zebrafish were placed in a solution of NFP with a concentration of 7.5 μM for 24 hours. It is important to note that this concentration was lethal to Tg(*dat:CFP-NTR*) zebrafish. Surprisingly, WT zebrafish were able to withstand this toxic concentration for more than 24 hours. They were able to survive the treatment, demonstrating that just like MTZ, the prodrug NFP acts in a specific manner via nitroreductase-mediated cell-specific ablation.

4.3 Drastic changes in the number of proliferating cells and neural stem cells undergoing proliferation following DAergic neuron ablation

To understand how the zebrafish brain can regenerate ablated DAergic neurons, it is essential to consider the fundamental cells that ultimately give rise to these specialized neurons. The adult zebrafish brain contains distinct progenitor niches where most of the proliferation happens. These highly prolific areas contain cells that are regenerative neural precursors as they have stem cell-like characteristics. One way the adult zebrafish brain might compensate for the ablated neurons is by increasing the number of neural stem cells undergoing proliferation. If this was the case, it would be expected to find more proliferative neural stem stems in the proliferation zones found along the rostro-caudal axis of the adult zebrafish brain. For this project, the olfactory

bulb and the telencephalon regions were examined. One important thing to consider when looking at changes in proliferation is the timepoint following cell ablation. This question is critical to address since the ablation technique in this project is a chemogenetic one. Most studies studying regeneration rely on physical measures to cause damage in order to trigger a possible regenerative response. For example, Kishimoto et al. investigated the regenerative nature of the telencephalon in adult zebrafish by causing a physical lesion in the dorsolateral region of the right hemisphere of the telencephalon (Kishimoto, 2012). They created this lesion by using a microneedle. They found that the injured area of the telencephalon was able to regenerate by 35 days post-lesion (dpl) (Kishimoto, 2012). However, one major drawback of this approach is the possibility of promoting undesirable global side effects. To understand how the telencephalon might self heal post-injury, they looked at the expression of PCNA at various time points following injury. They reported a significant increase in PCNA cells at 3, 7, 10 dpl in the lesioned group relative to the non-lesioned control. The greatest increase in PCNA^{+} cells was found at 7 dpl in both the ventricular zone and the medial pallium of the telencephalon (Kishimoto, 2012).

To examine if the same proliferation profile exists following DAergic neuron ablation via a chemogenetic approach, the telencephalon was immunostained at 1, 2, and 7 dpt. Results showed a significant increase in PCNA^{+} cells in the telencephalon at 1 and 7 dpt in the MTZ-treated group relative to the DMSO control. PCNA expression at 2 dpt was comparable to that at 1 dpt. That said, the highest spike in PCNA^{+} cells was found at 7 dpt in the ventricular zone of the telencephalon. These results confirm the previous observations reported by Kishimoto et al.

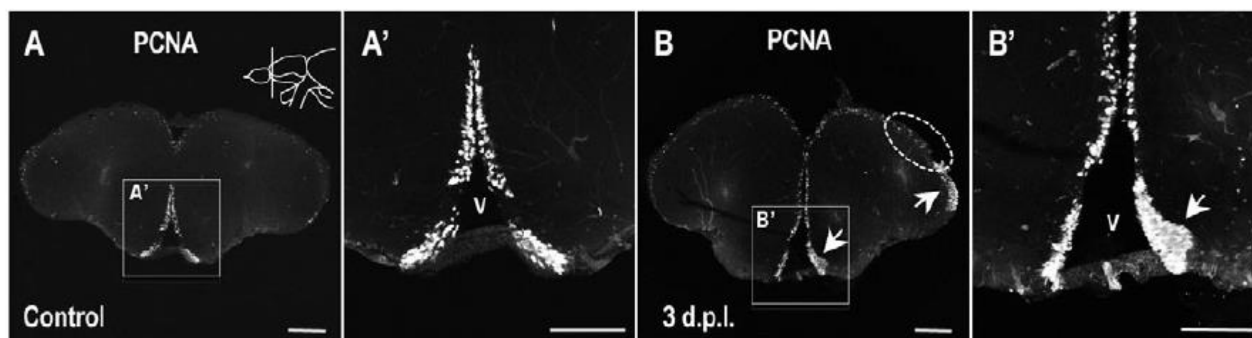


Figure 25. Increase in cell proliferation in the ventricular zone of the rostral telencephalon at 3 dpl in the lesioned group relative to the non-lesioned control.

(A) Coronal section of the rostral telencephalon immunostained with PCNA in the non-lesioned control **(B)** Coronal section of the rostral telencephalon immunostained with PCNA in the lesioned group. Panels (**A'**) and (**B'**) represent a magnified representation of panels (A) and (B). The dotted circle represents the injury site. V, ventricle. Scale bar: 100 µm. This figure was taken from Kishimoto et al. (2012).

Kishimoto et al. also reported a drastic increase in PCNA expression in the ventricular region in the rostral telencephalon at 3 dpl (**Figure 25**) (Kishimoto, 2012). A similar observation was noticed in the ventricular region of the rostral telencephalon at 7 dpt in the MTZ-treated group relative to the DMSO control (**Figure 17B'**). This could explain how the adult zebrafish brain might respond to DAergic neuron ablations in order to replenish the ablated ones. It was also noticed that there was an increase in PCNA levels at 1 dpt in the telencephalon. However, this was not statistically significant, and this could possibly be explained by the fact that not enough time has passed to trigger a sufficient cellular response. Comparing the number of $PCNA^+$ cells in the telencephalon for the MTZ treated fish at 1 dpt versus 7 dpt, results showed almost a 2.5-fold

increase at 7 dpt. Changes in PCNA levels were also noticed in the olfactory bulb region. There was a two-fold increase in $PCNA^+$ cells at 1 dpt and about a five-fold increase at 7 dpt. This is relative to the DMSO control at each time point. That said, the absolute number of PCNA cells in the OB remained drastically lower relative to the telencephalon at 1 dpt and 7 dpt. This could be explained by the fact that there are more proliferation zones located in the telencephalon compared to the OB (**Figure 3**).

One important aspect to consider is the nature of the cells undergoing proliferation during this regenerative process. To answer this question, the co-localization of $PCNA^+$ and $Sox2^+$ was considered. Observing $PCNA^+$ $Sox2^+$ cells in the OB region at 1 dpt, results showed a slight increase in the MTZ-treated group relative to the control, however, it was not statistically significant. This can be explained by the fact that the time point chosen was too early that not enough neural stem cells were able to proliferate. That said, there was a 2.5-fold increase in the number of $PCNA^+$ $Sox2^+$ in the OB of the MTZ-treated group at 7 dpt relative to the DMSO control. The difference here in terms of the number of $PCNA^+$ $Sox2^+$ was found to be statistically significant.

Similar trends were observed in terms of PCNA Sox2 co-localization in the telencephalon. At 1 dpt, there was a two-fold increase in $PCNA^+$ $Sox2^+$ cells in the telencephalon relative to the control. At 7 dpt, the number of $PCNA^+$ $Sox2^+$ cells markedly increased by almost five-folds relative to the control. Comparing the number of $PCNA^+$ $Sox2^+$ cells at 1 dpt versus 7 dpt in the MTZ treated group showed a three-fold increase at 7 dpt. Based on the number of $PCNA^+$ cells, it seems that more than half of them are neural stem cells. This hints at the possibility that the adult zebrafish brain is trying to restore the ablated DAergic neurons in the brain by accelerating the number of neural stem cells undergoing proliferation. From all the results analyzed in this section,

it seems that it takes about 7 days following the MTZ treatment to notice the greatest increase in $PCNA^+$ and $PCNA^+$ $Sox2^+$ cells. Understanding the ultimate fate of these proliferating neural stem cells would be helpful to identify the number of regenerated DAergic neurons.

4.4 Tracking the regeneration of new DAergic neurons at later time points

Another way to examine regeneration in the adult zebrafish brain is to follow the number of newly formed DAergic neurons. Since 7 dpt was determined to be the point were most DAergic neurons were ablated, the next aim was to determine the point when the number of DAergic neurons in the MTZ-treated group will be comparable to the control. It was previously reported that the number of CFP^+ in the olfactory bulb returned back to normal levels when compared to DMSO-treated fish at around 45 dpt (Godoy, 2020). To determine if the regeneration of DAergic neurons happens spontaneously or gradually over time, I decided to track the number of CFP^+ cells at 15 dpt. Surprisingly, the number of CFP^+ cells did not increase drastically at 15 dpt relative to 7 dpt in the MTZ-treated fish. This suggests that the regeneration of DAergic neurons occurs gradually and takes at least 40 days from the day of maximum ablation. Godoy et al. conducted a series of experiments to confirm that the newly formed CFP^+ cells were in response to neuronal damage. Results from a pulse-chase analysis showed that the number of $BrdU^+$ CFP^+ cells increased by 2.5-folds relative to the control at 45 dpt (Godoy, 2020). Bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BrdU) is another marker for proliferating cells (Taupin, 2007). This result confirms that the adult zebrafish brain triggers the formation of new DAergic neurons in response to its ablation (Godoy, 2020).

4.5 The impact of DAergic neuron ablations on swimming behaviour

One of the key signs of PD diagnosis is the appearance of distinct motor dysfunctions. To investigate the link between the loss of DAergic neurons in the brain and the associated motor effects, NFP and MTZ-treated fish were examined at 0 hpt and 7 dpt. Both groups displayed severe impairments in motor activity across three parameters immediately after the treatments relative to the DMSO control. NFP-treated fish travelled about half the distance travelled by the control fish. Interestingly, there was a significant decrease in total distance travelled in NFP-treated fish relative to MTZ-treated fish at 0 dpt. Overhead images displaying the path taken by NFP-treated fish show that they spend most of their time rotating around themselves in small circles. In addition, they tend to swim upwards towards the surface; however, their entire body seems to sink back to the bottom. Furthermore, most of the tracked path taken by NFP-treated fish is black colored, indicating that they tend to swim around with minimal movement. This suggests that NFP promotes severe motor impairments at 0 dpt. Analyzing the same treated fish at 7 dpt showed that there is some recovery in motor activity as the distance travelled by NFP and MTZ-treated fish was comparable to that of the control.

A similar trend was noticed when observing the average velocity of the treatment groups. It seems that NFP and MTZ-treated fish suffer from severe transient impairments in motor activity, and that is why they tend to show some recovery at 7 dpt. That said, NFP-treated fish still showed an increase in time spent in a stationary state relative to the control, albeit not statistically significant. Observing the overhead path images for the three treatment groups did not show any stark differences between each other at 7 dpt. Most of the path lines were green coloured, indicating normal swimming activity. This was unexpected as it was predicted that the motor impairments would last longer, especially since 7 dpt corresponds with the greatest ablation of DAergic

neurons. This could possibly mean that changes on the cellular level do not necessarily lead to long-term behaviour impairments.

Taken together, these results suggest that NFP has the strongest impact on motor activity when compared to MTZ and the DMSO control. In addition, it seems that MTZ and NFP-treated fish need at least 7 days following the initial treatment to show signs of motor recovery.

4.6 Future Directions

One important aspect that could be improved in the future is the route of drug administration. Instead of immersing the fish in a solution containing the drug, it would be beneficial to develop a method that will ensure that the drug gets injected in the area of interest. This could avoid the possibility of having undesirable side effects. For example, MTZ or NFP could be administered directly in the telencephalon region via cerebroventricular microinjection. This method has been established over the past couple of years and thus could deliver more specific and reliable DAergic neuron ablations (Bhattarai, 2017). That said, finding the optimal dose of MTZ to be administered might be challenging.

Another factor that could be added when examining DAergic neuron ablations is developing a method that could quantify the degree of cell death. For example, immunostaining cryosections obtained from MTZ or NFP-treated brains with anti-caspase-3 antibody in order to detect cell death via apoptosis (Karamitopoulou, 2007). Detecting apoptotic cells can also be supplemented by using the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay (Kyrylkova, 2012).

5. Conclusion

The general aim of this project was to further our understanding as to how adult zebrafish can effectively regenerate their DAergic neurons over time. Even though it would be challenging to extrapolate the results obtained from zebrafish to patients suffering from Parkinson's disease, it would serve as a good starting point to improve our approach when it comes to tracking common cell markers and conserved brain regions.

Our results were able to uncover the effects of the prodrug MTZ on brain regions like the olfactory bulb, telencephalon and pretectal area. In addition, we were able to test the efficacy of NFP, an alternative substrate to MTZ, for the first time on adult zebrafish. Our findings will potentially allow for novel alternative substrates to be explored, possibly causing more effective and specific ablations of DAergic neurons.

We also followed the expression of key cell markers like PCNA and Sox2 in the olfactory bulb and the telencephalon. This allowed us to track the degree and localization of cells undergoing proliferation, most importantly neural stem cells. Overall, we hope that this research project will advance our understanding as to how the adult zebrafish brain responds to DAergic neuron ablations and how this could potentially translate to targeting the symptoms seen in patients with Parkinson's disease.

6. References

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