Understanding Molecular Mechanisms Underlying Zebrafish Brain Regeneration

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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled "**Understanding Molecular Mechanisms Underlying Zebrafish Brain Regeneration**" submitted by Mr.Jaskaran Singh (Reg. No. MS15062) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

Dr. Santosh B. Satbhai | Dr. Sharvan Sehrawat | Dr. Rajesh Ramachandran (Supervisor)

Dated: May 4,2020

DECLARATION

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rajesh Ramachandran at the Indian Institute of Science Education and Research, Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Jaskaran Singh
(Candidate)

Dated: May 4, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rajesh Ramachandran
(Supervisor)

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Contents

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ABBREVIATIONS

1.RG- Radial glial
2.TBI- Traumatic Brain Injury
3.CNS- Central Nervous System
4.EMT- Epithelial to Mesenchymal Transition
5. GSK3 β- Glycogen synthase kinase 3 beta
6.ECM – Extracellular Matrix
7. Hdac- Histone deacetylase
8. VPA-Valproic acid
9. BrdU-Bromodeoxyuridine
10. PCNA-Proliferating Cell Nuclear Layer
11. dpi-days post-injury
12. RT-PCR-Reverse Transcription PCR
13. qPCR-Quantitative PCR
14. 30G- 30 Gauge
15.Sox2- SRY-related HMG-box gene 2
16. Ascl1a - Achaete-scute homolog 1
17. Mmp9-Matrix metallo protease 9
18. Mmp9-Matrix metallo protease 2
19. tgfbi- Transforming Growth Factor Beta Induced
20. Snai2- Snail Family Transcriptional Repressor 2
21. GS-Glutamate Synthetase

22. MG-Muller Glial
23.RNA- Ribonucleic acid
24.RGC- Retinal Ganglion Cell
25.APC- Adenomatous polyposis coli
26.TCF- T-cell factor
27. MGPC- Muller Glial Proliferative Cell
28. DNA- Deoxy Ribonucleic acid
29. PBS- Phosphate-buffered saline
30. PFA- Paraformaldehyde
31. MCT-Microcentrifuge Tube
32.DPEC- Diethyl pyrocarbonate

Abstract

A lot of people around the globe suffer from traumatic brain injuries and neurodegenerative diseases. Despite having many therapeutic advancements, the concept of neuroregeneration or brain repair still remains unclear. Whereas, Zebrafish, a teleost fish shows a robust regenerative response in any of the complex tissues including the brain, following an injury, which makes it an excellent model to study the molecular mechanisms underlying zebrafish brain regeneration. The concept of reprogramming and the proliferation of RG cells and stem cell niches, is the key regulatory mechanisms in the course of brain regeneration. This phenomenon requires a change in gene and protein expression. Therefore, identification of the molecular players in the regenerative process and its application in the non-regenerative species is of key relevance. It has been revealed by the various studies about the genes and proteins that take part in the CNS development and here in my study, I tried to depict the role of those genes and proteins such as *sox2, lin28, ascl1a and tgfbi*, especially in the proliferative phase in the injury-induced regenerative process. Also, the induction of the EMT transition factor snai2 is upregulated at 3dpi, which helps the proliferating cells to migrate to the site of injury. The role of wnt signalling seemed to be governed through a β-catenin independent manner in the proliferative phase of brain regenerative process. Blockade of GSK3 β resulted in excessive injury proliferation in the RG cells following an injury. The ECM proteases MMP2 and MMP9 were found to be upregulated at 3dpi. Combined blockade of ECM proteases MMP2 and MMP9 resulted in the significant decrease in the proliferation of the RG cells and downregulation of the intracellular genes such as *sox2, ascl1a, lin28, tgfbi* that are involved in the brain regenerative mechanism. This shows the utmost importance of ECM factors in the brain regenerative process

Chapter 1: Introduction

Brain disorders are most prevalent all over the globe due to the lack of therapies to cure these diseases. The most important subtype of brain disorders is Neurodegeneration. Neurodegeneration can again be further classified in Acute and Chronic neurodegeneration. Chronic neurodegeneration can give rise to various diseases such as Alzheimer's, Parkinson's, Huntington's etc whereas acute neurodegeneration can result from various reasons such as clots, accidents, brain strokes etc. The most important reason for a limited cure for neurodegeneration is the limited capacity of a mammalian brain to exhibit adult neurogenesis, which is only limited to Subventricular zone and Dentate gyrus in the mammalian brain(Altman & Das, 1965). On the other hand, some of the vertebrates such salamander and zebrafish can produce neuronal cells throughout their life cycle and can regenerate their lost neurons upon traumatic brain injuries. Around 6000 cells are born every 30 minutes in the adult zebrafish brain(Hinsch & Zupanc, 2007). As compared to 2 stem cell niches in mammals, zebrafish consists of 16 stem cell niches in their adult brain(Kaslin et al., 2009)(Zupanc, 2008), which is one of the most important factors that zebrafish can replenish its lost neurons in a situation of acute neurodegeneration(N. Kyritsis et al., 2012). As compared to zebrafish, there is a formation of gliotic scar in mammals after a traumatic brain injury(TBI) at the injury site which hinders the formation of neuroregeneration and neuronal cell integration(Fitch & Silver, 2008). I have also been seen that the astrocytes are the main cell type that comprises the gliotic scar formation and they share the same parent cell lineage with neuronal cell type(Bovolenta et al., 1992)(Faijerson et al., 2006). Therefore, in the process of neuroregeneration, there are some molecular mechanism that are working differently in zebrafish as compared to mammals, as in zebrafish there is such scar formation seen and the lost neurons are replenished by the dedifferentiation of existing radial glial cells in the vicinity and the proliferation of neural stem cell niches at the ventricular and periventricular zones(N. Kyritsis et al., 2012)(Jorstad et al., 2017). One of the most unexplored reasons for the mammals being not able to regenerate their lost neurons is the difference in cell death in brain regions of mammals and zebrafish. In zebrafish most of the neuronal cell death takes place by apoptosis which has a moderate inflammatory response to induce the regenerative machinery whereas in mammals most of the neuronal cell death occurs via necrosis which has a very high inflammatory response and thus along with the death neurons, this inflammation induces death in the neighbouring neurons and

thus resulting in the formation of gliotic scar at the injury site(Zupanc et al., 1998)(Liou et al., 2003). Different neurogenic responses are seen in the different parts of the zebrafish brain and even the different populations of the proliferating cells are seen sometimes in the same lobe itself, for example, the telencephalic lobe of zebrafish brain consists of different population of proliferating cells from dorsal to ventral surface that consists of positive radial glial cell markers and negative radial glial cell markers(Kizil, Kaslin, et al., 2012). Most of the genes that are involved in the developmental process of the brain are induced in the repair mechanism such as pluripotency inducing factors as Sox2 and Lin28 and pro-proliferative factor such as Ascl1a etc. Also, ECM proteins such as MMP2 and MMP9 plays a very crucial role in the induction of genes required in the repair mechanism inside the cells. By the mechanism of stem cell proliferation, migration of cells to the injury site and dedifferentiation of radial glial cells, the injury site is restored for the lost neurons. Therefore, understanding the molecular mechanisms underlying zebrafish brain regeneration could help us decipher strategies that would help to push restoration of lost neurons in the mammalian brain.

1.1 BRAIN ARCHITECTURE, INJURY, AND REGENERATION

1.1.1 Zebrafish Brain Architecture

Zebrafish brain consists of different lobes such as olfactory bulb, telencephalic lobe, optic tectum, cerebellum and medulla oblongata. The olfactory bulb has a very limited capacity to regenerate whereas other brain regions have an extensive capacity to replenish its lost cells after a traumatic brain injury(Schmidt et al., 2013). Different population of progenitors lies within the different region of the zebrafish brain and thus different molecular mechanisms are induced in response to the injury. The most desirable region to study the brain regenerative process is the telencephalic lobe as it consists of the most stem cell niches present in the zebrafish brain. The telencephalic lobe of zebrafish brain consists of ventricular zone, periventricular zone and the parenchymal region. In the unlesioned zebrafish brain, mitotic activity is only limited to the ventricular zone where most of the stem cell niches are located but after the injury, there is strong proliferation response seen in the ventricular zone along with the parenchymal region of the brain(Kizil, Kaslin, et al., 2012). The reactive proliferation of RG cells is seen in the ventral surface of the telencephalic lobe of zebrafish along with the non-RG cells in the dorsal surface of the lobe(Kizil, Kaslin, et al., 2012; Nikos Kyritsis et al.,

2012). RG cells have very long process that can reach up to the pial surfaces(Kriegstein & Alvarez-Buylla, 2009). The peak phase of the proliferation can be seen at 3dpi with the help BrdU and further neuronal cells can be marked using HuC/D(Kizil & Brand, 2011).

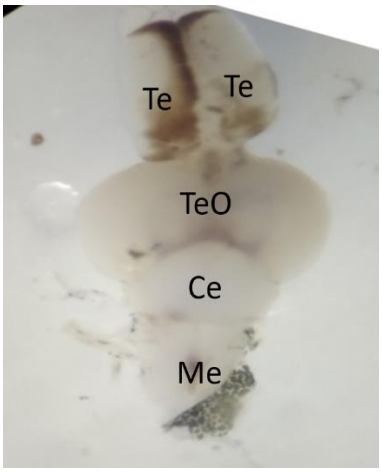


Figure 1.1.1: Zebrafish Brain Lobes. Te- Telencephalon, TeO-Optic Tectum, Ce-Cerebellum, Me-Medulla oblongata

1.1.2 Brain injury paradigms

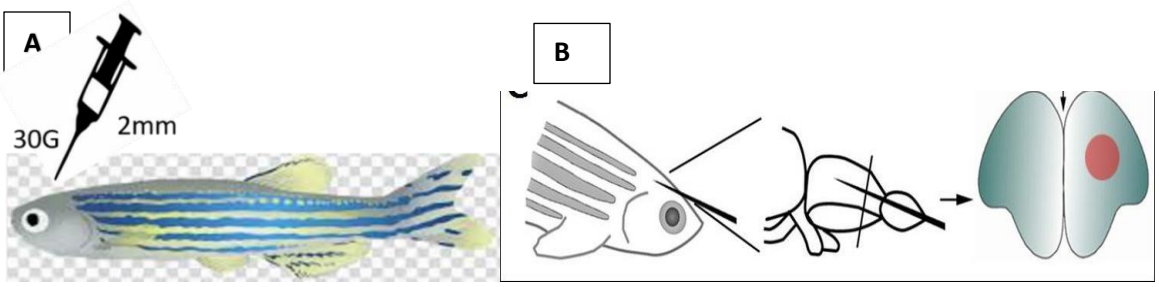
Different brain injury paradigms exist for injuring zebrafish brain, but irrespective of the method used, the regenerative response is almost the same providing the injury should be done in the same region.

A. Stab injury method: In this method, a 30G needle is taken and paced slantly on the zebrafish head near the eye region to injure the telencephalic lobe and is inserted 2mm deep dorso-ventrally as to break the skull and injure one part of the lobe. Then using fluorescence microscopy, we can see an injury line in the zebrafish brain. This method is mostly used as more than 99% of fishes survive after a stab injury.

B. Nostril injury method: In this method, a 30G needle is taken and is inserted along the rostrocaudal axis, 5-6 mm deep in the nostril of the zebrafish injuring the olfactory bulb as well as the telencephalic lobe(Kizil, Kaslin, et al., 2012). By taking transverse sections, we can see a hole like an injury structure under fluorescence microscopy. This method provides us with a 90% survival rate of the injured fishes. One advantage of this method is that it gives us a clearer vision of cytokines, chemokines, leukocytes and

proliferation cells accumulating around the injury site, with the help of confocal microscopy.

For my study, I preferred to choose the Stab injury method, due to its edge of survival rate over the nostril injury method and it does put us with any further hindrances in the study.



Reference: 1Volker Kroehne, Dorian Freudenreich, Stefan Hans, Jan Kaslin, Michael Brand; Development 2011 138: 4831-4841; doi: 10.1242/dev.072587

Figure 1.1.2: A)Stab injury B)Nostril injury(Kizil, Dudczig, et al., 2012)

1.2 Activation of developmental processes in Regeneration

Development of CNS consists of induction and the proliferation of the neural progenitors and the subsequent differentiation into the mature neurons. Neural induction is initiated at the early stages of the development to specify neuroectoderm in the developing CNS of vertebrates(Doniach & Musci, 1995). It has been shown that the neural induction relies upon signalling from many intrinsic such as Sox family genes and extrinsic factors as Wnt for the formation of complex neural architecture in the brain(Wilson et al., 2001)(Avilion et al., 2003). Also, these factors are found to be essential for the formation of the neural plate in the developing CNS. SoxB1 family genes are also found to be very essential to specify neuroectodermal fate(Avilion et al., 2003). All these intrinsic as well as extrinsic factors such as Sox2 and Wnt respectively play a crucial role in in the repair mechanism after a traumatic brain injury in the zebrafish brain. Blockage of these factor after traumatic brain injury may lead to severe impairment in the repair mechanism. One of the other major factors that drive the developmental process is the migration of neural progenitors and neural crest cells in the CNS to various location to make up the neural architecture. EMT(epithelial to mesenchymal transition) is very for the migration of cells to take place in which cells lose their polarity and cell-cell adhesion properties to migrate to specific directed location. In the adult zebrafish

brain, after a TBI, neural progenitors must adopt these properties to migrate to the injury site and thus replenish the lost neurons. Factors such as Snails that mark for the migratory phase of the cells are seen at the peak, after a TBI in zebrafish which indicates for the migration of cells at the injury site.

1.2.1 Sox2

Sox2 is one of the most important pluripotency inducing factors and has a vital role in the maintenance of neural progenitor properties in the vertebrate lineage(Masui et al., 2007). It is also regarded as stemness factor(Tanimura et al., 2013). It is found in the neural progenitor cells in the zebrafish embryo as well as in the neural stem cells in the adult zebrafish brain. Sox2 also acts as a repressor of different factors such as her1 and her3 that are involved in neuronal differentiation and thus it helps to maintain the neural progenitor pool in CNS(Schmidt et al., 2013). *Sox2*, along with three other factors, that are *oct4, c-myc and klf4* can be used to induce pluripotency in an adult cell and all these four factors are popularly known as Yamanaka factors. It is known that in zebrafish retina regeneration, Sox2 binds with the promoter sequence of *lin28* and thus upregulating its expression which in turn acts helps in the reprogramming of MG cells(Gorsuch et al., 2017). It is also seen that *sox2* expression is also necessary for the expression of *ascl1a* which is a pro-neural transcription factor involved in neurogenesis(Gorsuch et al., 2017). Although this protein has been well characterized and studied in the retina regeneration in zebrafish but its role in the repair mechanism of zebrafish brain remains largely unknown and is yet to be explored.

1.2.2 Ascl1a

Ascl1a is a proneural transcription factor and is expressed at the onset of neurogenesis in the zebrafish neural plate. Expression of *ascl1a* is controlled by the expression of *sox2*, which is upregulated in the regenerative mechanism of the zebrafish retina(Gorsuch et al., 2017). It has also been shown that ascl1a along with two other proneural factors that are myt11 and brn2 can directly reprogram the cells from fibroblast to specif neural cell types by bypassing the intermediary stage of pluripotency(Hinsch & Zupanc, 2007). Knockdown of ascl1a in after

zebrafish retinal injury leads in the blockage of MG proliferation and thus preventing the generation of retinal progenitors and further differentiated neurons, therefore ascl1a is very essential for quiescent MG cells to convert into actively dividing retinal progenitors for the regeneration to take place(Ramachandran et al., 2011). Although ascl1a has been extensively studied in retina regeneration but its role in the brain repair mechanism and its interaction with pluripotency inducing factor sox2, is yet to be discovered which can provide us with a more clear vision for the processes taking place in neuronal regeneration in the brain.

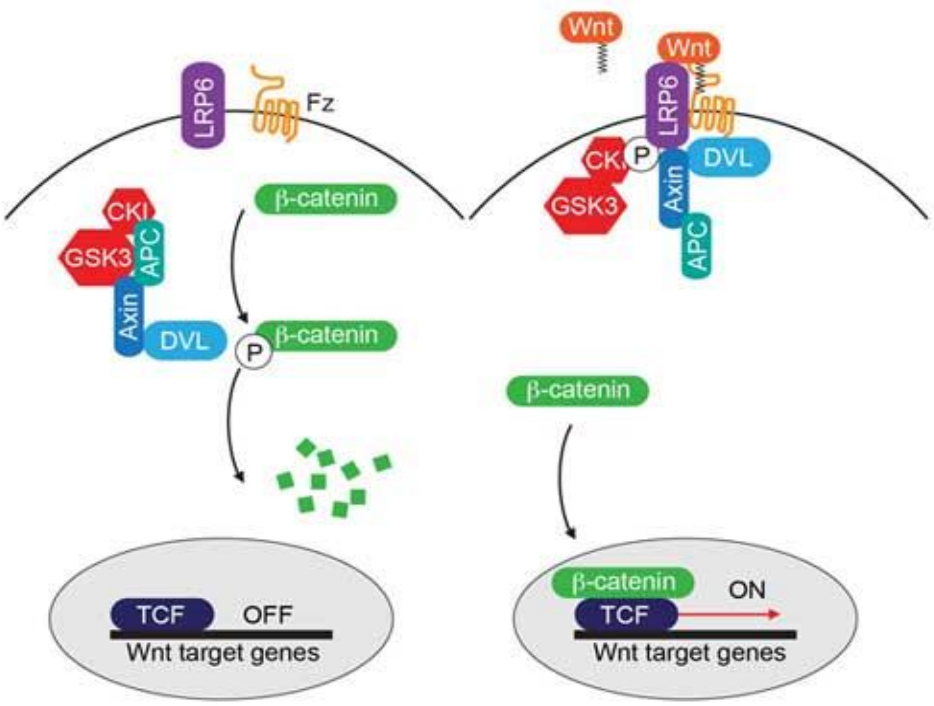
1.2.3 Lin28

Lin28 is an RNA binding protein and is mostly located in the cytoplasm but is also known to shuffle between nucleus. Lin28 can bind to let7 pre-micro RNA and then henceforth blocking the production of mature let7 micro RNA, which is involved in the differentiation of cells(Newman et al., 2008). Therefore lin28 in a way helps to maintain the progenitor pool of cells in case or development and repair mechanism. Lin28 is involved in the reprogramming of MG cells in the retina regeneration(Gorsuch et al., 2017). It is also important for the self-renewal of stem cells and is expressed in the early stages of development in the mouse embryonic stem cells. It is also seen that overexpression of lin28 in the retinal ganglion cells(RGC) can assist in the optic nerve regeneration(Wang et al., 2018). In developing mouse neural tube, lin28 can be seen to co-localize with sox2, giving a sight of its role in neural development(Ouchi et al., 2014). Furthermore, lin28 can also collaborate with oct4 and nanog to promote pluripotency. Most of the lin28 study has been done in zebrafish retina regeneration and in the developmental processes, which gives us a clue for its role in the brain repair mechanism, which is yet to be studied.

1.2.4 Wnt/ β-catenin signalling in regeneration

Wnt signalling is highly conserved among species. It has multiple roles in embryonic development from cell migration to cell proliferation. It is a signal transduction pathway in which a protein passes through the cell surface receptors to further activate specific genes inside the nucleus. Wnt signalling can be divided into two types: 1) Canonical wnt signalling and 2)Non-canonical Wnt signalling. Canonical signalling pathway involves the function of β-catenin whereas the non-canonical pathway is independent of it. In the canonical pathway, wnt protein binds to frizzled which in turns deactivates the destruction complex of β-catenin, that

includes APC, GSK3β and Axin, so that β-catenin accumulates in the cytoplasm and thus translocates to the nucleus to activate pro-proliferative genes that are essential in developmental and regenerative processes(De Robertis, 2010; Ramachandran et al., 2011). Wnt signalling is shown to have multiple roles in the development of vertebrate CNS which includes neural progenitor cell proliferation, neural differentiation. Its role has also been shown in post-embryonic neurogenesis, for neuronal progenitor cell differentiation in hippocampus and hypothalamus regions of the brain(Schmidt et al., 2013). It's a widely studied signalling pathway but yet its role in the brain repair mechanism is unknown and more its interaction with pro-proliferative and pluripotency inducing genes in the course of brain regeneration is yet to be found out.



Reference:Hitoshi Sawa1§ and Hendrik C. Korswagen2§;wormbook.org

Fig 1.2.4- Canonical Wnt/ β-catenin signalling: Binding of Wnt to Fz and LRP6 leads to inhibition of β-catenin degradation, and therefore β-catenin translocates to nucleus activating TCF family of transcription factors which in turn co-activates target genes.(*wntsignal_fig1.jpg (1577×1239)*, n.d.)

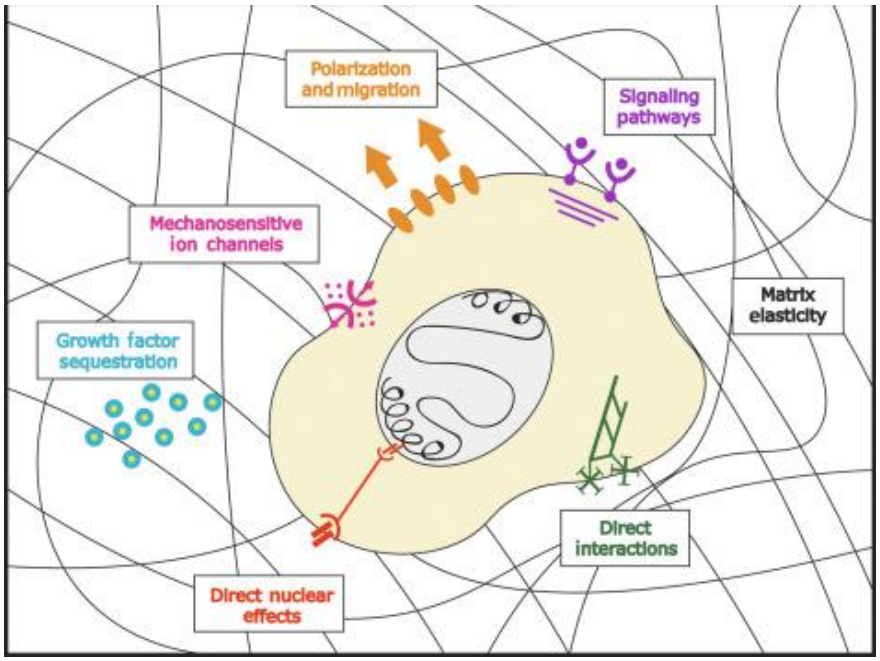
1.2.5 EMT protein Snai2 in regeneration

In the developmental processes, Snai2, which is zinc finger transcription factor, is mainly involved in the migration of neural crest cells. Mutated Snai2 can produce severe defects in the neural tube(Oram & Gridley, 2005). It mostly acts as a transcriptional repressor which binds to the E-box motifs and is known to repress the E-cadherin expression in the cell that marks for its epithelial state. Therefore it promotes the epithelial to mesenchymal transition (EMT) in the neural crest cells in the CNS that helps them to migrate to their specific target locations. It has also been found out that Snai2 can control the undifferentiated state of human epidermal progenitor cells(Mistry et al., 2014). In the regenerative mechanism, Snai2 plays a vital role for the proliferating cells at the stem cell niches to migrate at the site of the injury and replenish the lost cell types. It has been shown that the knockdown of the snail family genes drastically reduces the MGPC in the regenerating zebrafish retina after an injury(Sharma et al., 2019).

1.3 Role of ECM in regeneration

In the early developmental stages, the extracellular matrix (ECM) is secreted by embryonic cells. ECM provides with a microenvironment around the cell for cell growth, cell-cell signalling, cellular proliferation and differentiation cell morphogenesis etc. ECM secretes most of the growth factors that are essential for cell survival. The ECM architecture provides cellular integrity which also limits cell movements and diffusion of morphogens. therefore an ECM can also be regarded as morphogenetic code language for cells to act. The morphogens in ECM that comes in contact with the cell surface receptors can alter the behaviour of cell as cell-cell adhesive forces, cellular polarity, signalling between the cells, cellular migration, cellular proliferation and differentiation(Muncie & Weaver, 2018). ECM plays a vital role in cell migration which can also be seen the developmental stages of the CNS. Migration of neural progenitors is the key functional role in the CNS development in the embryonic as well as postnatal stages. Reelin and tenascin are the key components in the ECM that guide the migration of neural progenitors for the development of the cerebellum in the brain(Porcionatto, 2006). ECM also plays a very critical role in cell proliferation and differentiation as ECM contains a gradient of growth factors and

cytokines that guide the cell to proliferate in its surrounding(Schmidt et al., 2013). Studying the neuronal migration to specific target locations in contrast to ECM can help to resolve many neurological disorders due to neuro misplacement. ECM despite having its role in the developmental processes in the CNS, also plays a vital role in the repair mechanism. After TBI along ECM is supposed to expose certain growth factors that interact with the cell surface receptors for cellular growth and cellular proliferation to restore the lost cell types. But yet the role of ECM in CNS repair is to be studied which could provide us with the data of utmost importance in neuroregeneration.



Reference: M.Muncie†Valerie M.Weaver*‡;https://doi.org/10.1016/bs.ctdb.2018.02.002*

Fig 1.3: Mechanical and Biophysical properties of ECM(Muncie & Weaver, 2018)

1.3.1 Role of MMP2 and MMP9 in regeneration

Matrix metallopeptidases are zinc-containing endopeptidases that belong to a larger family of metzincin proteases. MMP's can degrade any kind of ECM proteins, therefore remodelling the extracellular microenvironment around the cells. They play a major role in cell migration, cell proliferation and differentiation. MMP2 and MMP9 are found to play a major role in metastasis.MMP9 is also upregulated during human respiratory epithelial healing and MMP9 knockdown mouse were unable to remove fibrinogen matrix during the wound healing process(Caley et al., 2015). MMP's are also found to essential for cancer metastasis for its property to degrade ECM, allowing primary tumour cells to evade out to form secondary tumours(Kleiner & Stetler-Stevenson, 1999). As one of the main events in regenerative processes is the remodelling of the extracellular matrix, therefore it suggests that MMP's have a crucial role to play in the repair mechanism. Temporal expression of MMP9 has been found to play a crucial role in the axolotl limb regeneration(Yang et al., 1999). It has also been shown that the absence of MMP9 significantly compromises the survival of regenerated cones in the adult zebrafish retina(Silva et al., 2020). Uncontrolled expression of MMP's is associated with the onset of many neurological disorders and CNS injuries. MMP's are shown to be promoters of retinal ganglion cell axonal outgrowths plus acting as guiding molecules, in mammals. MMP2 has a vital role in optic nerve regeneration in zebrafish(Lemmens et al., 2016). Single knockdown of MMP2 can result in decreased innervation of RGC axons in the optic tectum(Lemmens et al., 2016). Therefore, ECM remodelling with the help of MMP's can be a very useful finding to study the regenerative processes occurring in the brain.

1.4 The epigenetic basis of regeneration

Regeneration is a very controlled mechanism which involves highly coordinated expression of genes. Gene expression can be controlled in two major ways: 1) by transcription factors and 2) epigenetic modification. Epigenetic modification can alter the gene expression without a change in the DNA sequence. Epigenetic modification can be done by RNA associated gene silencing, DNA methylation or histone modification. Histone modifications involve the change in chromatin by transferring or removing acetyl or methyl groups to the amino acids on the histone proteins. These modifications can either activate or repress the expression of various genes in chromatin. Role of epigenetic modifiers is largely unknown in brain regeneration,

therefore its necessary to study the role of these modifiers in brain regeneration as they highly coordinate the process of regeneration by activating and repressing the expression of a gene.

1.4.1Histone deacetylases

These are the class of enzymes that regulate the expression of the gene that removes the acetyl group from the lysine amino acid on the histone protein which results in the more tightly packed DNA and thus becoming difficult for the transcription factors to access that gene and thereby suppressing the expression of that gene. HDACs are classified into three major groups: Class I, II and IV and are present in both nucleus and cytoplasm(Bhalla, 2005).

Valproic acid(VPA) is an inhibitor of the HDAC activity by binding to its catalytic centre(Göttlicher et al., 2001). it inhibits the HDACs to deacetylase the acetylated lysine on the histone proteins and thus the DNA remains in the euchromatin state and accessible by the TFs to drive the expression of various genes. It has a strong potency towards the HDAC class I enzymes(Göttlicher et al., 2001).

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal maintenance

- Zebrafish were maintained in water tanks attached to the automated water circulation system.
- pH: Physiological pH
- Temperature: 27°C of water
- Lightning: 14 hours of light followed by 10 hours of dark

2.2 Brain injury

1. Fish was anaesthetized using Tricaine methanesulfonate.
2. Then using a small wet tissue paper a stand was made in which the fish could be fitted as to hold the fish from the sides using the tissue, so that while injury the fish does not slip from the hand.
3. Then fish was placed with its dorsal side facing upwards.
4. A 30G needle was sterilized using 70% alcohol.
5. Then the head of the fish was gently held and with the other hand, the needle was placed on the head of the fish slightly towards one eye.
6. The needle was placed a bit slantly towards caudal axis and then the needle was pushed 2mm deep inside the brain penetrating the skull of the head to the telencephalic lobe.
7. Then the fish is immediately transferred to water providing a slight shake in water.

2.3 Brain Isolation

1. Take a petri dish and fill it with 1x PBS.
2. Place petri dish above an ice-filled container.
3. Now anaesthetize the fish and using a blade cut the head part of the fish and discard rest of the body.
4. Now put the head part in the 1x PBS.
5. Then using forceps remove the eyes and the skin surrounding it form both the side.
6. Now hold the head of the fish with its ventral side facing upwards using one forceps.

7. Then using another forcep, crush the skull part and open it by inserting forcep around lateral sides.
8. Whitish part of the brain will be visible, then gently remove the remaining debris from the side facing upwards.
9. Then take your forceps to the telencephalic part and remove the covering of skull form the ventral part.
10. Now slide your forceps in the cavity of the skull and slide it under the optic tectum region and take the brain out from the cavity.
11. Put the brain in Trizol, Laemmli buffer or PFA for RNA isolation, western blotting and immunostaining respectively.

2.4 RNA Isolation

1. Dissected brains were collected in 200µL of TRIZOL taken in an MCT.
2. Tissues were homogenized using a homogenizer.
3. After homogenizing, tissues were kept at room temperature for 10 minutes.
4. Then 0.2 volume(40µL) of chloroform was added and mixed gently for 15 seconds.
5. Then samples were centrifuged at 4°C at 12000 rcf for 15 minutes.
6. Then using cut tips aqueous phase was removed; collected in fresh MCT and 25µL of isopropanol was added.
7. Then the samples were gently tapped and kept at ice for 20 minutes.
8. After that samples were centrifuged at 4°C for 20 minutes at 10000 rcf.
9. Then the supernatant was discarded and 200µL of 80% ethanol was added in each tube.
10. After that samples were centrifuged at 4°C for 10 minutes at 7600 rcf.
11. Then the supernatant was discarded.
12. Pellet was kept for drying for about 15-20 minutes at RT.
13. Then samples were eluted in 15µL of DPEC water.
14. Samples were left in ice for about 30 minutes.
15. Then samples were given a short spin and checked on 1% agarose gel by gel electrophoresis.
16. Samples were stored in -80°C.

2.5 cDNA preparation

(Kit used – RevertAid First Strand cDNA Synthesis Kit by Thermo Fisher)

1. Following reagents according to the given volumes were used and added into a sterile tube:

a) Template RNA 2.5 µL

b) Primer (Oligo (dT)18 +Random Hexamer) 0.25+0.25 µL

2. All the contents in the tube were mixed thoroughly and then were incubated at 65°C for not more than 5 minutes. Then, the tubes were taken out and quickly transferred back on the ice.

3. Following components were added in the indicated order:

a) 5X Reaction Buffer 1µL

b) RiboLock Rnase Inhibitor 0.25µL

c) 10mM dNTP Mix 0.5µL

d) Revert-Aid M-Mul VRT 0.5µL

Total volume 5µL

4. All the contents were properly mixed, then centrifuged and then were incubated at the following temperatures

- 5 minutes at 25°C
- 60 minutes at 42°C
- 5 minutes at 70°C

5. The cDNA was then diluted accordingly with autoclaved Milli-Q water and then checked in 1% agarose gel by gel electrophoresis and then stored at -80°C.

2.6 Reverse Transcription Polymerase chain reaction (RT-PCR) using Taq Polymerase

1. The reaction mixture (10 µL volume)

10X buffer	0.5 µL
2.5mM dNTPs	1.0µL
Primers (forward + reverse)	0.2µL
Taq polymerase	0.1µL
Template	(as per standardized volume)
MQ Water	Rest

2. Reaction Parameters

Enzyme activation	95°C for 2 min
DNA denaturation	95°C for 20 sec
Primer annealing	60°C for 30 sec
Elongation	72°C for 30 sec
Final elongation	72°C for 7 min
Infinite hold	4°C

3. Then using gel electrophoresis, PCR products were checked on 1-2% agarose gels.

2.7 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

(Kit used for the reaction – KOD SYBR® qPCR Mix)

- Total volume of reaction mixture-5μL

Master Mix(KOD)	2.5μL
Primer(forward+reverse)	0.5μL
Template	1.0μL
MQ water	1.0μL

Reaction Parameters :

Enzyme activation	95°C for 2 min
DNA denaturation	95°C for 20 sec
Primer annealing	60°C for 30 sec
Elongation	72°C for 30 sec
Final elongation	72°C for 7 min
Infinite hold	4°C

Then using Microsoft excel, the data was analysed and plotted in graphical form.

2.8 Tissue fixation and Cryosectioning

1. Isolated brains were kept in 1XPBS for 10 minutes.
2. Then after the brains were transferred into an MCT containing 4% PFA and kept overnight at 4°C for tissue fixation.
3. Then the next day, serial washes in the given order were given for 45 minutes at RT on rotar for the dehydration of the tissue.
    a) 5% Sucrose 1ml
    b) 5% sucrose+20% Sucrose 800µL +400µL
    c) 5% sucrose+20% Sucrose 600µL +600µL
    d) 5% sucrose+20% Sucrose 400µL +800µL
    e) 20% Sucrose 1ml

The composition of the solutions that were used in the washes are :

a) 4% PFA in 1X Phosphate buffer (made DEPC water) :

- 2g PFA
- 5mL of 10X phosphate buffer
- Make up the volume to 50mL with DEPC water.
- Dissolve it by keeping in 65°C in a water bath and constant shaking after 10 minutes.

b) 5% sucrose:

- Dissolve 2.5g sucrose in 50mL of autoclaved water. Store at -20°C.

c) 20% sucrose:

- Dissolve 10g sucrose in 50mL of autoclaved water. Store at -20°C.

4. Then 400µL of OCT was added in 1ml 20% existing sucrose solution and rotated on rotar for 30 minutes.
5. Then with the help of cuvette, an aluminium block was made.
6. The aluminium block was filled with OCT and the brain from the MCT was taken out with the help of focep and transferred to the block.
7. Then the was brain was oriented in such a direction so that its dorsal side faced upwards.
8. Blocks were quickly transferred to -80°C and kept overnight.
9. Then the blocks were sectioned using Leica cryostat.

10. Transverse sections of 12 microns of the brain were taken on slides.
11. Slides were kept overnight for drying.
12. Then the slides were stored in -20°C.

2.9 Immunostaining

Day 1

1. Slides were taken out from -20°C and dried for 30 minutes.
2. Then three washed of 1XPBS were for 10 minutes each.
3. Then the slides were treated with 2N HCl at 37°C for 20 minutes.
4. Then two 0.1M Sodium borate washes were given for 10 minutes each.
5. Then sections were blocked using 5% BSA in 1X PBST (1XPBS + 0.1% Triton X) for 2 hours.
6. Then discard the BSA from the slides and incubate the slides with primary antibody of choice (PCNA, BrdU) with 400µL of antibody per slide(Antibody dilution:1:500 in 1% BSA in 1XPBST)
7. Incubate the slides overnight at 4°C.

Day2

1. The antibody was collected back in an MCT.
2. Three 1X PBST washes were given for 10 minutes each.
3. Incubate the slides with desired secondary antibody for 2 hours at RT(Antibody dilution:1:1000 in 1% BSA in 1XPBST) with 400µL of antibody on each slide.
4. Give three washes of 1XPBST for 10 minutes each
5. Check for the signals in a fluorescence microscope.
6. Then given two 1XPBS washes for 10 minutes each.
7. Then incubate the slides with DAPI for 90 seconds.
8. Give three washes of 1X PBS for 10 minutes each.
9. Discard the PBS and keep the slides for drying for about 15-20 minutes at RT.
10. Mount the slides with DABCO and keep it overnight at RT.
11. Next day image or store the slides in -20°C.

2.10 Double immunostaining

Day1

1. Slides were taken out from -20°C and dried for 30 minutes.
2. Then three washed of 1XPBS were for 10 minutes each.
3. Then sections were blocked using 5% BSA in 1X PBST (1XPBS + 0.1% Triton X) for 2 hours.
4. Then discard the BSA from the slides and incubate the slides with primary antibody GS(Glutamate synthetase) with 400µL of antibody per slide(Antibody dilution:1:500 in 1% BSA in 1XPBST).
5. Incubate the slides overnight at 4°C.

Day2

1. The antibody was collected back in an MCT.
2. Three 1X PBST washes were given for 10 minutes each.
3. Incubate the slides with desired secondary antibody for 2 hours at RT(Antibody dilution:1:1000 in 1% BSA in 1XPBST) with 400µL of antibody on each slide.
4. Give three washes of 1XPBST for 10 minutes each
5. Then one wash of 1XPBS was given for 10 minutes.
6. Then 4% PFA was overlayed on the slides for 15 minutes to fix the signal of GS.
7. The box was washed thoroughly so that no traces of PFA was left.
8. Two 1XPBS washes were given each for 10 minutes.
9. Then the slides were treated with 2N HCl at 37°C for 20 minutes.
10. Then two 0.1M Sodium borate washes were given for 10 minutes each.
11. Then sections were blocked using 5% BSA in 1X PBST (1XPBS + 0.1% Triton X) for 2 hours.
12. Then discard the BSA from the slides and incubate the slides with primary antibody of choice (PCNA, BrdU) with 400µL of antibody per slide(Antibody dilution:1:500 in 1% BSA in 1XPBST)
13. Incubate the slides overnight at 4°C.

Day3

1. The antibody was collected back in an MCT.
2. Three 1X PBST washes were given for 10 minutes each.
3. Incubate the slides with desired secondary antibody for 2 hours at RT(Antibody dilution:1:1000 in 1% BSA in 1XPBST) with 400µL of antibody on each slide.
4. Give three washes of 1XPBST for 10 minutes each
5. Check for the signals in a fluorescence microscope.
6. Then given two 1XPBS washes for 10 minutes each.
7. Then incubate the slides with DAPI for 90 seconds.
8. Give three washes of 1X PBS for 10 minutes each.
9. Discard the PBS and keep the slides for drying for about 15-20 minutes at RT.
10. Mount the slides with DABCO and keep it overnight at RT.
11. Next day image or store the slides in -20°C.

2.11Microscopy

1. Bright field Zeiss microscope was used for injuring and dissecting zebrafish brain.
2. Fluorescence microscope was used for checking signals .
3. Nikon confocal microscope was used for imaging.

2.12 Western Blotting

Sample preparation

1. Brains were dissected and collected in 100µL 2X Laemlli Buffer in a fresh MCT(2X Lamelle Buffer – 4mL of 10% SDS + 2mL of Glycerol + 1.2mL of 1M Tris-HCl (pH 6.8) + 2.8mL of MilliQ-water + 0.02% of Bromophenol blue. Store at 4°C).
2. Tissues were properly homogenized using homogenizer and pipette.
3. The sample was given brief vortexing along with ice incubation for 10 times.
4. Then the samples were heated at 95°C for 10 minutes.
5. Then samples were centrifuged 5000rpm for 10 minutes
6. Samples were stored at -80°C.

Day1

1. Resolving gel was casted.
   (12% Resolving gel – 2.5mL Resolving Buffer + 4mL 30% Acrylamide + 3.3mL MilliQ water + 100µL 10% SDS + 100µL 10% Ammonium Persulfate + 6µL TEMED)
2. Then after 20 minutes Stacking gel was casted.
   (Stacking gel – 625µL Stacking Buffer + 667µL 30% Acrylamide + 3603µL MilliQ-water + 50µL 10% SDS + 50µL 10% Ammonium Persulfate + 5µL TEMED).
3. Then the samples were taken out from -80°C and thawed.
4. Then the samples were loaded along with the protein ladder.
5. Then the gel was run at 25 Ampere for 3 hours.
6. After the run, the gel was cut out accordingly and washed with MQ water.
7. The PDFV membranes were cut according to the size of the gel and were transferred to methanol for charging for 5 minutes.
8. Then the membrane was washed with MQ water.
9. Then the gels were soaked in transfer buffer.
10. Then a sandwich was made with gel kept on pad and membrane above it.
11. The sandwich was transferred to the apparatus containing transfer buffer.
12. Then the transfer was set up at 70 volts for 90 minutes.
13. Then the membrane was blocked overnight in 10% skimmed milk at 4°C.

Day2

10. 0.01% PBST (For 200mL of 1X PBST, add 200µL of TWEEN20) washes were given for 15minutes, four times.
11. Then the blots were incubated with the primary antibody of choice for 3 hours at RT.
12. Three 0.01% PBST washes were given for 15 minutes each.
13. Then blots were incubated with secondary antibody for 2 hours at RT.
14. Three 0.01% PBST washes were given for 15 minutes each.
15. Blot was then developed in ImageQuant LAS4000.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Regulation of Epigenetic factor HDAC in the proliferative phase

HDACs role is very crucial for the regenerative mechanism to work in a very controlled manner. As it suppresses the gene expression by removing the acetyl group from the histone proteins, thereby suppressing the gene expression which in turn would be activating or suppressing the genes upstream of the pathway. In zebrafish brain regeneration, after traumatic brain injury, a peak of proliferative cells marked by BrdU is seen at 3dpi around the injury site. There to check for the regulation HDAC, a UC v/s 3dpi brain, was cryosectioned and was immunostained for BrdU and HDAC, that would provide us with the data of the events of the epigenetic modifiers taking place at the peak proliferative phase in an injured brain v/s control brain. The result revealed that on the Injury site, HDAC +ve cells were co-expressing with BrdU +ve cells whereas, in the uninjured brain, expression of HDAC and BrdU +ve cells was independent of each other. This suggests that HDAC has a crucial role in the proliferation of the cells around the injury site after a traumatic brain injury(Fig 3.1).

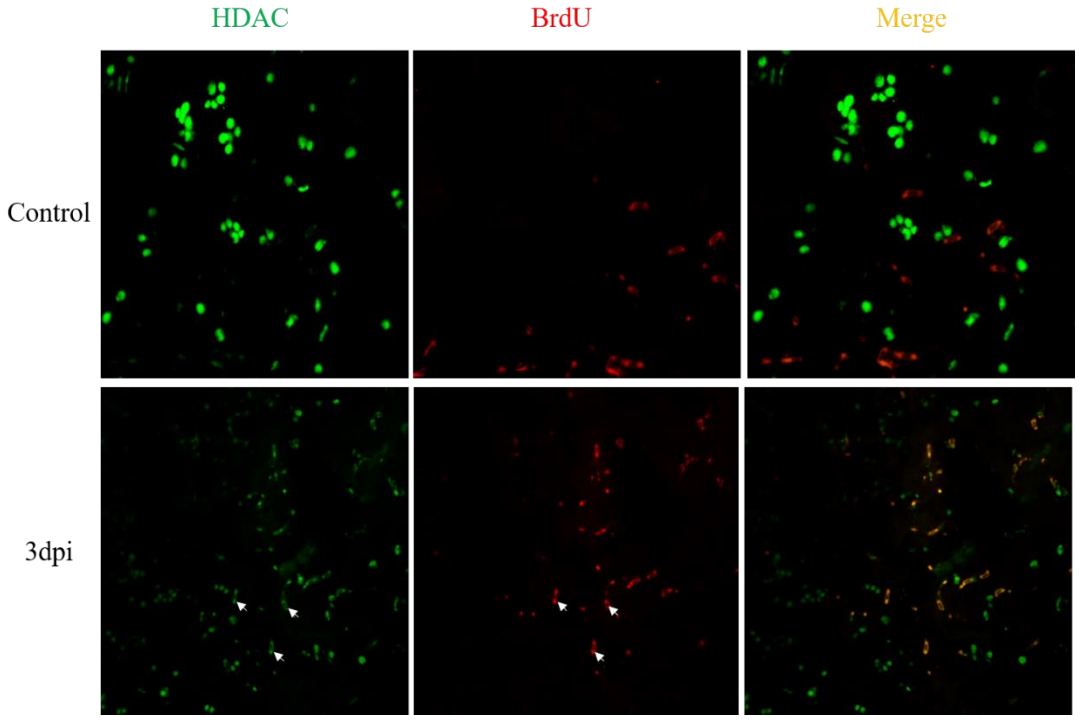


Fig 3.1: Colocalisation of HDAC-BrdU +ve cells in injured v/s control brain.

3.1.1 Blockade of HDAC by Valproic acid(VPA) results in scattered proliferation

Valproic acid blocks the action of HDAC by binding to its catalytic centre. It does not allow the HDAC to deacetylase the lysine amino acids and thus suppresses the expression of the genes, which may be positively or negatively regulating the proliferation of RG cells after traumatic brain injury in the regenerative process. So here 50µM of VPA concentration was used and fishes were dipped in 100 ml of water after injury. Control fishes were also treated with VPA to rule the possibility that VPA is having an exclusive effect than regeneration. It was found out that the VPA treated fishes show scattered proliferation all over the brain, whereas the proliferation was only limed to the ventricular zone and injury site, in injured fishes at 3dpi. So, the only conclusion that can be drawn here was that the HDAC was somehow limiting the proliferation in the brain after an injury, to rule out the formation of tumours or clumps of cells at the site. This conclusion can be supported by the fact that regeneration is a very controlled mechanism and its important to study the control of proliferation in the regenerative mechanism along with the study to induce it in the higher vertebrates.

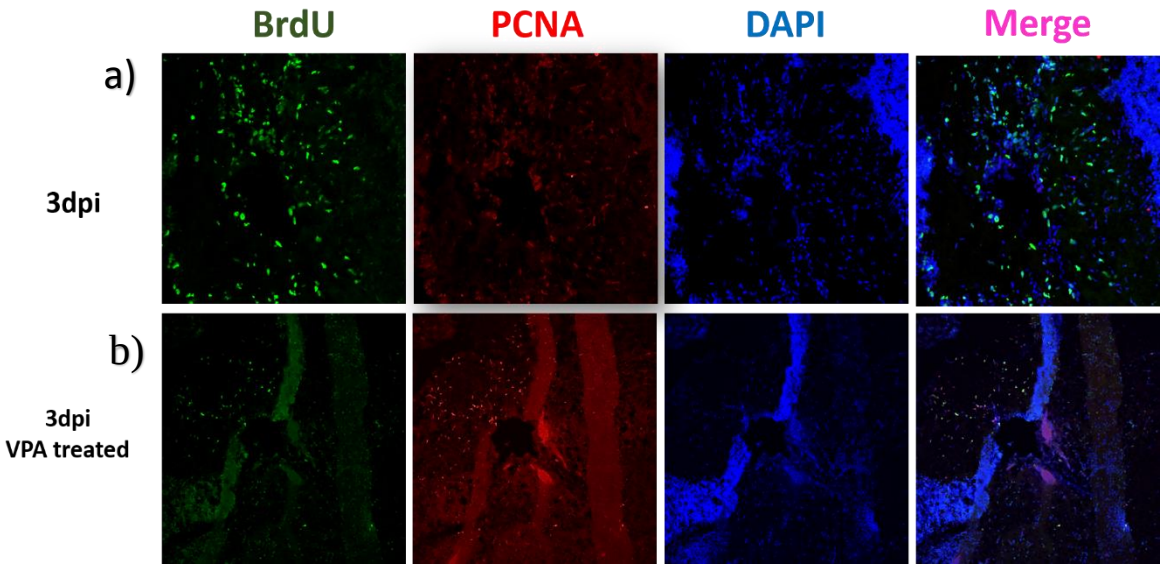


Fig 3.1.1: Blockade of HDAC by VPA a) Untreated VPA 3dpi fish with normal proliferation at the injury site b) VPA treated fish with scattered proliferation all over the brain.

3.2 Regenerative capacity of brain hindered in obese zebrafish

Zebrafish were fed five times a day for 3-4 months and made obese. Obesity is the main majorly evolving matter of concern as it leads to the origin of various diseases in the body from cardiac diseases to diabetes and many more. Many experimental proofs have been shown for the obese nature of the body to catch a disease faster. But not much has been studied about the obesity affecting the CNS and diseases related to it. It was observed that the proliferative capacity in the zebrafish brain after a traumatic brain injury is severely compromised as compared to the normal injured brain(Fig 3.2).

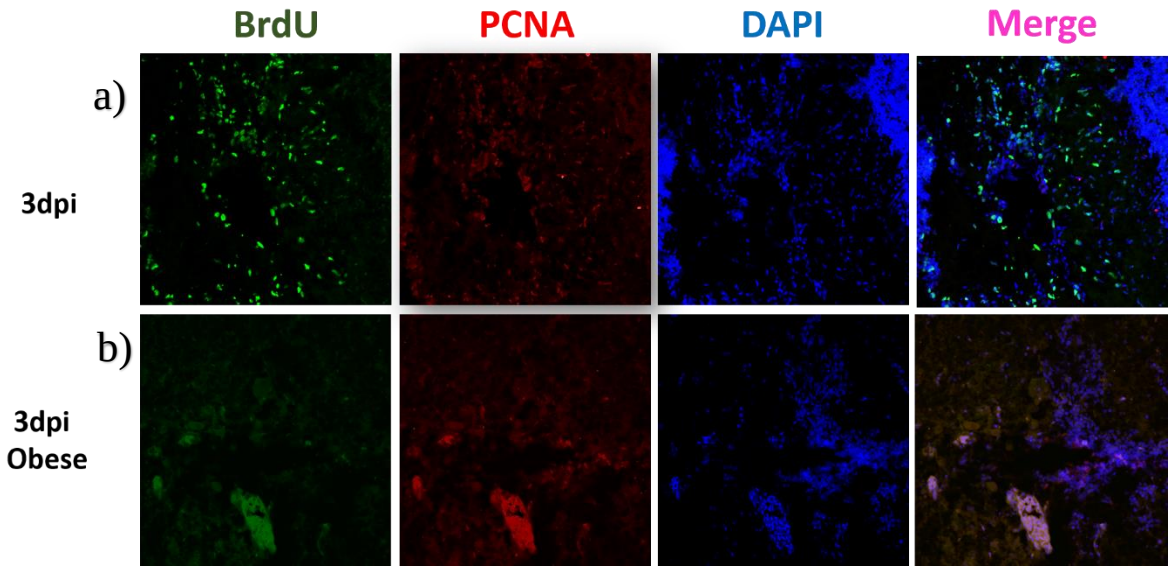


Fig 3.2: Significant decrease in proliferation in the obese zebrafish brain a) Normal zebrafish brain showing peak proliferation at 3dpi b) proliferation significantly reduced in the obese zebrafish brain.

3.3 Regulation of Ascl1a, Lin28 and Sox2 in brain regeneration

3.3.1 Upregulation of proneural factor Ascl1a in brain regeneration

Ascl1a is a proneural transcription factor expressed at the onset of neurogenesis. It is thought to control the expression of Lin28 in the developing CNS, which in turns derive let 7 micro-RNA expression. But the role *ascl1a* remains unknown in the brain repair mechanism. it was observed that in a time-course experiment using qPCR reaction that *ascl1a* shows a peak at 3dpi which is peak proliferative phase in the brain regenerative mechanism. Also, the fact was further confirmed by

western blotting experiment which in comparison to control showed a greater expression at 3dpi. So the conclusion comes out that the proneural factor *asc1a* also play a role in the repair mechanism, especially in the proliferative phase of the brain regeneration mechanism.

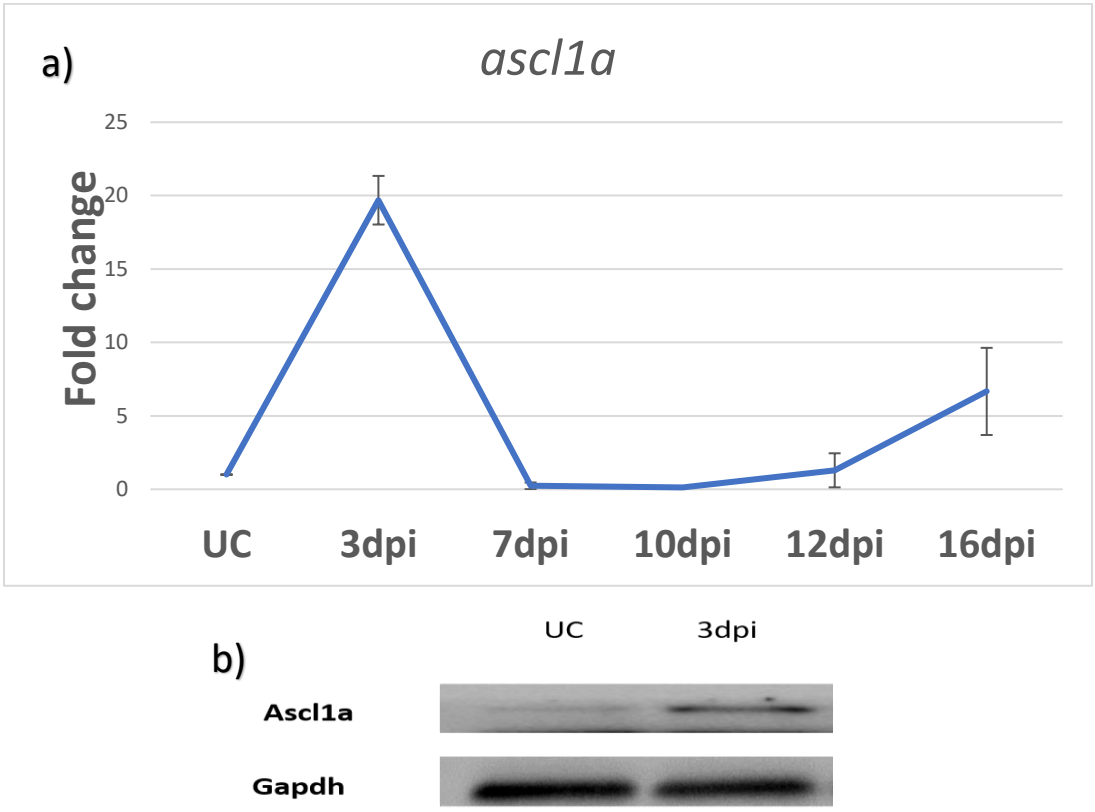


Fig 3.3.1: Temporal regulation of ascl1a in brain regeneration a) Peak of ascl1a observed at 3dpi in the qPCR data b) Higher protein expression of Ascl1a at 3dpi in comparison to UC.

3.3.2 Upregulation of Lin28 in brain regeneration

Lin28 is a binding protein that blocks the activity of mature let-7microRNA which is involved in the differentiation of the cells, therefore Lin28 helps to maintain the progenitor pool with self-renewal of the stem cells. Expression of *lin28* in the developing CNS is guided by the expression of *ascl1a*, and then *lin28*, in turn, blocks the expression of mature let-7 microRNA to stop the differentiation of the cells. Therefore in the brain, it was observed that after traumatic brain injury, the expression of *lin28* showed a peak at peak proliferative phase at 3dpi(Fig3.3.2a,b), where the expression of ascl1a was also

found to be the highest. Therefore, Lin28 apart from playing the role in the development of CNS is also involved in the brain repair mechanism and its expression could be guided by the proneural transcription factor Asc1a(3.3.2c). But further experiments needed to confirm this would be morpholino mediated gene knockdown of *ascl1a* and checking the expression pattern of *lin28* after TBI and vice versa.

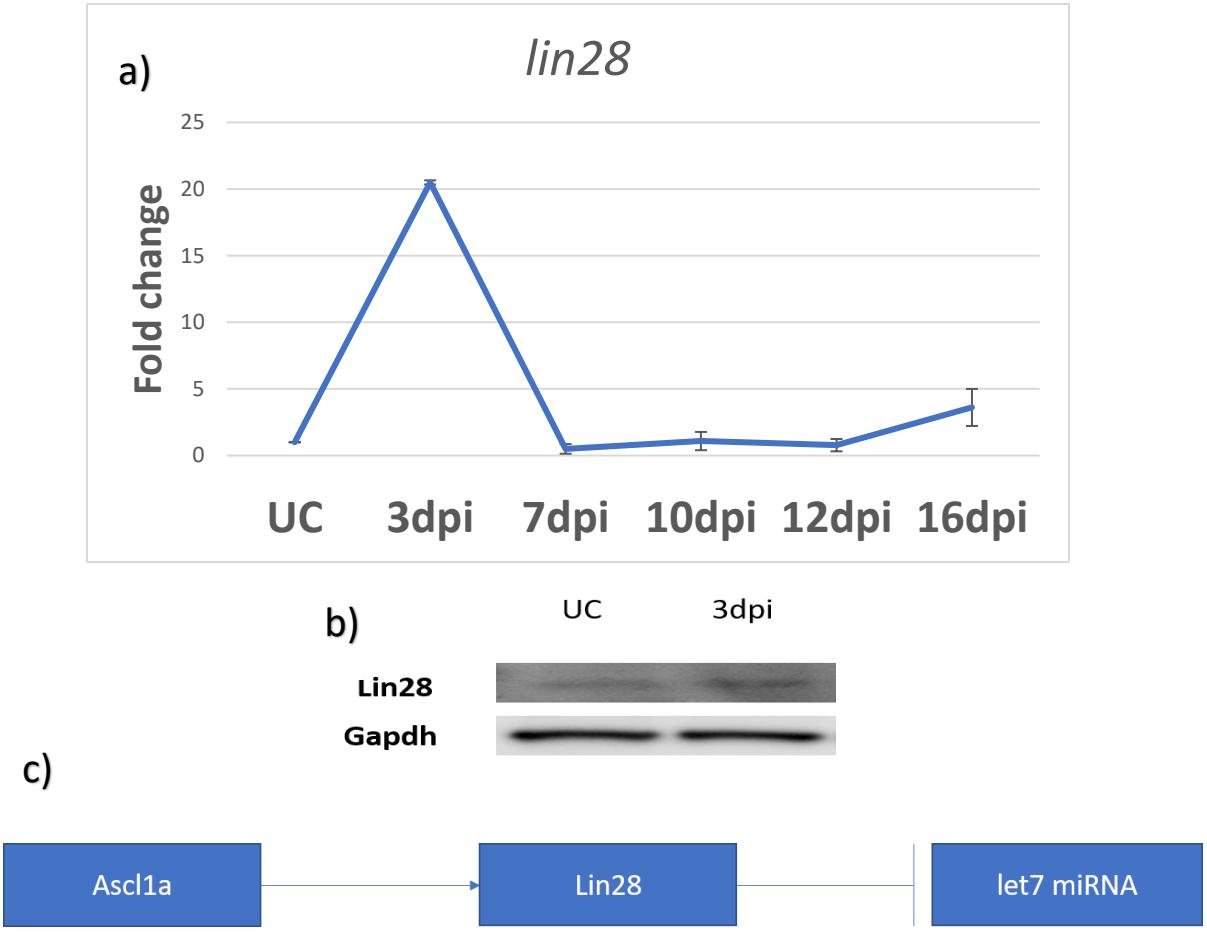


Fig 3.3.2: Temporal regulation of lin28 in brain regeneration a) Peak of lin28 observed at 3dpi in the qPCR data b) Higher protein expression of lin28 at 3dpi in comparison to UC c) Induction of lin28 by ascl1a which in turn blocks the production of mature let-7 microRNA.

3.3.3Upregulation of pluripotency inducing factor Sox2 in brain regeneration

Sox2 is a pluripotency inducing factor and is also known as stemness factor. It is found in the neural progenitor cells. It is also known to block differentiation by repressing factors like her1 and her3 and in zebrafish retina regeneration it binds with the promoter sequence of lin28 and upregulates its expression thereby helping in the reprogramming of the MG cells. As the role of sox2 has been not studied in brain regeneration, therefore I decided to check the temporal profile of zebrafish expression in brain regeneration. The peak of the *sox2* was observed at 3dpi which is peak proliferative phase and its peak correlates with the peaks of ascl1a and lin28. Also, this experiment was further confirmed at the protein level with the help of western blotting. Therefore, sox2 other than its role in the developing CNS has a major role in brain repair mechanism. Also, it could be possible that sox2 in brain regenerative mechanism could be regulating the expression of *lin28,* but the further knockdown experiment of sox2 is needed to confirm this.

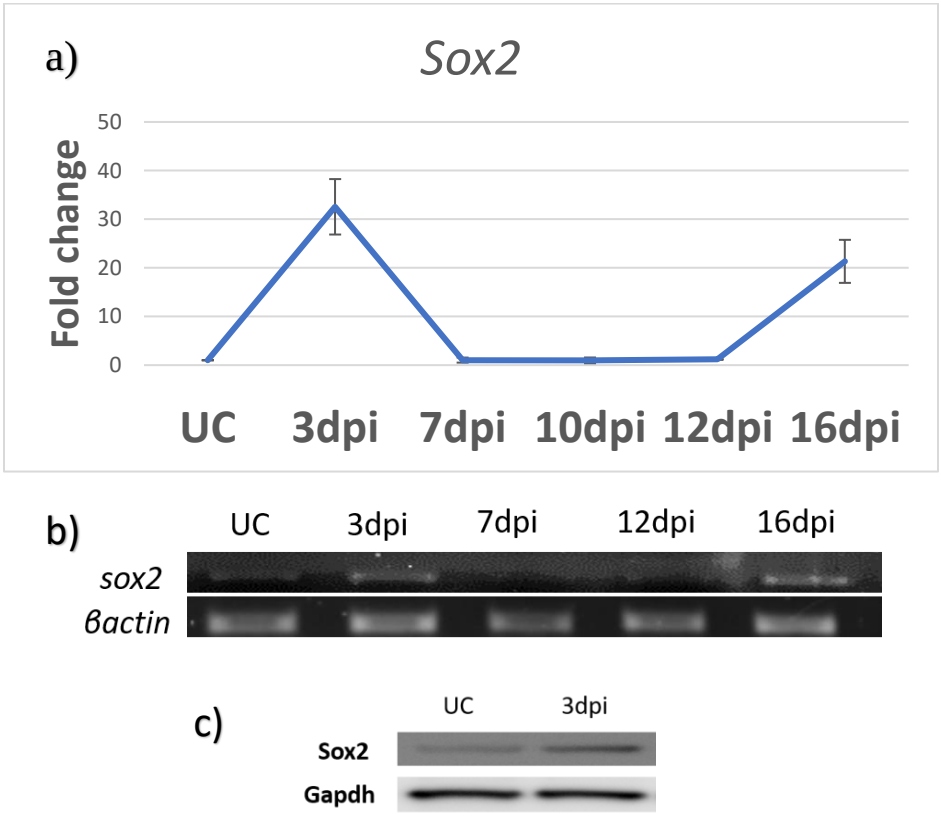


Fig 3.3.3: Temporal regulation of sox2 in brain regeneration a,b) Peak of sox2 observed at 3dpi in the qPCR and RT-PCR data c) Higher protein expression of sox2 at 3dpi in comparison to UC

3.4 Concentration-dependent increase in the number of proliferative cells due to the stabilisation of β-catenin in brain regeneration

Wnt signalling is known to perform various roles in developmental and regenerative processes. Its main role in the regenerative mechanism is to activate pro-proliferative genes involved in regeneration. Wnt signalling works via two pathways: a) canonical (β-catenin dependent) signalling pathway and b) non-canonical(β-catenin independent) signalling pathway. It can adopt any of the above pathways in the regenerative process. It was observed that in the injured brain as compared to the control brain, the level of the β-catenin was significantly low, via western blotting(Fig 3.4.1). But the number of proliferating cells are seen to be increased in the injured brain as compared to the control at 3dpi. Therefore, it can be concluded that the wnt signalling may be working through a non-canonical pathway which is independent of β-catenin.

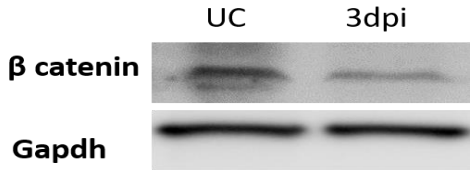
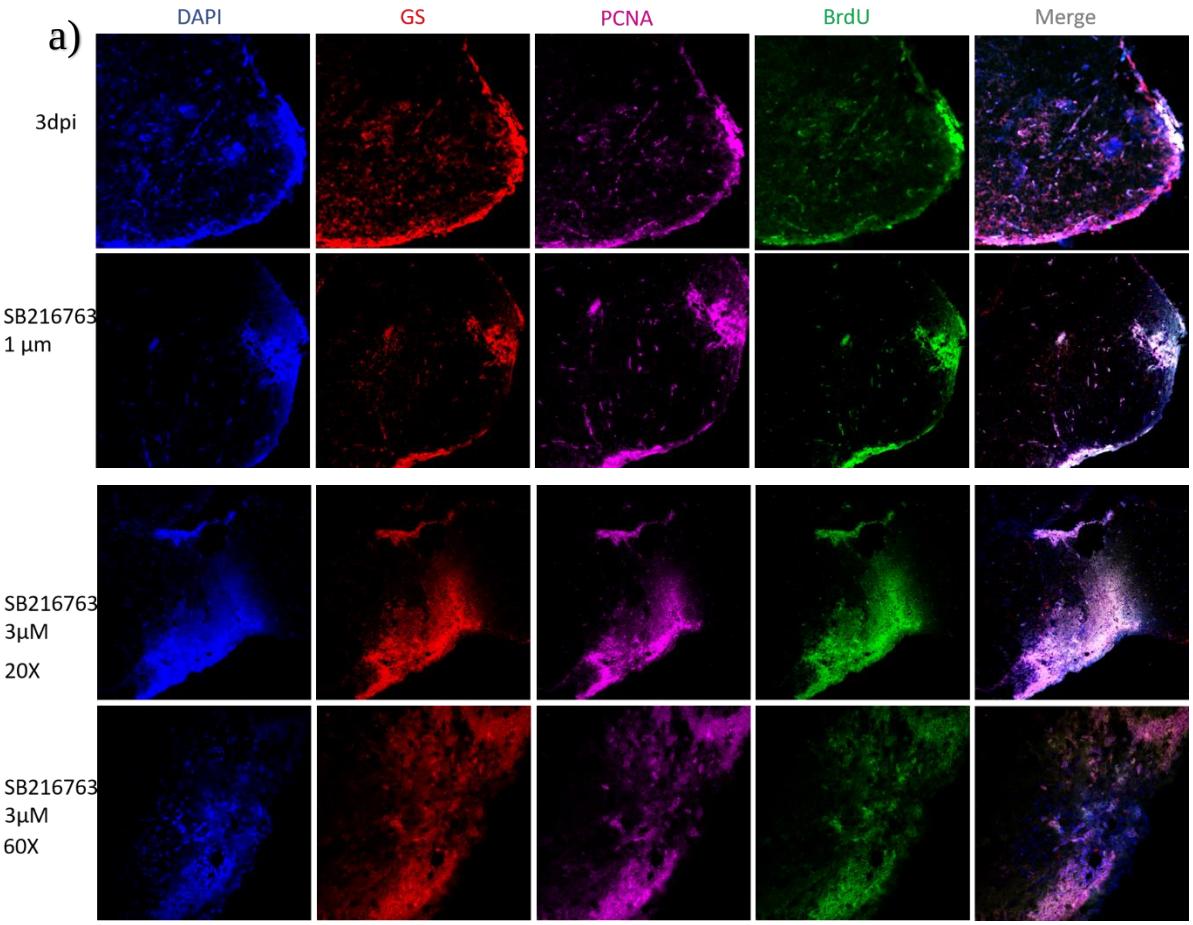


Fig 3.4.1: Significant decrease in the β-catenin protein level at 3dpi v/s UC

Therefore to confirm the fact of non-canonical signalling and checking the effect of stabilization of β-catenin, by inhibiting GSK3β by the drug SB216763 in a concentration-dependent manner (1μm,5μm), it was observed that the number of RG proliferative cells marked by using GS, BrdU/PCNA double immunostaining, has been increased significantly in a concentration-dependent manner(Fig 3.4.1 a,b). Therefore, it was concluded that the wnt signalling would be working via a non-canonical pathway and thus stabilising the β-catenin would also have triggered the canonical signalling which led to an excessive increase in the number of proliferating cells.



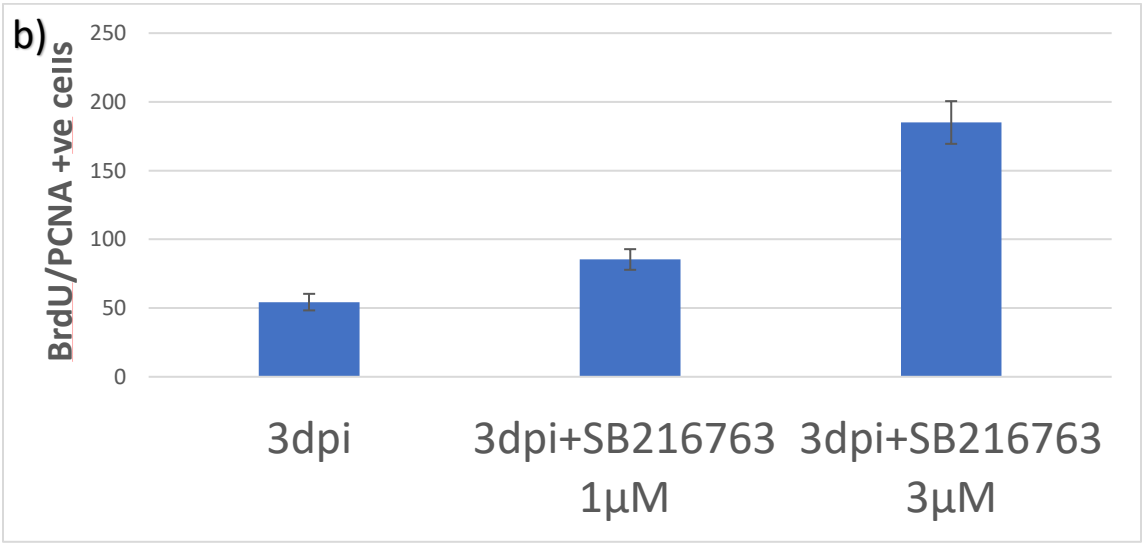


Fig 3.4.2: Concentration-dependent increase in the number of BrdU/PCNA +ve cells by inhibiting GSK3β a) Increase in BrdU/PCNA +ve cells in SB216763 treated fishes b) Graphical representation of the increase in the number of proliferating cells.

3.5 Snai2 upregulated in the proliferative phase of brain regeneration

Snai2 is responsible for the migratory action of the cells and is one of the factors responsible for EMT transition. As the stem cell niches are located at the ventricular zone, where neural progenitors proliferate and migrate to the site of injury, therefore to confirm the fact that the cells at ventricular zone are proliferating and are migrating to the site injury, the expression of the gene *snai2* was checked in the brain regeneration. It was observed that *snai2* showed a peak at 3dpi which is also a peak proliferative phase in zebrafish brain regeneration(Fig3.5). Therefore, it was concluded that cells at the ventricular zone migrate to the site of injury via EMT transition.

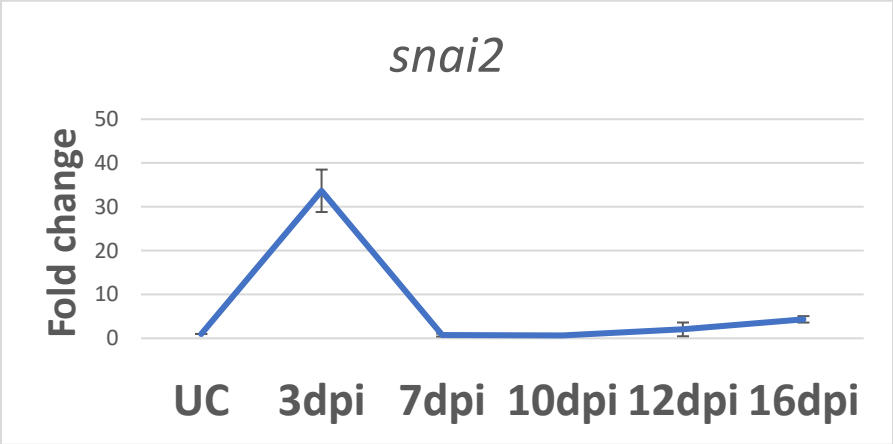


Fig 3.5: Peak of snai2 observed at 3dpi after traumatic brain injury in zebrafish

3.6 Regulation of MMP2 and MMP9 in brain regeneration

MMP's are proteases that cleave ECM, as to expose certain growth factors essential for the regenerative cells and also pave a way for migratory cells to reach the site of injury. Therefore, it is very essential to study the role of the extracellular signals and extracellular environment that are required in the course of regeneration. It was observed that both *mmp2* and *mmp9* show a peak at 3dpi, that is a peak proliferative phase(Fig 3.6 a,b). Therefore, it is safe to assume that MMP2 and MMP9 have a very essential role in the proliferative phase.

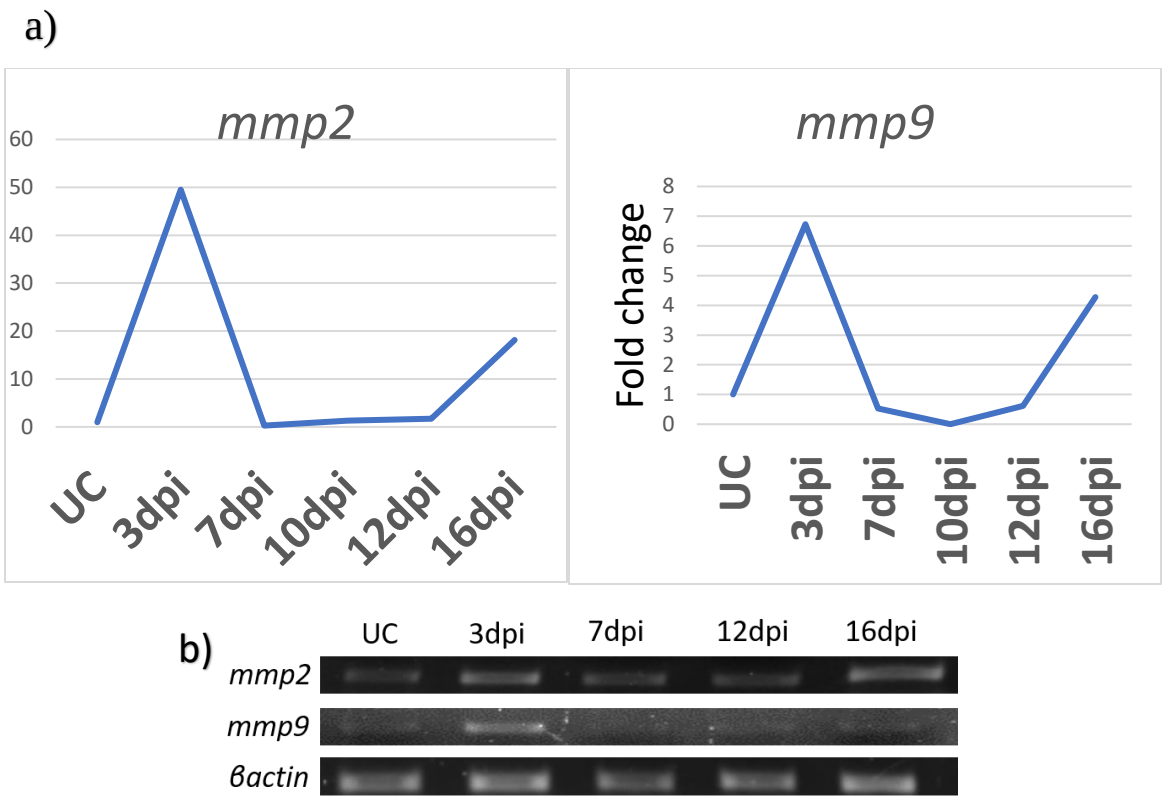
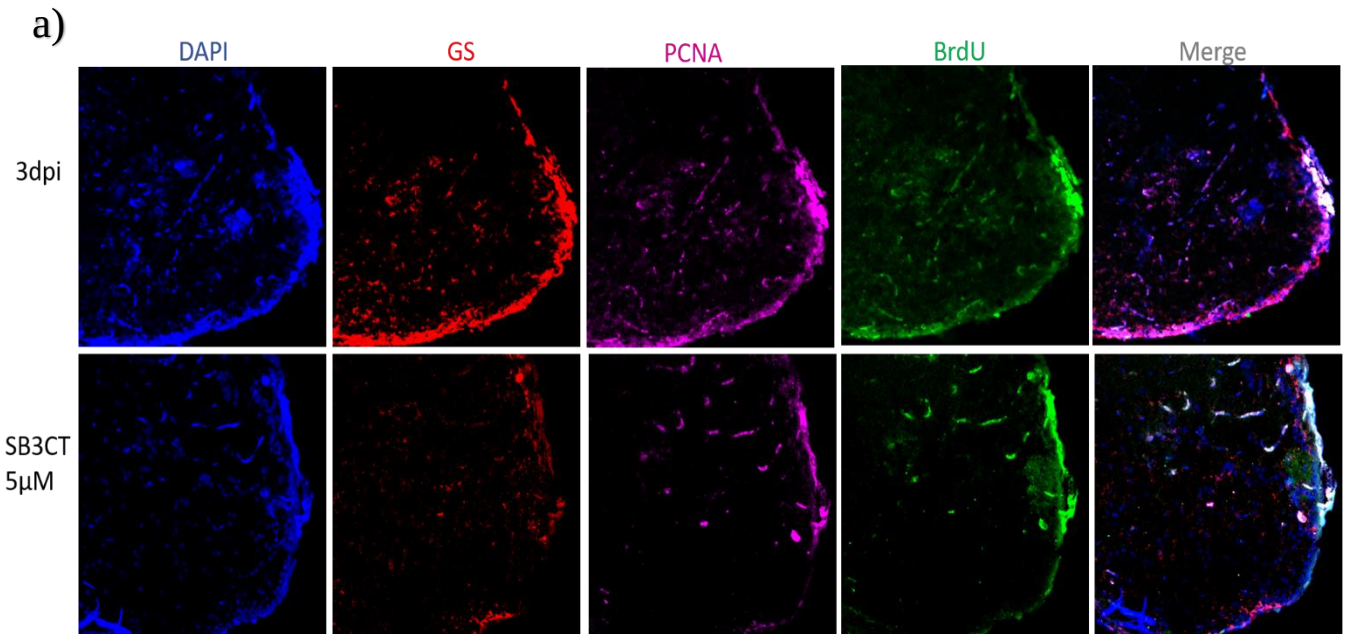


Fig 3.6: Temporal regulation of mmp2 and mmp9 in brain regeneration a) Peak of mmp2 and mmp9 observed at 3dpi in the qPCR data b) Peak of mmp2 and mmp9 observed at 3dpi in RT-PCR data

3.6.1 Combined blockade of MMP2 and MMP9 results in a significant decrease in the number of proliferative cells

MMP9 and MMP2 are very essential ECM proteases that are required in the course of the regenerative process but its role in the brain regenerative mechanism was still unknown. It was observed in a double immunostaining experiment that the proliferation of RG cells in the after the blockade of MMP2 and MMP9 via the drug SB3CT at 5μM, is significantly reduced. This concludes that extracellular signals and the extracellular environment around the cell have a very essential role to play in brain regenerative process after a traumatic brain injury. Combined blockade of MMP2 and MMP9 resulted in a significant decrease in the number of BrdU/PCNA +ve cells (Fig3.6.1).



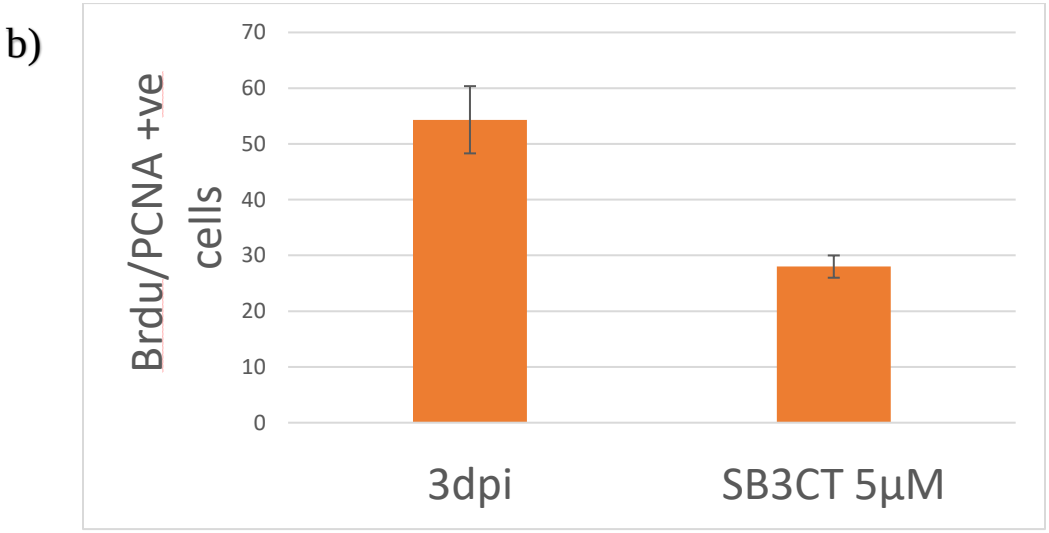
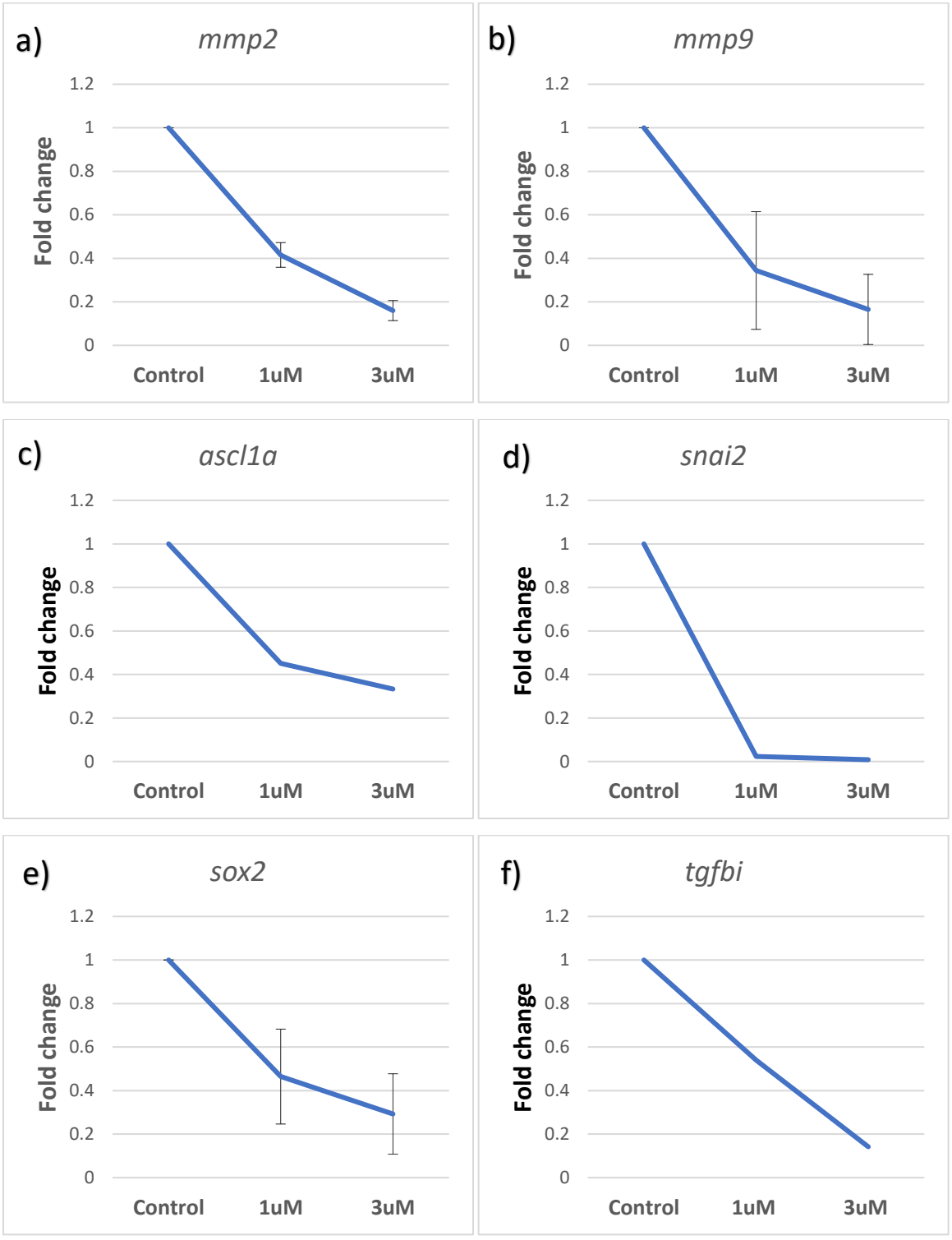


Fig 3.6.1: Decrease in the number of BrdU/PCNA +ve cells by combined blockade of MMP2 and MMP9 a) Decrease in BrdU/PCNA +ve cells in SB3CT treated fishes b) Graphical representation of the decrease in the number of proliferating cells.

3.6.2 Concentration-dependent decrease in the induction of *ascl1a, sox2, snai2* and *tgfbi* at 3dpi by the combined blockade of MMP2 and MMP9

The pro-neural transcription factor (ascl1a), pluripotency inducing factor (sox2), EMT transition factor(snai2) and pro-proliferative factor(tgfbi) are downregulated at 3dpi during the combined blockade of MMP2 and MMP9 in a concentration-dependent manner(Fig 3.6.2

c,d,e,f,g). This proves the fact that ECM factors as MMP2 and MMP9 have a very essential role in the brain regenerative process and are very essential for the induction of intracellular genes involved in the regenerative mechanism.



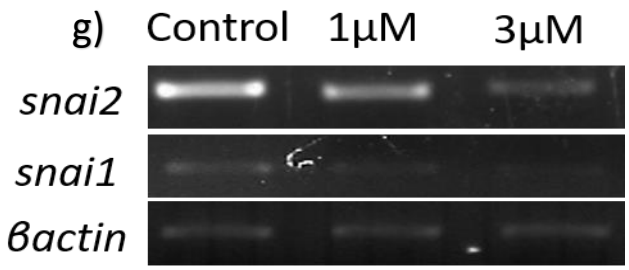


Fig 3.6.2 Concentration-dependent downregulation of intracellular genes at 3dpi involved in the brain regenerative process due to the combined blockade of mmp2 and mmp9 by SB3CT. a,b) qPCR data of concentration-dependent downregulation of the mmp2 and mmp9 at 3dpi as compared to control c) qPCR data of concentration-dependent downregulation of the pro-neural transcription factor coding gene ascl1a at 3dpi as compared to control d) qPCR data of concentration-dependent downregulation of the EMT factor coding gene snai2 at 3dpi as compared to control e) qPCR data of concentration-dependent downregulation of the pluripotency inducing factor coding gene sox2 f) qPCR data of concentration-dependent downregulation of the pro-proliferative factor tgfbi at 3dpi as compared to control. g)RT-PCR data of concentration-dependent downregulation of Snai family genes that are responsible for EMT transition at 3dpi as compared to control.

Conclusion

In the study of understanding molecular mechanisms underlying zebrafish brain regeneration, we have observed the role of HDAC, wnt/ β-catenin pathway, EMT factor as snai2, genes and proteins involved in the proliferative phase as ascl1a, sox2, lin28 and also the factors of ECM as MMP2 and MMP9. It was seen that the colocalization of the HDAC with the BrdU +ve cells in the 3dpi fishes as compared to the control fish brain, meant to play a significant role for the proliferation of RG cells to occur. Then the pro-neural factor ascl1a, reprogramming factor lin28 and pluripotency inducing factor was found to be upregulated during the peak proliferative phase i.e 3dpi, which signifies the induction of the developmental genes in the brain repair mechanism that are responsible for the induction of proliferation in the RG cells.EMT transition factor snai2 is found to be upregulated at 3dpi which signifies the migration of the proliferating cells to the injury site from the ventricular zone. Then it was seen that β-catenin is downregulated at 3dpi as compared to the control brain but upon the inhibition of GSK3β, a tremendous increase in the number of the proliferative cells are seen in a concentration-dependent member as compared to control 3dpi brain. This result gives a clue that wnt signalling may be acting through a non-canonical pathway and hence increasing the level of β-catenin produces excessive proliferation. Also the ECM proteases MMP2 and MMP9 are found to be upregulated at 3dpi and the combined blockade of both results in the significant decrease in the number of proliferative cells. Also, it was seen that combined blockade of MMP2 and MMP9 resulted in concentration-dependent downregulation of ascl1a,sox2,snai2 and tgfbi , from which we can conclude that extracellular signals through MMP2 and MMP9 are very essential for the intracellular gene to induce a proliferative phase in the regenerative mechanism of the brain.

FUTURE PERSPECTIVES

The study done is the preliminary insights into the molecular mechanisms underlying zebrafish brain regeneration. The future experiments that are to be done are devising a method for morpholino injection in the zebrafish brain and checking the effect of β-catenin knockdown on proliferation after TBI. Morpholino knockdown of ascl1a and checking the expression pattern of lin28 after TBI and vice versa. Doing a ChIP assay to find out the potential targets of mmp2 and mmp9 that are responsible to induce intracellular signalling. In-situ mRNA hybridization and FISH to check the localization of mmp2 and mmp9 in the brain region. Effect of Dexamethasone (Immunosuppressive agent) on MMPs in zebrafish brain after TBI and also checking the effect of regeneration in chronic v/s acute inflammation. Another interesting experiment to be done is to find out the effect of injury-induced different cell death types in the course of brain regeneration i.e difference in apoptosis and necrotic injury-induced cell death on the brain regenerative mechanism.

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PRIMER LIST

Table1: List of Reverse transcription primers used

Mentioned sequences are in 5'-3' direction

ascl1a RT Fwd	ATCTCCCAAAACTACTCTAATGACATGAACTCTAT
ascl1a RT Rev	CAAGCGAGTGCTGATATTTTTAAGTTTCCTTTTAC
sox2 RT Fwd	GAAAAACAGCCCGUACCGCATCAAGAGACC
sox2 RT Rev	GTCTTGGTCTTCCTCCGGCGTCTCTATGTCS
lin28a RT Fwd	TAACGTGCGGATGGGCTTCGGATTTCTGTC
lin28a RT Rev	ATTGGGTCCTCCACAGTTGAAGCATCGATC
tgfbi RT Fwd	CGCTGACCTCAACAAACTCATGAGAG
tgfbi RT Rev	TGGTCACTCACAATTTTAGGAGGCAG
mmp9 RT Fwd	GGAGAAAACTTCTGGAGACTTG
mmp9 RT Rev	CACTGAAGAGAAACGGTTTCC
snai1a RT Fwd	AGCTGGAATGTCAGAACGACACTTC
snai1a RT Rev	GTCTGACGTCCGTCCTTCATCTTC
snai2 RT Fwd	AGCACGTATTCGGGACTCATGAAGC
snai2 RT Rev	CAGGAAAACGGTTTCTCACCCGTG
β-actin RT Fwd	GCAGAAGGAGATCACATCCCTGGC
β-actin RT Rev	CATTGCCGTCACCTTCACCGTTC