Role of E2-2 in Brain Functions

B.S – M.S Thesis

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Under the guidance of
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Certificate

This is to certify that this dissertation entitled “Role of E2-2 in Brain functions” towards the partial fulfilment of the B.S - M.S dual degree programme at the Indian Institute of Science Education and Research, Pune represents the research carried out by Athira.D.P at NCBS Bengaluru under the supervision of Dr. Hiyaa.S.Ghosh, Assistant Professor, NCBS Bengaluru during the academic year 2016-2017.

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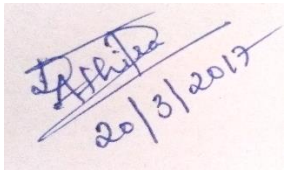

Date: 20 March 2017

Declaration

I hereby declare that the matter embodied in the report entitled “E2-2 in brain functions” are the results of the investigations carried out by me in, NCBS, Bengaluru under the supervision of Dr. Hiyaa.S.Ghosh and the same has not been submitted elsewhere for any other degree.

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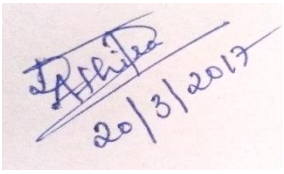


Abstract

E2-2 is a basic helix loop helix (bHLH) transcription factor that is classified under the family of E proteins. E2-2 is the only E protein that is continually expressed throughout the adult mouse brain. Earlier reports have established the importance of E2-2 in regulation of cellular morphology and cell fate maintenance of plasmacytoid dendritic cell (pDC), a myeloid cell-type of the immune system. Microglia are resident immune cells in the brain, and have a myeloid origin. Apart from their essential roles in immune protection against any infection or damage, importance of microglia in brain development, maintenance and homeostasis has recently been recognized. Our lab seeks to elucidate the molecular regulators that enable such diverse functionality of microglia under homeostasis and activation context. To this end, the lab is investigating the role of E2-2 in the regulation of microglia. Previous studies show that E2-2 mediated gene regulation can influence specific cellular properties that can be relevant to microglial biology. Furthermore, E2-2 is shown to be affected in multiple psychiatric disorders, some of which are also thought to be influenced by microglial dysregulation. The aim of the ongoing project in the lab is to gain a better understanding of the molecular regulation that underlie microglial function in the healthy brain and during pathologies. My project is part of the above mentioned study, where I have established primary microglia culture for *in vitro* studies. Alongside, I have also worked to standardize a laser microdissection protocol for isolating neural stem/progenitor cells from the hippocampal neurogenic region, the sub-granular zone (SGZ), in the adult brain.

Table of Contents

List of Figures

Acknowledgements

First and foremost, I thank Dr. Hiyaa Ghosh for her encouragement throughout the project tenure. She boosted up my morale and kept me motivated during multiple contamination eras. I am also very grateful to Vinaya and Rajit, for their immense help, be it teaching protocols, finding literature, frequent discussions or keeping things organized. I also thank all my lab members for their willingness to help, for all the meaningful discussions and mostly, for being curious and interested in my project. I would also like to thank the whole NCBS community for creating a friendly and vibrant environment as well as for their willingness to help whenever there was a necessity.

I express my immense love and thanks to my parents, Umbidi and Akshay for being on my side all throughout the journey.

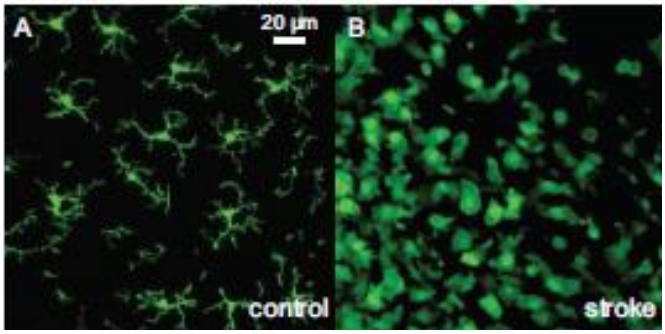
Chapter – 1

Introduction

Microglia

Microglia are unique cells in the central nervous system (CNS) that are critical for immune defence as well as homeostatic maintenance of the CNS. Microglia were first characterised by Rio Hortega towards the beginning of 20th century. Since then, the longstanding debate about the origins of microglia has been resolved only recently with lineage tracing studies establishing that microglia originate from the primitive yolk sac precursors. At the early embryonic stage, i.e. from day 9.5-10.5 in mice, CNS is colonized by microglia **(**Merad et al., 2010**)**. Microglia, under homeostatic conditions, have small cell body and long processes, whereas when they become activated they obtain an amoeboid morphology with relatively large cell body and shortened processes. Microglia has been shown to have functions like synaptic pruning during the course of development and in adult central nervous system (CNS) it has immune functions and maintenance of neurons. In conditions of trauma or injury, microglia are observed to become active and surround the injured neurons (Loane et al.,2010). Activated microglia secrete a multitude of noxious cytokines, chemokines, and oxygen radicals, and perform neuroprotective functions (Ransohoff and Perry et al., 2009). During steady-state microglia keep surveying their environment with theirs processes and contribute to maintaining the homeostatic conditions (Nimmerjan et al., 2005) Microglia activation is reported in a range of circumstances such as infection, injury and aging. Often microglia activation is accompanied with microgliosis (figure 1.1 a), a process whereby an accumulation and proliferation of microglia occurs at the affected site (Fischer et al., 2004).

a)



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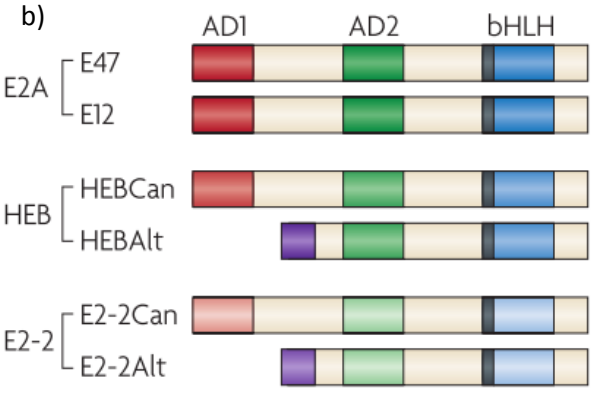


Figure 1.1 a) Microglia during resting phase (control) and during activated phase (Stroke) (Li,zhang et al.,2015) (b) E-proteins and their splice variants (Kee et al.,2009)

E proteins

E proteins are ubiquitously expressed class-1 bHLH transcription factors. They bind to the Ephrussi box (E-box) sequence CANNTG and hence the name E-proteins. E-box is a DNA response element found in some eukaryotes which acts as a protein-binding site and has been identified to regulate gene expression in muscles, neurons and other tissues. E-proteins function either by forming homo-dimers or hetero-dimers with other Helix-Loop-Helix (HLH) proteins. Class-1/2 bHLH family of transcription factors are highly conserved from invertebrates to humans, and are expressed in a wide range of tissues. E proteins are known to have regulatory effects on diverse biological processes like myogenesis, neurogenesis, B and T cell development (Engel et al.,2001) . E proteins include E12 and E47 (encoded by a single gene, E2A), HEB and E2-2 (Figure 1.1b). The activity of E proteins are known to be antagonised by the Id proteins (Id1-Id4) which converts them to non-functional heterodimers.

E2-2

E2-2 is a basic helix loop helix (bHLH) transcription factor which belongs to family of E proteins. Haplo-insufficiency of E2-2 causes Pitt Hopkin's Syndrome, a genetic disorder. Mutations in E2-2 is identified as a risk factor for schizophrenia through genome wide association studies (Steffanson et el.,2009). In mice E2-2 is expressed in the adult brain, including the Sub Granular Zone (SGZ) and Sub Ventricular Zone (SVZ), which are the two neurogenic regions of the adult brain (Flora et al., 2007). Daughterless (Da) in drosophila is the orthologue of E-protein in drosophila. It's been also shown that expressing human E2-2(TCF4) can rescue loss of function in Da mutant phenotypes in Drosophila. Both Da and E2-2 are shown to have similar functions in drosophila and mice respectively. They both function to restrict neurite branching and synaptic formation (Rozzario et al, 2016). Apart from the brain, E2-2 is most highly expressed in an immune cell type of myeloid origin called plasmocytoid dentritic cells (pDCs), where it functions as a master regulator for pDC development and maintenance (Cisse et al.,2008 ; Ghosh et al., 2010). Microglia

being of myeloid origin, we hypothesise that E2-2 has a role to play in microglial cells.

E2-2 in microglial homeostasis and function

The aim of our study is to explore the potential role of E2-2 in microglial homeostasis and function. Unpublished data from the lab shows that E2-2 is abundantly and continually expressed in the adult mammalian brain, however, little is known about its function. Also previous studies of E2-2 in PDCs suggest that E2-2 could potentially be relevant in the context of the dynamic structural and functional manifestation of microglia. Preliminary data from our lab shows that E2-2 is expressed in microglial cells (Vinaya.S, unpublished data). Given this, we hypothesized a role for E2-2 in microglial maintenance and/or function. To this end, we aimed to establish primary microglial culture to examine if E2-2 could influence microglial survival, proliferation and function.

E2-2 in adult neurogenesis

The preliminary data from our lab suggest a role for E2-2 in adult neurogenesis. Adult neurogenesis, a process by which new neurons are formed in the adult mammalian brain has been implicated in cognitive function. Adult neurogenesis takes place in two specific locations; the SVZ of the lateral ventricles and the SGZ in *dentate gyrus* (Figure 1.2) in the hippocampal formation. Data from the lab suggest that E2-2 plays a critical role in the hippocampal adult neurogenesis and influences specific learning and memory, such as pattern separation. In order to understand the targets of E2-2 during hippocampal adult neurogenesis, we aim to micro-dissect the hippocampal neurogenic region, the sub-granular zone of the dentate gyrus. SGZ is a two-three cell layer thin regions on the hilus side of the densely packed granule cell layer of the dentate, which is difficult to micro-dissect through conventional dissection methods. Therefore, we aimed to standardize Laser Capture Microdissection (LCM) for isolating SGZ from adult murine brain.

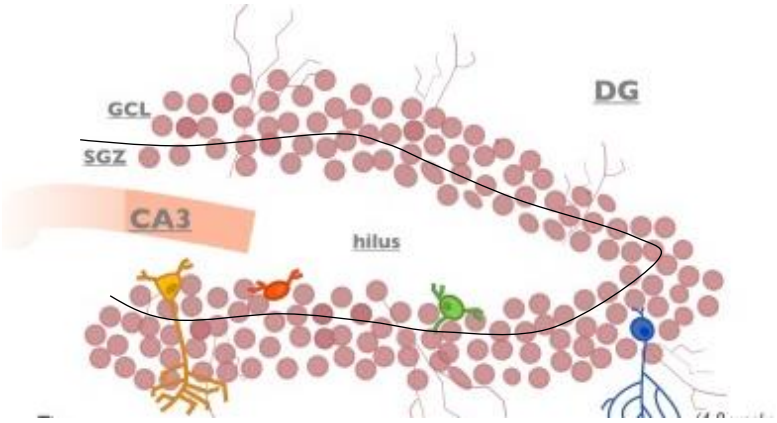


Figure 1.2 The SGZ layer in the *Dentate* g*yrus. (*Patricia et al.,2013) The black line marked along the DG shows the separation of SGZ from the glomerular cell layer (GCL) of the DG.

Objectives and Approach

To study *invitro* microglia in the aspect of proliferation and activation, firstly we scanned through a few available protocols and decided to use “A new technique to isolate primary microglia” (Moussad et al., 2010). This was simple as well as very similar to another protocol which our lab was already following for FACS technique for the isolation of microglial cells. The protocol was successfully tested with the help of Vinaya.S, Hiyaa Ghosh lab. The same was followed for all the cultures which were put up later, for various experiments.

To address the neurogenesis part, I have used the Laser Microdissection Microscope facility – Zeiss LCM scope at the CIFF facility, NCBS, Bengaluru.

Chapter – 2

Materials and Methods

Solutions

For microglia culture

DMEM-F12+ Glutamax(Gibco)

Hanks Balanced Salt Solution (HBSS) 10X (Gibco)

Fetal Bovine Serum (FBS) (Gibco)

Percoll (GE Healthcare)

Papain (Fluka)

GM-CSF (R&D Systems)

Enzyme solution – NaCl, KCl, NaHCO3, NaH2PO4, CaCl2, MgSO4, EDTA, Glucose, Cysteine

4% Paraformaldehyde Solution (To fix cells)

2 gm Paraformaldehyde in 50ml PBS

Heat solution to 60-65º C and stir till the paraformaldehyde dissolves.

For LCM

Polyethylene Naphthalate (PEN) slides

RNA Zap

DEPC water

LCM Blades

Adhesive caps

Blocking Solution (For IF)

10 % Normal Goat Serum(Gibco)

1% BSA(Sigma)

0.1% Triton(Sigma)

100 mM Glycine(Fischer)

Make up the volume with PBS

Antibody Solution(For IF)

1% Normal Goat Serum

0.1% BSA(Sigma)

0.1% Triton(Sigma)

100mM Glycine (Fischer)

Make up the volume with PBS

Antibodies(For IF)

Primary Antibodies:

1. Rabbit Anti - E2-2 Antibody, Dilution 1:2000
2. Rat Anti – GFAP Monoclonal Antibody (Invitrogen), Dilution 1:500
3. Guinea pig Anti – Iba 1 Antibody (SySy), Dilution 1:500

Secondary Antibodies:

1. Goat anti – rabbit IgG Alexa Fluor 568
2. Goat anti – guinea pig IgG Alexa Fluor 488
3. Goat anti – rat Alexa Fluor 647

RIPA Buffer (For western)

5mL 1M Tris pH 7.4 (Fischer)

5mL Triton X-100 (Sigma)

5mL 0.5M EDTA (Fischer)

75mL 1M Nacl (Fischer)

0.5g Deoxycolate (Sigma)

make volume upto 500mL

Others

Polystyrene with vented/filter cap 25mm tissue culture flasks, 75 mm tissue culture flasks(nulcon),24 well culture plates(genetix),frosted micro slides 75mm,24 mm (# 1.5) and 18 mm (# 1.5) circular coverslips (Bluestar), Fluoromount(southern biotech),Surgical instrument box(hospito),Stripettes(Corning),Cell strainers(genetix),Glutamax, DMEM-F12+ Acrylamide and Bisacrylamide (Sigma-Aldrich), TEMED (N,N,N’,N’-tetramethylethylenediamine, APS (Ammonium persulphate, Sigma Aldrich), HEPES, PET slides(Kindly provided by CIFF,NCBS), D-Glucose, HI FBS (Heat inactivated fetal bovine serum),1X Trypsin, 10X Trypsin (Sigma-Aldrich), 0.22 µm syringe filters (Merk), Insulin syringes , Syringes (20,50 and 10 ml)

Methods

Cre-Lox System

Cre-Lox is the tool that we are using to selectively delete E2-2 from microglia.

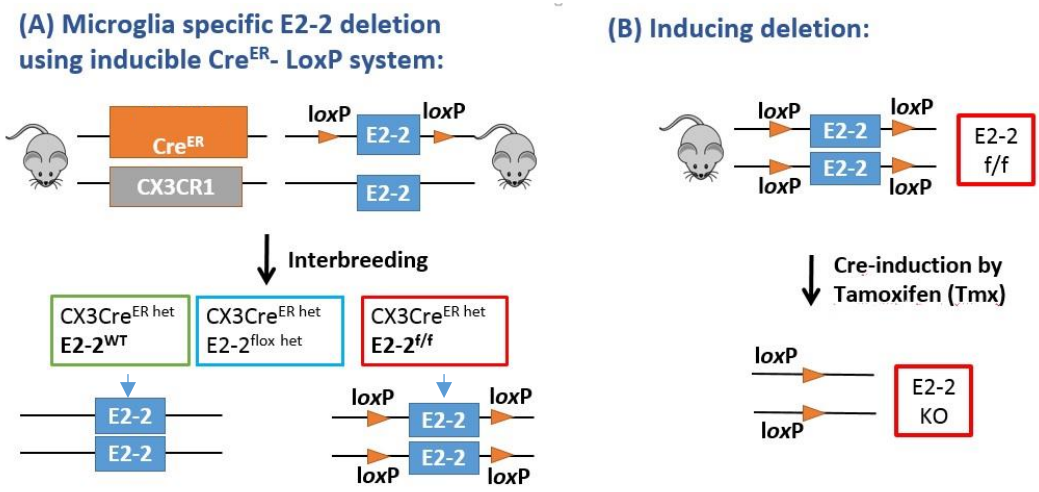


Figure 2.1 : How the Cre-Lox tool works : a) CreER/CX3CR1 animals are interbreed with E2-2 f/+ animals. Litter will be CreER/Cx3CR1; E2-2 +/+ (wildtype) or CreER/Cx3CR1; E2-2$^{f/f}$ (knock-out). These animals are administered tamoxifen to induce Cre-recombinase, which results in the deletion of E2-2 gene in case of E2-2$^{fl/fl}$. b) E2-2 deletion in CreER/Cx3CR1; E2-2$^{f/f}$ (KO) by the administration of tamoxifen.

The Cre recombinase with modified estrogen receptor (Cre ^{ER}) is under a CX3CR1 promoter which is specific to microglial cells. E2-2 locus is flanked by the loxP sites. When Cre-recombinase is activated by tamoxifen administration, the Cre recognizes the loxP sites inducing deletion of the flanked gene in a temporal and tissue specific manner (Figure 2.1). Tamoxifen was administered consecutively for four days (5mg/35gm) and the animal was sacrificed and cultured one month post first tamoxefin dose.

Whole animal perfusion and brain removal (for microglia culture)

1. Before beginning with the surgery, ensure that the tubing is free of air bubbles by running chilled PBS through the perfusion apparatus.

2. Prior to surgery administer a ketamine/xylaxine mixture via intraperitoneal injection (100 mg/kg body weight for Ketamine and 5-16 mg/kg body weight for Xylazine)

3. Once animal is fully sedated, confirm as no movement in response to toe-pinch, pin its forelimbs and hindlimbs to the surface for easy access to the body.

4. Make an incision through the integument just beneath the rib cage.

5. Carefully separate the liver from the diaphragm.

6. Make a small incision in the diaphragm to expose the pleural cavity

7. Place the scissors along one side of the ribs, carefully displacing the lungs and make a cut through the rib cage up to the collar bone. Make a similar cut on the contralateral side.

8. Lifting the sternum away, carefully trim any tissue connecting it to the heart.

9. Clamp the tip of the sternum with the haemostat and place the haemostat over the head, exposing the heart and all major vessels.

10. Pierce the left ventricle with a perfusion needle.

11. Immediately make an incision to the right atrium without damaging the descending aorta.

12. Switch on the perfusion apparatus and let chilled PBS+heparin(2U/mL) circulate through the mouse. Continue this until the liver looks visibly clear.

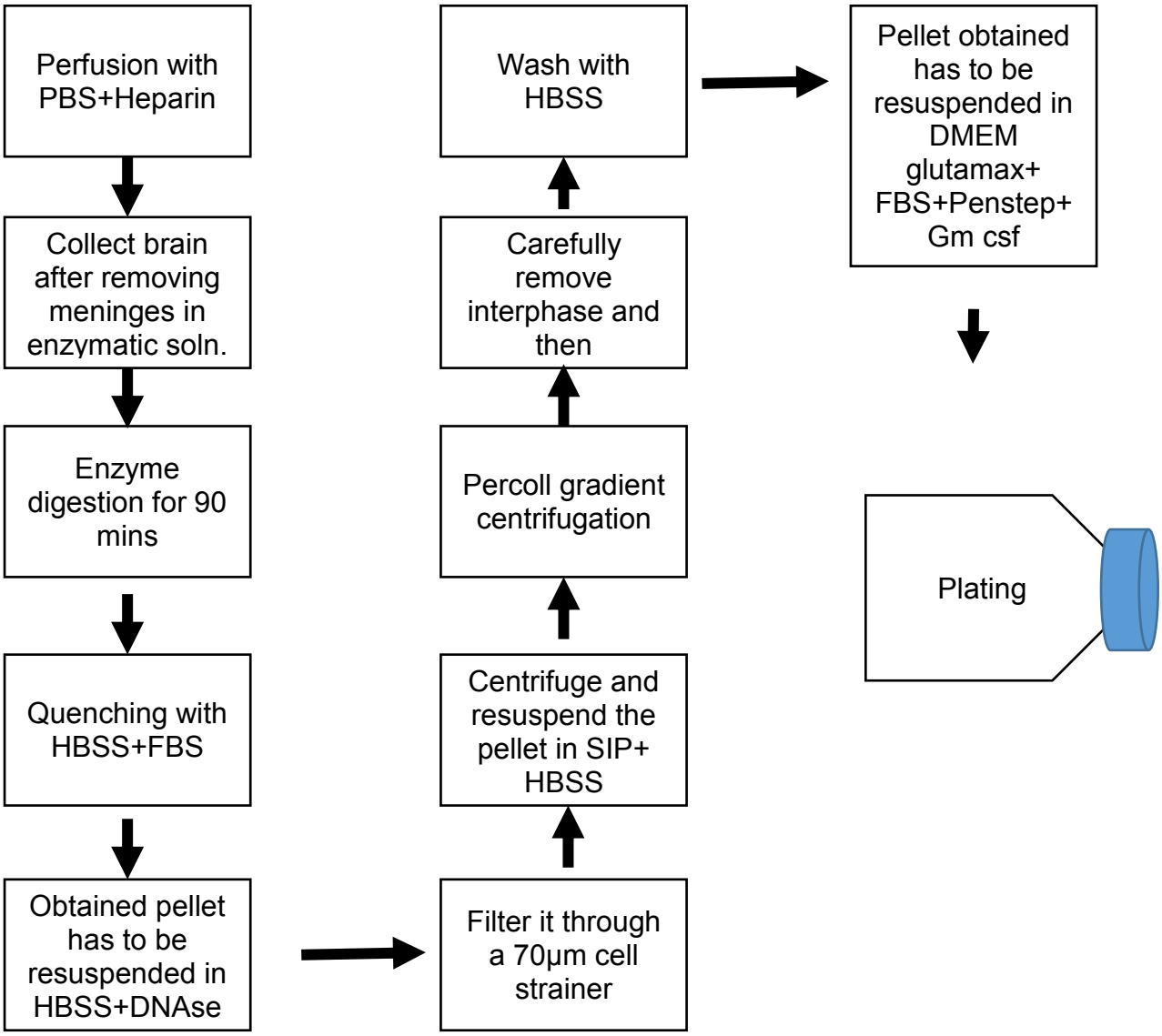
13. Close the outlet valve and remove needle from the heart.

14. Cut the neck of the mouse such that the brain is not disturbed.

15. Make a cut starting from base of the inter-parietal bone to the tip of the frontal bone in the skull and similar cuts on the sides. Remove the skull bones and expose the brain.

16. With a thin spatula, scoop out the brain gently and transfer it to the enzymatic solution

Microglia primary culture



Laser Micro Dissection

This protocol was standardised during the course of project for SGZ in adult mouse brain and is adapted from Zeiss LCM manual and Zhang et al., 2009.

Dissect the brain out from the mouse. Give it a PBS wash. Make sure that the PBS is chilled. This can be kept in an ice bucket till you start further procedures. Keep the cryo-sectioning chamber at -21ºC.The Brain should be embedded in OCT before sectioning. This should be kept on the mounting plate (figure 2.2). Freeze it so that

the OCT hardens, either in the -21ºC chamber or -80 ºC (till it hardens enough). For our experiment we keep it in -21ºC for at least half an hour.

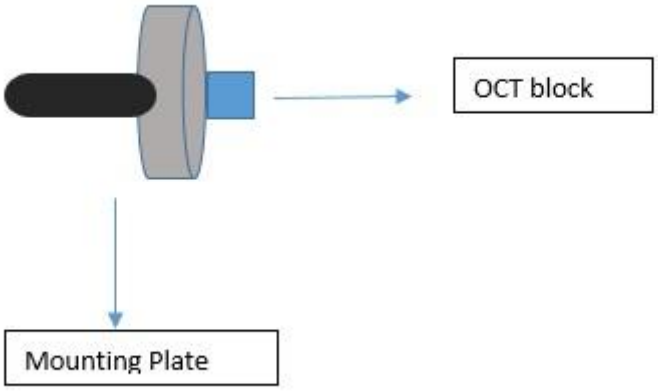


Figure 2.2: Brain mounted on a mounting plate of cryotome

Once the OCT block is hardened enough on the plate, start taking the cryo-sections in the range 10µm – 20µm. These sections can be directly mounted on to the PEN slides that were baked at 180º C for 4 hrs to make them sterile. The slides can be kept inside the chamber until the next section is taken. The slides have to be stained and dehydrated. Staining and dehydrating tissues helps in catapulting them better. Staining should be done in 4% cresyl violet solution.

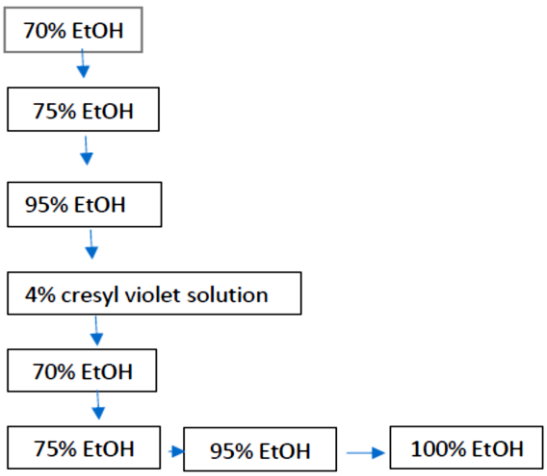


Figure 2.3 Steps for dehydration and staining of tissue (30 seconds each step)

Air dry the slides and keep them on the stage for imaging and taking sections. In the software, for 10µm sections, the energy point for the laser should be in the range of 65-74 and for 20µm this should be a little more. Focus of the laser can also be changed. This works the best in the range of 79-85. Every time you move to a new section slightly standardise these values for that section by marking and cutting a region outside our area of interest. Depending on the size of the sections the energy for catapulting can be changed.

Things to be taken care of:

1) The sections should not have traces of OCT as retained OCT in the slide makes it difficult to cut and catapult the sections.
2) Be as quick as possible while taking sections as when tissues are completely dried off, there was difficulty in cutting
3) Standardisation is required for every section as there will be heterogeneity in tissue and accordingly the values has to be changed.
4) Keep the things very very clean.
5) Always do cap-check to ensure that you have the ROI in the cap

RNA Extraction

- Pellet down the cells/ tissue at 200G for 5min.
- Re-suspend the cells in 1ml of trizol (see to it that no clumps are visible)
- Add 200 µl of chloroform, mix vigorously and spin at 12000g for 15 min. at 4C
- Collect only the upper colourless aqueous layer and transfer into a new tube. (the white particles in the interface is DNA and is to be avoided)
- Add equal volume of isopropanol and incubate in is for 5 min on ice.
- Spin at 12000 g for 5 min at 4C.
- remove the supernatant (A small white pellet must form at the bottom)
- Add 75 % ethanol and re-suspend gently.
- Spin at 12000 g for 5 min at 4C.
- Discard the supernatant and air dry (~10 min)
- Add RNase free water according to the pellet size (generally 10-20 µl).

- Check for RNA quantity and quality by *nano* drop reading.
- Immediately use for cDNA prep (RT PCR) or Store at -20C (do not freeze thaw. multiple times)

RT PCR

- The following is for 1 reaction.

Step 1: add the following

- 1ul of random primers:(1:15dilution of 1ug/µl stock from Sunil Lab)
- 1ul of 10uM dNTP
- 2ug of RNA (try to get at least 1ug)
- Make up 10ul with Nuclease free water. the reaction mix can go upto 13µl

Place the tube in PCR machine:

- 65C ---> 5min
- 4C ----> hold

Meanwhile make master mix of:

- 4ul 5X buffer
- 1ul of 0.1M DTT
- 1ul of Superscript-III
- 3/4 µl of NF water

Add the above mix to the reaction mix (total volume 20ul) and proceed to RT reaction:

- 25C ---> 5 min
- 50C ---> 45 min
- 70C ---> 15min
- 4C ----> hold

Store this cDNA at -20C and use by diluting according to need.

qPCR

- Reaction mix: 0.5 µl of primer mix + 5ul SYBR Green +0.5-1 µg of diluted cDNA + NF water make up 10uL
- Make the reaction and follow the steps given below for the PCR programme:

Step 1

- 95 ---> 10 min

Step 2 (45 cycles)

- 95C ---> 15sec
- 60C ---> 15 sec
- 72C ---> 10 sec

Step 3 (melting/ optional)

- 95C ---> 1min
- 55C ---> 30sec
- 95C ---> 30 sec

Check for the beta-Actin value which must be less than 20

Protein Extraction

- Add 2 volumes of RIPA buffer (with Protease inhibitor) to the cell pellet
- Homogenize with syringe (Insulin syringe)
- Sonication 3min 10sec on 10 sec off pulse(36% amplitude)
- Centrifugation at 12500 rpm
- Collect the supernatant
- Concentration estimation in *nano* drop

Bradford Assay

- Bradford stock: 5x

  Make 1x working solution by diluting it with distilled water.
- Prepare protein standards in buffer ranging from 0.1-1.4 mg/ml using BSA
- Add 5ul of protein standards to separate wells in 96 well plate, keep one blank (only buffer)
- Prepare unknown sample with an appx. con b/w 0.1-1.4 mg/ml
- To each well add 250 µl of Bradford reagent
- Take the reading in a multi-well plate reader spectrophotometer

Intensity analysis and quantification

Confocal images were taken using LSM 780 microscope and were analysed using ImageJ software.

All the graphs were plotted using Graphad-Prism5. Unpaired t-test was used to check the significance.

Chapter-03

Results

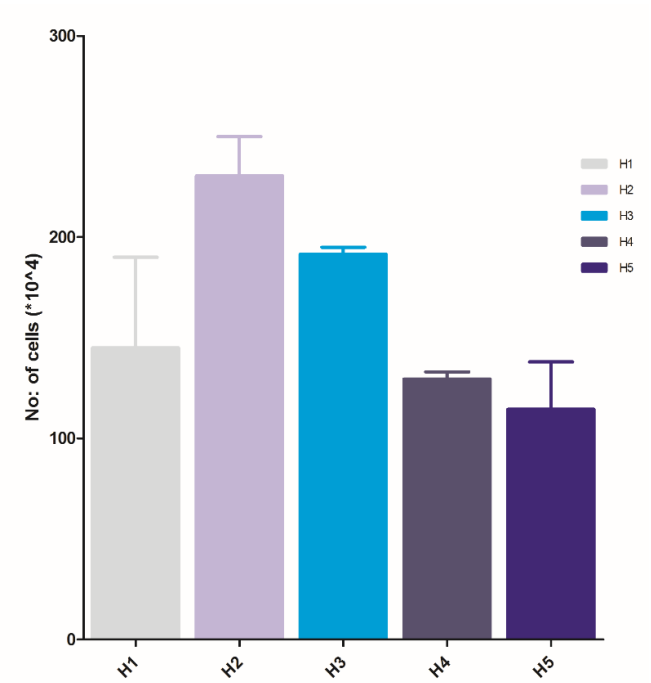
1) Establishment of primary microglia culture

First the standardization of culture with respect to yield and purity had to be done. The additional step of removing the meninges was added to (Moussad et al.,2010) as it will help to remove the macrophages present in the blood brain barrier. The microglia primary culture is a floating culture and therefore, the harvests can directly be collected along with the media. Later the cells can be pelleted down. These pellets were stored in -80°C after flash freezing. The cultures were continuously monitored. After seeding, once the cells starts to attach, half the media is changed and after a week media is changed again. In 2 weeks the adhered cell layer will become more than 95% confluent if supplemented with Gm-csf (for non Gm-csf cultures it usually takes 2.5-3 weeks) and from then the harvests are collected.

The questions that were addressed:

1) - cell yield, viability and purity of culture
   - confirm E2-2 expression and dynamics

a)



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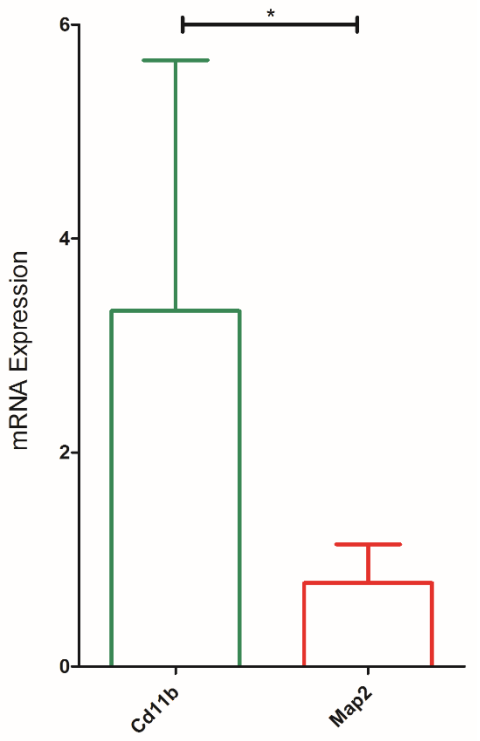

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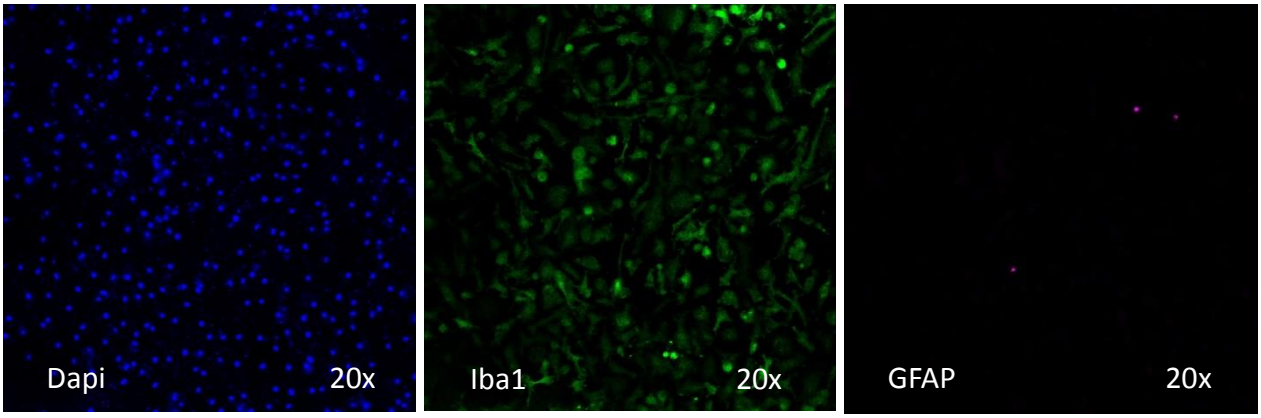


Figure 3.1: a) Shows the compiled cell number from two-6month old mice b) qPCR data compiled from WT cultures. Cd11b is a microglia marker, Map2 marks neurons. c) Immunofluorescence data from Harvest-3 of 6m old WT mouse Dapi marks the nucleus, Iba1 marks microglia and GFAP marks the astrocytes.

In order to figure out the harvest from which the maximum number of microglial cells can be obtained with highest purity, first set of cultures were established. For every harvest, the cell count was taken using a haemocytometer**.** The values obtained were plotted using Graph pad Prism (Figure 3.1a). Only viable cells which appear as bright circles in the haemocytometer was counted. Almost 100 percent of the obtained cells were viable across all the age groups. To make sure that the cells obtained were microglia, IF and qPCR (Figure 3.1b,c) analysis were also done.

2) Effect of Granulocyte macrophage colony stimulating factor (Gm- csf) in E2-2 expression

Colony stimulating factors (CSFs) are lineage specific growth factors for hematopoietic cells in the bone marrow (Clark and Kaman et al., 1987). But introducing CSFs have shown to increase the yield in *invitro* cultures and in some cases they are even shown to be essential for proliferation and survival. Gm-csf is a growth factor that is known to increase proliferation of microglial cells. CSFs are found to function as growth factors and immune regulators of human foetal microglial cultures (Lee et al., 1992, 1993). They are also established to promote proliferation, survival and effector function of *in-vitro* rat and mouse microglia (Suzumura et al., 1990). Gm-csf has been reported to alter microglial immune properties and morphology. It transitions microglia into a dendritic cell (DC) like phenotype (Nilufer et al.,2007).

As we know from literature that Gm-csf can induce increased proliferation of microglial cells, we thought of looking at the E2-2 expression of the same in comparison with cultures which didn't had Gm-csf in it. If at all E2-2 had to play a role in proliferation, there will be an increased expression of E2-2 in the cultures. To address this 6 cultures were established from 3month, 6month and 12 month old mice. Each age was a set which had two cultures each – one supplemented with Gm-csf and the other lacking Gm-csf. Now, first and foremost it had to be checked if there is an increase in yield in the cultures with Gm-csf. This was estimated via taking the cell counts from the harvests (Figure 3.2 a). Also a fold change of E2-2 was estimated for the respective cultures using qPCR(3.2 b)..

a) b)

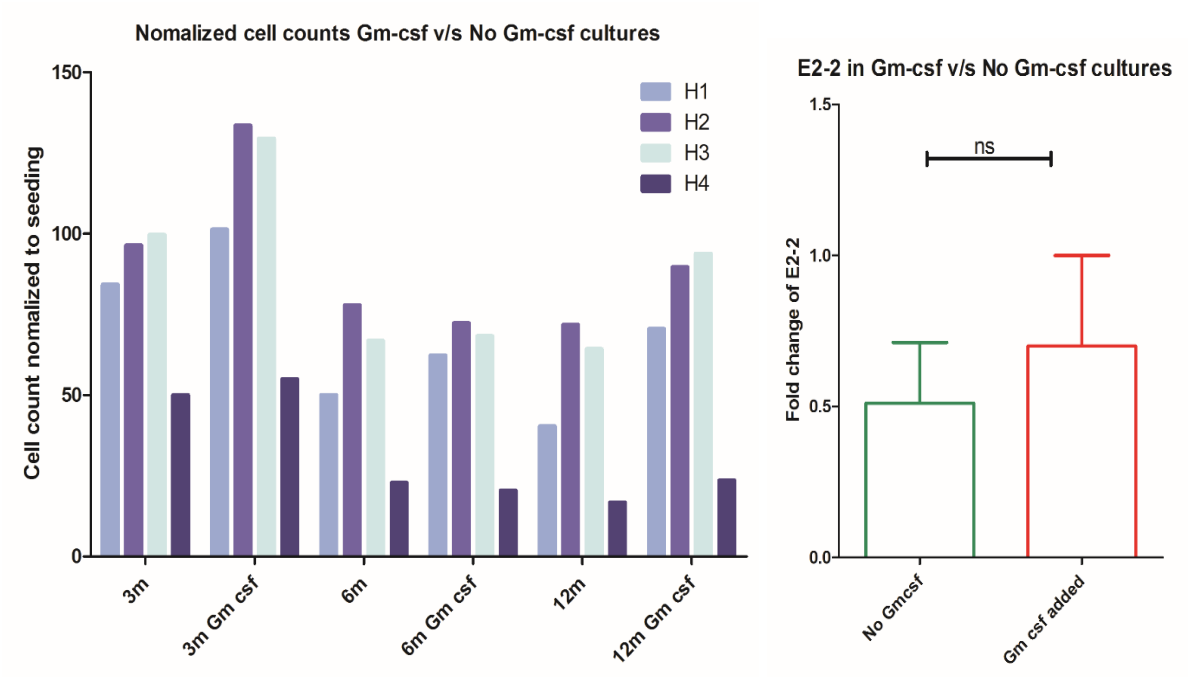


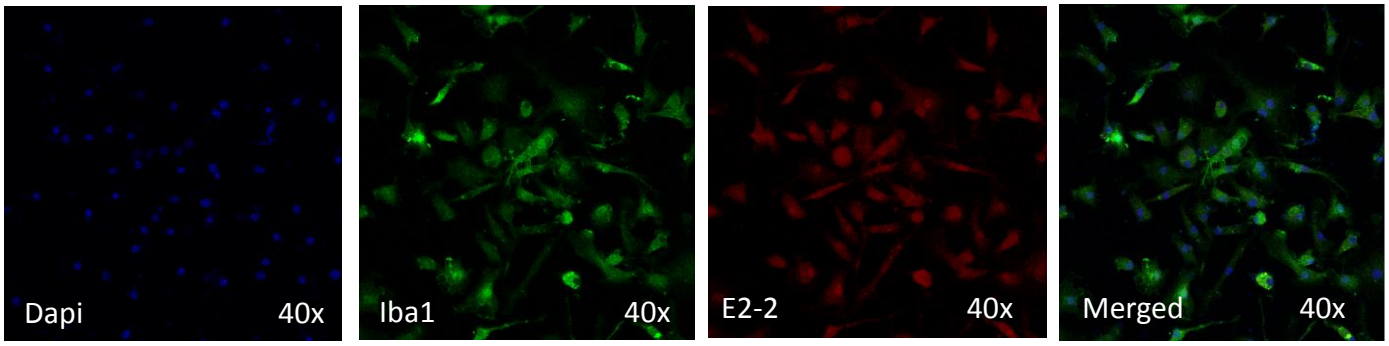
Figure 3.2 a)Normalized yield obtained from Gm-csf –no Gm-csf set b) qPCR data compiled from 6m and 12m old Gm-csf – no Gm- csf cultures

3) Role of E2-2 in microglia proliferation

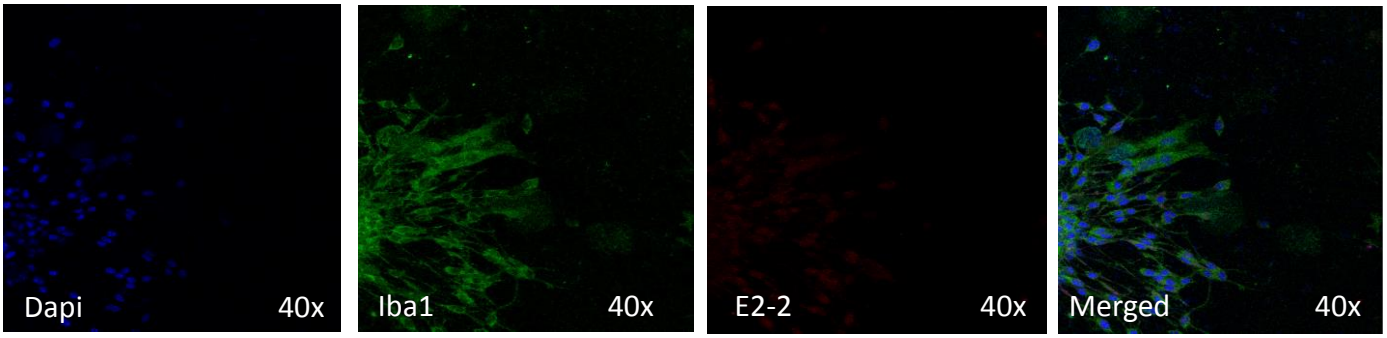
Second goal was to confirm E2-2 deletion in *in-vitro* cultures as that was important to figure out the role E2-2 in microglia. To achieve this 3 new sets of microglial culture was put up. Each set comprised of 2 cultures, one wildtype (WT) and the other knock-out (KO). Harvest 3 from all the sets were seeded, fixed and imaged in LSM

780 confocal microscope (Figure 3.3a). These images were used for intensity analysis. Intensity of E2-2 from 3month old and 6month old cultures were measured using ImageJ. The same was analysed and plotted using Graph pad Prism (Figure3.3b). The same harvest pellet was used for qPCR analysis to confirm the deletion of E2-2 (Figure 3.3 c).

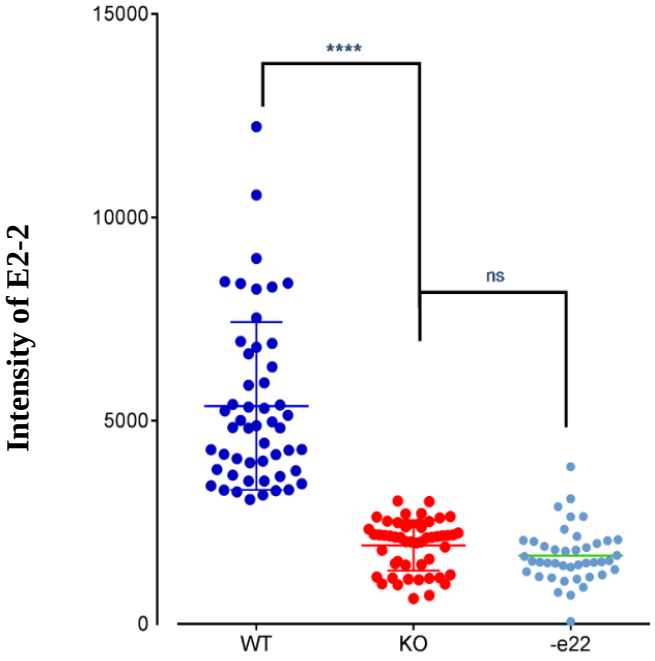
a) WT



KO



b)



c)

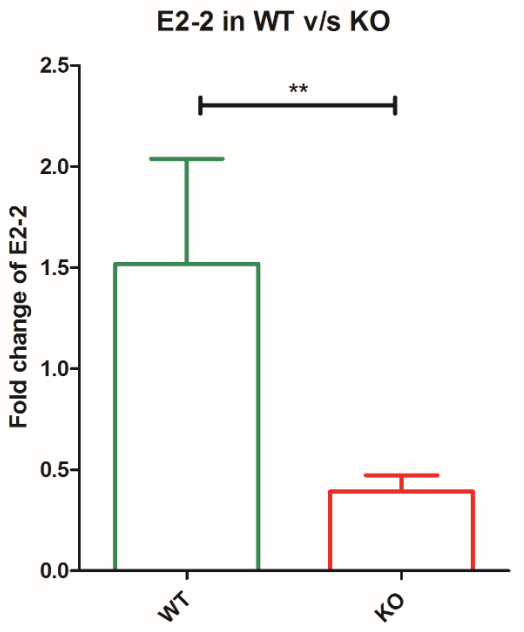


Figure 3.3 : Confirming E2-2 deletion in *in-vitro* cultures a) IF images of microglial cultures belonging to 6month old mice, both are images of harvest 3. b) Intensity analysis for E2-2 from the IF images. This is compiled data from four mice 6m,3m harvest 3.c) the qPCR data for the respective set.

The cell counts from the same set was taken and analysed to see if there is any change in yield, to examine effects on cell proliferation/survival (Figure 3.4).

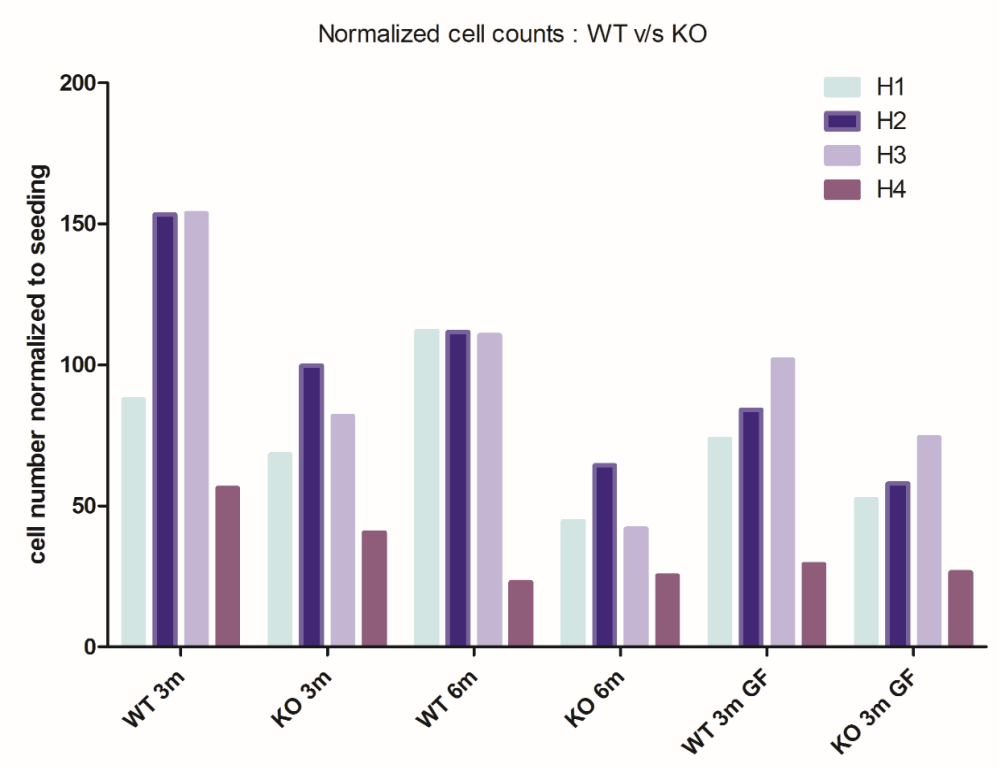
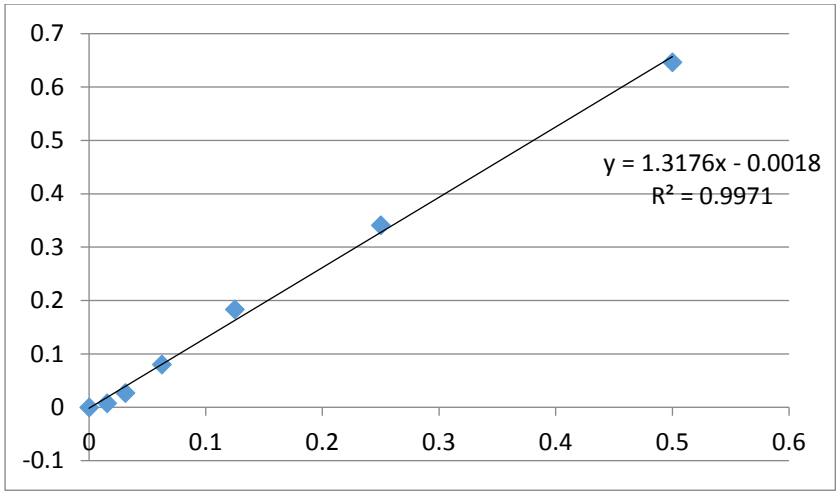


Figure 3.4 Normalized yield obtained from harvests in KO-WT set

4) Protein extraction and Western blot standardisation of the cultured cells

Protein extraction and western blotting are required to look into the details about levels of E2-2 in the cultured cells. Bradford standard curve was plotted after taking the absorbance values of the BSA standard made (Figure 3.5a)

a)



b)

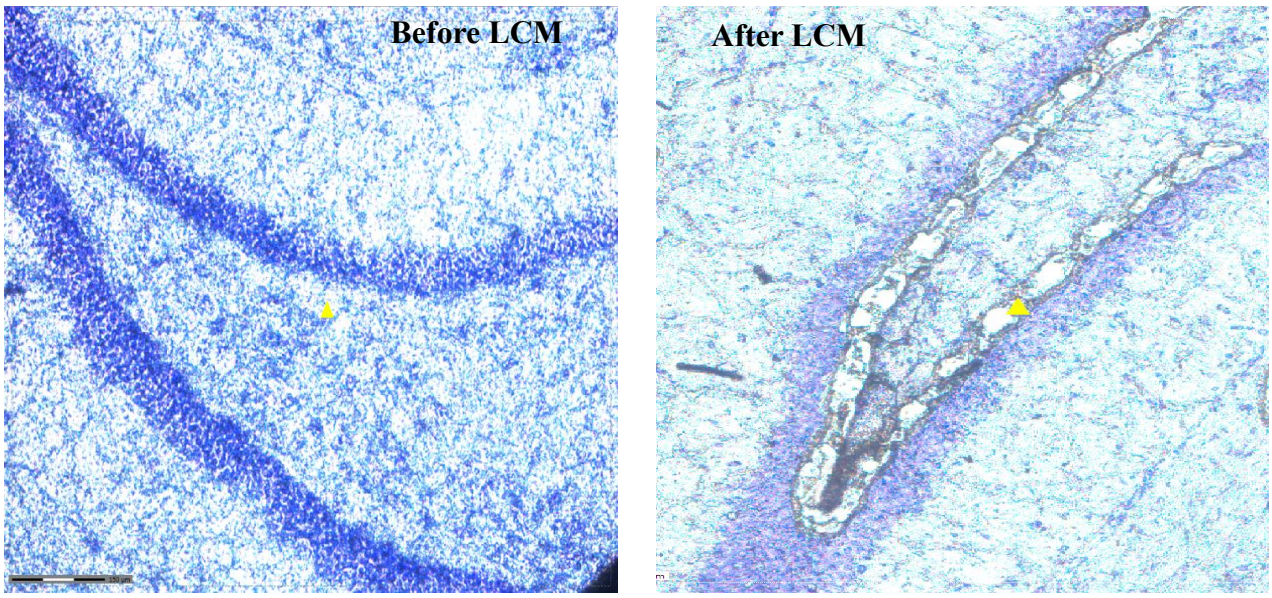
	V1	V2	Average	Sample-Buffer	x=(y+0.001)/1.317	mg/ml	to get 50ug
Buffer	**0.5062**	**0.5137**	**0.50995**	**0**	**0.000759301**	**0.015186**	
microglia	**0.9087**	**0.9703**	**0.9395**	**0.42955**	**0.326917236**	**6.538345**	**7.64719545**

Figure 3.5 a) BSA standard curve. b) Table with the concentration of protein obtained

5) Standardisation LCM and checking the quality of obtained tissue

Laser Capture micro-dissection and catapulting (LCM) is a powerful tool which can be used to micro-dissect tissue and even single cell. The advantage of this technique is that it allows us to study tissue/cell specific properties. The technique was initially standardised according to our tissue. Parameters like delta (difference between cutting and lifting energy), energy and focus were tweaked according to the heterogeneity in tissue. Step by step, the quality of tissue after dehydrated-staining and LCM were checked and the quality of RNA obtained in both cases was almost similar. Further, SGZ was dissected out (Figure: 3.6a). qPCR analysis was used to check the Ascl1 expression, which is a bHLH transcription factor expressed in Neural Progenitor cells(NPCs), in the neurogenic (SGZ) region and compared with the mature neuron layer of *dentate gyrus* (Figure 3.6b).

a)



b)

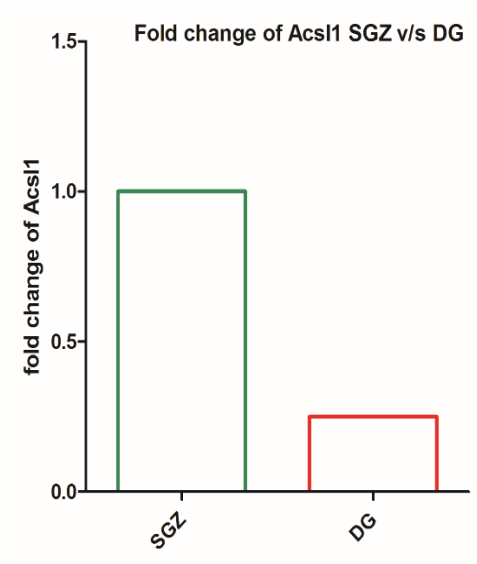


Figure 3.6 a) LCM images of dissecting out SGZ b) qPCR data showing difference in the the fold change of Acsl1 in SGZ and DG

Chapter-04

Discussion and future directions

Through this study I have firstly tried to establish the *invitro* part of culturing microglia as it helps us in achieving a lot of cells which can be used for induction assays to activate certain receptors like TLRs and thereby to study the role of E2-2 in microglia. The preliminary *invivo* data from our lab suggests the presence of E2-2 in microglia and differential expression of E2-2 in mice grown under different pathological conditions (Vinaya.S, unpublished data). To further confirm this we are trying to analyse the same on primary microglial cultures as well. And from the cultures that were grown now we know about the best harvests to be used for analysis, with respect to their yield and purity. The preliminary data of this study shows that harvests 2 and harvest 3 are mostly the best. Also the purity of the cells obtained is almost 100 percentage from the IF data, as there were no GFAP positive cells and all DAPI positive were Iba1 positive too. The qPCR data shows that the culture is free from neuronal contamination as well. From the cell viability test using trypan blue, almost 100 percent viability was observed from almost all the cultures irrespective of age, gender and pathogen free environments.

Gm-csf - no Gm-csf cultures were also put up to see if a growth factor is showing effects on E2-2 expression. But from our observations, we couldn't see a significant difference between the yield obtained from the cultures with and without Gm-csf. The qPCR data also didn't show a significant change in the expression of E2-2 between the cultures supplemented with Gm-csf and the cultures which had no Gm-csf. When 3 month old cultures without Gm-csf (n=2) and 3 month old cultures (n=3) were compiled together, the cell counts obtained from both looked almost similar (Figure 4.1). Hence, this part of the result has to be further verified by increasing the number of animals. Western blot analysis of all the sets will further be a confirmation of the above results and would help in proper quantification of the protein levels. For this, steps till protein extraction has been standardised. Because E2-2 is a nuclear protein a couple of buffers were tried for the efficient extraction including RIPA, and RIPA appeared to work the best for E2-2 extraction. Also E2-2 intensity analysis from the Gm-csf no Gm-csf cultures will help us to get a better picture of E2-2 expression in the growth factor supplemented cultures.

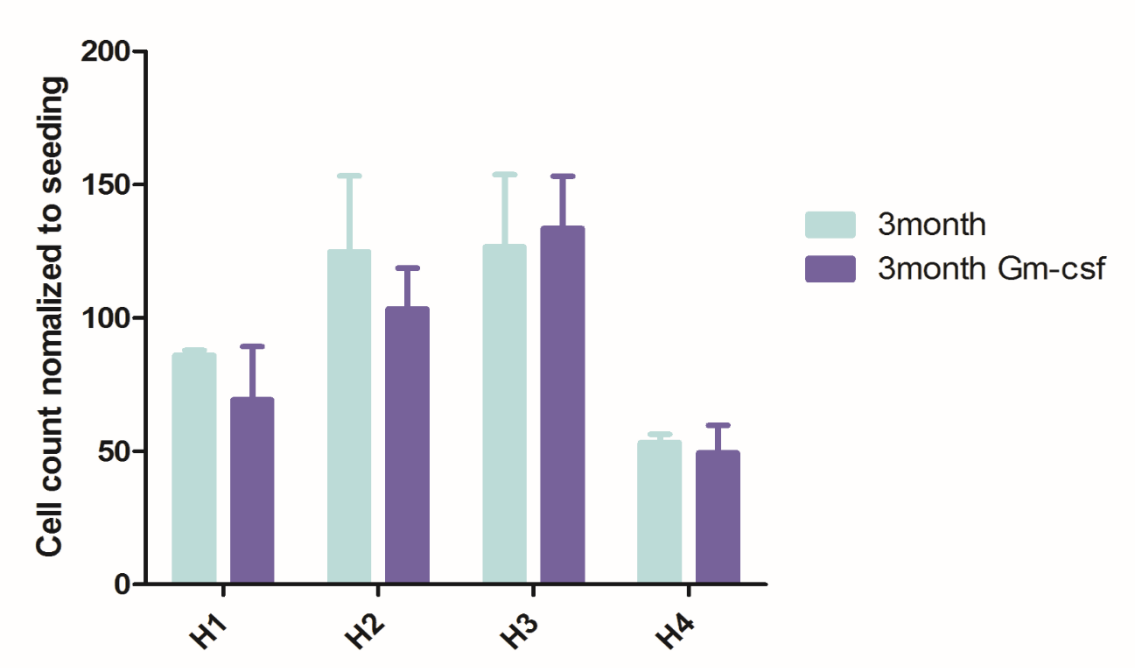


Figure 4.1 Compiled cell counts from Harvests of 3month old cultures with and without gm-csf

The third part of the study was to confirm the E2-2 deletion in the knock-out mice via *invitro* cultures. The data obtained from IF was further analysed with a control (where primary antibody was not added). The analysis showed that E2-2 is deleted in the cultured microglial cells from the KO mice. The qPCR analysis on the same set was also done to confirm the same. Together these suggest that E2-2 may modestly influence microglial proliferation *in vitro*. Because when we compare WT and KO, the yield of microglial cells in WT was more in comparison to KO. This might be because in the WT mice cultures as E2-2 is present, it is assisting proliferation and helping to increase the microglial yield. Similarly in KO as there is no E2-2 present, proliferation is slower. But here one thing that we didn't knew was that, is it that the proliferation rate which is different in WT and KO set or is it that more number of cells are dying in KO than in WT. From the cell viability test using Trypan blue, almost 100 percent viability was observed in both the cultures, suggesting that the proliferation is more.

Standardization of LCM for extracting SGZ from adult mouse brain was performed. The standardization part for parameters like energy, delta and focus are achieved for the respective experiment. These are the necessary elements for a successful Laser

Micro-dissection and catapulting. The quality of the RNA extracted after LCM was checked using trizol extraction of RNA and qPCR. The qPCR data supports the presence of more NSCs in SGZ than in DG. Further, SGZ from KO and WT mouse will be collected using LCM technique and RNA-seq will be done on the collected tissue to know the transcriptional targets of E2-2.

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