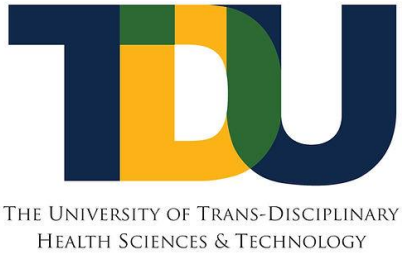
INVESTIGATING THE ROLE OF TRANSCRIPTION FACTOR 4 (TCF4) IN ADULT NEURAL STEM CELLS

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY



FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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UNDER THE GUIDANCE OF

DR. HIYAA S. GHOSH

NATIONAL CENTRE FOR BIOLOGICAL SCIENCES

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013

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DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "Investigating role of Transcription Factor 4 (TCF4) in adult neural stem cells," submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr. Hiyaa S. Ghosh, at National Centre for Biological Sciences- TIFR, Bengaluru. I also wish to inform you that no part of the research has been submitted for a degree or examination at any university. References, help, and material obtained from other sources have been duly acknowledged.

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Place: Bengaluru Signature of the Candidate

Date: 03.09.2023

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CERTIFICATE

This is to certify that the work in this thesis, "Investigating role of transcription factor 4 (TCF4) in adult neural stem cells" submitted by Mr. Mohammad Shariq was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help, and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

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List of acronyms (in alphabetical order)

bHLH	basic-helix-loop-helix
CIS	Chronic immobilization stress
CNS	Central nervous system
CSF	Cerebrospinal Fluid
DEG	Differentially expressed genes
DG	Dentate Gyrus
DIV	Day *in vitro*
ECM	Extracellular matrix
EGFP	enhanced GFP
GC	granule cells
GFAP	glial-fibrillary acidic protein
GO	Gene ontology
icKO	inducible-conditional Knock Out
ID	Intellectual disability
IPC	Intermediate proliferating progenitors
IVC	individually ventilated cages
MBP	myelin-basic protein
ND	Neurodegenerative disease
NLR	Novel location recognition
NPC	neural progenitor cells
NSC	Neural stem cells
OHT	hydroxy-tamoxifen
PCR	Polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PFA	Para formaldehyde
PTHS	Pitt Hopkins syndrome
RGL	Radial glia like cell
RMS	rostral migratory stream
SGZ	Sub granular zone
Shh	Sonic hedgehog
SVZ	Sub ventricular zone
TF	Transcription factor
TMX	Tamoxifen
WT	Wild Type

Synopsis

The phenomenon of production of new neurons in adulthood is called adult neurogenesis. The brain was considered a post-mitotic organ for a long time[1] until Gopal Das and Altman discovered proliferating cells in adult rat brains in the 1960s[2–4]. Further, studies confirmed the functional integration of these adult-born neurons and their association with learning[5]. In 1998, a study showed the first evidence of adult neurogenesis in the human brain[6]. Later, subsequent studies supported the existence of adult neurogenesis in human[7–11], but recent findings created a controversies regarding existence of adult neurogenesis in human[12–15]. Soon after, this controversy was counteracted by number of studies which showed robust evidence of persistent adult neurogenesis in human[16–19]. These discrepancies raised important technical questions, regarding human brain sample sensitivity towards method of preservation and histological detection. Subtle variations in preservation can lead to variability of results in studying adult neurogenesis in human brain.

Adult neural stem cells (NSC) reside in specific locations in the adult brain called neurogenic niches. The adult mouse brain has two major neurogenic niches: the sub-ventricular zone (SVZ)[20] and the sub granular zone (SGZ)[21–23]. In SVZ, the neural progenitors are positioned on the sub-ventricular wall lining. These NSC give rise to neuroblasts which migrate to the olfactory bulb via the rostral migratory stream, where they finally differentiate[24] into inhibitory neurons and participate in the olfactory circuit[25]. SGZ is present within the dentate gyrus in the hippocampus. Hippocampal neurogenesis in SGZ is conserved across mammalian species[22,26–32] and found in the adult human brain[6–8,10,11,16–18,] . In adult hippocampal neurogenesis, the adult NSC give rise to newborn excitatory granule neurons, which ultimately integrate and contribute to the functioning of the hippocampal circuitry[34–36]. Adult hippocampal neurogenesis is vital for learning memory[37,38], mood regulation[30], and has been implicated in pattern separation[31], and stress resilience[39].

Adult neurogenesis is sensitive to intrinsic and extrinsic environmental conditions[40,41]. Hormones like corticosterone have a negative impact on adult neurogenesis[42], whereas testosterone is known to enhance neurogenesis in birds[43,44]. Other than systemic factors, adult neurogenesis is also sensitive to stress[45], neurodegeneration[19], and voluntary exercise[41]. Adverse conditions like stress, aging, and neurodegeneration reduce adult neurogenesis[19,45–48]; in contrast, enriched environmental conditions and voluntary exercise enhance the production of newborn neurons[21,41]. All these evidences suggest a dynamic molecular machinery in adult neural stem cells for active regulation of adult neurogenesis in these various contexts. However, the molecular principles for regulating adult neurogenesis during varied physiological conditions largely remain elusive.

To better understand the molecular regulation in adult neural stem cells, we investigated the role of the transcription factor Tcf4, which showed high expression in the SGZ region in preliminary data from our lab. Also, studies have shown that Tcf4 can influence super-enhancers in neural stem cells[49], and regulate NSC differentiation in SVZ[50]. The essential role

of Tcf4 in brain development has been established by multiple studies[50,51]. These studies carried out in embryonic brains and cell lines demonstrate a regulatory role of Tcf4 in neural stem cells. Data from our lab suggested that Tcf4 continues to be expressed in adult NSC, but its function in adult NSC remained unknown. Tcf4 is clinically associated with neurodevelopmental and psychiatric disorders. Haplo-insufficiency of Tcf4 causes Pitt-Hopkins syndrome (PTHS). This neurodevelopmental disorder manifests as developmental delay, intellectual disability (ID), and cognitive deficits[52]. In addition, genome-wide association studies in Schizophrenia patients have identified Tcf4 as a high-risk allele where mutations were found in the intronic regions of the Tcf4 locus[53]. To understand the significance of continual expression of Tcf4 in the adult neural stem, we adopted an inducible loss-of-function strategy to generate inducible conditional Tcf4 deletion/knock-out (icKO), specifically in adult neural stem cells.

The chapter 2 of my dissertation describes our observations regarding inducible loss-of-function of Tcf4 in adult neural stem cells in vitro[54]. The chapter 3 consists of preliminary observations regarding the physiological relevance of Tcf4-mediated regulation of adult neural stem cells during physiological stress.

List of publications

1. **Shariq, M**., Sahasrabuddhe, V., Krishna, S., Radha, S., Nruthyathi, Bellampalli, R., ... & Ghosh, H. S. (2021). Adult neural stem cells have latent inflammatory potential that is kept suppressed by Tcf4 to facilitate adult neurogenesis. *Science Advances*, *7*(21), eabf5606.

2. Sarkar, D., **Shariq, M**., Dwivedi, D., Krishnan, N., Naumann, R., Bhalla, U. S., & Ghosh, H. S. (2021). Adult brain neurons require continual expression of the schizophrenia-risk gene Tcf4 for structural and functional integrity. *Translational psychiatry*, *11*(1), 494.

Chapter 1

Introduction

Neural stem cells in adult brain

Adult neurogenesis is the process of production of functionally active neurons which integrate and contribute to the functions of existing circuitry in adult brain[55]. For a long time, it was believed that production of new neurons ceases after embryonic development and no neurons are born after birth[1]. In 1959 Sidman et.al used radioactive [H^3] thymidine labelling to study cell proliferation and migration during embryonic brain development[56] . Presence of new neuron was first reported using [H^3] thymidine labelling method in a 3-day old developing mouse brain in ependymal region[57]. Soon after that for the first time Joseph Altman and team demonstrated the presence of dividing cells in a series of findings using [H^3] thymidine labelling technique in various regions of brain which includes dentate gyrus in the hippocampus, olfactory bulb and neocortex of adult rat brains[2–4]. Unfortunately, this discovery failed to grab the attention of neuroscience community mainly because their identification of new neuron was completely based on morphological evidence, and lacked neuron-specific immuno-histochemical markers. In 1984 first functional association of new born neurons with learning were demonstrated in seasonal song birds. Studies in the last decade of 20th century using techniques like *in-vivo* BrdU labelling, retroviral tracing and *in-vitro* culturing of neural stem cell from adult brain, with confirmation of neuronal marker expression have established the presence of adult neural stem cells in adult brain of both human and rodent system[6,31,32,58]. Some recent studies challenged existence of adult neurogenesis in human[12–15], which are later counteracted by more robust evidences proving existence of persistent adult neurogenesis in adult human brain throughout life[16–19].

Adult neural stem cells (adult NSCs) are capable of self-renewal, and differentiation to form various neural cell types such as neuron, glia and oligodendrocyte. They reside in specific location in adult brain, where they divide and give rise to new born neuron or glia. Adult neurogenesis occurs in two neurogenic niches; the sub ventricular zone (SVZ) in the lateral ventricles[20] and the sub granular zone (SGZ) in the dentate gyrus (DG) of hippocampus[21,23,59]. Some studies suggest existence of adult neurogenesis in hypothalamus[60] and striatum[9,61]. The mechanism of new neuron production is different in the two major neurogenic niches. In SVZ the adult NSCs are called as type B1 cells[62–64]. Neural stem cell populations can be recognised using the marker *Nestin* [65]. *Nestin* is only expressed by NSCs that are active in the SVZ; quiescent NSCs do not[66]. To reach the olfactory bulb, the neuroblasts move along a route known as the rostral migratory stream (RMS)[24,67]. They develop into interneurons once they get to the olfactory bulb, they integrate into olfactory circuitry[25].

In SGZ, adult NSCs are known as radial glia like cells (RGLs) or type1 cells and they reside in the first 2-3 layers of dentate gyrus. RGLs express glia marker GFAP and NSC marker *Nestin*, and exhibit specific morphological features such as radial projection of a single process across the granule cell layer, branching into the molecular layer of the dentate gyrus. Unlike SVZ, in SGZ both quiescent and activated RGLs expresses *Nestin* and it is widely used as a marker for NSCs[68,69]. Upon activation, SGZ adult NSCs form intermediate proliferating progenitors (IPC) or type2 progenitors, which after a few rounds of proliferation gives rise to neuroblasts.

Neuroblast transit to form immature neurons which mature into granule cells integrating into the DG circuit [35,36] (Figure 1[70]).

Adult neurogenesis in SGZ of dentate gyrus is conserved across the mammalian species including human[16–18,26–28,71]. RGLs are not homogenous but have been reported of various subtypes, categorized by morphology and differential response to stimuli[72–74]. A study by Gebara et. al. classified RGLs into two types; type α and type β[73]. Type α cells have long primary processes projecting into the granule cell layer, whereas the type β cells have short radially projecting branched processes[73]. They also showed a difference in their proliferation capacity[73].

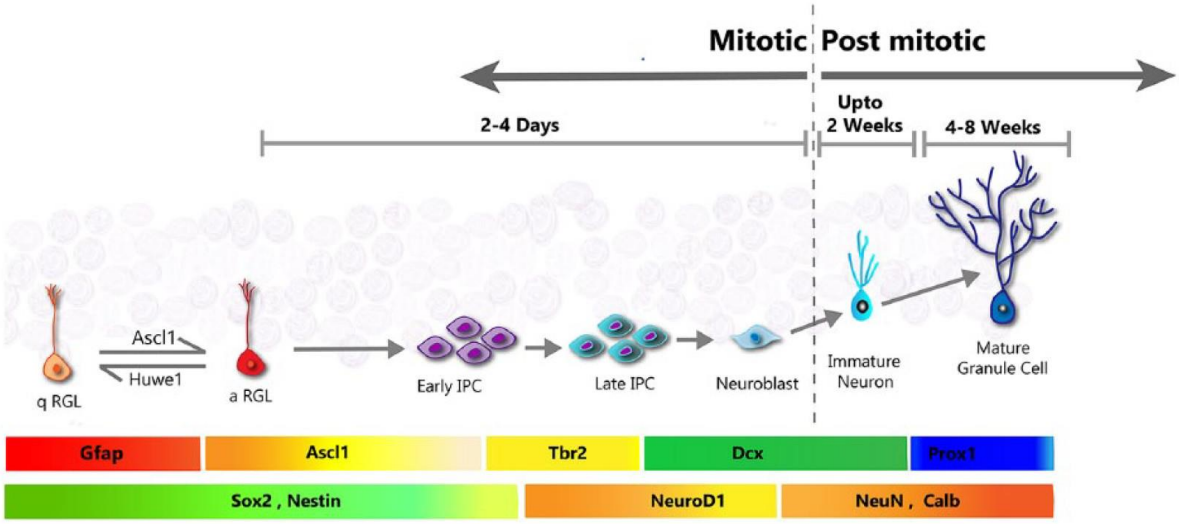


Figure 1: Overview of progression of adult hippocampal neurogenesis (Ghosh et al.2019)

The adult neural stem cells are known as radial glia like cells (RGLs). RGLs exist in quiescent and activated state. Upon activation, RGLs give rise to intermediate proliferating progenitors (IPCs). IPCs rapidly proliferate and differentiate into neuroblast. The neuroblasts subsequently develop into immature neurons, then mature neurons, and finally the mature granule neurons integrate into functioning circuits. Each stage of this progression is marked by specific markers.

Factors affecting adult neurogenesis

The process of adult neurogenesis is highly sensitive and responsive to environmental conditions[21,47,75]. It is dynamically regulated by physio-pathological conditions, cell intrinsic factors like transcriptional and epigenetic regulators, inflammation and cell-extrinsic factors like hormones[19,42,43,76–79]. The microenvironment of adult neural stem cells plays an important role in regulating the process of adult neurogenesis. This microenvironment, also called the neurogenic niche comprises of glial cells[80,81], endothelial cell[82], vasculature[82] and neuronal innervations[83]. In addition to neurogenic niche, several signaling pathways also participate in adult NSC regulation. The adult mammalian brain has limited neurogenesis, but stroke can activate a latent neurogenic program in striatal astrocytes through reduced Notch1

signaling[61]. Blocking Notch signalling triggers neurogenesis[84] in both the striatum and medial cortex, even in the absence of stroke. In the hippocampal sub granular zone, Notch1 signalling is required to maintain undifferentiated cells and ensure continuity of adult neurogenesis.[85]. Bone morphogenetic protein (BMP) signalling increases in the aged murine hippocampus, leading to decreased neural progenitor cell proliferation[86], but attenuation of BMP signalling promotes neural stem cell proliferation and subsequent neurogenesis in aged mice[87]. In the adult hippocampus, BMP signalling regulates the tempo of neural progenitor cell maturation by directing their transition between states of quiescence and activation[86]. Thus the microenvironment provides signals that regulate the maintenance, proliferation, and neuronal fate commitment of the stem cell population in the adult mammalian brain.

Cell intrinsic factors regulating adult neural stem cells

Cell intrinsic transcriptional and epigenetic regulation are important for determining the fate of adult neural stem cells. A key transcription factor, Sox2, is highly expressed in neural precursor cells, including RGLs and proliferating progenitors, and is involved in regulating the Notch and Shh signaling pathways that are crucial for maintaining the neural stem cell pool[88,89]. Recent studies have shown that Sox2 interacts with a nuclear pore protein, Nup153, to help maintain adult neural stem cell identity[77]. In addition to Sox2, other transcription factors from the bHLH family, such as Mash1(Ascl1), Olig1, Olig2, Neurog1, and Hes5, are also expressed in adult neural stem cells and play a role in regulating quiescence and proliferation[90]. Previous research on embryonic neural stem cells has revealed that oscillations in bHLH factors (Ascl1, Hes1 and Olig2) expression patterns are important for regulating proliferation, with sustained expression leading to differentiation into various neuronal cell types[91].

Physiological Stress

Stress is a physiologically aversive condition, which disrupts the homeostasis both at cellular and functional level. It can be caused by change in environmental conditions, which include both psychological and physical stress like social isolation, defeat, trauma etc.[92]. Studies have shown that stress inhibits the proliferation of neural progenitors[45–47] and effects the dendritic complexity of neurons[93], which eventually leads to behavioral deficits[94]. As a treatment for stress-related depressive conditions, antidepressants have been widely used[95]. Studies conducted in animal models show that anti-depressants prevent the reduction in hippocampal volume caused by stress, as well as promote production of new born neurons[96]. The functioning of these antidepressant drugs is directly co-related with activity of newborn neurons[97]. Recent studies demonstrate the important functions of newborn neuron in establishing stress resilience[39]. Newborn neurons inhibit the mature granule neurons responding to stress stimuli for stress resilience. Promoting the production of newborn neurons by inducible deletion of pro-apoptotic gene iBAX leads to amelioration of effects caused by stress[98–100]. Stress also disrupts the body's thermal regulation[101] and triggers

systemic inflammatory molecules like cytokines[102]. Increase in body temperature in stress is detected by thermal receptors on adult NSC, which eventually results in decreased neurogenesis[103]. Similarly, inflammation is known to be detrimental for neurogenic progression, and systemic inflammatory molecules are known to affect the survival and proliferation of neural progenitors[40,104].

Taken together, while stress disrupts adult hippocampal neurogenesis, by decreasing NSC proliferation and effecting dendritic morphology, the adult born neurons are required for stress resilience response[39]. However, how exactly stress regulates the adult neural stem cells, and mediates potential adaptive response from the adult neurogenic program for imparting stress-resilience remains to be understood.

Functional relevance of adult neurogenesis

Adult hippocampal neurogenesis contributes to the hippocampal functioning, where new-born neurons contribute to hippocampus-based learning and memory[31,105,106] . Increase in production of newborn neurons by voluntary exercise, enriched environment and genetic manipulation is known to enhance hippocampal-dependent spatial memory and object recognition[21,39,41]. A very specific function of the newborn neurons is to impart the ability of pattern separation[31,107]. Pattern separation allows an individual to differentiate between two very similar contexts and encode non-overlapping neural representation of the given context[34]. Altered adult hippocampal neurogenesis has been associated to various psychiatric diseases including depression and epilepsy[108,109]. So, these new born neurons play pivotal role in learning-memory, pattern separation, stress coping and contribute towards normal brain functioning. Ablation of adult neurogenesis or inhibiting newborn neuron activity results in attenuation of antidepressants' effects[30,110]. In traumatic brain injury (TBI) animal models, cognition is impaired and pharmacological interventions to increase neurogenesis improves functional performance. This suggests enhancement in production of newborn neurons can partially mitigate the effects of TBI[111]. Adult neurogenesis declines with aging, which is concomitant with cognitive decline[69]. More adult NSC transit to quiescence with aging and re-activation of these quiescent adult NSC by pharmacologic intervention improves cognition, by enhancing adult neurogenesis[69]. Thus, knowing the underlying molecular regulation of adult NSCs under diverse pathophysiological situations including stress, ageing, and neurodegenerative illness will improve our understanding of adult NSCs' function and potential, and will aid in the design of better therapeutic intervention strategies.

Transcription Factor 4 (Tcf4) in neurogenesis

Tcf4 is a basic-helix-loop-helix transcription factor belonging to the E-protein family, that binds to hexanucleotide E-boxes in the genome [112]. It is widely expressed during embryonic brain development and continues its expression in adult brain throughout the lifespan[113,114].

Tcf4 is required for proper brain development[51]. It plays crucial function in NSC differentiation[50,115]. Recent study in induced pluripotent stem cells identified Tcf4 as master regulator in Schizophrenia patients at neuro-developmental stages[116]. In addition, Tcf4 binds to mediator complex in NSC and regulate NSC functions[49].

Haplo-insufficiency of Tcf4 causes Pitt-Hopkins syndrome, which manifests as developmental delay, hyperventilation, and cognitive deficits[117]. Genome-wide association study identified mutations in the 5' intronic regions of Tcf4 in Schizophrenia patients, marking Tcf4 as a high-risk allele[53]. Heterozygous Tcf4 models demonstrate defects in intrinsic excitability[114] and cognitive dysfunction[52,118], whereas in-utero Tcf4 overexpression leads to disruption of columnar arrangement in developing cortex[119]. However, these Tcf4 loss-of-function and gain-of-function studies were performed in constitutively altered genetic models, which lack cell-type specificity for probing Tcf4 function in the adult brain.

Given Tcf4's regulatory functions in the developing brain and its connection to functional regulation in NSCs, these observations suggest that it would be crucial to comprehend Tcf4's role in adult brain NSCs. Here, our goal was to understand Tcf4's role in adult brain stem cells, for which we employed cell-type and age-specific genetic modification using inducible CreER-LoxP loss-of-function tool.

Chapter 2

Investigating Tcf4's role in adult neural stem cells

Publication

Shariq, Mohammad, et al. "Adult neural stem cells have latent inflammatory potential that is kept suppressed by Tcf4 to facilitate adult neurogenesis." Science Advances 7.21 (2021): eabf5606.

Tcf4 deletion strategy in adult brain neural stem cells

Data from our lab showed that Tcf4 is continually expressed in adult NSC [54]. Therefore, we set out to investigate its function. To this end we used conditional inducible CreER-LoxP model for Tcf4 loss-of-function in adult mice (Fig. 2.1 A-B). This model allows us to delete the gene of interest in a cell-type specific manner by using cell-type specific promoter-driven Cre expression. We used *Nestin*CreER, since *Nestin* is specifically expressed in neural stem cells , providing cell type-specific control for genetic manipulation. Tamoxifen-mediated Cre activity enables temporal control over the deletion. The transgenic mouse has LoxP sequences flanking the target gene, which Cre-recombinase recognises and uses to cause recombination to delete the target gene.

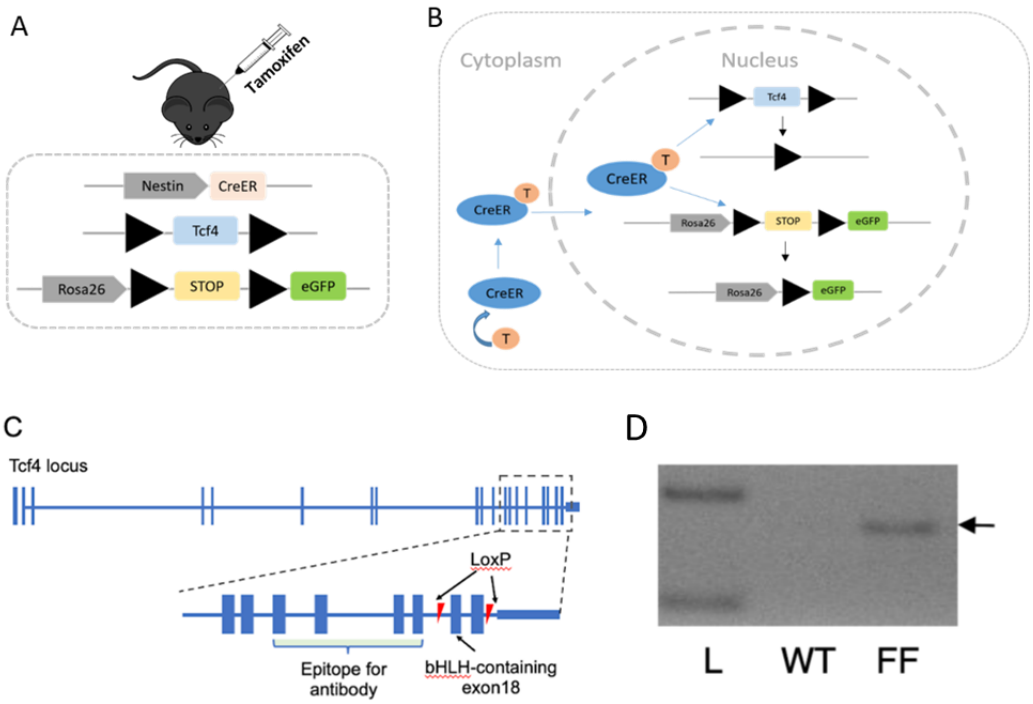


Figure 2.1: Schematic of CreER-LoxP transgenic model. (A) Conditional inducible Tcf4 knockout mouse model with proxy eGFP reporter. (B) Mechanism of tamoxifen action, tamoxifen(orange) binds to Cre (blue) on ER sites which facilitates its translocation inside the nucleus. Cre recombinase detects LoxP sites and deletes the target gene by recombination. Cre-mediated recombination in the cell is reported by eGFP expression. (C) Diagram of the Tcf4 locus showing the N-terminal area containing epitopes recognised by the antibody, and the LoxP sites at the C-terminus flanking the bHLH domain (Sarkar.et al. 2021). (D) Representative PCR genotyping demonstrating recombination for the Tcf4-loxP region in NSC of KO animals.

Tcf4 deletion in adult neural stem cells leads to deficit in spatial memory

Reduced adult neurogenesis is known to affect hippocampus-based cognition such as contextual and spatial memory[37,38]. Data from our lab showed decrease in adult neurogenesis upon Tcf4 deletion in adult NSC[54]. Therefore, we investigated the impact of Tcf4 deletion–mediated reduction of adult neurogenesis at the behavior level. For this, we performed novel location recognition (NLR) test, a hippocampal adult neurogenesis–dependent[120] memory task. In this cognitive test, spatial memory is assessed. The mice were trained to remember the location of two identical objects placed within an arena where both the objects were placed near a wall having a sticker as visual anchor. During training the mice explore the identical objects. On test day, the mice are re-introduced in the same arena, but one of the

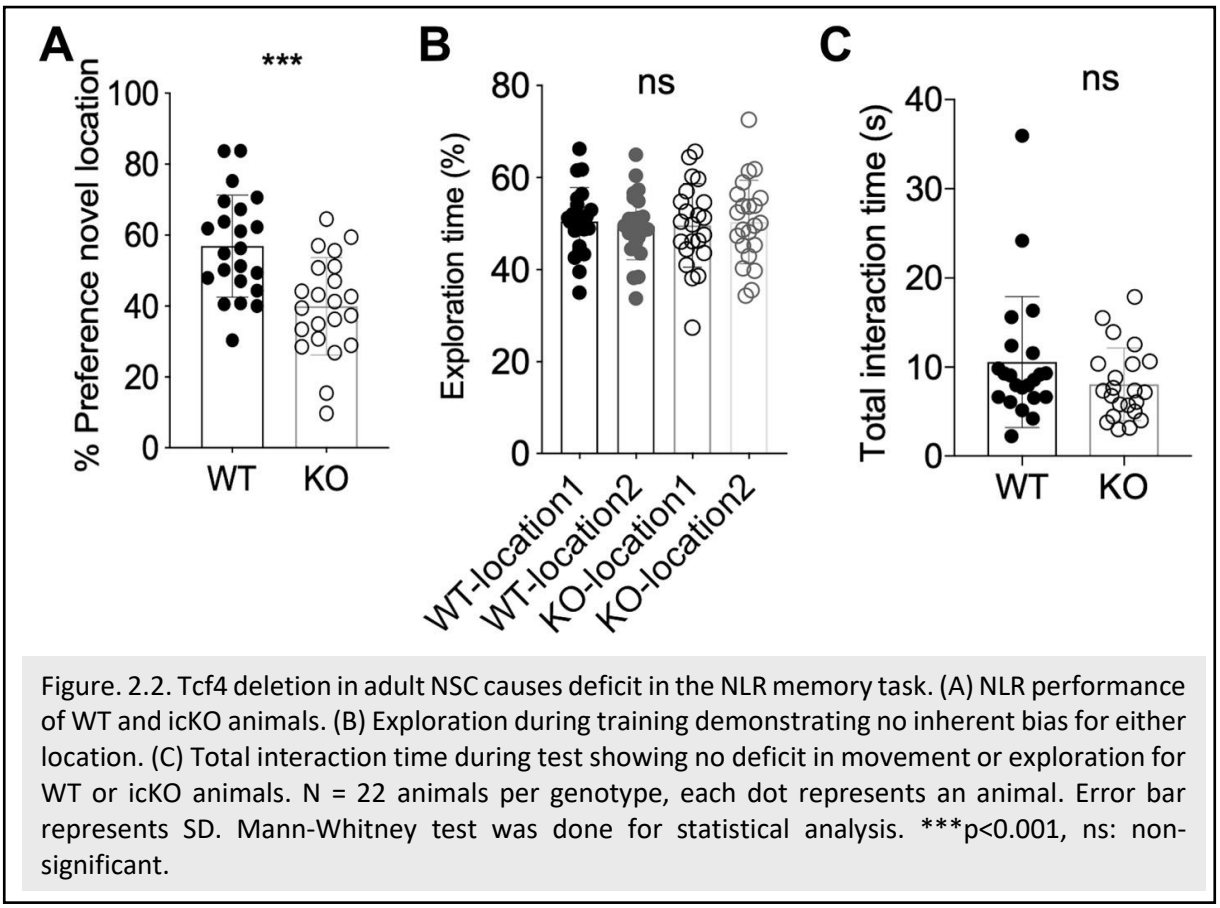


Figure. 2.2. Tcf4 deletion in adult NSC causes deficit in the NLR memory task. (A) NLR performance of WT and icKO animals. (B) Exploration during training demonstrating no inherent bias for either location. (C) Total interaction time during test showing no deficit in movement or exploration for WT or icKO animals. N = 22 animals per genotype, each dot represents an animal. Error bar represents SD. Mann-Whitney test was done for statistical analysis. ***p<0.001, ns: non-significant.

objects is displaced to a novel location. Mice have an innate tendency to explore novelty. So we expect the mouse with normal cognition to have spatial memory of the previous location of objects and to identify the displaced object's location as novel, and hence spend more time exploring the displaced object (now at a novel location).As shown in Fig. 2.2A, the icKO mice did not show a preference for the newly located object, indicating poor spatial memory . As shown in Fig. 2.2 B and C, total exploration time did not vary for either of the locations during training and the total time of exploration during test did not vary between WT and icKO mice. This indicates that the mice did not have bias for any location during training and had no defects in their ability to explore.

Tcf4-deleted neural progenitors acquire myeloid-like inflammatory cell state

Data from our lab indicated that Tcf4 deletion in *Nestin*-expressing progenitors of the adult brain affected hippocampal adult neurogenesis by affecting the IPC population[54]. Therefore, we next investigated the effects of Tcf4 deletion in proliferating IPC. To understand the cell-intrinsic effects in IPC, while avoiding potential secondary effects from its niche, we isolated hippocampus from the WT and icKO brains for neurosphere culture and induced Tcf4 deletion in progenitors *in vitro*. Consistent with the *in vivo* data demonstrating reduction in proliferation in DG[54], the Tcf4-deleted progenitors formed significantly lower number of neurospheres (Fig. 2.3A), which were also smaller in size (Fig. 2.3,B and C), indicating loss of proliferative capacity. Furthermore, the icKO neurospheres often exhibited spreading (Fig. 2.3B) and did not give rise to secondary neurospheres (Fig. 2.3 D and E), indicating loss of self-renewal capacity.

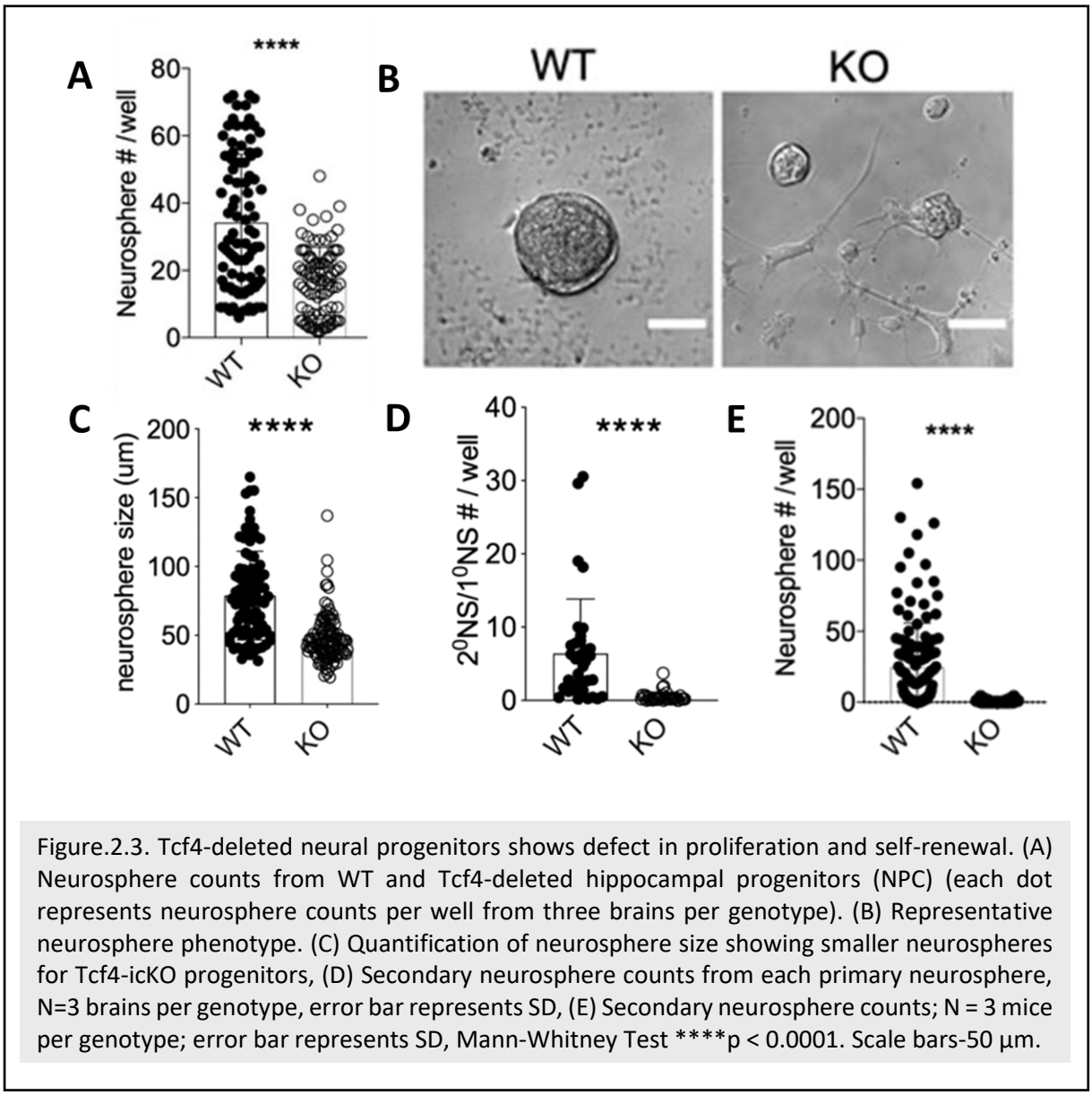
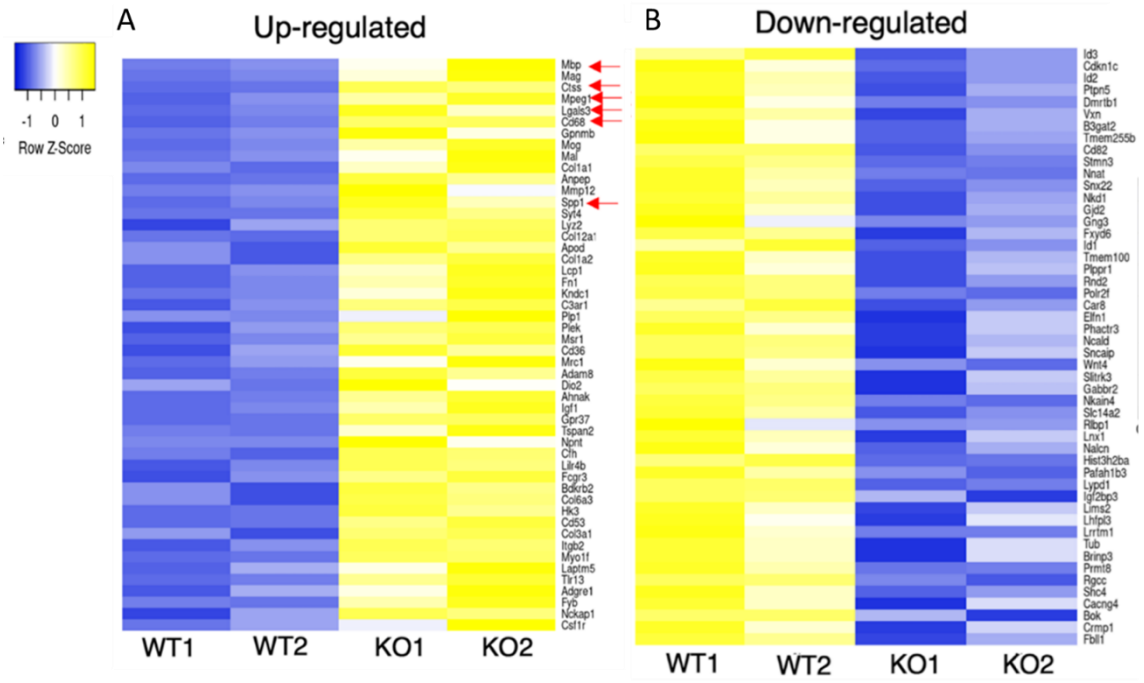


Figure.2.3. Tcf4-deleted neural progenitors shows defect in proliferation and self-renewal. (A) Neurosphere counts from WT and Tcf4-deleted hippocampal progenitors (NPC) (each dot represents neurosphere counts per well from three brains per genotype). (B) Representative neurosphere phenotype. (C) Quantification of neurosphere size showing smaller neurospheres for Tcf4-icKO progenitors, (D) Secondary neurosphere counts from each primary neurosphere, N=3 brains per genotype, error bar represents SD, (E) Secondary neurosphere counts; N = 3 mice per genotype; error bar represents SD, Mann-Whitney Test ****p < 0.0001. Scale bars-50 µm.

To elucidate the molecular effect of Tcf4 deletion in neural progenitors, we performed RNA sequencing (RNA-seq) of NPC from neurospheres, which gave 740 differentially regulated genes (DEGs) between WT and Tcf4-icKO NPC (Fig 2.4). Unexpectedly, the transcriptomic data revealed that Tcf4 deletion in hippocampal proliferating progenitors resulted in an up-regulation of myeloid inflammatory gene signature, with Lgals3 (Galectin-3) and CD68 being two of the top up-regulated genes (Fig. 2.4 A and E). Analysis of gene ontology of the top 50 significantly up-regulated genes in icKO progenitors identified "inflammatory response" and "extracellular matrix organization" as the prominent up-regulated functions of the icKO progenitors (Fig.2.4C). Conversely, cell cycle and neuronal differentiation pathways were down-regulated in the icKO progenitors (Fig. 2.4D). Given the up-regulation of myeloid and inflammatory genes in icKO progenitors, we further confirmed protein expression of Lgals3 (Fig. 2.5A) and CD68 (Fig. 2.5B), in WT and icKO progenitors. Lgals3 is a gene implicated in inflammatory process in multiple tissues, including the brain[121–123]. It promotes pro-inflammatory pathways in conditions like arthritis[121] and atherosclerosis[122]. Lgals3 has also been implicated in brain pathological conditions which are associated with inflammation like Alzheimer's, stroke and ischemia[124]. CD68 is a prominent lysosomal marker expressed by myeloid cells. Expression of these proteins in the Tcf4-deleted progenitors further confirmed their myeloid inflammatory transformation.



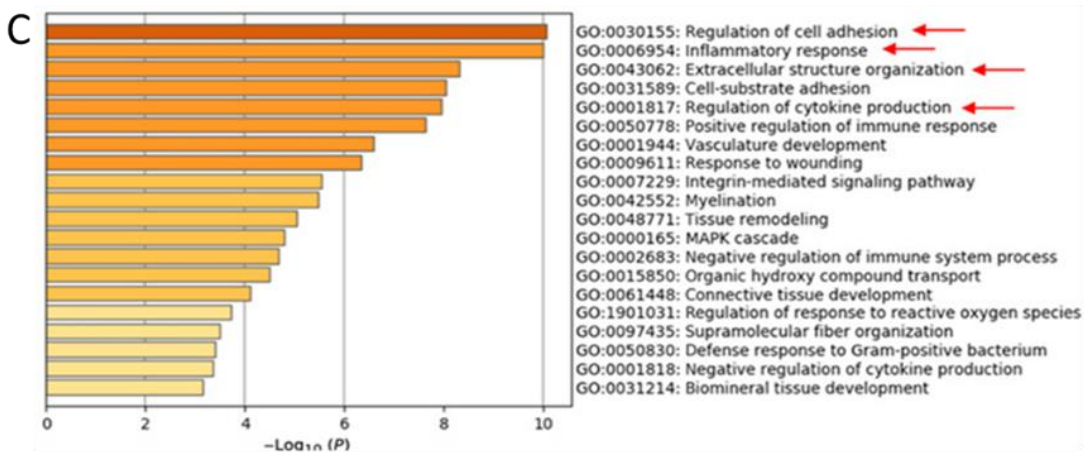


Figure. 2.4. Tcf4-deleted neural progenitors gain myeloid inflammatory potential

(A and B) Heat map showing top-50 differentially expressed genes in WT versus icKO neurospheres. (C and D) Enriched ontology cluster analysis for the top 50 up-regulated and down –regulated genes from (A and B) in icKO progenitors respectively. (E) Volcano plot showing differentially expressed genes in WT versus icKO neurospheres, ($p<0.05$, green dots). FC:fold change.

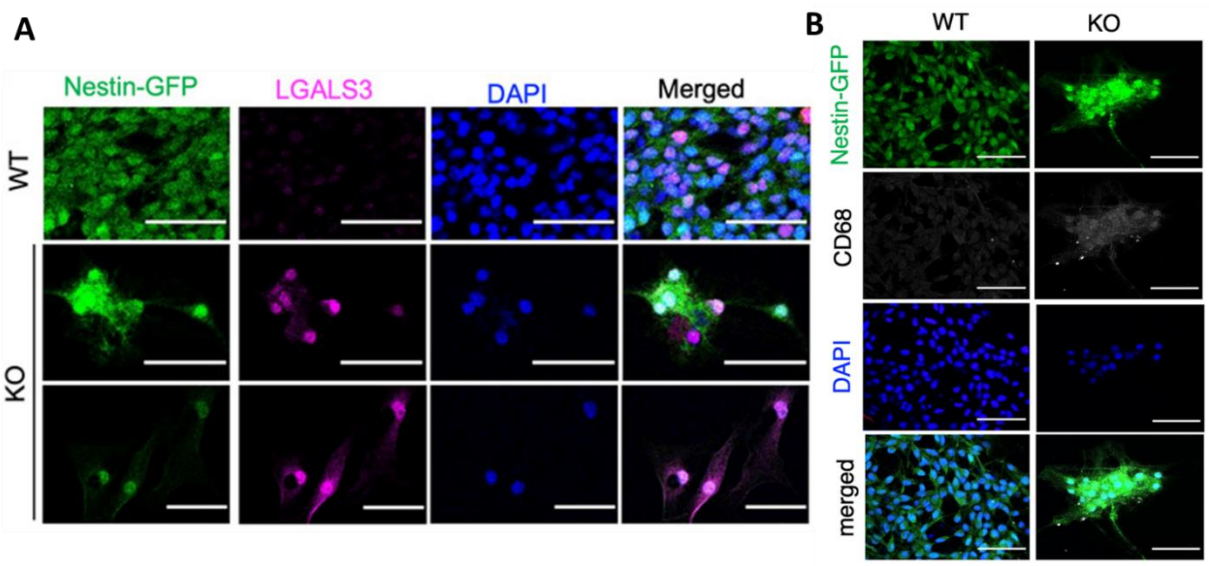


Figure. 2.5 Expression of myeloid markers in Tcf4-deleted neural progenitors.
(A) Representative images of genetically traced (GFP+) WT and icKO NPC for the expression of LGALS3. (B) Representative images showing CD68 expression in genetically traced (GFP+ve) icKO-progenitors, N=3 brains per genotype. Scale bar=50µm.

Tcf4-deleted hippocampal neural progenitors show aberrant differentiation potential

Given the spread-out morphology in icKO neurospheres, we also examined the differentiation potential of the NPCs in the absence of Tcf4. Consistent with the transcriptome data (Fig. 2.4E), the icKO progenitors indeed showed aberrant differentiation, expressing the neuronal marker Tuj1 along with the oligodendrocyte gene myelin-basic protein (MBP) and the myeloid gene CD68, two of the top up-regulated genes detected in RNA-seq of the icKO progenitors

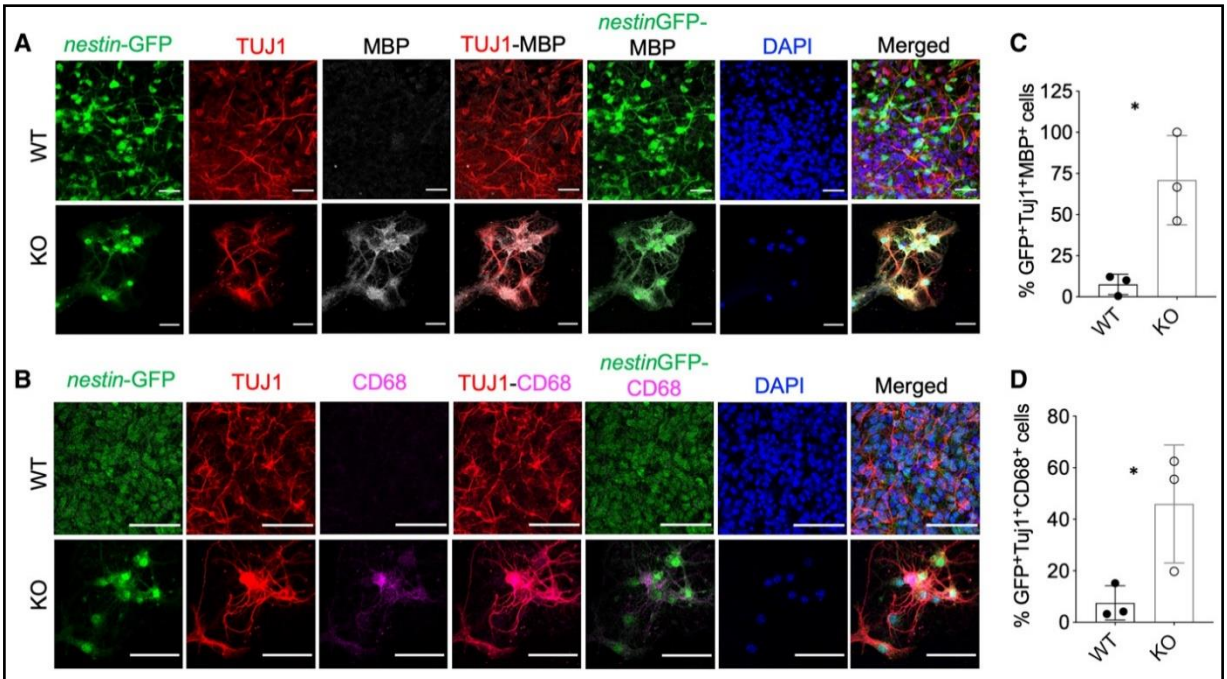


Figure. 2.6 Tcf4-deleted hippocampal neural progenitors show aberrant differentiation potential. (A and B) Representative image for differentiation phenotype of neural progenitors, demonstrating co-expression of neuronal gene Tuj1 and oligodendrocyte gene MBP (A) and Tuj1 and myeloid gene CD68 (B) in genetically traced GFP+Tcf4-deleted icKO progenitors. (C and D) Quantification of percentage of aberrant cells among the lineage traced progenitors. Each dot represents average counts from an animal; error bars represent SD. Mann-Whitney Test *p< 0.05. Scale bars, 50 µm.

(Fig. 2.6, A to D). Furthermore, differentiated icKO progenitors presented a stalky morphology when compared with the thin long processes seen in the differentiated WT progenitors (Fig. 2.6, A and B). Interestingly, *Nestin* lineage tracking data from our lab shows no fate diversion of progenitors to microglia. Thus, even though the icKO progenitors showed up-regulation of several myeloid genes, including CD68 which is also expressed by the brain-resident myeloid cell microglia, the Tcf4-deleted NPC do not become microglia.

Collectively, icKO of Tcf4 in neural progenitors results in decreased proliferative and self-renewal capacity. Tcf4 deletion in progenitors trigger a novel latent inflammatory potential, where they acquire myeloid signatures and display aberrant differentiation.

Tcf4-deleted neural progenitors produce detrimental factors that adversely affect niche cells

The evidence for inflammatory gene expression of Tcf4-icKO progenitors (Fig. 2.4 and 2.5), suggested that the icKO progenitors may produce factors that could cause inflammation in DG. To test if Tcf4-deleted progenitors could adversely affect its niche cells, including the possibly undeleted NPC in icKO brain SGZ, we designed a co-culture experiment using *Nestin*CreER-Tcf4 flox; flox-STOP-flox-eGFP mice for neurosphere cultures. In this co-culture experiment, in order to uniquely identify the WT and Tcf4 icKO neurospheres, we used Cre+, Tcf4 Flox-Flox (FF) mice, with flox-STOP-flox-eGFP cassette as proxy reporter for Cre activation. WT neurosphere received vehicle-based treatment, while icKO neurosphere received tamoxifen-based treatment. In the co-culture experiment, WT and icKO NS were able to be distinguished from each other because only deleter neurosphere would produce eGFP upon induction, while non-deleter WT would be non-GFP neurosphere. For this, we induced Tcf4 deletion in progenitors from icKO brain DG in only half of the progenitors, while the other half was vehicle-treated to mimic undeleted progenitors in the icKO-DG (reported as WT for this experiment). We adopted this co-culture regime after validating that tamoxifen treatment does not have its own non-specific effect on NPC, by comparing neurospheres from tamoxifen-treated WT with vehicle-treated Tcf4-FF progenitors, which showed no difference in either genotype (Fig. 2.7).

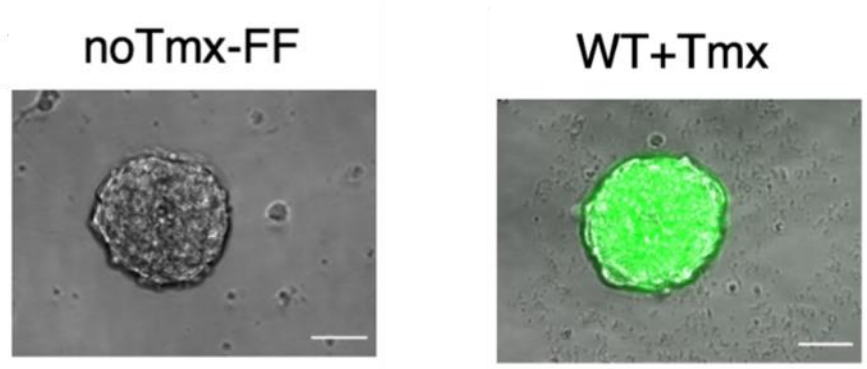


Figure 2.7 Comparison of vehicle and tamoxifen treated controls in NS. Representative image of tamoxifen-treated WT NS versus vehicle-treated Tcf4-flox (FF) neurosphere, demonstrating no difference. Scale bar-50µm.

Once the icKO neurospheres appeared green because of Cre-induced recombination and eGFP expression (while vehicle-treated controls remained GFP-; labeled as WT for this experiment), WT and icKO progenitors were put together in a single well for co-culture to investigate the effect of icKO progenitors on its neighboring cells. The vehicle-treated Tcf4-FF progenitors (WT) and the tamoxifen-treated Tcf4-flox progenitors (icKO) are shown as control from the same brain (Fig. 2.8A, left). The hydroxy-tamoxifen (OH-Tmx)–induced GFP expression in icKO progenitors allowed us to discern the Tcf4-undeleted (WT) progenitors from the Tcf4-deleted (icKO) progenitors in the same well. As shown in Fig. 2.8A, the health of the WT neurospheres (white arrows) was severely compromised when co-cultured with icKO progenitors (green arrows) in the same well, indicating that Tcf4-deleted progenitors not

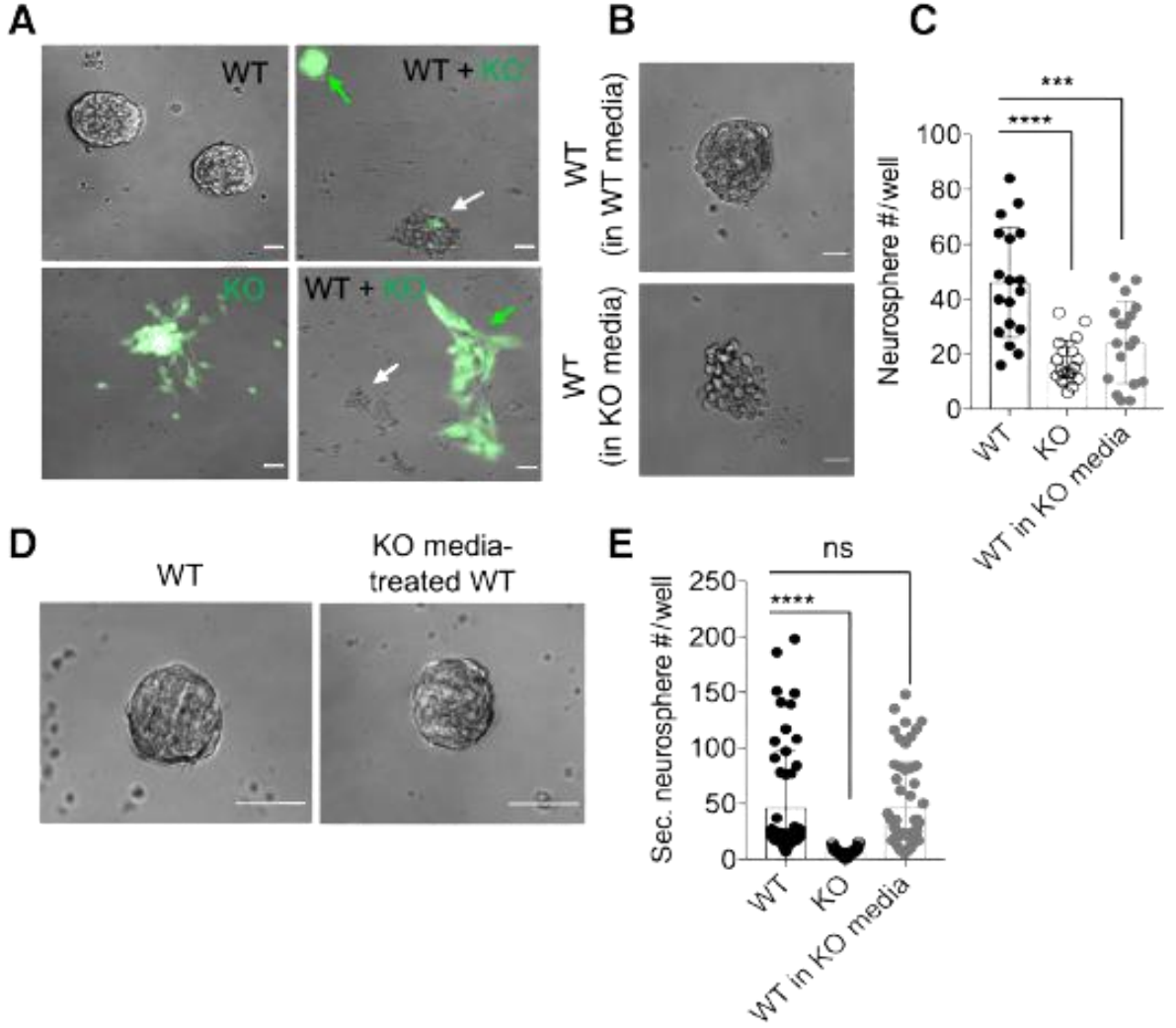


Figure. 2.8. Tcf4-deleted neural progenitors produce detrimental factors that adversely affect niche cells. (A) Representative neurosphere images from WT, icKO, and WT-icKO co-culture. Green arrows show Tcf4-deleted neurospheres, and white arrows show WT neurosphere in co-culture wells. (B) Representative images showing WT neurospheres in normal media versus in media from icKO cultures. (C) Neurosphere counts from (B). (D) Representative secondary neurosphere demonstrating recovery of icKO media–treated WT neurospheres when returned to normal media. (E) Neurosphere counts of (D). Three brains per genotype; error bar represents SD, Mann-Whitney Test, ****p< 0.0001, ***p<0.001. Scale bars-50 µm.

only have a cell-intrinsic detrimental effect on themselves but also have a detrimental effect on its neighboring cells.

Lastly, to test whether the influence of icKO progenitors is possibly through extracellular/secreted factors, we separately cultured progenitors from WT and icKO brains treated with Hydroxy-tamoxifen (OH-Tmx). We then transferred the culture media from the icKO neurospheres onto the WT neurospheres, to examine potential adverse effects of secreted factors from icKO-NPC on WT progenitors. This indeed resulted in deterioration of the health and number of the WT neurospheres (Fig. 2.8, B and C). However, the adverse effect of icKO neurosphere media on WT progenitors was reversible, since upon returning to normal media, the WT progenitors were rescued from "unhealthy" phenotype and formed neurospheres that were normal in morphology and numbers, comparable to WT neurospheres in normal media (Fig. 2.8, D and E).

We also examined whether cell death and related factors in icKO NPC culture could be responsible for the adverse effects of icKO media–secreted factors. For this, we performed cleaved–caspase-3 staining of the neurosphere cultures, which showed no cell death in WT or icKO neurosphere cultures (Fig. 2.9A). In contrast, as a positive control, the etoposide-treated cultured cells showed positive staining for cleaved caspase-3 (Fig. 2.9B). This further confirmed that the genetic deletion of Tcf4 in hippocampal NPCs also has a "cell-extrinsic" effect manifested through the production of extracellular factors by the icKO progenitors, which could adversely influence its niche cells. This is in addition to the "cell-intrinsic" effects of Tcf4 deletion in NPC that is manifested as its myeloid transformation, affecting the progenitors' proliferation and differentiation potential (as shown in Figs. 2.3,2.6).

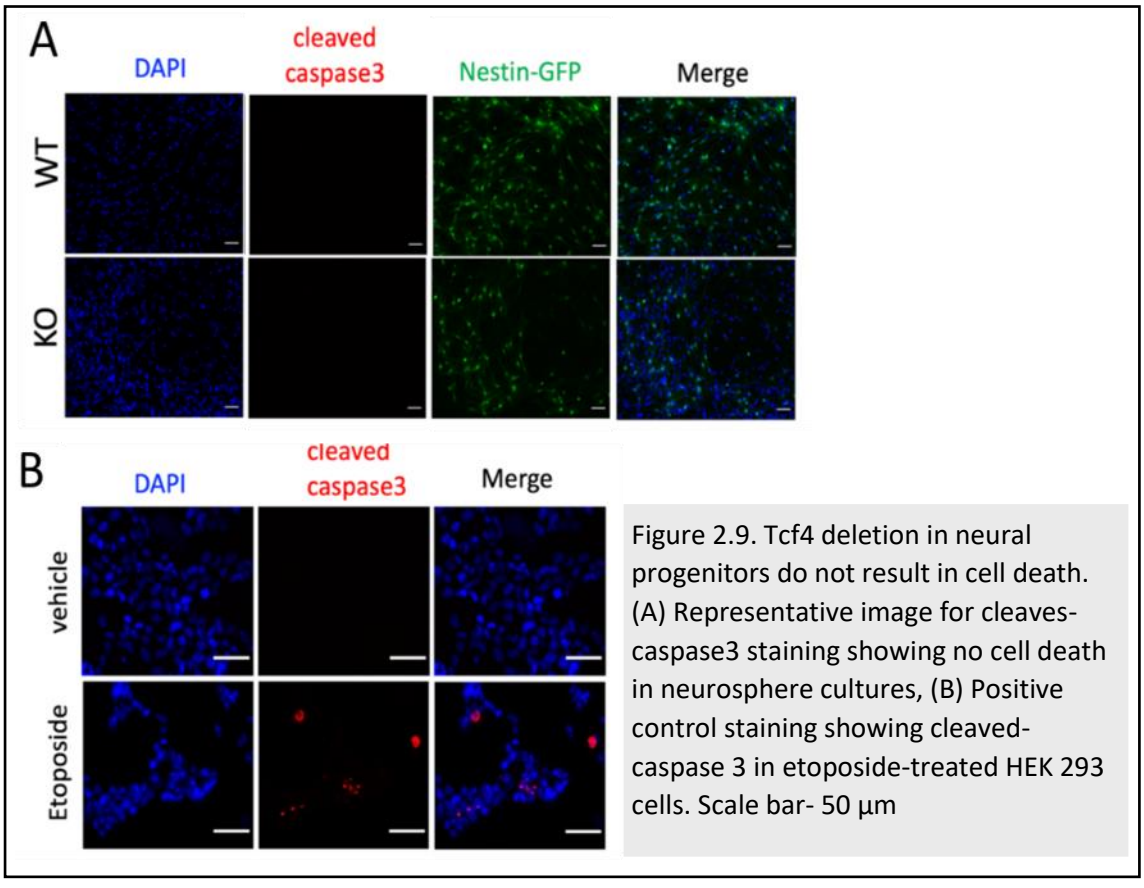


Figure 2.9. Tcf4 deletion in neural progenitors do not result in cell death. (A) Representative image for cleaves-caspase3 staining showing no cell death in neurosphere cultures, (B) Positive control staining showing cleaved-caspase 3 in etoposide-treated HEK 293 cells. Scale bar- 50 µm

Chapter 2: Discussion

In recent years, the neural stem cells of the adult brain have been characterized in depth with regards to their transcriptome and proteome, thereby detailing the molecular regulations that underlie adult NSC maintenance[125,126], differentiation, and fate potential[68,127]. Specifically, studies examining the potential of the adult hippocampal progenitors have revealed that the fate of these progenitors is restricted to neuron, astrocyte, and oligodendrocyte lineages with a bias towards neuronal differentiation[128,129]. Investigating the functional relevance for the high expression of a cell fate regulatory transcription factor Tcf4 in the adult neurogenic niche, we have uncovered an unexpected aspect of the adult NSC. Our study reveals that Tcf4, which is highly expressed in the adult neurogenic cells, facilitates hippocampal adult neurogenesis by proactively suppressing an inflammatory potential of the neural progenitors. Transcriptome analysis of Tcf4 deleted *Nestin*-expressing neural progenitors revealed that several genes that are known to be expressed in myeloid cells, such as Ctss, Mpeg1, Lgals3, CD68, and Spp1 were up-regulated in neural progenitors upon Tcf4 deletion, indicating their transformation into a myeloid inflammatory state. Lgals3 is highly expressed by a variety of myeloid cells including macrophage and neutrophils and has been implicated in immune-modulatory functions including chemotaxis and extracellular matrix regulation[130,131]. Corresponding to the up-regulation of Lgals3 in icKO NPC, its binding partner Lgals3bp was up-regulated in the icKO brain DG, as shown in transcriptomic datasets. Consistent with the myeloid inflammatory gene signature of the icKO progenitors, we were able to detect protein expression of the inflammatory gene Lgals3 and the lysosomal marker CD68, both of which are known to be expressed in myeloid cells, in the icKO progenitors. Consistent with this, we further confirmed that the icKO progenitors indeed produce extracellular factors that detrimentally affect its niche cells. Together, our observations demonstrate that Tcf4 deletion in adult NSC adversely affects the process of adult neurogenesis in two ways; on one hand, it abolishes the proliferative and differentiation capacity of the NPC, while on the other hand, it causes inflammation in the niche via the NPCs' inflammatory transformation. Given that inflammation is already known to adversely affect mammalian adult neurogenesis[40,78,104], the inflammatory transformation of adult NPC likely affects adult neurogenesis further in the icKO brain. This two-pronged effect of Tcf4 deletion in adult NSC can be understood as a cell-intrinsic effect that leads to abolition of the neuronal fate potential by transforming the progenitors into a myeloid-like inflammatory cell state and a cell extrinsic effect due to production of detrimental factors as a result of their transformation, thereby further affecting the niche cells. The importance of Tcf4 in the CNS is underscored by its implication in neurodevelopmental (PTHS) as well as psychiatric (SCZ) disorders. While several studies have demonstrated the role for Tcf4 in brain development and embryonic neural stem cells, the underlying mechanism, and targets of Tcf4 in the embryonic brain remain poorly understood. Furthermore, Tcf4's role in the postnatal brain, where it continues to be abundantly expressed, has only started to emerge, implicating Tcf4 in myelination[132] and neuronal activity[114,118,133,134]. However, Tcf4's potential function in the adult neural stem cell remains unexplored. It is important to note that new transcriptomic

dataset of the adult hippocampal NSC reveals that the adult NSC diverge from the embryonic NSC at a very early postnatal stage, around postnatal day 5-7 [128]. Given this, the molecular regulators, and the possibilities of fate potential for adult NSC are likely to be different from the embryonic NSC, especially given the environment-responsive functions of adult hippocampal NSC. In this regard, our study reveals a completely unprecedented aspect of adult NSC, its latent myeloid inflammatory potential, and implicates Tcf4 in suppression of this detrimental potential. Injury-induced neurodegeneration and inflammation have been shown to induce NSC proliferation and neurogenesis in zebrafish[135]. However, a quest for similar response in mammalian model systems has largely met with disappointment. Analysis of human samples from neurodegenerative disorders such as Huntington's disease[9] and Alzheimer's disease (AD)[19] demonstrates a decline in adult neurogenesis under neurodegenerative conditions. Since parenchymal inflammation is known to be concomitant in these neurodegenerative diseases, this further emphasizes that the mammalian adult brain NSC respond adversely to inflammation. However, the source of inflammation and its potential molecular link to reduction in adult neurogenesis remain unclear. Our observations provide the first evidence that (i) inflammation can be triggered cell intrinsically by adult neural progenitors residing in the DG and (ii) that Tcf4 is a cell-intrinsic regulator of adult NSC that proactively suppresses the inflammatory potential of adult NSC, to facilitate normal adult neurogenesis. The revelation of a latent inflammatory potential of the adult NSC may have broader implications beyond the field of adult neurogenesis, in the wider context of aging, neurodegeneration, neuro-inflammation, and regenerative approaches where all the effectors are not yet fully understood. For instance, although adult neurogenesis is known to decline with age and in stress, what triggers the aging NSCs to adopt quiescence remains poorly understood. Whether aging RGL adopt an inflammatory state that affects their functions remain elusive. In the context of regenerative research, the potential of neural stem cell is only CNS-friendly neural cells; however, the presence of a latent inflammatory property within these cells would be a critical aspect to keep in consideration. In the case of neurodegenerative disorders, while a reduction in adult neurogenesis is observed at the very early stages of AD[19], the triggers for the degenerative process in AD remain unknown. A potential involvement of a CNS- intrinsic trigger for inflammatory processes contributing to neuro-inflammation and neurodegeneration would be an important question to explore. Our observations provide evidence that adult NSC residing in the hippocampus harbors an inflammatory potential that is proactively suppressed, thereby shedding light on to a previously unknown potential source of inflammation within CNS. However, future studies identifying potential physiological contexts for the inflammatory transformation of adult NSC will be needed to provide further insights into the physiological and clinical relevance of our findings.

Chapter 3

Investigating physiological relevance of Tcf4 mediated regulation of adult neural stem cells

Stress is a key contributor to various psychological disorders like depression and Post Traumatic Stress Disorder (PTSD)[136]. Childhood trauma, loneliness, work-related stress, socio-economic position, and sickness are all factors that contribute to stress pathology[137]. Stress adversely affects the social well-being as well as impacts cognitive functions[94,138,139]. In stress condition hypothalamic-pituitary axis (HPA) orchestrates the release of glucocorticoids in circulation, which later triggers inflammation in circulatory system by elevation of pro-inflammatory molecules[92]. Inflammation is detrimental for brain function. Systemic inflammatory molecules are known to negatively regulate neurogenesis and blocking inflammation helps in restoration of neurogenesis[78,104]. Interestingly our data revealed a novel latent inflammatory potential in adult NSC, but how and if this latent inflammatory potential is useful in physiological conditions remains unknown.

In order to examine the physiological relevance of the latent inflammatory potential in adult NSC, we examined adult NSC in a stress model. Although adult neurogenesis is known to deteriorate in response to physiological stress, the manifestation of stress stimuli in adult NSC and its influence on the neurogenic program, remains largely elusive. To address this and to study the potential role of Tcf4 in adult NSC during stress response, we investigated a chronic immobilization stress paradigm in mice.

Characterization of Tcf4 expression in adult neural stem cells in chronic immobilization stress (CIS)

For this study we adapted the chronic immobilization regime from published protocols[94,140]. Animals were divided and housed separately into “control” and “stress” groups. The “stress” animals were subjected to chronic immobilization stress in decapicones, daily for six hours. In order to gain insight about the dynamic effect of stress on adult neural stem cells and the inflammatory state of the neurogenic niche, we performed a time course of varied stress duration.

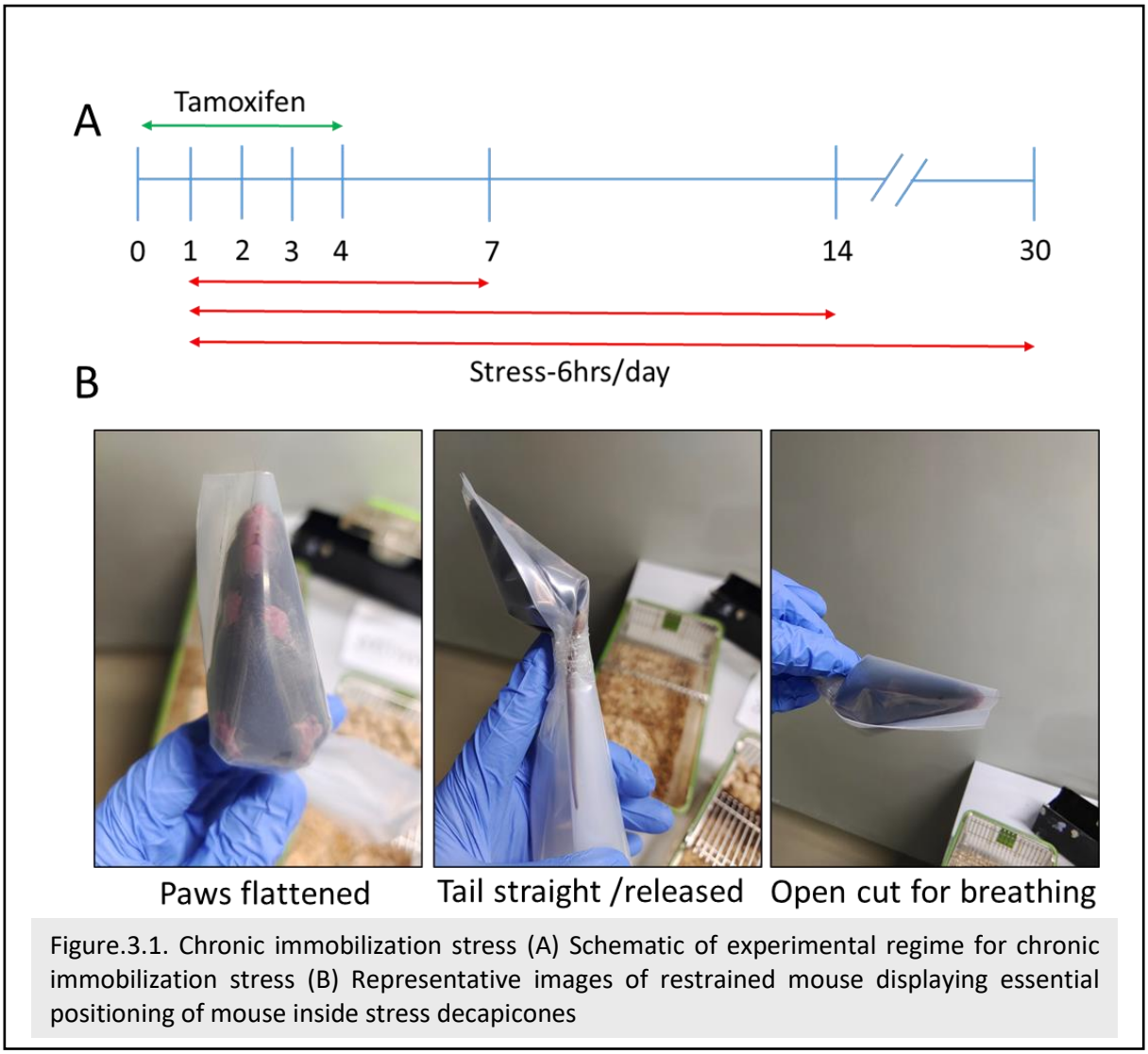


Figure.3.1. Chronic immobilization stress (A) Schematic of experimental regime for chronic immobilization stress (B) Representative images of restrained mouse displaying essential positioning of mouse inside stress decapicones

Tcf4 expression in neural stem cells upon stress

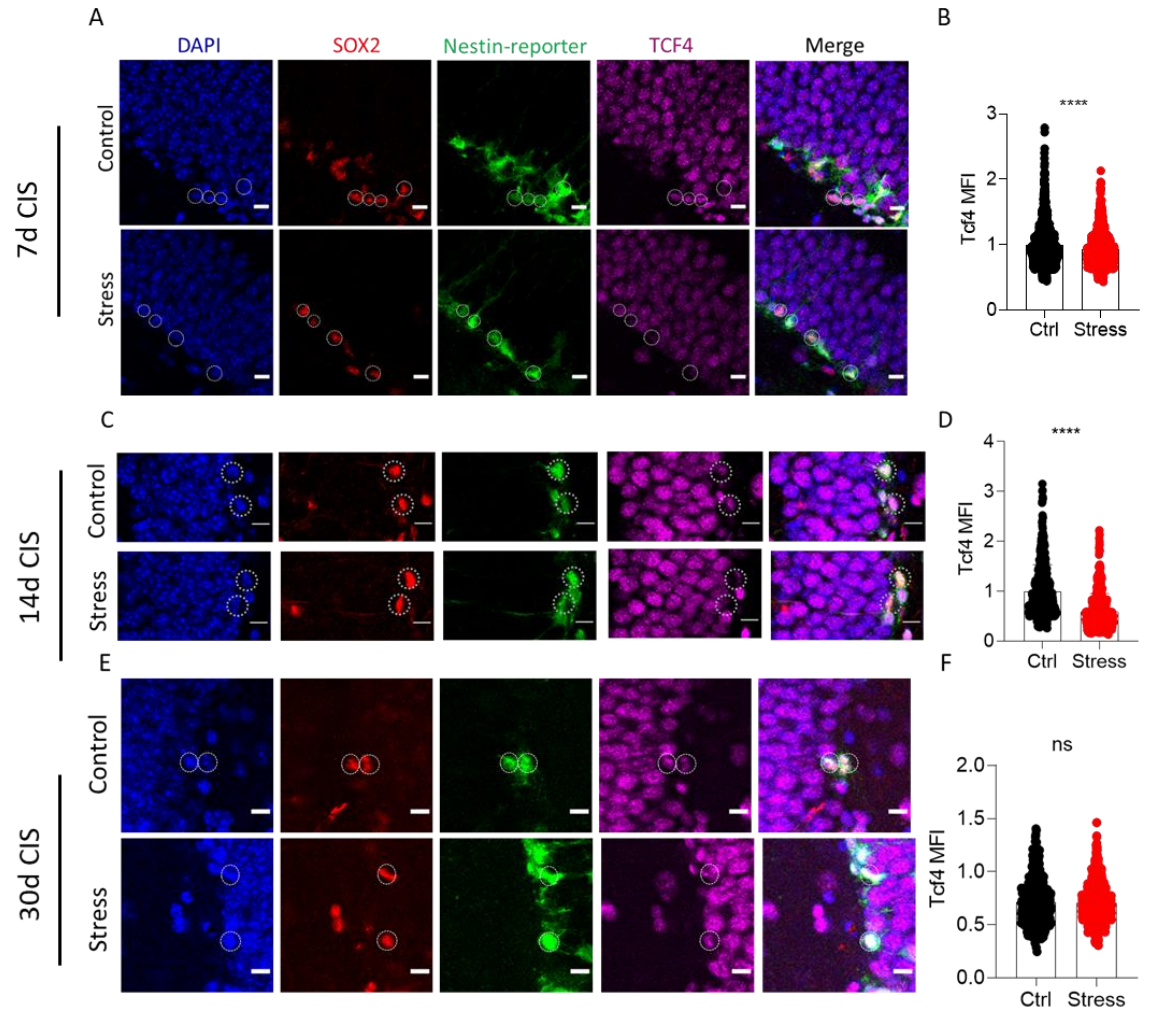


Figure.3.2. Tcf4 expression in adult neural stem cells during the course of chronic immobilization stress. (A, C and E) Representative image of genetic-reporter labelled Sox2+ve neural progenitor for Tcf4 expression. (B, D and F) Quantitation of mean fluorescence intensities of Tcf4 of (A, C and E), each dot is a repoter-Sox2+ve neural progenitor. Scale bar 10µm. Counts are from three to four mice, each dot represents a repoter-Sox2+ve neural progenitor and error bar represents SD, Mann-Whitney test; ****p < 0.0001 and ns: non-significant. 7d CIS animals had Ai9Td reporter, whereas 14d and 30d CIS animals had RCE-GFP reporter.

We were interested to examine the effect of stress on the dynamics of Tcf4 expression in adult neural stem cells. For this, we first labelled the adult neural stem cells using inducible indelible labeling of *Nestin* expression, using a tamoxifen inducible Rosa-flox-Stop-flox-eGFP reporter *Nestin*. The tamoxifen induction was started one day prior to start of stress and continued for the stipulated dosage for 4 days.

We next quantified Tcf4 expression in genetically labelled reporter and Sox2 double positive cells (marking the neural precursor cells) from confocal images of immuno-stained brain sections in the SGZ of the hippocampus. Tcf4 fluorescence intensity was quantified across three time points during CIS, that is 7 days, 14 days, and 30 days of CIS.

Interestingly we observed that upon shorter stress duration, that is 7 days and 14 days, the neural progenitors showed significant decrease in Tcf4 expression in stressed animals. Whereas, we observed no difference in Tcf4 expression in neural progenitors of animals which underwent CIS for 30 days. The dynamic pattern of Tcf4 expression raises the possibility that molecular adjustments are being made in response to the duration of stress exposure. This finding suggests that adult NSC may display a latent inflammatory potential during the early phase, which the decrease in Tcf4 may spark. This finding emphasizes the complex molecular mechanisms that control the stress response in adult NSC. It also highlights the importance of further research into the underlying molecular pathways behind this process.

Stress triggers inflammatory signature in neural progenitor's cells

Our observations in Chapter 2 demonstrated that absence of Tcf4 in adult neural progenitors triggers inflammatory state by secreting inflammatory factors. Furthermore, our data show decrease in Tcf4 expression upon stress. So, we asked if a decrease in Tcf4 expression brought on by stress in adult neural progenitors also leads to a comparable inflammatory trigger.

We therefore performed the neurosphere assay after stressing the animals for 7 days, in order to examine the impact of stress on neural progenitors from a cell-intrinsic perspective. Cell proliferation is known to be reduced by stress[45,46]. Interestingly, our data demonstrate that stress has a non-uniform effect on the proliferation of neural progenitor's cells; we observe a decrease in neurosphere proliferation in one animal, whilst another animal exhibits proliferation at levels comparable to controls (Fig. 3.3D).

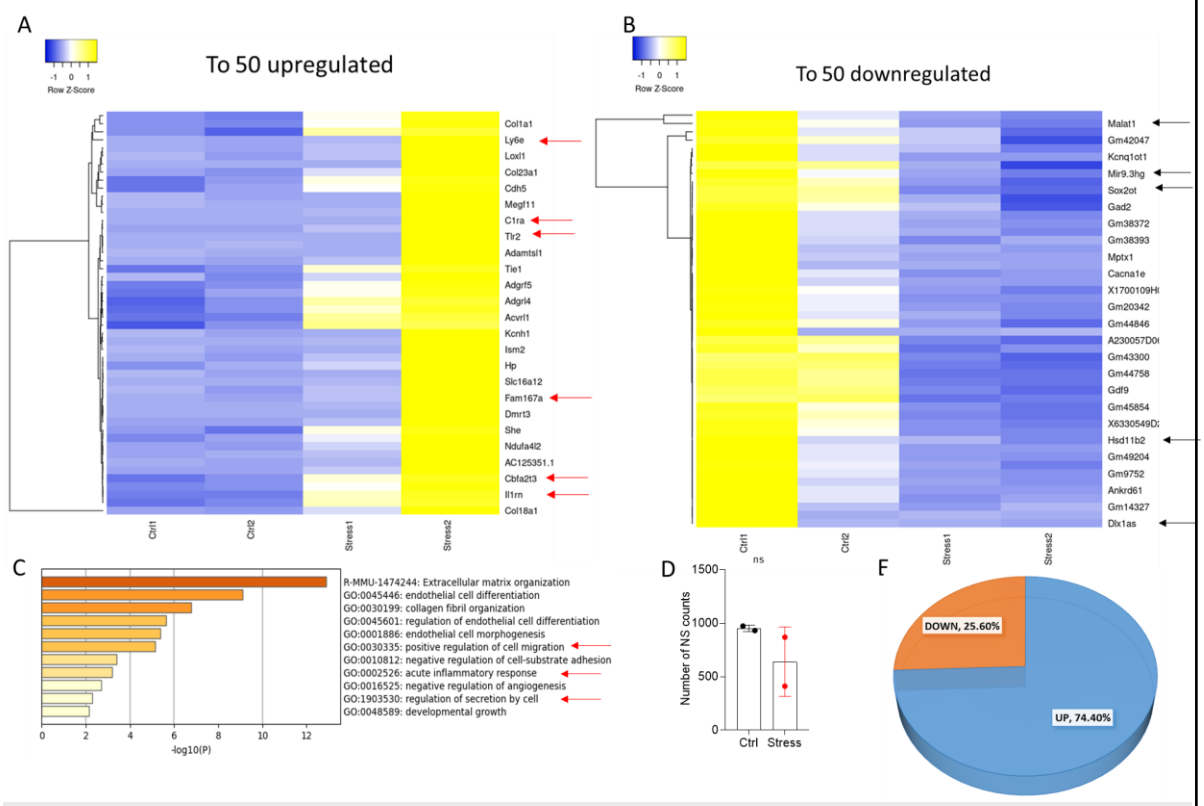


Figure. 3.3. Stress triggers latent inflammatory potential in neural progenitors.
(A and B) Heat map showing top-50 differentially expressed genes in Controls versus Stress neurospheres. (C) Enriched ontology cluster analysis for the top 50 up-regulated genes from (A) in stress progenitors. (E) Pie chart representing percentage up- or downregulated genes among total DEG in neurospheres after 7 days of chronic immobilization stress. Error bar represents SD, Mann-Whitney test; ns= non-significant

Next, we carried out RNA sequencing on control and stressed neurospheres to look at the molecular regulation in neural progenitors under stress. In conjunction with the decline in Tcf4 expression, we discovered 796 differentially regulated genes, of which 593 genes were upregulated and 203 genes were downregulated (Fig. 3.3E), suggesting Tcf4's suppressive effects, as demonstrated by Sarkar et al[141]. Top upregulated genes were associated with extra-cellular matrix organization, endothelial cell regulation and inflammation (Fig.3.3C).

In accordance with our earlier discovery, a decrease in Tcf4 in neural progenitor cells causes the expression of genes linked to inflammation, such as Ly6e[142], Megf11[143], and Tlr2[144](Fig. 3.3A). This shows that the stress-induced reduction in Tcf4 in neural progenitor's initiates latent inflammatory potential. Long non-coding RNAs including Malat1, Mir9.3h, and Sox2ot (Fig.3.3B), which are involved in cell proliferation regulation[145146147], were the top downregulated genes.

One thing to keep in mind is that we also notice here that the heat map signatures amongst stress animals are not uniform, which highlights the biological variations among stress animals and calls for the addition of more experimental animals to have decisive certainty.

Inflammation in the neurogenic niche upon stress

Our observations in Chapter 2 demonstrated that absence of Tcf4 in adult neural progenitors triggers inflammatory state by secreting inflammatory factors. Furthermore, evidence from our lab show that microglia in the neurogenic niche of the dentate gyrus are activated when Tcf4 is deleted in adult neural stem cells (NSCs)[54], as shown by upregulation of CD68 in microglia. So, we asked if a decrease in Tcf4 expression brought on by stress in adult neural progenitors also leads to a comparable inflammatory trigger. By examining microglia activation markers as a proxy for inflammation in neurogenic niche.

We observed microglial activation in the initial phase of stress (7-14 days), whereas in the prolonged stress regime (30 days) we observed a reduction in microglial activation markers. Again, this suggests that downregulation of Tcf4 in adult neural stem cells (NSCs) during the early stages of stress may lead to its possible inflammatory transformation, activating microglia. In chronic stress environments when Tcf4 expression is the same, there might not be a trigger for microglial activation from the adult NSCs' latent inflammatory potential. Iba1 and CD68 activation marker expression, however, is significantly downregulated. A recent study demonstrated that global Iba1 deletion reduces microglial motility and ramification and also alters their phagocytic capacity[148]. According to our data, the decrease in microglial marker brought on by prolonged stress exposure may be contributing to impaired microglial functioning.

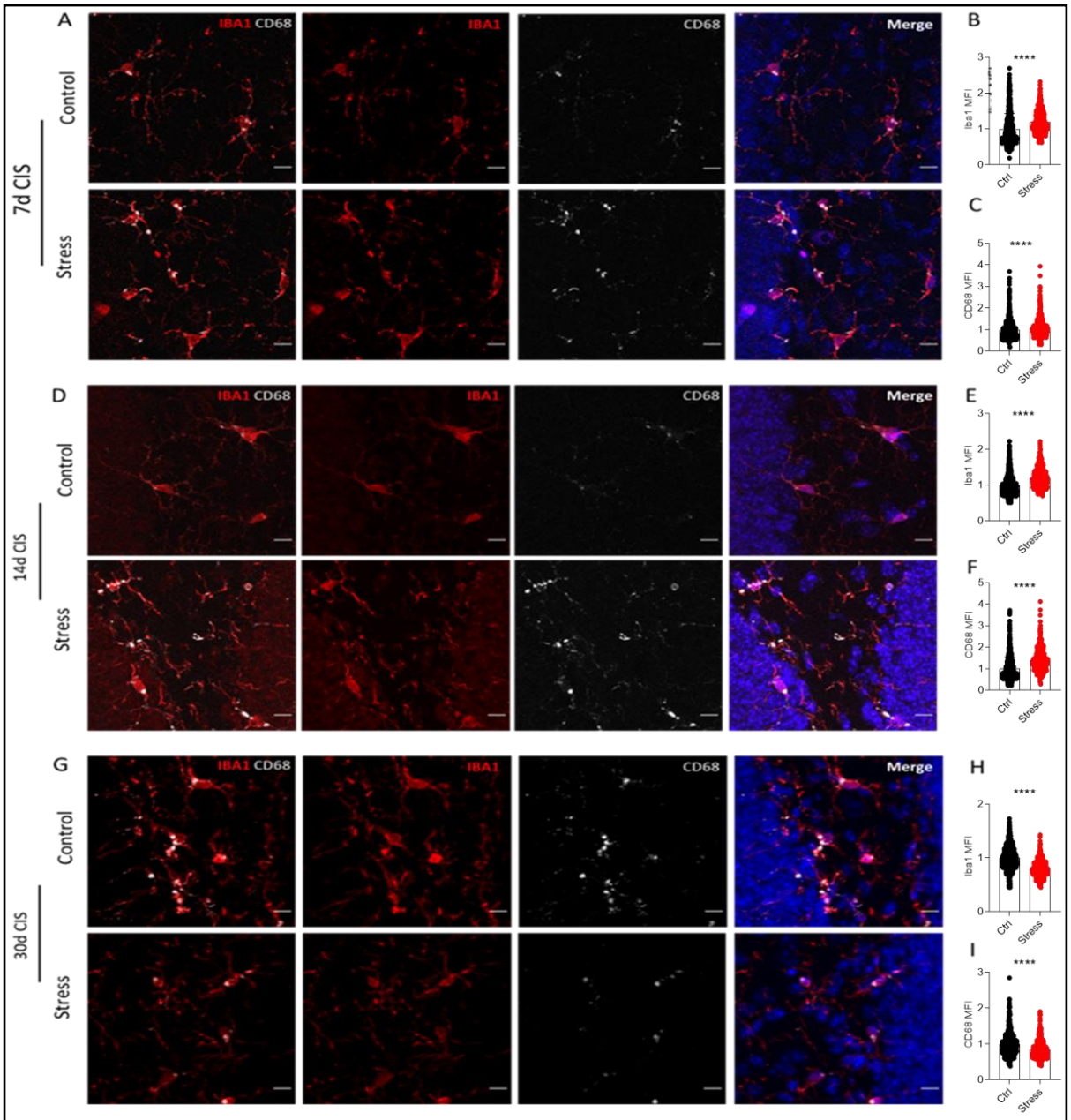


Figure.3.4 Microglial activation in response to stress during the course of chronic immobilization stress. (A, D and G) Representative image of microglia stained with Iba1 and CD68. (B-C, E-F and H-I) Quantitation of mean fluorescence intensities of Iba1 and CD68 of (A, D and G).(J and K) Iba1 and CD68 mean fluorescence intensity (MFI) in stress group normalized to control across the stress regime. Scale bar-10µm. Counts are from three to four mice, each dot represents a microglia and error bar represents SD, Mann-Whitney test; ****$p < 0.0001$.

Impact of stress on the adult hippocampal neurogenic program

Nestin positive neural precursors cells comprises RGLS and IPCs in the SGZ. These precursor cells are dedicated to the decision of neuronal fate, where they develop into neuroblast (proliferating population) and immature neurons, and finally these immature neurons mature into granule neurons, and they functionally integrate into the DG circuit and participate in hippocampal functioning. Usually, it takes 4-6 weeks for a neural precursor to develop into a mature granule neuron. The majority of cells die during the neuroblast/immature neuron stage, and only a small number of cells survive to the mature granule neuron stage to contribute to dentate gyrus function[70].

Stress is known to inhibit the adult neurogenic program[45–47,94,149]. Not only it causes reduction in proliferation of neural precursors and immature neurons, but also affects the dendritic morphology of newborn neurons[93]. These pertubation caused by stress to adult neurogenesis results in impaired behaviour associated with cognition and mood regulation.Recent studies show that enhancement in production of new born neurons alleviates some of the effects of stress[100]. The newborn granule neurons inhibit the activity of mature granule cells in ventral DG that respond to stress stimuli, enabling stress resilience response[39]. Newborn neurons are also required for the effective action of antidepressant drugs in case of depressive disorders[97]. Investigating the changes in the neurogenic progression that occur during the first and protracted phases of stress exposure is essential to understand the role of the neurogenic program during the course of stress.

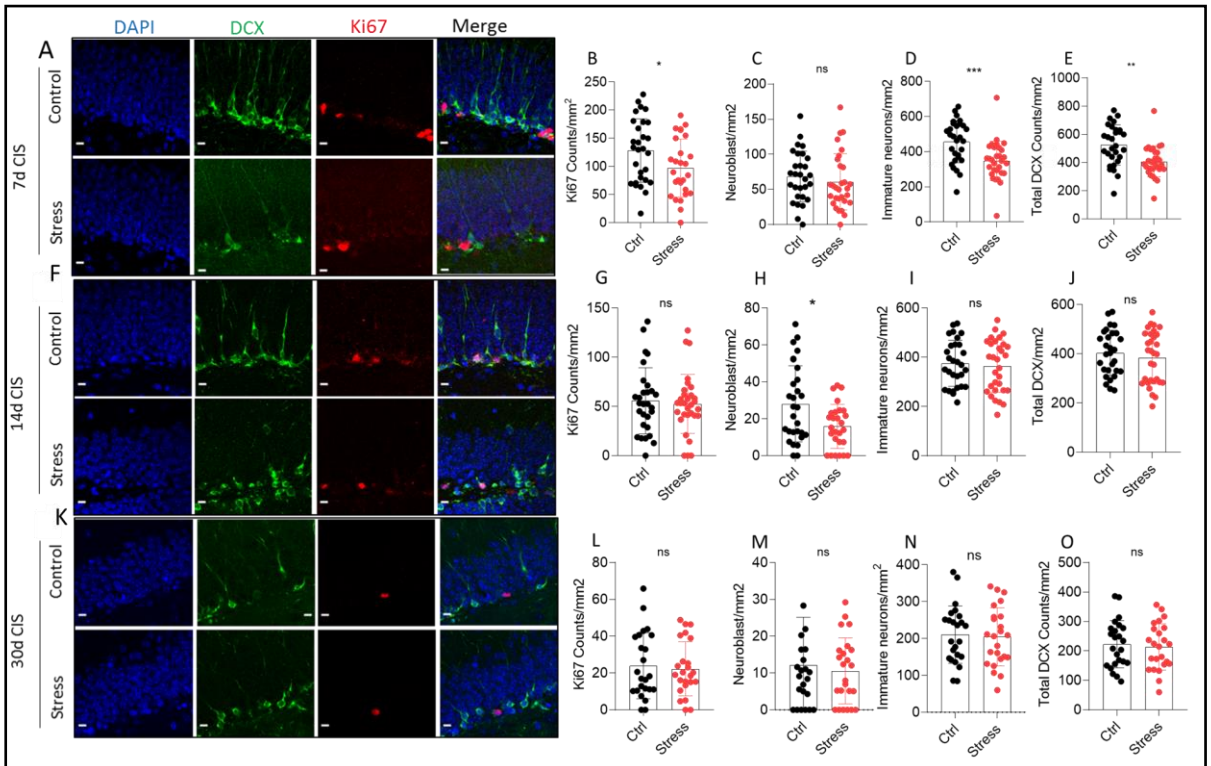


Figure.3.5. Impact of chronic immobilization stress on neurogenic population in adult brain hippocampus (A, F and K) Representative images of 7 days, 14 days, and 30 days CIS for Ki67 and DCX staining. (B-E) Quantification from (A), (G-J)Quantification from (F), (L-O) Quantification from (K) for total proliferation (Ki67+ve), neuroblast (Ki67/DCX+ve), Immature neurons(Ki67-DCX+) and total DCX+ cells (neuroblast plus immature neurons). Scale bar - 10µm. Counts are from three to four mice, each dot represents an image frame from a brain, and error bar represents SD, Mann-Whitney test; *P<0.05, **P < 0.01, and ***P < 0.001 and ns= non-significant.

To study the effect of ongoing chronic immbolization stress on adult hippocampal neurogenic population, we examined the following cell populations within the SGZ: total proliferating cells, neuroblast cells and immature neurons. In order to identify these specific populations we stained the brain sections with Ki67 to identify total proliferating cells. We identified neuroblast as Ki67+DCX+ cells, immature neurons as Ki67-DCX+ cells and also counted total DCX+ve cells which includes both neuroblast and immature neurons. In acute phase, at 7 days of CIS, we observed significant decrease in total proliferation (Fig3.5B) and no difference in neuroblast cell population, but immature neurons and total DCX+ve population is decreased in stress when compared to controls (Fig3.5D and E).. Whereas we do not see any difference in total proliferating population, , immature neurons or total DCX+ve cells in 14 days and 30 days of stress (Fig3.5G-O). But, we observe decrease in neuroblast population in 14 days of stress (Fig3.5H).

Effect of chronic immobilization stress on hippocampus-dependent cognition

Hippocampal adult neurogenesis is very sensitive to environmental stimuli. It plays important role in learning and memory, pattern separation and stress resilience. Depending on the type of environmental stimuli, adult neurogenesis can be modulated in positive or negative manner. Enriched environment and voluntary exercise is known to enhance production of newborn neurons[41,107]. Whereas adverse conditions like ageing, stress and neurodegeneration has a negative impact on adult neurogenesis[150,19,75].

To test the effect of chronic stress on hippocampal functioning, we decided to test spatial memory using Y-maze. In Y-maze test, animals were trained to explore one arm of Y-maze, while the other arm was kept closed. In the test-phase both the arms were open and mice

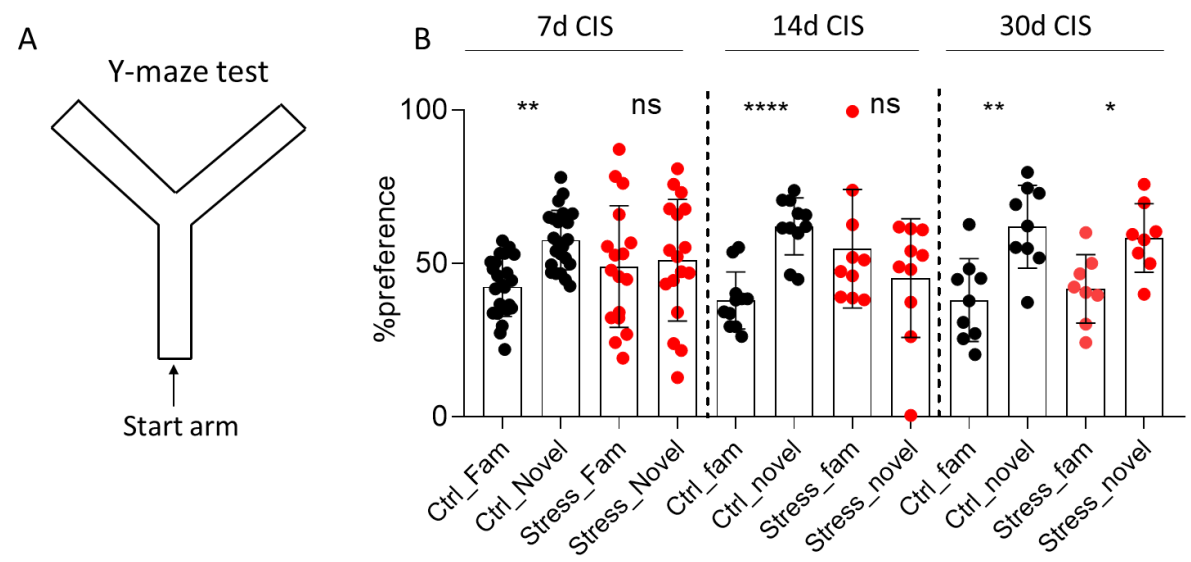


Figure.3.6. Hippocampus dependent spatial memory test in 7 days, 14 days, and 30 days of chronic immobilization stress. (A) Diagram of Y maze setup used for testing spatial memory. (B) Percentage preference for novel arm during test shows deficit in spatial memory.7d CIS (Ctrl N=23 and Stress N=17), 14d CIS (Ctrl N=11 and Stress N=10) and 30d CIS (Ctrl N=9 and Stress N=8) each dot is an animal ,Mann-Whitney test, ***p<0.0001, ns= non-significant. This test includes WT animals from two transgenic background (NestinCreERT2 and DcxCreERT2).

were allowed to explore. The arm which was closed during training becomes the novel arm during test-phase, while the arm which was open is used as the familiar arm. Mice have an innate tendency to prefer novelty, so a mouse with normal spatial memory will remember the training arm as familiar and prefer to explore the novel arm during the test-phase. The amount of time a mouse spends with the novel or familiar arm is calculated as percentage preference for familiar or novel, wherein a reduction in preference for novel arm exploration is taken as an indicator of defict in spatial memory.

We observed that in shorter stress regimes (7 days and 14 days) the stress mice showed deficits in spatial memory, demonstrating reduced preference for novel arm exploration. Suprisingly, when we perofrmed the same test on 30 days stressed animals, they showed no deficits in spatial memory, performing as good as controls, indicating rescue of memory deficit at d30

Chapter 3: Discussion

Stress is a physiological state that arises from exposure to aversive environmental stimuli, and can disrupt the body's normal homeostatic functions[151]. Stress is known to negatively impact the proliferation in the adult dentate gyrus[45–47,96], which can in turn affect adult neurogenesis and mood and memory[30,94,139]. However, recent research has shown that newborn neurons in the dentate gyrus of the hippocampus play a critical role in the brain's ability to cope with stress[39]. Thus, an elucidation of how stress influences cells in neurogenic niche would be critical to elucidating the role of adult NSC in stress resilience.

Our data from chapter 2 show that adult neural stem cells have latent inflammatory potential, that is kept suppressed under the regulation of a transcription factor Tcf4. Tcf4 deleted neural stem cells acquire inflammatory state and cause inflammation in neurogenic niche[54]. But the physiological relevance of this latent inflammatory potential remains elusive. We hypothesized that Tcf4-mediated regulation of adult neural stem cells may play a role in stress induced regulation of adult neurogenesis.

In this study, first we examined Tcf4 expression in adult neural stem cells during a long period of chronic immobilization stress. Interestingly, we observed dynamic expression of Tcf4 in adult neural stem cell during different stages of stress. During the initial phase of chronic immobilization (7-14 days), we observed a reduction in Tcf4 expression in Sox2 positive progenitors in the Dentate Gyrus. By day 30, the difference in Tcf4 expression between controls and stress animals was no longer present. The normalization of Tcf4 expression during longer stress duration suggests that with prolonged stress exposure the neural progenitors potentially undergo some molecular adaptation to bring back normal functioning. Next, we asked, whether the reduction in Tcf4 expression in Sox2+ progenitors could trigger inflammation. To investigate this, we performed RNA-Seq of neurospheres made from control and stressed animals. Gene-Ontology of top-50 upregulated genes show pathways associated with extra-cellular matrix organization, endothelial cell regulation and inflammation. This includes Tlr2, which is involved in regulating IFN-β and IL-6 production in conditions of viral infection[144,152,153], Fam167a is known to activate NF-κB in chronic myeloid leukemia and also been associated with systemic sclerosis[154,155], cbfa2t3 is a co-regulator of cancer stem cell proliferation and differentiation in acute myeloid leukemia[156,157] and Megf11 is involved in breast cancer re-occurrence[143,158].These genes are associated with progression and regulation of inflammatory pathways in disease. We also, observed downregulation of long-non coding RNAs like Malat1, which plays important functions in cell proliferation[145,159], Mir9.3h[146], and Sox2ot[147,160,161] are involved in cancer cell proliferation. These findings are consistent with Tcf4-KO studies, which show that the loss of Tcf4 triggers an inflammatory transcriptome and reduces proliferation. In a similar vein, we see that acute stress also reduces Tcf4 expression in neural precursors, inhibits proliferation, and manifests an inflammatory transcriptome. This decline in long non-coding RNA, which is linked to the regulation of proliferation, validates our findings that stress inhibits proliferation. Next, we asked, whether this decrease in Tcf4 induced inflammatory signature leads to inflammation in the neurogenic

compartment. Inflammation is known to be detrimental for progression of adult neurogenesis[40,54,78,104,162]. Microglia are the first responder to any inflammation in the brain parenchyma[163], and microglial activation is often used as an indicator of inflammation in brain[164]. Activated microglia upregulates expression of genes like Iba1 and CD68, so we checked these markers to examine microglial activation in SGZ during different stages of chronic immobilization stress. Our findings show microglial activation in the initial phases of stress (7 and 14 days). Whereas, in prolonged stress, we observe downregulation of microglial activation markers. Activation of microglia is in coherence with our observation of reduced Tcf4 expression, which could potentially trigger an inflammatory transformation of the adult NSC in the initial phase of stress. However, the decrease in microglial activation markers can be possibly inferred as microglial dysfunction due to prolong exposure to stress[148] . We hypothesize that a reduction in Tcf4 expression could trigger the latent inflammatory state in adult neural stem cells, which in turn could produce secretory factors to cause inflammation in the neurogenic niche, eventually leading to microglial activation.

Our findings suggest that a reduction in Tcf4 may be responsible for upregulating inflammatory genes in neural progenitors. We also assessed the impact of stress on progression of adult neurogenic program, by quantifying total proliferating cells, neuroblasts and immature neurons. Immature neurons play a vital role in dentate gyrus functioning. Immature neurons are distinctive from mature granule neurons with regards to high excitability, low input specificity and low GABAergic inhibition [165]. This functional distinction of immature neurons helps the mature granule neurons of dentate gyrus for sparse decoding. Our data shows that 7 days of acute stress affects total proliferating cells and causes decrease in immature neurons. However, we do not see any difference in neural precursor cells, and immature neurons in mid and prolonged period of stress (14 and 30 days). To test the effect of this on cognition we performed hippocampus-dependent spatial memory test. We observed spatial memory deficits in 7 days and 14 days stress group animals; however, 30 days stress group showed no deficit in spatial memory, suggesting that the spatial memory deficit that occurs due to stress at early time points is ameliorated when the stress is continued for a prolonged period. Interestingly, the rescue of cognition, coincided with reinstatement of Tcf4 expression in adult NSC.

Together, our results demonstrated a dynamic pattern of Tcf4 expression in adult neural progenitors, suggesting a relationship between stress-induced regulation of Tcf4 and potential inflammatory transcriptome in adult progenitors, during the initial phase of stress. This in term seems to modulate the hippocampal neurogenic program, potentially for an adaptive response in longer duration of stress. Further investigation will be needed to better understand the adaptive changes in the neurogenic program triggered by Tcf4-mediated regulation of adult NSC during stress.

Overall, this body of work highlights a possible role for Tcf4 in adult NSC and the hippocampal neurogenic program during stress response.

Methods and materials

Mice

All animals were housed, bred, and used according to the protocols approved by the Institutional Animal Care and Ethics Committee. The *Nestin*-CreERT2 transgenic line[166] was a generous gift from R. Hen, Columbia University Medical Center, New York, USA. For conditional targeting of Tcf4 in *Nestin*-expressing progenitors in the adult brain, Tcf4-flox line [167,168] was crossed with *Nestin*-CreERt2 line to produce *Nestin*-CreERt2; Tcf4f/f (icKO) or *Nestin*-CreERt2; Tcf4+/+ (WT). For genetic lineage tracking in neurosphere studies, the Rosa-*flox*-STOP-*flox*-eGFP reporter from The Jackson Laboratory (strain 32037-JAX) was further crossed on to *Nestin*CreERt2; Tcf4 flox line. All animals were bred and kept at the Animal Care and Resource Centre.

Mice treatment

Tamoxifen (Sigma-Aldrich) was used at 5 mg/35 g body weight once a day for 4 days for *Nestin*-Cre induction in WT and icKO adult animals at the age of P45-60. Hydroxy-tamoxifen treatment (dose?) was used to induce the eGFP expression and Tcf4 deletion in neurosphere cultures, and mice were euthanized as per IAEC approved protocol for molecular studies at various time points

Immunostaining

Animals were deeply anesthetized and transcardial perfusion was performed with chilled phosphate-buffered saline, followed by 4% paraformaldehyde (PFA). Brains were kept in 4% PFA for overnight fixation at 4°C, and 30-µm sections were sliced using vibratome. For immunostaining, floating sections were first blocked (10% normal goat serum, 1% bovine serum albumin (BSA), 0.1% Triton X-100, and 100 mM glycine) for 1 hour at room temperature (RT) and stained with primary antibody (in 1% normal goat serum, 0.1% BSA, 0.1% Triton, and 100 mM glycine) overnight at 4°C. Sections were then washed and incubated with fluorophore-conjugated secondary antibody for 1 hour at RT in dark, before mounting and imaging. Fluoromount-G mounting media (Southernbiotech #0100-01) was used for mounting. Primary antibodies used were DCX (Millipore #2253 and Abcam #18723), CD68 (AbD Serotec #MCA1957), Tcf4 (Abcam #217668), Lgals3 (Invitrogen #50-5301-80), MBP (SySy #295004), Tuj1 (Promega #G7121), Sox2 (Abcam #79351, #97959) , Ki67 (eBioscience #14-5698-82) and cleaved caspase-3 (Cell Signaling Technology #9664).

Confocal imaging and analysis

Sections were imaged using a Leica SP8 confocal microscope at 20x, 40x or 63x objective. Images were analyzed using Fiji and Imaris. For cell number quantitation in Fiji, individual cells of interest were marked as region of interest (ROI) by going through the z-stacks of individual images. The cell numbers were then normalized with the area of DG blades for that section, as marked using Fiji ROI function. For Imaris counts analysis, the spot module was used. for. At least three to four coronal sections from each mouse were analyzed, and three to five mice per genotype/group for each experiment were used. For neurosphere cultures, cells were quantified from multiple images from individual animal's neurosphere culture and number of neurosphere per well. For altered morphology quantification, well-separated GFP+ Tuj1+ cells whose primary process could be properly identified to mark as stalky were counted. Unpaired student's t-test or Mann-Whitney test was used for statistical analysis for all immunofluorescence-based quantification.

Neurosphere culture

For *in vitro* deletion and functional assays, *Nestin*-CreERt2/TCF4f/f or wt brains of P10-13 mice were used, since the number of neurospheres obtained from 2- to 3-month-old mouse brain was very limiting. The use of P10-13 brains for neurosphere cultures was justified on the basis of recent transcriptomic revelation[128] establishing that neural progenitors from P5 to adulthood cluster together as one group with regard to their transcriptome. We adapted previously published protocol[169]for hippocampal neurosphere culture as follows: Hippocampi from individual brains were micro-dissected and digested in PDD [papain (2.5 U/ml), Dispase II (1U/ml), and DNase I (10 µg/ml)] for 30min in a shaker incubator at 37°C and 170 rpm with intermittent trituration. The digestion was stopped by adding complete Neurobasal media (Neurobasal supplemented with B27, GlutaMAX, and Anti-Anti). The cell suspension was strained through a 40-µm sieve and washed in wash buffer (30 mM glucose, 2 mM Hepes, and 26 mM NaHCO3 in 1× Hanks' balanced salt solution) by centrifuging cells at 130g for 5 min at 21°C. The cell pellet was resuspended in 20 ml of complete Neurobasal media, along with EGF and FGF (20 ng/ml), and seeded in a flat-bottom 96-well plate. For Cre-induced deletion in neurosphere cultures, 0.5 µM 4-OH-Tmx (Sigma-Aldrich H7904-25MG) was added in cell suspension at the time of seeding. The cells were incubated in a tissue culture (TC) incubator with 5% CO2 for 5 to 10 days. Neurospheres were counted at DIV7 and presented as counts per well for each brain. For immunofluorescence studies, neurospheres were transferred on Poly-D-Lysine–coated sterile coverslips in a 24-well plate and incubated for 48 hours in neurosphere growth media. The cells were then fixed in 4% PFA for 15 min at RT and stained.

Secondary neurosphere

For secondary neurosphere culture, primary neurospheres were collected and spun down at 200g for 10 min. The cell pellet was then gently triturated in neurosphere growth media using a 200-µl tip. The single-cell suspension was then spun down and resuspended in fresh neurosphere growth media supplemented with growth factors and seeded into a fresh 96-well plate. Secondary neurospheres were counted at DIV5.

Differentiation of neurosphere

Neurospheres were gently triturated to make single-cell suspensions as stated above. The single-cell suspension was then plated on Poly-D-Lysine–coated sterile coverslips in a 24-well plate. After 1 hour of incubation in the TC incubator, when the cells had adhered on to the coverslip, the medium was replaced with fresh differentiation medium without the growth factors EGF/FGF. The differentiation culture was allowed to grow for 3 days in the TC incubator. Cell was fixed at DIV3 in 4% PFA for 15 min at RT and stained for immunofluorescence studies.

Co-culture experiments

For co-culture experiments, cultures from *Nestin*-CreERt2/TCF4f/f brains, with or without 4OH-Tmx, were used for detection of WT and icKO neurospheres when co-cultured in the same well, based on tamoxifen-induced GFP expression only in icKO. First, the neurospheres cultures were allowed to grow until DIV6 with (icKO) or without 4OH-Tmx (WT). At this point, the 4OH-Tmx–treated wells showed GFP+ve neurospheres, whereas the vehicle-treated were not GFP+ve. The neurospheres were then gently collected in a tube and centrifuged at 130g for 5 min in culture media for wash. The cell pellets from WT and icKO tubes were then resuspended very gently in fresh media, to not dissociate the spheres, and seeded together for co-culture. Co-cultured WT and GFP-expressing icKO neurospheres were counted and imaged 2 days after the co-culture was set up. WT and icKO neurospheres in “no-mix/pure” culture wells were used as controls. The number and morphology of neurospheres in the co-cultures were compared with “pure”-cultured WT neurospheres in the same experiment.

Media swap experiments

Brains from *Nestin*-CreERt2/TCF4f/f and *Nestin*-CreERt2/TCF4wt mice were cultured with 4OH-Tmx as described above, except that each brain was seeded in 12 wells of a 24-well plate. On DIV6 to DIV7, neurospheres from six WT wells and six icKO wells were gently collected in a tube and spun down at 200g for 10 min. The supernatant was collected in a fresh tube and labeled as WT or icKO media. The icKO media was then used to resuspend the WT neurosphere pellet and the WT media used for icKO neurospheres. The transfer and resuspension were done very gently using a 200-µl tip to avoid dissociation of neuropsheres. The neuropsheres with exchanged media were then seeded into a fresh 24-well plate, to

avoid effects of any residual media from the original cultures. For comparison of neurospheres in exchanged media with those in the original media, controls from the same experiment were used. For this, the remaining six WT wells' and six icKO wells' neurospheres were similarly collected and spun down but resuspended in their own media. These were then plated in the same fresh 24-well plate as the "exchanged media" neurospheres. All the replated neurospheres were allowed to grow for 48 hours, after which they were counted and imaged for analysis. Furthermore, the WT, icKO, and "media-exchanged"-WT neurospheres were put for secondary neurosphere culture in fresh neurosphere growth media to examine whether the affected WT neurospheres treated with icKO neurosphere media were able to recuperate when put back in normal media.

RNA sequencing

RNA was isolated from neurospheres , using a Qiagen Micro Plus kit as per the manufacturer's instructions. Sample quality control, library preparation, and RNA-seq were done by the Next-Generation Sequencing facility at the institute. RNA quality was assessed using the Bioanalyzer. cDNA libraries were prepared by using New England Biolabs stranded mRNA library prep kit as per the manufacturer's instructions. PolyA selection method was used to enrich mRNA. Library quality was analyzed with the Bioanalyzer. Next-generation sequencing of libraries was performed at the Next-Generation Sequencing facility at the institute on the Illumina HiSeq 2500 platform for 1 × 100 bp at ~20 to 25 million reads per sample.

RNA- sequencing data analysis

The public server at usegalaxy.org was used to analyze the sequencing data. Briefly, after cutting the adapter sequence and initial quality check for sequence reads, Hisat2 was used to align the reads to Mus musculus mm10 reference genome, and feature counts were generated. For differential gene expression, the DESeq2 tool was used, and the output was annotated using "annotateMyID" feature in Galaxy. This differential gene expression output was used for volcano plot using R. Normalized feature counts were used to plot heatmap using the web tool "heatmapper." Enriched ontology cluster analysis was done using the public web tool at metascape.org[170]).

Behaviour assays (Novel Location Recognition and Y-Maze spatial memory test)

The animals were housed in individually ventilated cages with easy access to food and water with 14 hours of light and 10 hours dark cycles, respectively. Before the start of experiment, animals were individually handled for 5 min for 3 days.

Novel Location Recognition Test

On day 1, animals were acclimatized with the arena in which the experiment was to be performed. For this, the animals were released individually in arena for 5 min and then placed back into home cages. On training day (day 2), two identical objects were placed in the box near a wall with a triangular black sticker used as a visual anchor. The training was performed for 10 min, where animals were released individually into the arena in the corner of wall opposite to the objects and allowed to explore the objects freely for 10 min. After training, animals were placed back into their home cages. On the test day (day 3), one of the two objects were displaced to a new location (novel location) with respect to the previous location. The animal was then reintroduced in the arena as during training session and allowed to explore the objects for 5 min. NLR was done 9 to 11 days after tamoxifen treatment. The training and test sessions were recorded with top head camera, and the videos were manually analyzed for exploration time at each object as a proxy for location recognition. The time spent exploring the old versus newly located object was calculated. For this, the time spent in active sniffing of the object was taken as exploration. Rearing with head above the object level and climbing onto the object were not counted as "exploration." Percentage exploration was calculated as (time spent on one object/total time spent on both objects) × 100. Statistical analysis was done using Mann-Whitney test.

Y-maze spatial memory test

On day 1, animals were acclimatized with the arena in which experiment was to be performed. Both training and test performed on same day, with an interval of 1.5 hours. Animals were trained on Y maze with one arm closed and mice were allowed to explore the open arm for 5 min and after 1.5 hours post training, animals were reintroduced into y maze with both arms open for exploration for 5 min. In test the arm which was closed during training will become novel arm and other is familiar arm. The training and test sessions were recorded with top head camera, and the videos were analyzed using ANYMAZE software. The time spent exploring the novel vs familiar arm is quantified. Percentage exploration was calculated as (time spent in one arm/total time spent on both arms) × 100. Statistical analysis was done using Mann-Whitney test.

Chronic immobilization stress (CIS)

CIS is an established, as described in Jung, S, 2020; Yun, J 2010; Woo, H, 2018[94,140,171], and was adapted from previously published studies. Briefly, after three days handling for acclimatization with the experimenter, mice were divided into control and stress groups. The stress group mice were individually immobilized in decapicones (with breathing perforation at the tip of the decapicone), and kept in horizontal position for up to 6hrs every day for the duration of the experiment. The decapicone was placed in the behaviour room work-station, outside the IVC rack in a fresh bedding cage, and away from other animals for avoiding vocal distress cues to other animals. Animals of both gender were used for this paradigm.

Statistical Tests

Students' T test and Mann-Whitney test was performed for all quantifications. Graph Pad Prism was used for plotting the graphs and statistical analyses.

Table of resources

Materials	Company	Catalogue
Neurosphere culture		
Neurobasal medium	Invitrogen	21103-049
50X B27 supplement	ThermoFisher	17504-044
GlutaMAX supplement	ThermoFisher	35050-061
Antibiotic-Antimycotic (100X)	ThermoFisher	15240-062
HBSS (10X)	ThermoFisher	14185-052
Papain	Sigma	P4762
Dispase II	Sigma	D4693
DNase I	Sigma	10104159001
EGF (mouse)	ThermoFisher	PMG8041
FGF (mouse)	ThermoFisher	PMG0035
Poly-D lysine	Sigma	P6407
Glucose	Fisher Scientific	15405
Sodium Hydrogen Carbonate	Qualigens	Q14015
HEPES buffer	Invitrogen	15630-080
4-Hydroxy-Tamoxifen	Sigma Aldrich	H7904
RNA-Sequencing		
RNeasy plus micro kit	Qiagen	74034
Stranded mRNA library prep kit	NEB	N/A
Antibodies		
DCX	Millipore/ Abcam	2253/18723
CD68	AbD Serotec	MCA1957
TCF4	Abcam	217668
LGALS3	Invitrogen	50-5301-80
MBP	SySy	295004
TUJ1	Promega	G7121
Cleaved caspase-3	CST	9664
KI67	eBioscience	14-5698-82
SOX2	Abcam	79351, 97959
Goat anti-rabbit IgG, Alexa Fluor 647	Invitrogen	A21245
Goat anti-rabbit IgG, Alexa Fluor 568	Invitrogen	A11011
Goat anti-mouse IgG, Alexa Fluor 647	Invitrogen	A21235
Goat anti-mouse IgG, Alexa Fluor 568	Invitrogen	A11031
Goat anti-guineapig IgG, Alexa Fluor 647	Invitrogen	A21450
Goat anti- guineapig IgG, Alexa Fluor 568	Invitrogen	A11075

Goat anti- chicken IgG, Alexa Fluor 488	Invitrogen	A11039
Goat anti- chicken IgG, Alexa Fluor 568	Invitrogen	A11041
Goat anti- chicken IgG, Alexa Fluor 647	Invitrogen	A21449
Miscellaneous reagents		
Tamoxifen	Sigma Aldrich	T5648-5g
Corn Oil	Sigma Aldrich	8001-30-7
Ethanol	Merck	00983
Normal Goat Serum	Gibco	16210072
Bovine Serum Albumin	Sigma Aldrich	A9418-50G
Triton X-100	Sigma Aldrich	X100-100ml
Glycine	Fisher Scientific	12835
Etoposide	Sigma Aldrich	E1383
DMSO	Hi-Media	MB058
Mouse strains		
B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J	JAX	007914
STOCK Gt (ROSA)26Sortm1.1(CAG-EGFP)Fsh/Mmjax	JAX (MMRRC)	032037-JAX
Nestin-CreERT2 transgenic	Rene Hen Lab	DOI: 10.1016/j.neuron.2011.05.022
Tcf4 Flox	Bergqvist et al., EuroJImmuno, 2000	
Equipment		
Confocal Microscope	Leica	SP8
Hiseq 2500	Illumina	SY-401-2501
Bioanalyzer	Agilent	Bioanalyzer 2100
Software		
Imaris	Bitplane	Version 9.1.2
Fiji	Schindelin et al. Nat. methods,2012	https://imagej.nih.gov/ij/
ANYMaze	Stoelting Co.	Version 6.32
GraphPad Prism	Dotmatrics	n/a

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