

# COX-1/EP1R Inhibition Ameliorate Deficits in Adult Neurogenesis and Social Interaction via Alleviating NLRP3/ IL-1 $\beta$ Activation During Hypobaric Hypoxia

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## Research

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# Abstract

## Background

Low oxygen environments like hypobaric hypoxia (HH) are common nodes to various diseases: characterized by neuroinflammation, which is detrimental to the structural and functional aspects of hippocampal circuitry. Various hypoxic conditions also lead to elevation of NLRP3 mediated neuroinflammation that may contribute to cognitive deficits. Components of neurogenic niches like microglia and astrocyte are largely affected by neuroinflammation; however, a systematic investigation of the impact of NLRP3 mediated neuroinflammation on components of neurogenic niche during hypoxia (HH) remains elusive.

## Methods

In this study, we simulated cerebral hypoxia via decreasing partial pressure of oxygen(HH). The effect of hypobaric hypoxic (1, 3 and 7 day at 25000 ft) on social memory, anxiety, adult neurogenesis change in the inflammatory milieu in DG was explored in detail. We explored the efficacy of COX-1 inhibitor (Valeryl salicylate, 5mg/kg/day,i.p), and EP1R antagonist (SC19220, 1mg/kg/day,i.p) on NLRP3 mediated neuroinflammation and associated maladies during HH.

## Results

We observed that HH exposure induced alteration in social and anxiety-like behavior post 7 day exposure along with perturbation in levels of BDNF, Serotonin and adult neurogenesis in the DG right from day 1. Moreover, significant elevated NLRP3, caspase-1, and IL-1 $\beta$  levels are observed during HH from day 1. Concomitantly, a notable increase in the COX-1/EP1 pathway in both activated microglia and astrocyte in DG was evident after 3HH exposure. Pharmacological COX-1 inhibitor and EP1 antagonist counteract the detrimental effects of HH exposure on social memory, adult neurogenesis, and NLRP3 inflammasome induction.

## Conclusions

Thus, our data showed induction of the COX-1/EP1 pathway in glial cells is detrimental to adult neurogenesis and social memory, raising possibility that the COX-1/EP1 pathway as a plausible target for inflammasome related neurogenesis impairment.

## Highlights

- HH for 7days exhibits deficits in social and anxiety like behavior in rats
- Deficits in adult neurogenesis followed by reduction in BDNF, Serotonin and pCREB
- HH induce NLRP3 signaling followed by glial cell activation and neuroinflammation
- EP1R at downstream of COX-1induced pathology in glial cells during HH

- COX-1/EP1R inhibition mitigate HH induced detrimental effects

## Background

The adult brain continuously generates new hippocampal neurons, especially in the subgranular zone (SGZ) of the dentate gyrus (DG) each day. Neuronal progenitor cells NPCs migrate to the granule cell layer of DG, where they attain maturation, integration with the neuronal circuitry and contribute to behavioral response. The formation and integration of these adult neuronal cells are negatively regulated by environmental factors like- stress, aging, inflammation, glucocorticoids and glutamate toxicity [1]. One of such factor is hypoxia that has been shown to regulate adult neurogenesis in various brain ailments differentially. A decrease in partial pressure of oxygen leads to cerebral hypoxia that elicits psychological symptoms like Déjà vu, emotional lability, hallucinations, depression, anxiety, and suicidal ideation contribute to ill mental health experienced at altitude [2, 3,4]. Exposure to chronic hypobaric hypoxia reduces serotonin levels in the brain [5], which is pivotal for mood, emotion regulation, anxiety, and social behavior [6,7]. Neuroinflammation is one of the emerging causal factors contributing to behavioral alterations accompanied with deficits in adult neurogenesis. Sub-granular (SGZ) zone in DG represents neurogenic niche or local microenvironments that permit and support neurogenesis. Microglia, astrocyte, and endothelial cells are the critical components of the neurogenic niche. Microglia continuously survey the healthy brain in ramified morphology. In response to stress, there are functional and morphological changes in microglia as they are activated [1, 8] and alter neurogenesis by secreting proinflammatory cytokines, neurotoxic substance like nitric oxide(NO), ROS, arachidonic acid (AA) and its metabolites. Microglia make a significant contribution to the niche by balancing levels of pro- versus anti-inflammatory conditions, which can facilitate or suppress neurogenesis [1]. Microglia in co-ordination with progenitor cells regulate neurogenesis via phagocytosis in the adult hippocampus [1]. Our previous study reported that hypobaric hypoxia activates microglia, astrocytes, and endothelial cells followed by neuroinflammation and neurodegeneration in the DG but how microglia regulate adult neurogenesis during hypobaric hypoxia is yet to be explored. The NLRP3 inflammasome, also known as cryopyrin or NALP3, has an essential role in the damage caused by microglial mediated inflammation. NLRP3 induced drastically during hypoxia and associated disorder like stroke, cerebral ischemia, neonatal hypoxia [9,10].

One of the molecules that have drawn interest in cerebral hypoxia is COX due to its predominant localization in microglia, neuron and endothelial cells. Its ability to synthesize prostaglandins immediately, raise the possibility of its involvement and potential factors that may affect adult neurogenesis in the DG. Indomethacin, which blocks both COX-1 and COX-2 found to restore neurogenesis and decrease microglial activation after irradiation [11] indicates role of PG pathway. The relative contribution of the isoform of COX, COX-1, and COX-2 on adult neurogenesis largely depends on the model system and extent of neuroinflammation [12]. PGE2 produced by enzymatic reaction of COX works via its four receptors EP1, EP2, EP3, and EP4 in the brain. There are various behavioral paradigms associated with EP1 expression in the brain. EP1 receptor pharmacological and genetic deletion make animals susceptible to behavioral disruption upon environmental and social stress [13]. The effect of COX-1 specific inhibition on adult neurogenesis and its active PGE2 downstream receptor is far from

being clear specially during HH. Recent research has focused on inhibiting the activation of inflammasomes and thus reducing the maturation of proinflammatory cytokines such as interleukin (IL)-1 $\beta$  and IL-18. NSAIDS like Fenamate that inhibit COX-1 and COX-2 are reported to be specific NLRP3 blocker that can cross BBB and FDA approved [14]. Recently COX inhibitors are reported to inhibit NLRP3 activation in various modalities like pancreatitis, ischemia [15, 16,17].

Despite light shed on deficits of hippocampal-dependent tasks during HH exposure [18, 19, 20, 21] the effect of HH exposure on social interaction in rats is still an enigma. Effect of HH exposure on neuro-inflammation has been discussed earlier with primitive evidence but how glial cells activated and leads to perturbation of neurogenic niche is still unknown. The role of NLRP3 mediated inflammation in adult neurogenesis, social and anxiety like behavior during HH exposure is still very much vivid. The effect of COX-1 specific inhibition on adult neurogenesis and its active PGE2 downstream receptor is far from being clear specially during HH. The findings led to the proposal that microglial COX-1 via EP1 receptor induces NLRP3 mediated neuroinflammation, activation of glial cells, and perturbation of neurogenic niche in DG, culminates into defective social behaviour followed by elevation in anxiety during HH.

## Methods

### Experimental animals

Adult male *Sprague Dawley* rats of weight 230-280 grams were used for each experiment in this study. Optimal housing conditions (22  $\pm$  2  $^{\circ}$ C, 54–60% humidity, 12 h light-dark cycle) were provided. Experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the Indian Government and were approved by our Institutional Animal Ethics Committee (IAEC) IAEC/DIPAS/2015-22. Food pellets (Lipton India Ltd., India) and tap water were provided *ad libitum*. Animals were habituated well with the experimenter before behavioural study and sacrifice. Adult rats were randomly assigned into one of the following 7 groups: cage control (CC), 1 day of HH(1HH), 3 days of HH(3HH), 7 days of HH (7HH), 7HH with COX-1 inhibitor (7HH + ValS), and 7HH with EP1 R antagonist (7HH + SC19220). Specific groups and number of animals used in each experiment are mentioned in results and figure legends respectively.

### High altitude hypoxia chamber

The animals were stimulated with high altitude pressure using a specially designed animal decompression chamber (Seven Star Model). The ideal conditions like atmospheric pressure 282 mmHg, PO<sub>2</sub> 59 mm Hg, 28-30 $^{\circ}$ C, and relative humidity of 55-60% were maintained [7, 22, 23]. Rats were habituated at an altitude of 4572 m (15,000 ft) for 24 h prior to continuous exposure to HH at an altitude of 7620 m (25,000 ft) for 1, 3, or 7 days. A 12hr day-night cycle was adequately maintained. Food and water were provided *ad libitum* to rats. The schematic representation of experimental design provided in **Supplementary fig. 1**.

### Treatments

## Valeryl Salicyclate

Valeryl salicyclate, (V2289, 083K4622V, Sigma Aldrich, USA), a derivative of aspirin was injected intra-peritoneal at the dose of 5 mg/kg body weight intraperitoneally once daily for 7days as COX-1 inhibitor. The salt inhibitor was first dissolved in DMSO and then diluted in 0.9% NaCl to obtain the final concentration that was 20%. The selection of valeryl salicyclate dose was inconsistent with the previous usage in rats. [21, 24].

## BrdU hosphor

Rats from all groups were injected with BrdU (50021G, HMBG1870V), Sigma Aldrich, USA), a thymidine analog (50 mg/kg/day, i.p.), which incorporates into the genetic material during S-phase of cell division. BrdU was dissolved in sterile saline maintained at physiological pH (0.007N NaOH) [25, 26, 27].

## SC19220

SC19220 (8065, 125k1609V, Cayman Chemicals, USA) was given at the dose of 1mg/kg/day, i.p. to the rats. The dose was standardized from the range available in the literature [28, 29, 30].

## Assessment of social interaction behavior

Social recognition and novelty preference were evaluated using a three-chambered apparatus with minor modifications that appropriately fit the size of *Sprague Dawley* rats [31, 32, 33, 34]. This apparatus was made of Plexiglas dimensions: 50 cm x 50 cm x 40 cm and divided by walls, with an open middle section that allowed free access to each chamber. We habituate animals for 10 min daily with the whole instrument till rat explored it all (max. for 3 days). The movement of the experimental animals was recorded using SMART 3.0, PANLAB, Harvard Apparatus, USA. During trial 1, the experimental rat was allowed to explore between the chamber containing the wired cage (Empty) and another identical chamber containing stranger rat 1(Social). During trial 2, that was for another 10 min. The experimental rat was allowed the explore the chambers containing stranger rat 1(familiar) and stranger rat 2 (novel), and time spent in both the chamber was quantified. Social tendencies of test rat quantified using this test the principle behind the parameters a) the time spent with social stimuli versus empty enclosure and b) preference for novel versus already explored familiar stimuli. Contact behaviour was considered as the number of direct (active) contacts between the experimental rat and the wired enclosure housing or not housing the Stranger 1 or stranger 2, for each chamber individually. Direct contact between the experimental rat and the containment wired cup, or stretching of the body of the experimental rat in an area of 3-5 cm around the cup is counted as direct contact. After habituation rats were exposed to 7HH exposure and then tested again for same parameters.

## Elevated plus maze

Elevated plus maze (EPM) is a widely used test for measuring anxiety-related behaviour in rats [35, 36]. EPM is a plus-shaped instrument made up of four elevated arms 10cm wide and 50cm long,

perpendicular to each other, the two opposite closed-arm are equipped with 30 cm high walls. An overhead IR camera, coupled to a tracking system, ANY-Maze software (Stoelting, USA), was used to track the rat in the EPM. During this behavioral test, the rat was placed at the center of the apparatus to explore evenly lit all the arms freely maximum for 5min. The time spent in the open arm by the rat was calculated; to determine the anxiety level in the rat.

## **Immunohistochemistry**

Animals from all the groups were anesthetized deeply with urethane injection (1.2 g/kg body weight), fixed using ice-cold 4% paraformaldehyde (PFA) [18, 19]. IHC experiments were performed in 30µm, free-floating, coronal sections for every antibody. BrdU staining required pre-treatment with the solution (2 M HCl + Triton X-100 for 45 min at RT) for DNA denaturation and washing with PBS (pH 8.5) afterward. Denaturation followed by incubation with a rat anti-BrdU primary antibody details provided in Table 1.

Coronal brain section of the 30µm section of the dorsal hippocampus from the bregma -3.14 to -4.30 for all the staining protocol. Each section was washed with PBST (PBS with 0.1% Tween-20. Heating for 10 min was done for epitope retrieval in sodium citrate solution at (pH 6.0). Blocking was for 2 h in blocking buffer comprising 0.03% Triton X-100, 10% goat serum in PBS followed by incubation in an appropriate primary antibody for 40 h at 4<sup>0</sup> C followed by Fluorophore-conjugated with secondary antibodies (Alexa Fluor 488 and Alexa Fluor 633, Invitrogen, USA) for 2h at RT.

## **Sholl analysis**

Microglial morphology was analysed using a previously published protocol [21, 37]. Microglial cells in the DG region were selected (6–8 cells/section; n = 6) on 30µm hippocampal tissue sections prefixed with 4% PFA. Concentric circles were drawn using the ImageJ concentric circles plugin. Circles were centered on the soma by manually determining the radii, which were then increased by 2µm with every circle. Sholl analysis was performed using the ImageJ Sholl analysis plugin. These data were quantitated using the following parameters:

Nm = process maximum (the maximum number of intersections for the cell); Cr = critical value (the distance from the soma where the Nm occurred); maximum branch length (µm, the maximum radius at which a branch intersection occurred); and Np = number of primary branches (the number of branches originating from the microglial soma). The ramification index (Nm/ Np) was calculated to quantify cell branching density. The cell soma area was also calculated using ImageJ.

Skeleton analysis was performed on images stained with DAB, as described previously. The following sequence was followed: Grey Scale-Subtract Background-Binary-Skeletonize-Analyse Skeleton. The ImageJ Skeletonize plugin was used to quantitate microglial cell process endpoints and calculate the summed microglial process length.

## **Microscopy**

The dorsal hippocampus was taken for BrdU or DCX positive immune cells counting in 30µm sections as described in the protocols before [ 26, 27, 38] using Olympus BX51TF microscope, Japan. The three subpopulations of DCX positive immune-reactive cells based on their maturity were counted individually. The mean intensity values of both left and right hippocampus were considered for analysis.

## RT PCR

Rat hippocampal tissue was collected and weighed (30-50mg). Homogenized in 1000 µl TRIZOL reagent (**Sigma Aldrich, USA**) at RT for 15min. Chloroform in the quantity of 250µl per 1000 µl TRIZOL reagent was added in the hippocampal lysate. Samples were vortex vigorously for the 30s and kept at RT followed by centrifuge at 12,000 rpm at 400 C. for 5min. Centrifugation lead separation of the solution in three phases. After the separation of the aqueous phase, RNA was precipitated using isopropyl alcohol. A gel-like RNA pallet was visible on the bottom or side of the wall and stored at -80°C. Samples were made sure to have a 260/280 ratio of more than 1.8. The purity of total RNA regarding size, distribution, and integrity was evaluated by the denaturation using denaturing agarose gel electrophoresis. cDNA was prepared by using the RT2 first-strand cDNA synthesis kit (Qiagen, USA). It was then stored at -15 to -30°C freezer for further use.

The RT2 Profiler for rat neurogenesis and neuroinflammation (PARN-404, PARN-011-Qiagen, USA) performed according to the provider's protocol. The data was analyzed using Gene Globe Data Analysis Centre facilitated software-based tool available at Qiagen website ([www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page](http://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page)). The spreadsheet displayed Delta Ct values was uploaded and normalized with the values of housekeeping genes (at least 2 out of 5 were selected) . The values of fold change were used for comparison between groups.

## Western immunoblotting

A standardized protocol for Western blotting was performed [19, 21] on hippocampal tissue. The membrane was immune blotted with primary antibodies in **Table 1**. The blots were probed using HRP phosphor secondary antibody and developed with enhanced chemiluminescent (ECL) kit (Abcam, USA) in Molecular imager Chemidoc imaging system (Bio-Rad). Densitometry analysis to quantitate the band intensity of the blot was performed using ImageJ software (NIH).

## High performance liquid chromatography (HPLC)

The Serotonin (5HT) levels in hippocampal tissue were measured using ion-pairing reversed phase HPLC-ECD. Tissue samples were sonicated in the ice-cold 0.05 M (500 µl/100 mg tissue) perchloric acid buffer and were centrifuged for 5 min at 10,000 rpm. Supernatants were filtered with syringe filter (0.45 µm, Millipore, USA) and stored at -80 °C for further use. To estimate 5HT levels by HPLC-ECD, 50 µl of the aqueous lysate was injected into a C18 reverse phase column (Waters, USA) from an auto sampler (Water 717, USA) using an HPLC pump (Waters 515, USA). The column pressure was maintained at 1800 psi. The mobile phase comprised of vacuum-degassed 8.65 mM heptane sulphonic acid, 0.27 mM Ethylene

Diamine Tetra-acetic Acid (EDTA), 13% acetonitrile, 0.4% triethylamine and 0.2% phosphoric acid with flow rate at 1 ml/min in isocratic flow mode. The run time was set for 20 min, and peaks were identified in the sample by electrochemical detector. Compounds were identified based on the retention time matching with standards.

## Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, San Diego, CA, USA). Data were analyzed using ANOVAs to test differences of means between experimental groups mentioned with the results. All statistical tests were considered statistically reliable at a p-value of <0.05.

## Results

### Chronic HH exposure caused reproducible change in social interaction as well as anxiety-like behaviors

HH exposure exhibits detrimental effects on body weight at 3HH ( $p<0.01$ ), 7HH ( $p<0.05$ ) as well on food intake at 3HH ( $p<0.05$ ) and 7HH ( $p<0.01$ ) as compare to control **Supplementary Fig. 2**.

Previous studies have shown vulnerability in hippocampal-dependent tasks, so we sought to examine whether hippocampal based social memory is affected. The behavioral schematic setup was representing two trials, each consisting of 10 min (**Fig. 1a**). Representative track-plots are provided for both trial 1 and trial 2 from control and 7 days HH exposed rats showings differential movement in the chamber (**Fig. 1b**). Statistical analysis showed that time spent in the chamber containing social stimuli were markedly lower in 7HH exposed ( $F_{1,18} = 7.764$ ,  $p<0.01$ ) animals. In contrast, this time was significantly higher with empty enclosure ( $p<0.05$ ) as compared to control rats (two-way ANOVA, Bonferroni's multiple comparison test, **Fig. 1c**,  $n=10$ ). However, contact behaviour that represents the direct exploration of two stimuli, was also altered in 7HH exposed rats as there was a decrease in contact behavior with social stimuli ( $F_{1,18} = 7.709$ ,  $p<0.05$ ) and it increases when rats explored with an empty enclosure. Quantification of Trial 2 parameters revealed that both stressed (7HH) and control animals spent almost similar time in the chamber with the novel stimuli, but 7HH stressed animals spend more time with the familiar animal compared to control ( $F_{1,18} = 6.225$ ,  $p<0.05$ , **Fig. 1d**). However, the direct contact behaviour of test rats from 7HH group was lower ( $F_{1,18} = 12.51$ ,  $p<0.05$ ) with novel stimuli and higher with familiar ( $p<0.05$ ).

During many psychiatric disease states, anxiety and social deficits are co-expressed as the baso-lateral amygdala and the ventral hippocampus are reported to share conventional circuitry and robust reciprocal connections. In line with this information we next evaluated anxiety-like behaviour using EPM.

Representative track plot shown for reference as lesser track movement of rats recorded in open arm (**Fig.1e**). Exposure to 7HH reduced the time spent in open arm significantly (one-way ANOVA,  $F_{3,36} = 11.35$ ,  $p<0.01$ , **Fig.1f**, Tukey's post-hoc test,  $n=10$ ) as compared to CC but no significant reduction observed at 1HH and 3HH exposure.



Together, these experiments suggest 7 days of HH exposure diminished social interaction behaviour in rats by reducing the discrimination between social stimuli as the hippocampus being one of the affected brain regions. Along with social interaction, anxiety-like behaviour was also up-surged after 7HH exposure.

### HH exposure diminishes expression of BDNF, Serotonin and pCREB in DG

Given that BDNF and Serotonin has been demonstrated to regulate mood associated behavior in rat, we investigated the effect of HH exposure on their expression. We found that the expression levels of BDNF decreased significantly in temporal manner straight from 1HH (one-way ANOVA,  $F_{3,20} = 9.108$ ,  $p < 0.01$ , **Tukey's post-hoc test, Fig. 2 a, b, n=3**), 3HH ( $p < 0.01$ ) and 7HH ( $p < 0.01$ ) exposure in DG **indicated by representative IHC images as well**. We also found decrease in mRNA levels of BDNF as well **Fig 3 A, Fig 3 B**. Consistent with this observation also we found decrease in BrdU+ BDNF+ cells (Unpaired t-test,  $F_{5,5} = 2.336$ ,  $p < 0.01$ ,  $n=3$ ) in DG as compare to control after 7HH exposure indicated deficits in adult neurogenesis (**Fig.2 c, d**). Simultaneously we also observed striking difference in the expression of Serotonin (5HT) in the DG (Unpaired t-test,  $F_{5,5} = 1.374$ ,  $p < 0.01$ ,  $n=6$ , **Fig.2 e**) as well its levels in the hippocampus measured by HPLC (Unpaired t-test,  $F_{5,5} = 2.351$ ,  $p < 0.01$ ,  $n=6$ , **Fig.2 f**). Furthermore, we found that HH exposure for 3 days (one-way ANOVA,  $F_{3,20} = 13.39$ ,  $p < 0.05$ , **Tukey's post-hoc test, Fig. 2 g, h, n=3**) and 7 Days ( $p < 0.001$ ) significantly reduced the pCREB positive nuclei in the DG region. mRNA expression of Creb1 also decreased after 1HH, 3HH and 7HH exposure in hippocampus **Fig 3 B**.

Overall, these results suggest that HH exposure upsets the microenvironment of the DG by altering the levels of BDNF, Serotonin and pCREB in day dependent manner that might associated with deficits in social and elevation of anxiety like behavior.

### HH exposure proved to be detrimental for neurogenesis in adult rat brain

We next investigated the effect of stressful HH exposure on the proliferation and maturation of adult neuronal cells in the DG as BDNF, Serotonin and pCREB are very well-established influencers of adult neurogenesis that too regulate associated behaviors like social interaction and anxiety. Using RT<sup>2</sup> profiler array of neurogenesis, we explore the mRNA expression of different functional classes of genes. Fold value that was more than 2 was considered for statistically significance. There was generalized down-regulation of different functional classes like Cell adhesion molecule, cell cycle, apoptosis, cell differentiation, cytokines, growth factors, neuronal migration, signal transduction, synaptic function, synaptogenesis and axogenesis, transcription factors and co-factors, that regulate adult neurogenesis such as related molecules in mRNA levels after 1HH (viz. Adora1, Alk, Bdnf, Bmp2, Bmp4, Cdk5r1, Cdk5rap2, Chat, Creb 1, Dcx, Dlg4, Dll1, Dvl3, Drd2, Egf, Fgf2, Gdnf, Grin1, Hey1, Hey2, Map2, Mdk, Neurod1, Neurog1, Notch1, Notch2, Nrg1, Nrp1, Pax2, S100b, Sox2, Sox8). 3HH exposure also decreased **adult neurogenesis related molecules mRNA levels (viz. Adora1, Adora2a, Apbb1, Apoe, App, Bdnf, Bmp2, Bmp4, Cdk5r1, Cdk5rap2, Creb 1, Dcx, Dlg4, Dll1, Drd2, Dvl3, Ep300, Egf, Fgf2, Grin1, Hey1, Hey2, Map2, Mdk, Mef2c, Ndn, Neurod1, Notch1, Notch2, Nrg1, Nrp1, Olig2, S100a6, Sod1, Sox2, Sox8)**. mRNA

expression of adult neurogenesis also showed declined expression (viz. Adora1, Adora2a, Apbb1, Apoe, App, Bdnf, Bmp2, Bmp4, Cdk5r1, Cdk5rap2, Creb 1, Dcx, Dlg4, Dll1, Drd2, Dvl3, Ep300, Egf, Fgf2, Gdnf, Grin1, Hey1, Hey2, Map2, Mdk, Mef2c, Ndn, Neurod1, Notch1, Notch2, Nrg1, Nrp1, Olig2, S100a6, Sod1, Sox2, Sox8) (**Fig. 3A and Fig. 3B, n=3**).

Representative immunofluorescence images are shown in **Fig. 4a**, represents a marked reduction in BrdU positive (+ve) cells after time-dependent HH exposure, which is significantly reduced at 7 days of exposure (one-way ANOVA,  $F_{3,20} = 6.928$ ,  $p < 0.01$ , **Tukey's post-hoc test, Fig. 4b, n=6**). Endogenous neuronal proliferation marker Ki-67, showed marked reduction at 7 HH exposure ( $F_{3,20} = 4.033$ ,  $p < 0.05$ , **Fig. 4 a and b**). The idea of designing this experiment is to know the change in the phenotypic expression of Neuronal Proliferation cells (NPCs), labeled with BrdU, that can differentiate into astrocyte (GFAP) and neurons (SOX2, NeuN). No. of NPCs labeled with BrdU+SOX2+ is significantly depleted upon 7HH exposure (unpaired t-test,  $F_{5,5} = 4.616$ ,  $p < 0.01$ , **Fig. 4 c and d, n=3**), indicating deficits in neurogenesis. Whereas no significant difference was found in NPCs (BrdU+ GFAP+), indicating no alteration in gliogenesis (**Fig. 4 c and d**). 7HH exposure markedly reduced the BrdU+ DCX+ cells ( $F_{5,5} = 3.534$ ,  $p < 0.01$ ) and DCX+ NeuN+ cells ( $F_{5,5} = 4.034$ ,  $p < 0.001$ ), indicating a decrease in overall surviving neurons in DG (**Fig. 4 e**). There was no significant difference observed for BrdU+ NeuN+ cells as well as the mean intensity of NeuN (mature neuronal population) data not shown here. Immuno-micrographs describe the sub-population of immature neuronal marker DCX positive cells at the proliferative stage, Intermediate stage, and post-mitotic stage in DG (**Fig. 4 f**). Dramatic reduction in DCX cells at proliferative stage (one-way ANOVA,  $F_{3,20} = 17.56$ ,  $p < 0.001$ , Tuckey's post-hoc test), Intermediate stage ( $F_{3,20} = 14.50$ ,  $p < 0.001$ ), and at post-mitotic stage ( $F_{3,20} = 8.813$ ,  $p < 0.01$ , **Fig. 4 g**) cells straight after 1HH exposure. This reduction continued at 3HH ( $p < 0.001$ ), ( $p < 0.001$ ), ( $p < 0.01$ ) and at 7HH ( $p < 0.0001$ ), ( $p < 0.0001$ ), ( $p < 0.01$ ) **Fig. 4 h, i and j** respectively. We also observed significant decrease in the mRNA expression of DCX at 1HH, 3HH and 7HH exposure as compare to control **Fig 3 a, Fig 3 b**. No significant difference was observed in NeuN mean intensity **supplementary Fig 3**.

We inferred this results as 7HH exposure procreate deficits in generation of NPCs, immature neurons as well maturation of neurons whereas no change was observed in mature population of neurons and in gliogenesis. The observed deficits in neurogenesis are supported by mRNA expression of genes that contribute to different functional class associated with neurogenesis.

### **HH evokes NLRP3 Inflammasome mediated neuroinflammation and microglial activation**

To assess the expression of inflammatory cytokine and their receptors we used RT<sup>2</sup> profiler for inflammatory cytokines and receptors. Quantitative PCR array analysis revealed modulation of mRNA expression. mRNA expression of pro-inflammatory genes like CXCL1, CXCL12, CXCR2, IFNG, VEGFA, CSF3, IL1B, CCR1, CCR8, CCR5, IL10RA, LTb which related to decrease in neurogenesis and microglial activation were shown to be upregulated. On the other hand mRNA expression of genes like IL1A, IL1RN, BMP2, CD40LG that are anti-inflammatory and positively regulate neurogenesis are decreased after HH exposure. We previously reported that temporal exposure of HH activates microglia and astrocytes in the

DG followed by up-regulation of pro-inflammatory cytokines right from 1 day of exposure. Fold value that was more than 2 was considered for statistical significance (**Fig 5**, n=3).

Induction of pro-inflammatory cytokine lead us to further investigate the role of other neuro-inflammation associated mechanisms that may contribute to such pathophysiology we explored the expression of NLRP3/NFkB pathway. Here, we observed inflation in the phosphorylation in NFkB at its subunit 536 in hippocampus after 3HH (one-way ANOVA,  $F_{3,12} = 7.436$ ,  $p < 0.05$ , Tuckey's post-hoc, test n=3, **Fig 6 a, b, c**) and 7HH ( $p < 0.05$ ) exposure. We found a striking upsurge in the expression of levels of IL1 $\beta$  immediately after 1day (one-way ANOVA,  $F_{3,16} = 16.85$ ,  $p < 0.01$ , n=3, **Fig 6 a, b, c**), 3day ( $p < 0.01$ ) and continued till 7day ( $p < 0.001$ ) in the hippocampus. We also found increase in the mRNA levels of IL1 $\beta$  shown in the RT-PCR panel ( **Fig 5**). In consistence with that we also observed significant increase in the NLRP3 expression in the DG after 1HH (one-way ANOVA,  $F_{3,20} = 12.15$ ,  $p < 0.05$ , n=3, **Fig 6 a, b, c**), 3HH ( $p < 0.001$ ) and 7HH ( $p < 0.001$ ) exposure compare to control. We next evaluate the levels of downstream molecule that is caspase-1 and it also showed up regulation of the expression after 3HH (one-way ANOVA,  $F_{3,12} = 9.246$ ,  $p < 0.05$ , n=3, **Fig 6 a,c**) and 7HH( $p < 0.01$ ). To access the activated microglia stained with CD-68 we co-label them and we found elevation in the number of Iba+CD-68+ cells in the DG after 3HH exposure (one-way ANOVA,  $F_{5,5} = 3.266$ ,  $p < 0.05$ , n=3, **Fig 6 d,e**) as compare to control (CC) group.

Overall, theses results indicate elevation in the NLRP3 signalling indicated by induction in expression of IL1 $\beta$ , Caspase-1 as well NLRP3 expression in the DG. Simultaneously phosphorylation of NFkB and microglial activation observed which indicate elevation of neuro-inflammation in hippocampus. mRNA expression up-regulation of proinflammatory cytokines and their receptor right from day 1 strengthen these observations.

### Differential expression of E-type prostanoid receptors after time-dependent HH exposure

We earlier observed induction in the PGE2 conc. And associated neuroinflammation in the hippocampus and plasma, but its active downstream receptor was not explored during HH. We next checked the expression of all PGE2 receptor expression in hippocampus. Representative immunoblot of EP1 receptor and its quantitative analysis revealed its marked increase at 3HH (one-way ANOVA,  $F_{3,12} = 4.693$ ,  $p < 0.05$ , **Fig. 7 a**)  $p < 0.05$ ) and at 7HH exposure ( $p < 0.05$ ). Expression of another prostanoid receptor EP2 was (one-way ANOVA,  $F_{3,12} = 3.887$ ,  $p < 0.05$ , **Fig 7 b**) up-regulated after 7 day of HH exposure as indicated by representative immunoblot. **Whereas**, no significant difference was found in the expression of EP3, and EP4 receptors in the hippocampal tissue lysate after time-dependent exposure to HH represented with immunoblots and quantitative analysis (**Fig 7 c and d**).

These data indicate that HH differentially regulate expression of PGE2 G-protein coupled EP receptors. Only EP1 and EP2 receptors were reproducibly up-regulated after 3 and 7 days of HH exposure respectively, whereas expression EP3 and EP4 receptors were unaltered in the hippocampus after HH exposure at any day.

## **PGE2 receptor EP1 is evident in the activated microglia as well as in astrocyte during HH exposure.**

Induction in the EP1 expression in hippocampus lead us to examine its expression in the cell types in DG. Microglia and astrocyte activation was also prominent in the DG during HH exposure which opens the possibility of EP1R expression in these cell types. So we next performed a co-labelling experiment and found a significant increase in the CD-68+ EP1R+ (yellow) cells in the DG after 3HH exposure (Unpaired t-test,  $F_{5,5} = 1.202$ ,  $p < 0.01$ , Fig. 8 a, b) in the DG. Similarly, we found an interesting observation where EP1R+ GFAP+ (yellow) cells were significantly more in DG after 3 days of HH (Unpaired t-test,  $F_{5,5} = 2.806$ ,  $p < 0.001$ , Fig. 8 c, d) exposure as compare to control.

Taken, together we can say EP1R expression in the DG contributing to the activation of glial cells and influencing neurogenic niche during HH exposure.

## **COX-1 dependent PGE2 response via downstream EP1 receptor is critical for the induction of social interaction deficits during HH exposure.**

There are COX-1 and EP1 deficiency reported to abolished social avoidance and impulsive behavior under acute social and environmental stress. We found significant increase in protein expression of EP1R in hippocampus after 7HH exposure. The Tukey's post-hoc test revealed the considerable increase in EP1R expression after 7HH and was markedly decreased after treatment with valeryl salicyclate (ValS), COX-1 inhibitor ( $F_{3,12} = 8.444$ ,  $p < 0.05$ , **Fig. 9 a**) and SC19220, EP1R antagonist ( $p < 0.01$ ). We previously reported increase in COX-1 expression during 1HH, 3HH and 7HH of exposure. Significant positive correlation in the expression of COX-1 and EP1 was observed (Pearson correlation,  $r = 0.6722$ ,  $p < 0.01$ , **Fig. 9 b**). This striking result lead us to further carried out social interaction test in order to understand how pharmacological inhibition of COX-1 and EP1 receptor during HH affect social behaviour. Exposure of rats to HH for 7days ( $p < 0.001$ ) significantly reduced the time spent with social stimuli. Treatment with ValS (Two-way ANOVA,  $F_{3,40} = 8.778$ ,  $p < 0.05$ ) and SC19220 ( $p < 0.01$ , **Fig. 9 c**) during 7HH increased time spent with social stimuli. It is clearly indicated in (**Fig. 9 d**) that direct contact of test rat with social stimuli was significantly elevated after treatment with ValS ( $F_{3,40} = 4.679$ ,  $p < 0.05$ ) and SC19220 ( $p < 0.05$ ), which was reduced in 7HH ( $p < 0.05$ ) exposed rats. During Trial 2, 7HH exposure decrease time spent with novel animal and both treatments showed increased time spent with novel animal although this difference was not significant. 7HH group animals spent significantly higher time with familiar rat (Two-way ANOVA,  $F_{3,40} = 6.664$ ,  $p < 0.05$ , **Fig. 9 e**,  $n=6$ ), whereas treatment with ValS ( $p < 0.05$ ) and SC19220 ( $p < 0.05$ ) significantly reduced the time spent in the chamber containing a familiar rat. Bonferroni's post hoc test clearly depict that (**Fig. 9 f**) contact behavior of test rat with novel stimuli was significantly reduced ( $F_{3,40} = 6.498$ ,  $p < 0.05$ ) after 7HH exposure and treatment with ValS and SC19220 increased it (not significantly). Contact behavior with familiar was increased in rats exposed to HH for 7 days, which was reduced after treatment with both inhibitors, although not significant. Time spent in the open arm of EPM was significantly lower in 7HH (One-way ANOVA,  $F_{3,20} = 7.508$ ,  $p < 0.01$ ) exposed group and increased after administration of ValS ( $p < 0.05$ ) and SC19220 ( $p < 0.05$ , **Fig. 9 g**) during 7HH exposure.

Interpretation can be drawn from these results that COX-1/EP1 axis is evident during HH exposure right from 1 day. COX-1 inhibition reduced the expression of EP1 R indicate it to be the active downstream receptor and their strong correlation strengthen this. We could also found that 7HH induced alteration in social behavior in rats was reproducibly mitigated after COX-1 inhibitor (ValS), and EP1 antagonist (SC19220) probably by reducing anxiety like behavior.

### **Administration of COX-1 inhibitor and EP1R antagonist differentially rescued HH induced alteration in expression of BDNF, Serotonin and pCREB.**

Beneficial effect of ValS and SC19220 on social interaction as well on anxiety like behaviour and alteration in levels of BDNF, Serotonin and pCREB during HH lead to this experiment. We tested the effects of ValS and SC19220 on neurotrophic factor like BDNF and we found significant up-regulation in the expression of BDNF after COX-1 inhibition ( $F_{3,12} = 8.613$ ,  $p < 0.05$ , Fig. 10 a) during 7HH exposure as compare to only 7HH group. EP1R antagonism also increased BDNF expression as compare to 7HH but the elevation was not significant. On the other hand Serotonin ( $F_{3,20} = 2.243$ , Fig. 10 b) levels did not significantly increase in the hippocampus after both the treatments during 7HH. Number on pCREB positive nuclei which was drastically reduced after 7HH ( $p < 0.001$ ) exposure significantly rescued after administration of ValS ( $F_{3,20} = 10.43$ ,  $p < 0.01$ ,  $n=3$ , Fig. 10 c) and SC19220 ( $p < 0.01$ ) during 7HH.

Taken together, results showed that the inhibition of COX-1 and antagonist of EP1R rescued the perturbation of hippocampal microenvironment at some extent by elevating the expression BDNF and pCREB in DG but levels of serotonin in the hippocampus remained unchanged.

### **Inhibition of COX-1 as well EP1R pathway during HH stress boosts neuronal cell proliferation in DG**

To test the hypothesis that the ameliorative behavioural effects of COX-1 inhibitor (ValS), and EP1 antagonist (SC19220) could be associated with adult hippocampal neurogenesis, we further tested their efficacy on HH induced deficits of neuronal proliferation in DG. Representative photomicrographs of progenitor cell marker BrdU and Ki-67 as well as DCX labelled neurons among groups are displayed (**Fig. 11 a and b**). Quantitative analysis of the number of BrdU labeled cells represents that 7HH exposure (One-way ANOVA,  $F_{3,20} = 8.876$ ,  $p < 0.01$ , **Fig. 11 c**) severely reduced their number in DG, treatment with ValS ( $p < 0.05$ ) and SC19220 ( $p < 0.01$ ) elevated their number. Similarly, Ki-67 labeled cells were also decreased after 7HH exposure ( $p < 0.05$ ), but successfully increased after treatment with ValS ( $F_{3,20} = 4.327$ ,  $p < 0.05$ ) and SC19220 ( $p < 0.05$ ) during 7HH exposure indicating increase in proliferative cells in DG upon treatment. DCX labeled neurons at the proliferative stage were significantly reduced at 7HH exposure ( $p < 0.001$ ), increased after treatment with ValS ( $F_{3,20} = 10.50$ ,  $p < 0.05$ ) and SC19220 ( $p < 0.01$ , **Fig. 11 d**) significantly. Similarly, DCX labeled cells at the post-mitotic stage were also dramatically reduced ( $F_{3,20} = 14.54$ ,  $p < 0.001$ ) upon 7HH exposure, this reduction was mitigated after treatment with ValS (not significant) as well with SC19220 ( $p < 0.01$ ) indicating rescue of immature neuron at proliferative and post-mitotic stage but not at intermediate stage.

Representative immunomicrograph indicating higher number of co-labelled cells (BrdU+DCX+ (NeuN+DCX+) in DG after treatment with ValS and SC-19220 (**Fig. 11 e**) reflecting amelioration of maturation deficits caused during 7HH stress. No difference in (BrdU+NeuN+) as well (BrdU+ GFAP+). Although, BrdU+SOX2+ cells were decreased after 7HH exposure ( $p<0.01$ ) and significantly rescued after injection of ValS (One-way ANOVA,  $F_{3,20} = 9.676$ ,  $p<0.05$ ) and SC19220 ( $p<0.01$ , **Fig. 11 f**) to the rats during 7HH indicating increasing in new-born neurons in DG. 7HH exposure ( $p<0.001$ ) dramatically decreased (BrdU+ DCX+) cells in DG but when ValS ( $F_{3,20} = 8.466$ ,  $p<0.05$ ) and SC19220 ( $p<0.05$ ) were administered during HH exposure it significantly increased their number. Similarly, DCX+ NeuN+ cells were markedly reduced after 7HH exposure ( $p<0.001$ ) which was significantly mitigated after ValS ( $F_{3,20} = 7.791$ ,  $p<0.05$ ) and SC19220 ( $p<0.05$ , **Fig. 11 g**) treatment clearly indicate a rescue of surviving neurons in DG. Administration of ValS and SC19220 during HH mitigated the changes in mRNA expression of genes that contribute to different functional class associated with neurogenesis with differential efficacy. Details of their expression are represented as line graphs.

The cumulative result shown here clearly indicate that treatment with ValS and SC19220 ameliorated neurogenesis deficits procreated by 7HH exposure by rescuing NPCs, new-born neurons, immature neurons and their survival in DG. Elevation of expression of BDNF and pCREB after COX-1 inhibitor and EP1R antagonist during HH exposure might be helping in rescue of adult born neurons in DG. Thus providing experimental evidence of critical role COX-1/EP1 axis in regulating BDNF dependent adult neurogenesis during HH condition.

### **Pharmacological inhibition of COX-1 and EP1R during 7 days of HH exposure reverts glial activation in DG**

Activation of COX-1 as well EP1R promoted neuro-inflammation as well as microglia mediated neurotoxicity, we also reported COX-1 dominance in microglia in HH condition. EP1 gene ablation or selective antagonist shown to blunt microglial mediated inflammatory response. We planned our next experiment to answer the question about how COX-1/EP1R antagonism during HH can modulate morphology of microglia and astrocytes as they are prominent contributor to neurogenic niche. Representative immune-micrographs of Iba-1 and GFAP staining in DG among different groups (Fig. 12 a and b). Sholl analysis revealed different parameters like Critical value ( $F_{3,16} = 5.928$ ,  $p<0.05$ ), maximum branch length ( $F_{3,16} = 10.61$ ,  $p<0.01$ ), number of primary branches from soma ( $F_{3,16} = 13.2$ ,  $p<0.0001$ ), microglia process maximum ( $F_{3,16} = 10.61$ ,  $p<0.001$ ), decreased after 7HH exposure in DG clearly indicate activation of microglia during this stress.

Administration of ValS and SC19220 during 7HH exposure significantly ameliorated alteration in Critical value ( $p<0.05$ ), maximum branch length ( $p<0.01$ ), number of primary branches from soma ( $p<0.01$ ,  $p<0.0001$ ), microglia process maximum ( $p<0.05$ ,  $p<0.01$ ) indicate their ameliorative effects on microglial activation. Other parameters like Shoenen ramification index ( $F_{3,16} = 6.066$ ,  $p<0.01$ ), microglial soma area ( $F_{3,16} = 8.276$ ,  $p<0.01$ ), and number of branch endpoints ( $F_{3,16} = 10.76$ ,  $p<0.001$ ), were also dramatically altered after 7HH exposure. Pharmacological intervention with ValS and SC19220 during

7HH exposure also rescued parameters like Shoenen ramification index ( $p<0.05$ ,  $p<0.05$ ), microglial soma area ( $p<0.05$ ,  $p<0.01$ ), and number of branch endpoints ( $p<0.05$ ,  $p<0.001$ , Fig. 12 c) respectively. This indicates that ValS and SC19220 blunted microglial activation during 7HH.

We also checked the astrocyte intensity via GFAP staining in DG where exposure to HH for 7days ( $F_{3,20} = 6.612$ ,  $p<0.01$ ) significantly elevated the mean intensity of GFAP in DG, this was significantly mitigated after treatment with ValS ( $p<0.05$ ) and SC19220 ( $p<0.05$ ). Similarly, Number of resting astrocytes in DG was also reduced after 7HH exposure ( $F_{3,20} = 7.368$ ,  $p<0.01$ ) but ValS and SC19220 was not able to increase them significantly. The number of activated astrocyte were also increased considerably after 7HH exposure ( $F_{3,20} = 6.612$ ,  $p<0.01$ ) in DG, administration of ValS ( $p<0.01$ ) and SC19220 ( $p<0.01$ , Fig. 12 d) reverted their activation significantly.

Taken together, we can conclude that ValS and SC-19220 effectively rescue activation of microglia and astrocyte followed by amelioration of neuroinflammatory response. This may lead to the conclusion that COX-1 inhibition as well EP1R antagonist protecting the perturbed neurogenic niche via blunting the activation of glial cells as both COX-1 and EP1 R expression was evident in both cells specially microglia.

### **HH induced NLRP3/NFkB mediated neuroinflammation was reverted by specific COX-1 and EP1R inhibition**

HH exposure upsurge the expression of NLRP3/NFkB pathway and pro-inflammatory cytokines in the DG and COX-1, EP1R established to increase during inflammatory stimuli, so we next investigated the ability of COX-1 inhibitor and EP1R antagonist to blunt neuroinflammation during HH condition. We found elevation in the phosphorylation of NFkB at subunit 536 at 7HH ( $F_{3,12} = 6.021$ ,  $p<0.01$ ,  $n=3$ ) exposure which was significantly reduced after administration of ValS ( $p<0.05$ ) and SC19220 ( $p<0.05$ , Fig. 13 a). NLRP3 expression was drastically up surged in the DG at 7HH exposure ( $F_{3,12} = 7.560$ ,  $p<0.01$ , Fig. 13 b) which was significantly reduced upon administration of COX-1 inhibitor ( $p<0.05$ ) and EP1R antagonist ( $p<0.05$ ) for 7days during HH exposure. Pro-inflammatory cytokine like IL-1 $\beta$  ( $F_{3,12} = 9.694$ ,  $p<0.01$ , Fig. 13 c), IL-6 ( $F_{3,12} = 11.09$ ,  $p<0.01$ , Fig. 13 d) and TNF- $\alpha$  ( $F_{3,12} = 7.497$ ,  $p<0.01$  Fig. 13 e) elevated after 7HH exposure which was significantly ameliorated after treatment with ValS ( $p<0.01$ ) and SC-19220 ( $p<0.01$ ) in DG.

These results indicate that HH induced neuroinflammation was mediated by NLRP3/NFkB pathways which was mitigated after treatment with COX-1 inhibitor and EP1R antagonist. Reduction of pro-inflammatory cytokine was visible when COX-1 and EP1R elevation blunted after treatment with both pharmacological agents.

## **Discussion**

In this study we explored social interaction behavior and downstream molecular mechanisms under chronic HH exposure. Social memory is, in part, stored in the hippocampus and reported to affect during

environmental stress like heat, sleep deprivation, and neonatal hypoxia [39, 40, 41, 42]. HH induced deficits in social interaction behavior at 7HH exposure which is consistent with the growing understanding that external stress may also cause such deficits. Although neuroimaging studies suggest that multiple brain regions are impacted by HH exposure, the hippocampal formation is frequently identified as a site of injury during HH [43, 44]. In rats, some early cytotoxic lesion and studies concluded that the hippocampus is dispensable for recognizing a familiar conspecific [45, 46]. HH induced alteration in hippocampus might be the reason behind alteration in social interaction behavior in rats. Another interesting observation where we found anxiety-like behavior in rats as indicated by tract-plots during social behavior, more movement in the chamber, but lesser contact behavior with the novel subject. Neurodegeneration in both the amygdala and hippocampus after HH exposure have well established [18, 47] which make rats vulnerable to anxiety. During many psychiatric disease states, anxiety and social deficits are co-expressed [48, 49, 50]. Our observation that HH exposure for 7days severely affects the social interaction behavior and induce anxiety like behavior in rats provided a unique opportunity to explore underlying molecular mechanism in HH. We tried to address this question by examining effects of HH on microenvironment of hippocampus particularly DG. BDNF and Serotonin both regulate development and plasticity of neuronal circuits that are involved in mood dependent behavior like social interaction and anxiety [ 51, 52, 53]. We found dramatic decrease in their expression upon exposure to HH that might be causing deficits in the above mentioned behavior. Phospho CREB directly regulate BDNF signalling and that also found to be decreased during HH [54]. As we found disturbed hippocampal microenvironment and very less information available regarding the effect of HH on stages of adult neurogenesis in rats. We next evaluated and found decrease in neuronal progenitor cells upon 7HH exposure in DG. However, immature neurons showed marked reduction from day 1 of the exposure. After 7HH exposure, reduction or delay in the maturation of new-born neurons of the DG region observed. Overall, HH exposure at 25000ft elicits detrimental effect on progenitor, proliferative, immature neurons in DG. In other hypoxia-related studies like intermittent hypoxia witnessed contradictory contributions towards the generation of new-born neurons in DG, whereas ischemia majorly suppresses adult neurogenesis [55, 56, 57]. Whereas, there is a study where number of Ki-67 and DCX+ hosphor cells elevated upon high altitude exposure in rats at 3450m via elevation in VEGF signaling [58]. Similarly, another report where hypobaric hypoxia exposure at 11000m for 3hr elevated the expression of NeuroD1 in the hippocampus [59]. The discrepancy can be explained as altitude in our condition is 7260m and for 1, 3 and 7days, which is harsher for neuronal generation in DG and VEGF elevation reported to be in pathological range [19]. Like other stresses, HH exposure also increases glucocorticoids and corticosterone in the bloodstream and brain that might be one of the reasons behind the impairment in adult neurogenesis [60, 61]. Decrease in hippocampal BDNF, Serotonin and pCREB directly or indirectly causes deficits in adult neurogenesis in subgranular zone (SGZ) in rodents we observed similar string of event during HH exposure [ 62, 63, 64].

There are reports where proliferation blocker induced decline in BrdU and BrdU/NeuN labelled cells in DG abolished the effect of social interaction [65, 66] suggests an association of adult neurogenesis and social behavior in rats, which was further validated in our study. Decrease in hippocampal BDNF,



Serotonin and pCREB directly or indirectly causes deficits in adult neurogenesis might be the causal factors behind the alteration in social memory and induction in anxiety like behavior after HH exposure [62,64,67,68]. Increase in adult neurogenesis in DG improves social interaction behavior with reduced anxiety [67, 69]. Besides, alteration in neuronal progenitor proliferation and dendritic development of new-born neuron resulted in elevated anxiety like behavior [70]. Decrease in adult neurogenesis similar strings of events observed in our study.

Neuro-inflammation specifically via NLRP3 leads activation of glial and causes deficits in adult neurogenesis both observation were evident in our stress [68, 71]. NLRP3 and NFkB signalling pathways were shown to activate upon HH exposure [22] supports our observation where we showed their up regulation in DG. Reduction in BDNF dependent neurogenesis was simultaneous with induction in NLRP3 dependent inflammation similarly shown earlier [72]. Dramatic increase in levels of pro-inflammatory cytokines followed by microglial activation made it imperative to target inflammatory pathways in an attempt to rescue brain neurogenic capability. COX pathway is one of the major pathways for the contribution to the process of inflammation evident by its presence in microglia and neuron both [73]. In this study, an interesting result observed where COX-1 inhibitor also decreased the expression of the EP1 receptor at 7HH exposure. We found strong correlation in the expression of COX-1 and EP1R, so we thought COX-1 might be using PGE2 receptor EP1 for its downstream effects. There are several supporting literature regarding the role of COX-1 mediated PGE2 synthesis in the induction of social avoidance. Moreover, EP1 is critical for the anxiogenic effect of repeated social defeat [74]. Both COX-1 and EP1 antagonist reduced the anxiety-like behavior similarly with the study where EP1 deficient mice showed decreased anxiety like behavior.

Fenamate (NSAIDS) which block COX-1 also were found to effective in suppressing NLRP3 induced neuroinflammation and associated memory deficits in Alzheimer's disease in rodent models [14]. ValS also reduced the expression of NLRP3 during HH. During LPS induced neuroinflammation, COX-1 gene deletion reported to regulate hippocampal neurogenesis positively [12] during HH COX-1 inhibition rescued neurogenesis deficits at some extent. There are findings, which also reported that PGE2-EP1 signaling suppresses midbrain dopamine neurons and regulates impulsive behaviors under acute stress [75,76]. Interestingly, We found that the COX-1 specific inhibitor (ValS) and EP1 (SC19220) antagonist rescued social interaction deficits at 7HH exposure. We earlier reported COX-1 dominance in glial cells specially microglia and in this study we found dominance of EP1 R in glial cells. COX-1 and EP1 induction in activated microglia is reported to upsurge  $Ca^{2+}$  levels thus disrupts  $Ca^{2+}$  homeostasis leads to neurodegeneration and deficits in integration and maturation of newborn neurons in DG [77,78]. Therefore, it is plausible that antagonism of COX-1 / EP1 could mitigate disturbance in  $Ca^{2+}$  levels and rescued number of NPCs in the DG after injection of ValS and SC19220 to the rats during 7HH. There are not many studies that can elaborate on the effect of COX-1 on DCX labeled neuron directly, but COX-2 being an inflammatory marker has shown to be both negatively and positively regulating DCX expression in DG [79,80]. Importantly, depletion of intermittent progenitor cells due to LPS induced microglial activation rescued after COX-1 and COX-2 or only after COX-2 inhibitor [81,82]. To best of our knowledge,

it is the first study that has directly shown the effect of COX-1 inhibitor on DCX labeled neurons in DG. We earlier reported COX-1 neurotoxicity to be microglial dependent, hence COX-1 inhibition reverted microglia activation and rescued adult neurogenesis deficits. Similarly SC19220 also decreases microglia and astrocyte activation in DG, supported by an observation where EP<sup>-/-</sup> mice showed ~70% reduction in hippocampal microgliosis [83]. NLRP3/ NFkB mediated neuro-inflammation was also decreased after COX-1/EP1R blockade results in decreased microglial activation and associated neuroinflammation. These observations are inconsistent with the findings where alleviation in expression of NLRP3/NFkB reduced microglial activation and proinflammatory cytokines [84,85]. Both ValS and SC19220 rescued microglial and astrocyte activation as their (COX-1/EP1) predominance in those cells thus ameliorated neurogenic niches perturbations. Consistent with our observation, there is a fascinating study where, EP1<sup>-/-</sup> mice suppressed microglial activation and rescued BrdU positive cells [86]. Our observation that EP1 antagonism effectively decreased pro-inflammatory cytokines in DG strengthen this premise further.

## Conclusion

The present study demonstrates the following significant phenomena: The exposure to HH alters social behavior along with increase in anxiety like behavior in rats. HH disturb the microenvironment by altering the expression of BDNF, Serotonin and pCREB followed decreased proliferation of neuronal progenitor as well maturation of new born neurons in DG. HH exhibits upsurge in the NLRP3/ NFkB signalling pathway in the hippocampus particularly in DG accompanied by activation of glial cells and inflammatory milieu culminate in neurogenesis deficits. Along with COX-1, PGE2 receptor EP1R is up-regulated in the hippocampus specifically in the glial cells in DG and their inhibition during 7HH exposure rescued alteration in the levels of BDNF, pCREB. Both pharmacological approach mitigated adult neurogenesis deficits, decreased activation of glia cells, NLRP3, NFkB signaling molecules and associated neuroinflammation, ultimately rescued social interaction deficits along with reduced anxiety like behavior. COX-1/ EP1 specific inhibition proved more beneficial in HH induced pathology, provides therapeutic alternatives to non-selective nonsteroidal anti-inflammatory drugs (NSAIDs), which has side effects in prolonged use. Various modalities that can enhance neurogenesis can be suggested to people before the ascent to high altitude, hence minimize alteration in associated behavior, like mood, social interaction, and cognition.

## List Of Abbreviations

**BrdU:** 5-bromo-2'-deoxyuridine

**COX:** Cyclooxygenase

**CREB:** cAMP response element-binding protein

**DCX:** Doublecortin

**DG:** Dentate Gyrus

**GFAP:** Glial fibrillary acidic protein

**HH:** Hypobaric Hypoxia

**1HH:** 1day exposure at hypobaric hypoxia

**3HH:** 3 day exposure at hypobaric hypoxia

**7HH:** 7 day exposure at hypobaric hypoxia

**7HH + SC19220:** 7HH with EP1 R antagonist SC19220

**7HH + ValS :** 7HH with COX-1 inhibitor Valeryl salicyclate

**Iba-1:** Ionized calcium binding adaptor molecule 1

**NLRP3:** NLR Family Pyrin Domain Containing 3

**PFA:** Paraformaldehyde

**PGE2:** Prostaglandin E2

## Declarations

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N/A

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## **Contribution**

KR and GC conceived and coordinated the study. GC, GK, Kroy, PK, SA, BT and KR performed the experiments. KR, GC, GK, KK, UP analyzed the experiments. KR, GC wrote the manuscript.

## **Ethics approval and consent to participate**

Human volunteers did not participate in the study. Animal experiments were conducted after the approval from the Animal Ethics Committee.

## **Consent for publication**

Not applicable.

## **Conflicts of interest**

All the authors declared that they have no conflicts of interest.

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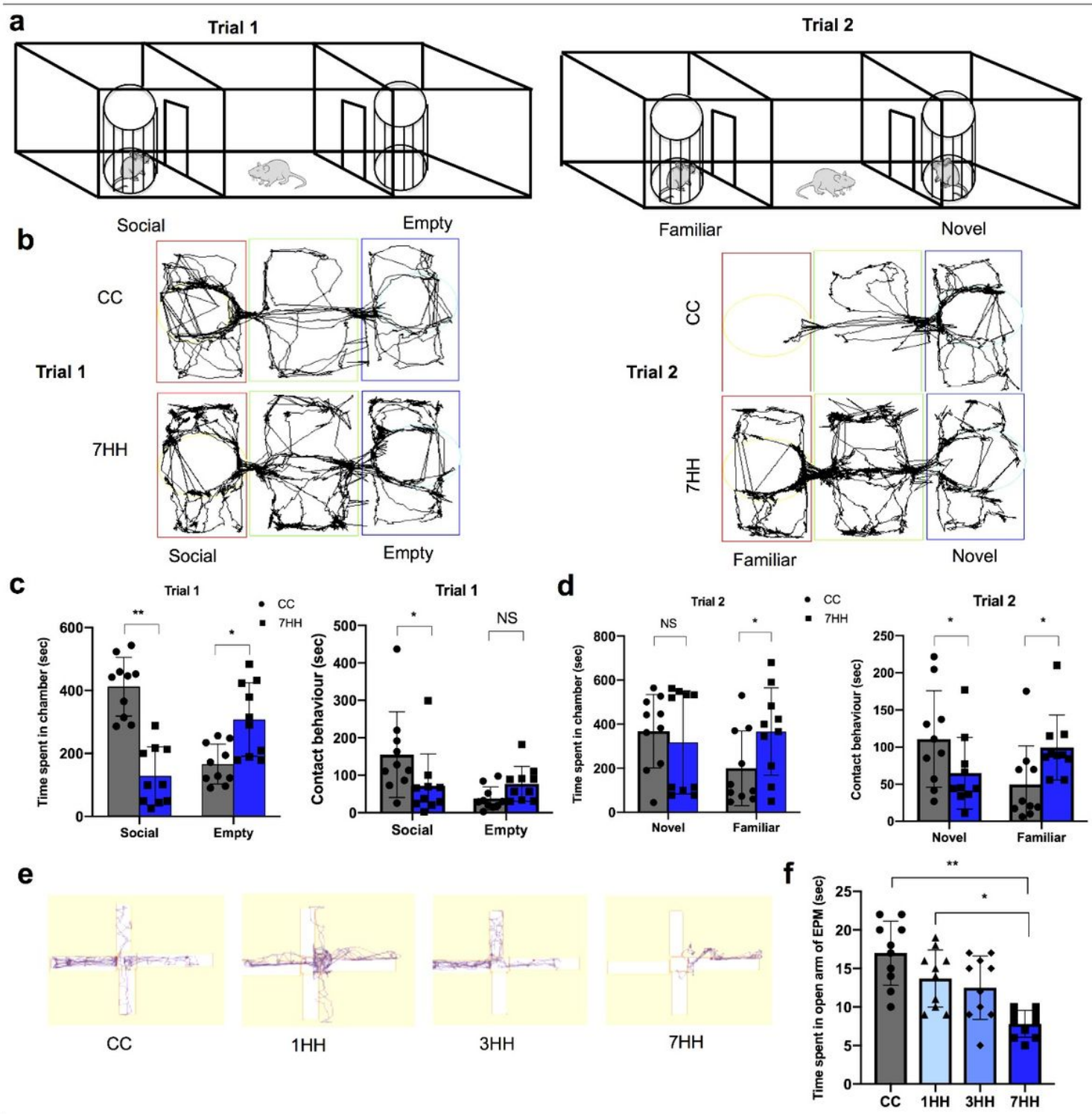
## Tables

**Table 1: List of primary antibodies used in immunohistochemistry and western blotting experiments**

Antibody Name	Host	Catalog Number	Provider	Dilution	Incubation
Anti-EP1	Rabbit (Rb)	ab140987	Abcam	1:1500 (WB)	24h,4°C
Anti-EP2	Rabbit (Rb)	ab167171	Abcam	1:1500 (WB)	24h,4°C
Anti- EP3	Mouse (Ms)	ab16152	Abcam	1:1000 (WB)	24h,4°C
Anti- EP4	Rabbit (Rb)	ab93486	Abcam	1:1000 (WB)	24h,4°C
Anti-IL1b	Rabbit (Rb)	ab9722	Abcam	1:1500 (WB)	24h,4°C
Anti-Iba1	Rabbit (Rb)	ab178846	Abcam	1:500 (IHC)	48h,4°C
Anti-COX1	Rabbit (Rb)	ab109025	Abcam	1:1000 (WB)	24h,4°C
Anti-COX1	Mouse (Ms)	ab695	Abcam	1:500 (IHC)	24h,4°C
Anti-TNF a	Rabbit (Rb)	ab6671	Abcam	1:1000 (WB)	24h,4°C
Anti-GFAP	Rabbit (Rb)	G9269	Sigma Aldrich	1:500 (IHC)	24h,4°C
Anti-COX2	Goat (Gt)	ab23672	Abcam	1:500 (IHC)	24h,4°C
Anti-NeuN	Mouse (Ms)	ab104224	Abcam	1:500 (IHC)	24h,4°C
Anti-Ki67	Rabbit (Rb)	ab16667	Abcam	1:250 (IHC)	24h,4°C
Anti DCX	Rabbit (Rb)	ab207175	Abcam	1:400 (IHC)	24h,4°C
Anti GFAP	Goat (Gt)	ab53554	Abcam	1:500 (IHC)	48h,4°C
Anti IL-6	Mouse (Ms)	ab9324	Abcam	1:1000 (WB)	24h,4°C
Anti BrdU	Mouse (Ms)	Mab4072	Millipore	1:250 (IHC)	24h,4°C
Anti Actin	Mouse (Ms)	ab8227	Abcam	1:1500 (WB)	24h,4°C
Anti NLRP3	Rabbit	NBP2-12446	Novus	1:1500	24h,4°C

			Biologicals	(WB)	
				1:300 (IHC)	
Anti NF- $\kappa$ B p65	Rabbit (Rb)	ab16502	Abcam	1:1500 (WB)	24h,4°C
Anti NF- $\kappa$ B p65(hosphor S536)	Rabbit (Rb)	ab86299	Abcam	1:1500 (WB)	24h,4°C
Anti Caspase-1	Rabbit (Rb)	ab108362	Abcam	1:1500 (WB)	24h,4°C
<b>Secondary antibodies</b>					
Alexa Fluor 594	Rabbit (Rb)	A11037	Invitrogen	1:2000 (IHC)	2h,RT
Alexa Fluor 488	Rabbit (Rb)	ab150077	abcam	1:2000 (IHC)	2h,RT
Alexa Fluor 594	Mouse (Ms)	A32740	Invitrogen	1:2000 (IHC)	2h,RT
Alexa Fluor 488	Mouse (Ms)	A32723	Invitrogen	1:2000 (IHC)	2h,RT

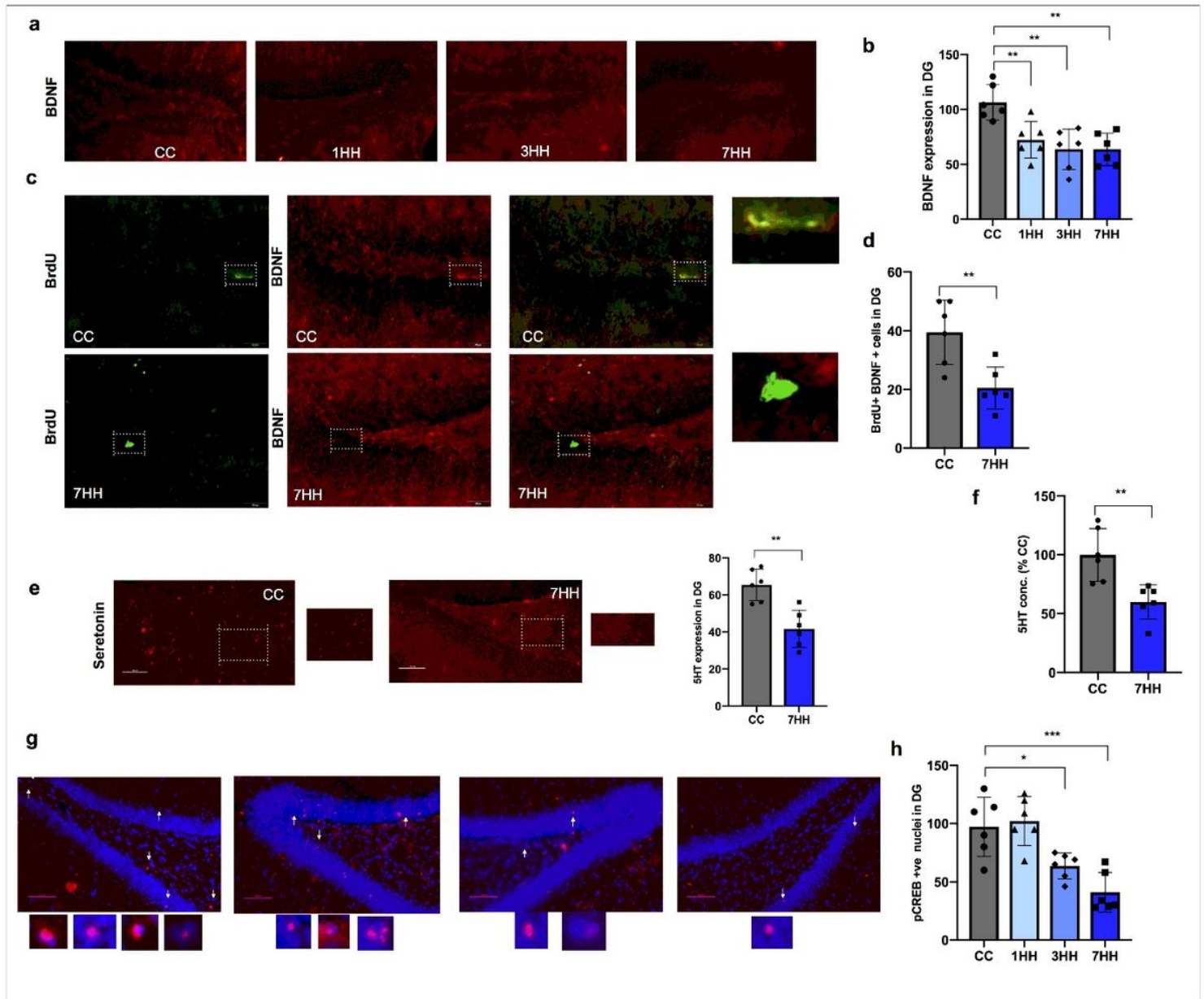
## Figures



**Figure 1**

Chronic HH exposure modulates social interaction behavior as well as anxiety-like behaviors. [A] Schematic representation of the three-chamber apparatus utilized to test for sociability in rats. The test was recorded in two trials for 10 min individually after 7HH exposure as compare to control. [B] Representative track plots of CC and 7 day exposed rats in the three-chamber apparatus recorded using Panlab SMART 3.0 software. [C] Statistic analysis graphs (Two-way ANOVA) represent time spent in the chamber and direct contact behaviour with social (Stranger 1) stimuli versus the empty enclosure during

trial 1. [D] Statistic analysis (n=10) graphs represent time spent in the chamber and contact behaviour with novel (Stranger 2) stimuli verses familiar (Stranger 1) during trial 2. [E] Behavioral representative track plot of Elevated plus maze (EPM) recorded with ANY-MAZE [F] Quantifications (One-way ANOVA, n=10) displayed as Time spent in the open arm of EPM in seconds among different groups. \*p < 0.05, \*\*p<0.01, \*\*\*p<0.001 versus control.

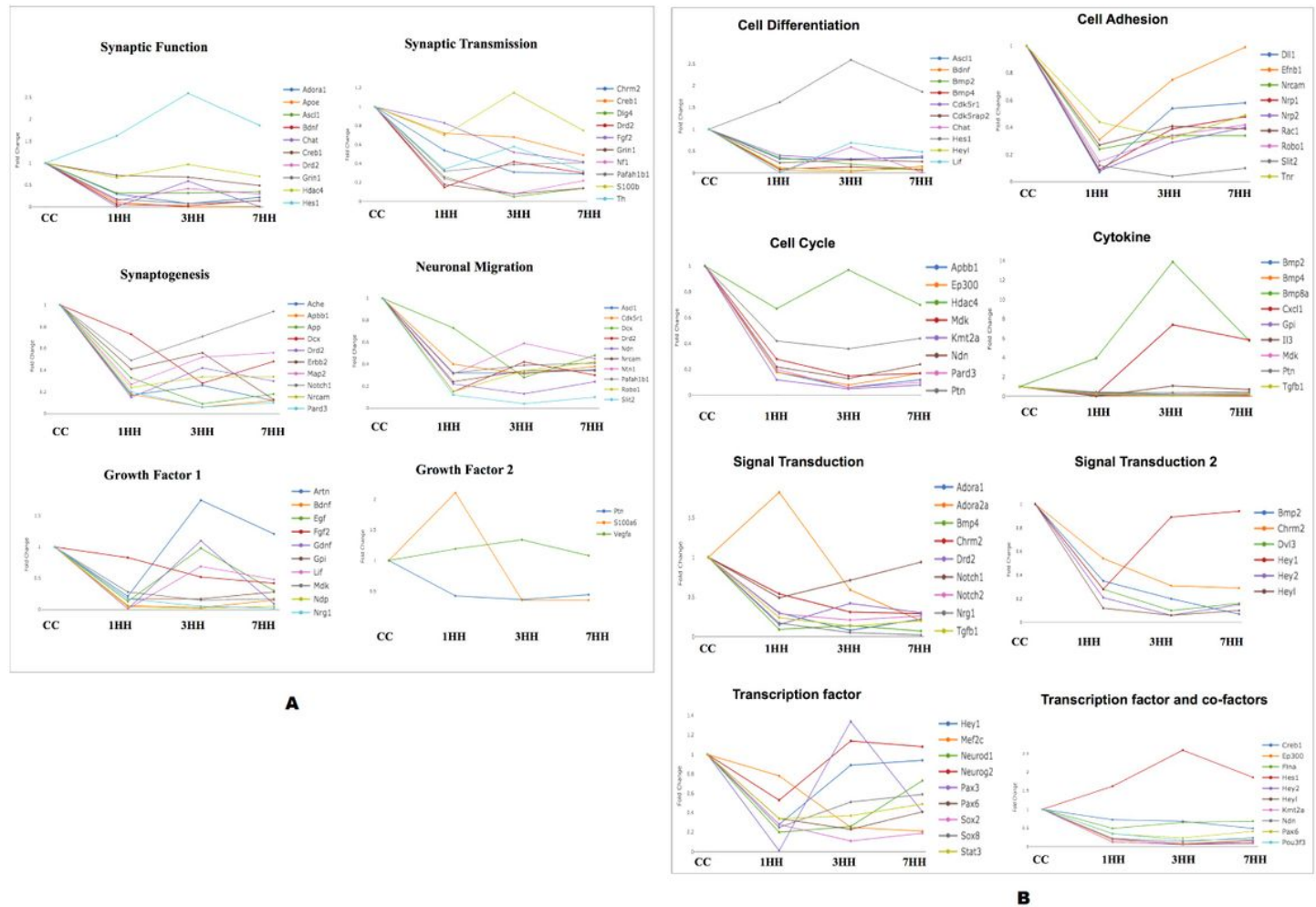


**Figure 2**

HH exposure alters expression of BDNF, Serotonin and pCREB in hippocampus. [A] Representative IHC images and [B] statistical analysis of BDNF expression (mean intensity) in the DG region after 1HH, 3HH, and 7HH exposure (Scale bar, 100  $\mu$ m). [C] Co-labelling images of BrdU (green), BDNF (red) and BrdU+ BDNF+ (yellow) in DG during control (CC) and 7days(7HH) group, Scale bar, 50  $\mu$ m. [D] Unpaired t-test analysis of BrdU+ BDNF+ cells compared between CC and 7HH groups. [E] Representative IHC images and statistical analysis of Serotonin expression in DG at CC and 7HH condition (Scale bar, 100  $\mu$ m). [F]

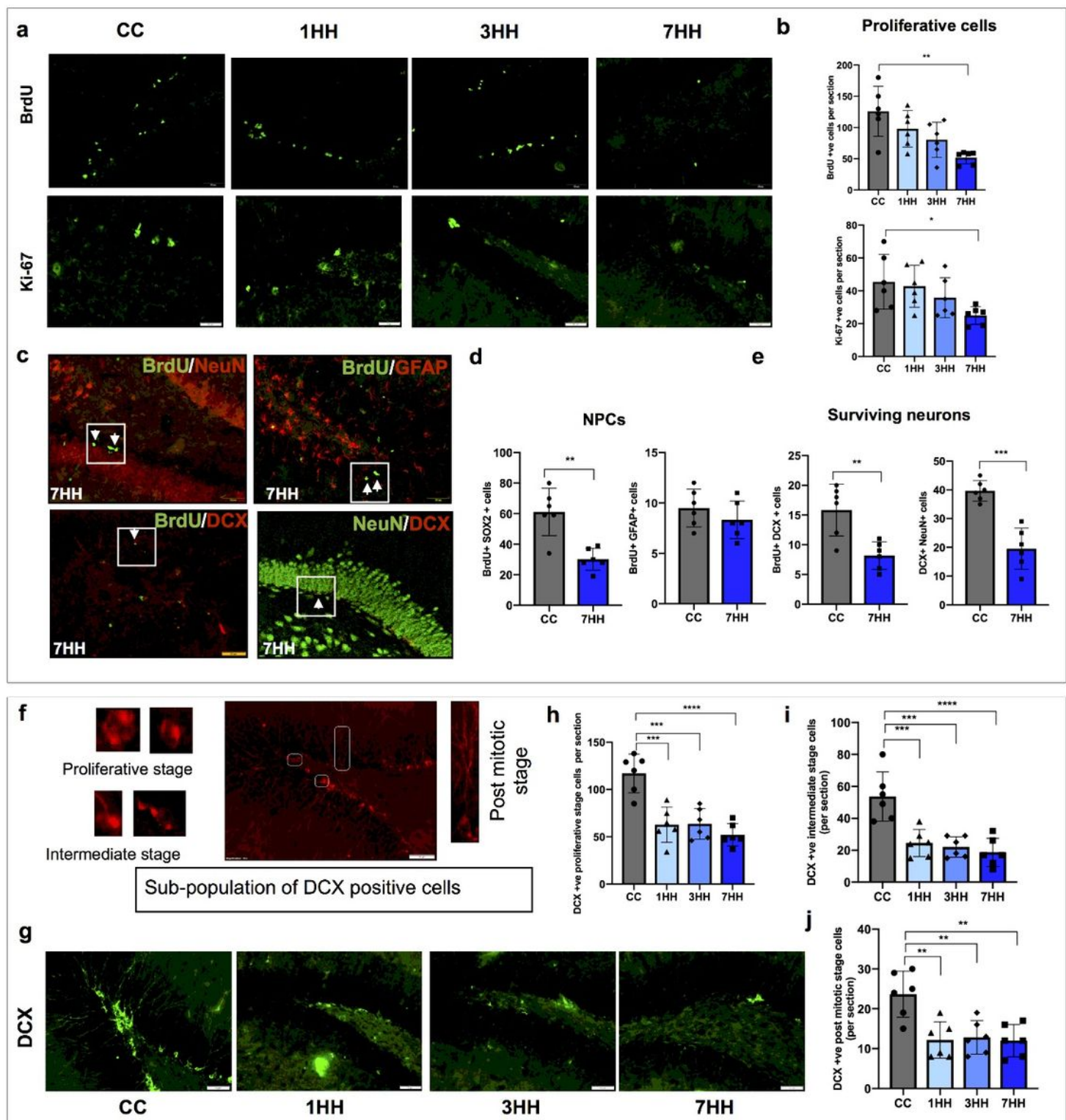


Levels of Serotonin in hippocampal lysate measured by HPLC at CC and 7HH condition. [G]  
 Representative IHC images and [H] statistical analysis of pCREB positive nuclei(pink) co-labelled with DAPI in DG showing decrease among HH groups compare to CC (Scale bar,100  $\mu$ m). \*p < 0.05, \*\*p<0.01, \*\*\*p<0.001 versus control.



**Figure 3**

3A: HH exposure induced perturbation of mRNA expression different functional classes of genes that regulate neurogenesis. Quantitative PCR array analysis revealed modulation of mRNA expression that contributes to different functional classes of genes like Synaptic function, Synaptic transmission, Synaptogenesis, Neuronal migration, and Growth factors upon exposure to 1 day HH, 3 day HH and 7 day HH as compared to CC. mRNA expression of genes like Bdnf, Adora1, Drd2, creb1, Egf, Fgf2, Chrm2, Dcx, Erbb2, Slit2, Nrg1, Lif, S100a6 down-regulated in temporal manner . 3B: HH exposure induced perturbation of mRNA expression different functional classes of genes that regulate neurogenesis. Quantitative PCR array analysis revealed modulation of mRNA expression that contributes to different functional classes of genes like Synaptic function, Synaptic transmission, Synaptogenesis, Neuronal migration, and Growth factors upon exposure to 1 day HH, 3 day HH and 7 day HH as compared to CC.

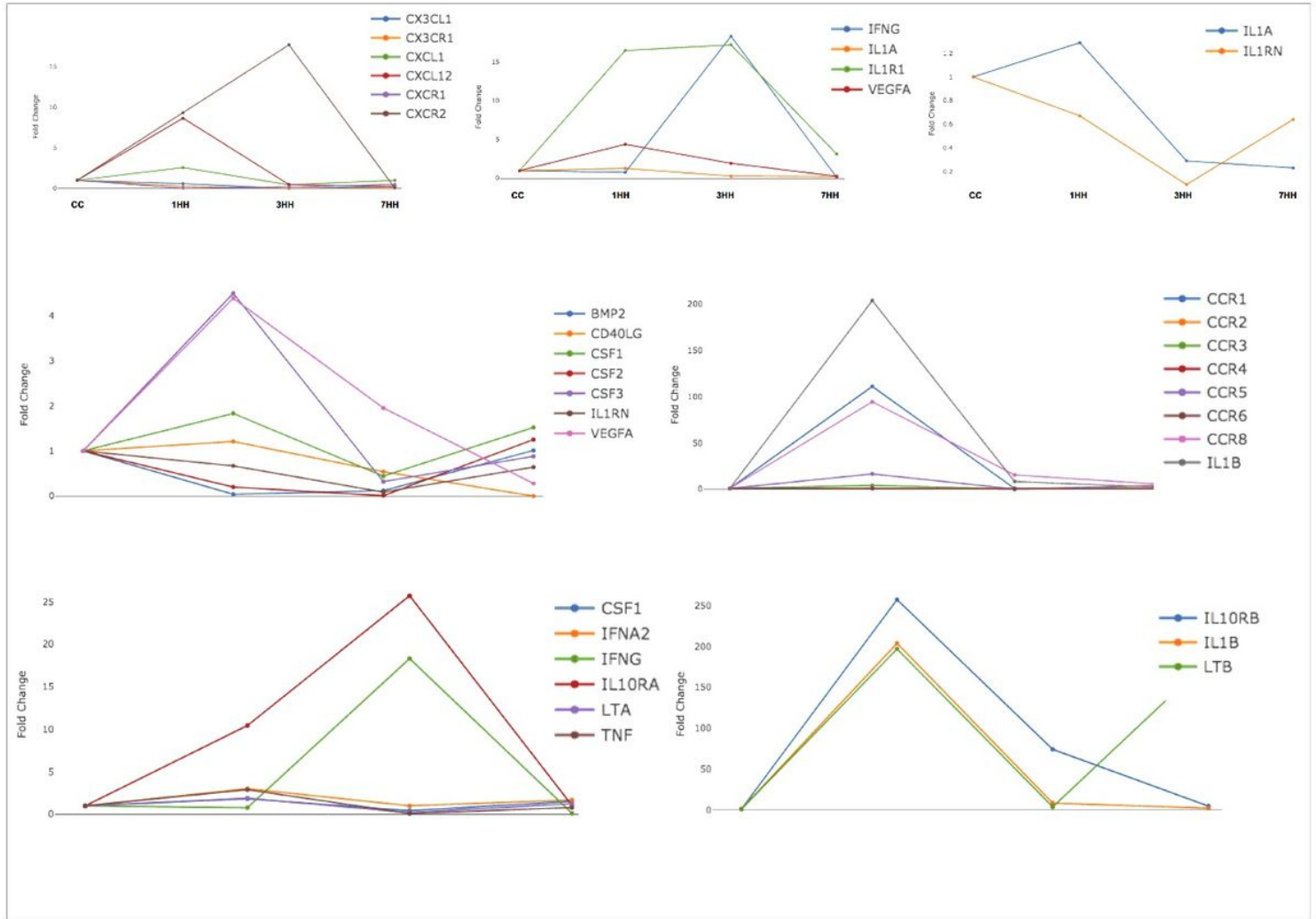


**Figure 4**

HH exposure elicits alteration in adult neurogenesis in the dentate gyrus. [A] Representative IHC images of BrdU and Ki-67 immuno labelled cells in the DG region after 1HH, 3HH, and 7HH exposure, respectively. [B] Statistical analysis graph (One way ANOVA) showing a decline in BrdU and Ki-67 labelled cells indicating decrease in proliferative cells in DG. [C] Representative co-labelled IHC images of neurogenesis markers (BrdU+NeuN+, BrdU+GFAP+, BrdU+DCX+, NeuN +DCX+) showing lesser co-labelling at 7HH exposure. [D]

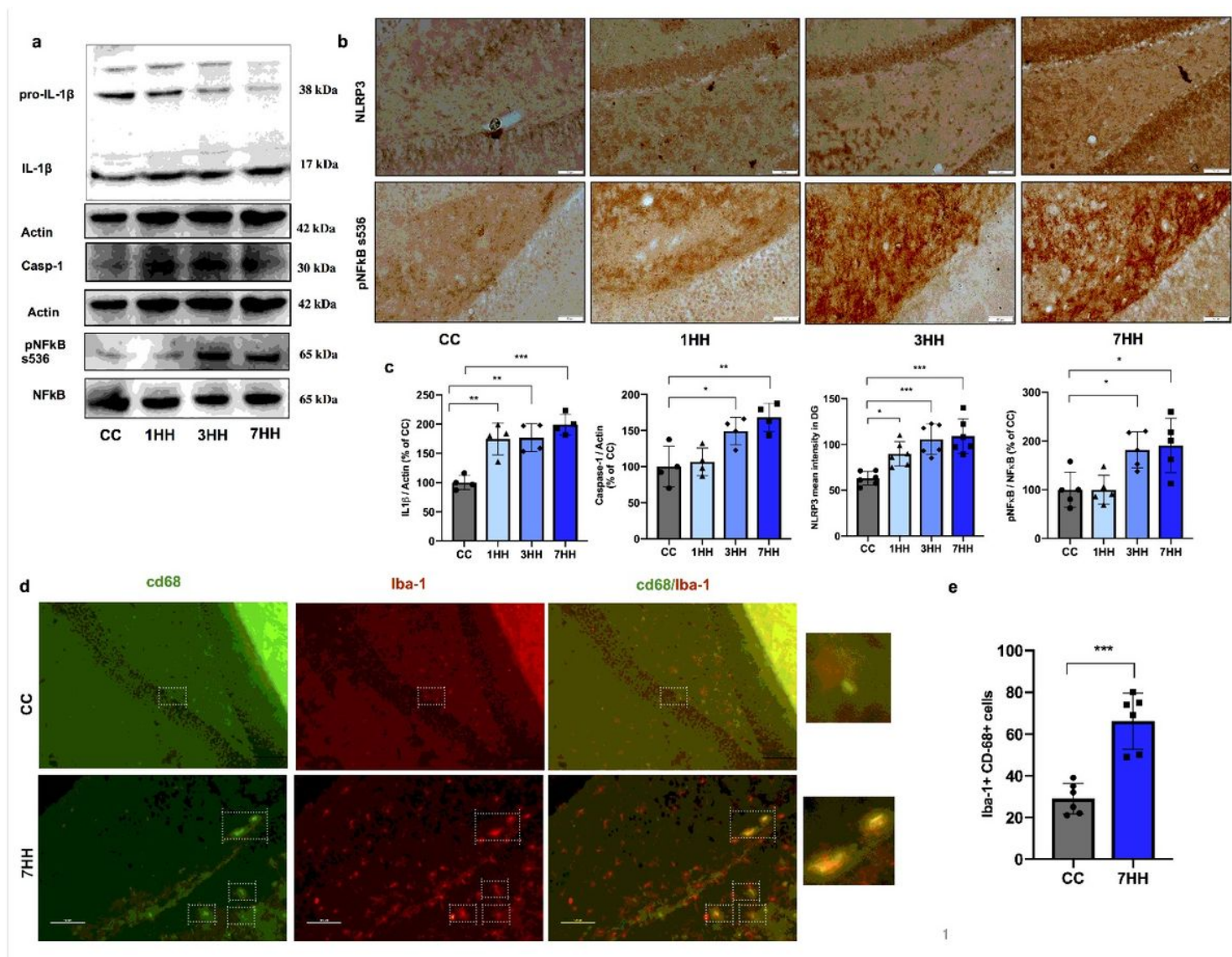


Reduction in neuronal proliferating cells at 7HH indicated by BrdU+SOX+ population but no difference in BrdU+GFAP+ cells. [E] Decrease in surviving neurons at 7HH indicated by BrdU+DCX+ and DCX+NeuN+ in DG as compare to control.[F] Sub-population of DCX +ve cells and [G] expression of DCX temporally in DG. Statistical graphs (n=6) showing decline in [H] DCX +ve cells at proliferative, [I] intermediate [J] post mitotic stage after 1HH, 3HH and 7HH exposure. Scale bars: 50  $\mu$ m, data displayed as mean  $\pm$  SEM. \*p < 0.05, \*\*p<0.01, \*\*\*p<0.001 versus control.



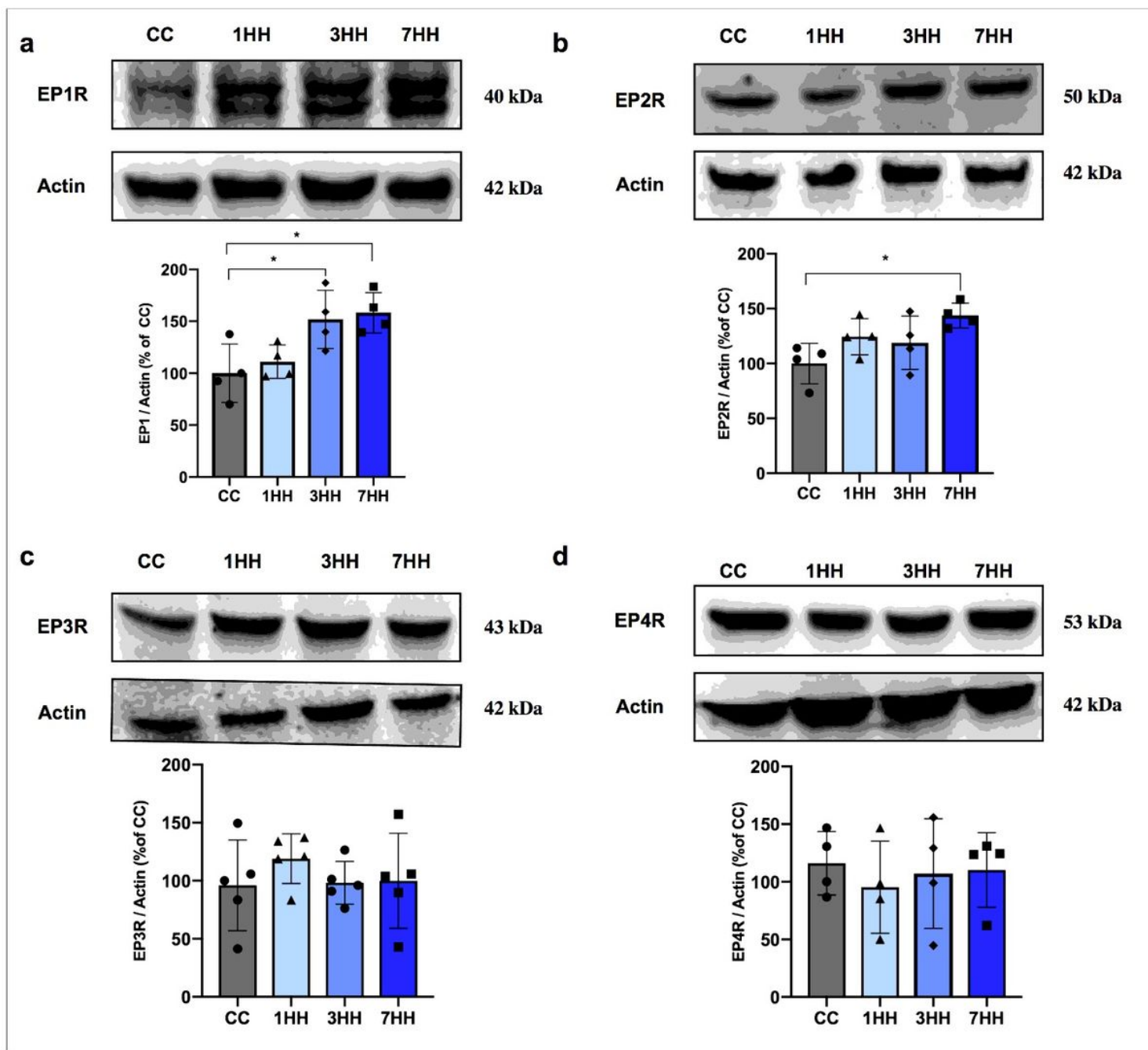
**Figure 5**

HH exposure perturb the mRNA expression of pro-inflammatory and anti-inflammatory cytokines and their receptors in hippocampus. Quantitative PCR array analysis revealed modulation of mRNA expression. mRNA expression of proinflammatory genes like CXCL1, CXCL12, CXCR2, IFNG, VEGFA, CSF3, IL1B, CCR1, CCR8, CCR5, IL10RA, LTB, IL1A, IL1RN, BMP2, CD40LG shown during 1HH, 3HH and 7HH exposure.



**Figure 6**

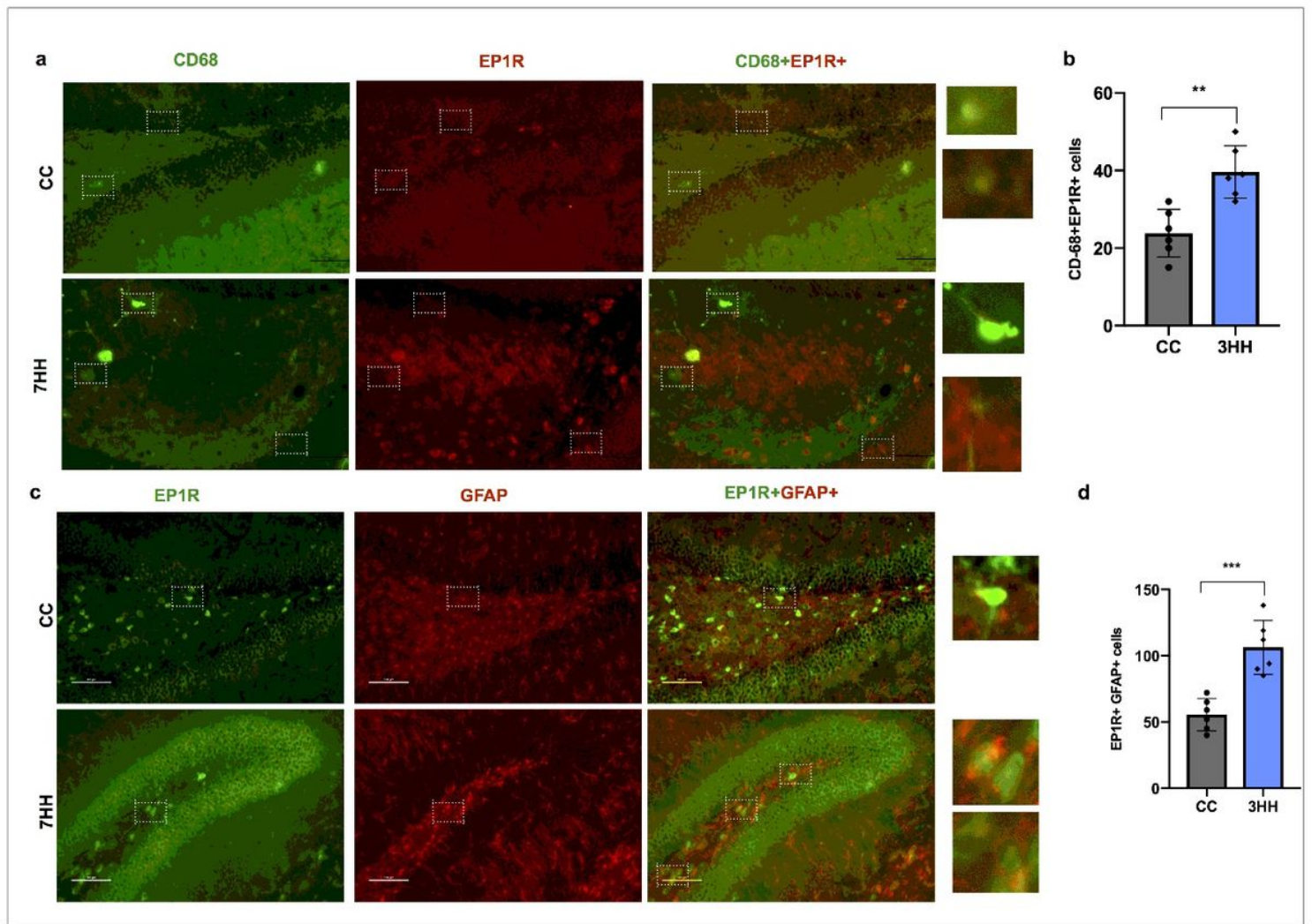
HH exposure upsurge NLRP3/NF $\kappa$ B associated neuroinflammation in hippocampus. [A] Representative Immunoblots of IL-1 $\beta$ , Caspase-1, pNF $\kappa$ B, NF $\kappa$ B among CC, 1HH, 3HH, 7HH in hippocampus. [B] Representative IHC images of NLRP3 and pNF $\kappa$ B in DG among CC, 1HH, 3HH, 7HH showing increasing pattern. [C] Statistical analysis of IL-1 $\beta$ , Caspase-1, pNF $\kappa$ B, NF $\kappa$ B among CC, 1HH, 3HH, 7HH representing upsurge in expression during HH temporally. [D] Representative IHC images [E] Unpaired t-test analysis of Iba-1+(red) CD68+(green), Iba-1+CD68+ (yellow) cells in DG compared between CC and 7HH group (Scale bar, 100  $\mu$ m). Data displayed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control.



**Figure 7**

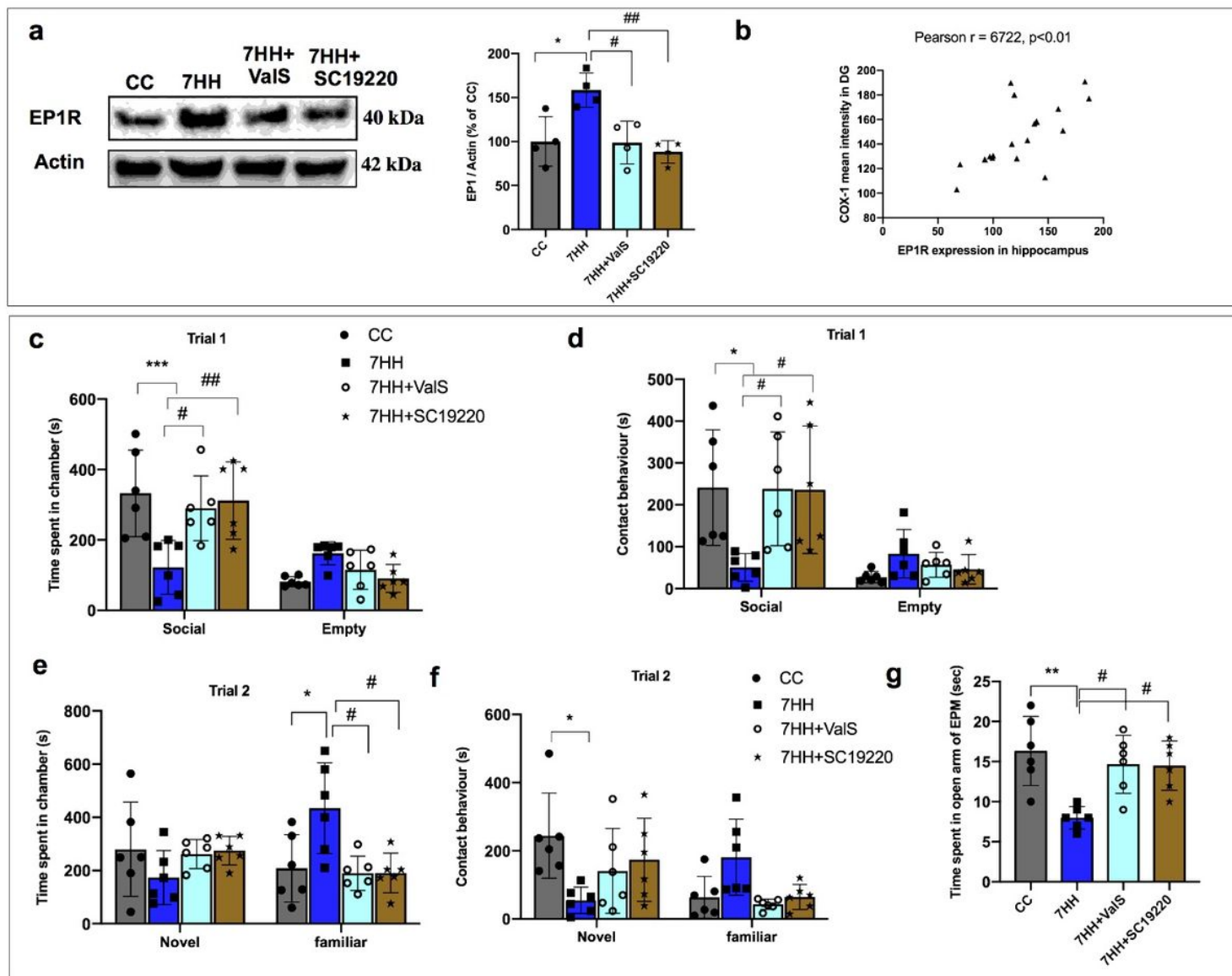
HH differentially modulated prostanoid GPCR receptors -Time course of EP1-4 receptors after HH exposure [A] A representative immunoblot of EP1 expression in the hippocampus over time in response to the HH Box plot showing EP1 expression increased significantly after 7HH exposure ( $p < 0.05$ ). [B] A representative immunoblot of EP2 expression, normalized with actin, is increased significantly after 7HH exposure. [C] EP3 expression and [D] EP4 expression in the hippocampal lysate was not statistically significant over time.





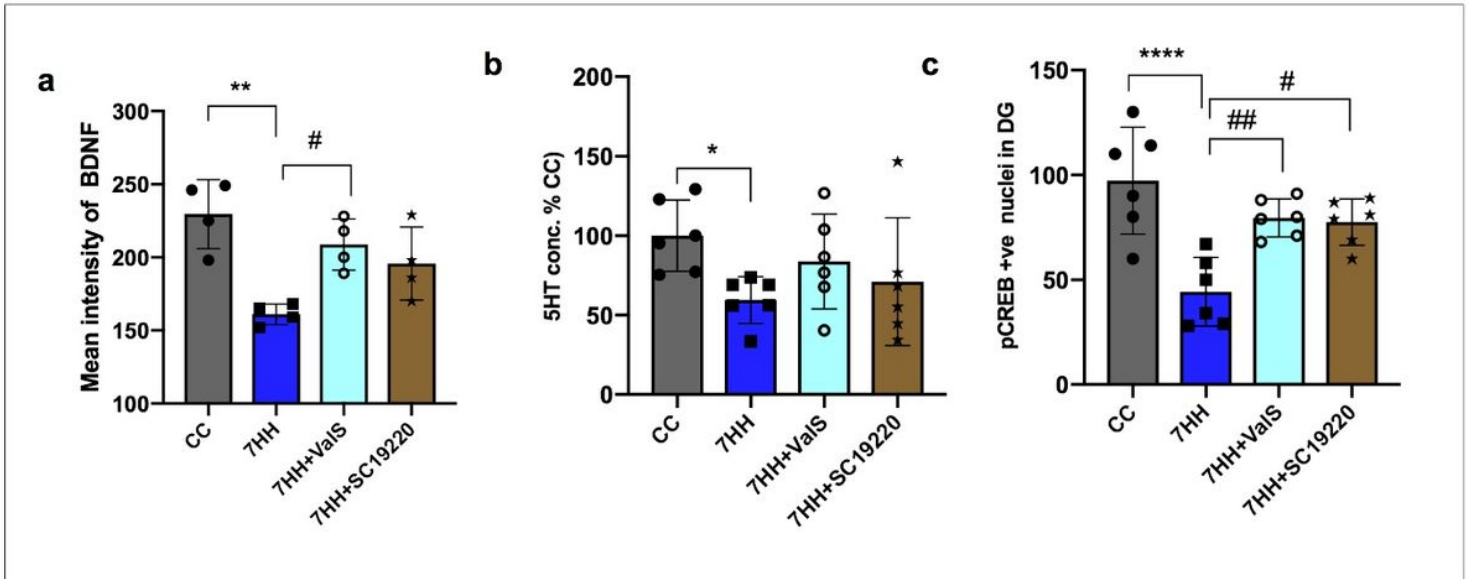
**Figure 8**

Elevation of EP1R expression in glial cells during HH exposure [A] Representative Immunoblots showing CD68+ (green) EP1R+ (red) cells and colabel CD68+IEP1R+ (yellow) in DG at CC and 3HH condition. [B] Statistical analysis showing increase in CD68+EP1R+ at 3HH exposure as compare to control. Similarly, EP1R+ (green) GFAP+(red) co-labelled EP1R+GFAP+ (yellow) cells number increase after 3HH exposure in DG shown in [C] IHC images and [D] Unpaired t-test between both groups (Scale bar,100  $\mu$ m). Data displayed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control.



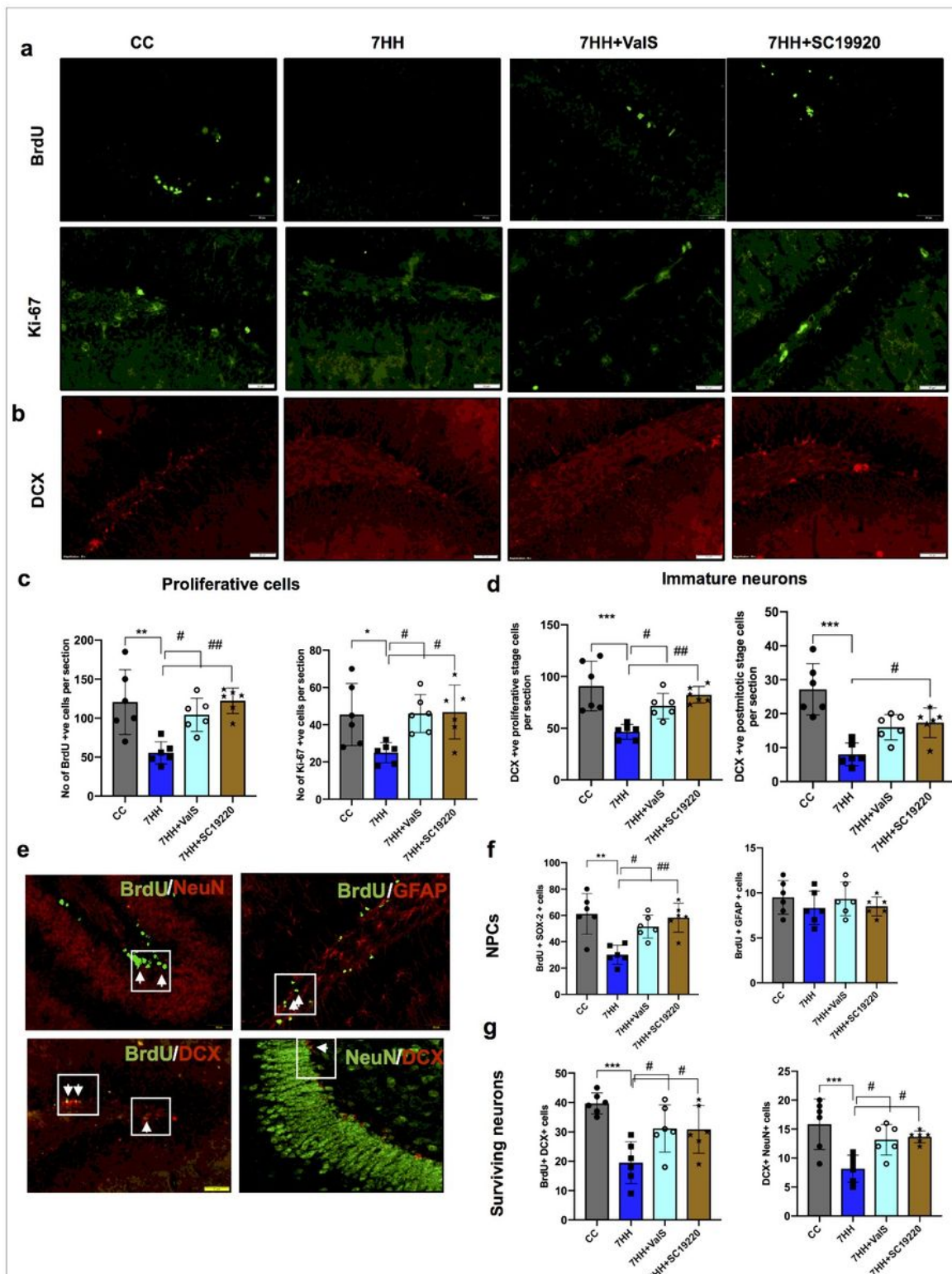
**Figure 9**

COX-1 dependent PGE2 synthesis via downstream EP1 receptor is critical for the induction of social interaction deficits during HH exposure. [A] Representative immunoblot and statistical graph (One way ANOVA,  $n=3$ ) showing expression of PGE2 receptor EP1 among groups, decreased with a specific COX-1 inhibitor (Valeryl salicyclate) and EP1R antagonist (SC19220) respectively. [B] Pearson's co-relation graph of COX-1 mean intensity in DG and EP1R expression in hippocampus [C] Statistical analysis (Two-way ANOVA,  $n=6$ ) indicating that treatment with ValS and SC19220 increased time spent in chamber [D] and direct contact with social (stranger 1) enclosure whereas no difference with empty enclosure in Trial 1. [E] During Trial 2, time spent in chamber and [F] contact behaviour with familiar (stranger 1) was reduced, whereas contact behaviour with Novel (stranger 2) was increased after treatment. [G] Time spent in open arm of EPM shown after pharmacological inhibition as compare to 7HH and control group. For all panels: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control, # values are in comparison to 7HH group. CC, Cage Control, 7HH: 7 day Hypobaric Hypoxia, 7HH+ ValS: valeryl salicyclate treated during 7 day Hypobaric Hypoxia, 7HH+ SC19220: SC19220 treated during 7 day Hypobaric Hypoxia.



**Figure 10**

Pharmacological inhibition of COX-1 and EP1 during HH differentially rescued BDNF, Serotonin, pCREB levels in hippocampus. One-way ANOVA analysis revealed that [A] mean intensity of BDNF in DG which decrease at 7HH, rescued after COX-1 inhibition(ValS) and EP1 antagonist (SC19220). [B] 5HT concentration measured by HPLC in hippocampus decreased after 7HH exposure and no effect from pharmacological intervention. [C] pCREB positive nuclei in DG increased after COX-1 inhibition (ValS) and EP1R antagonist (SC19220) during 7HH. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus control, # values are in comparison to 7HH group.



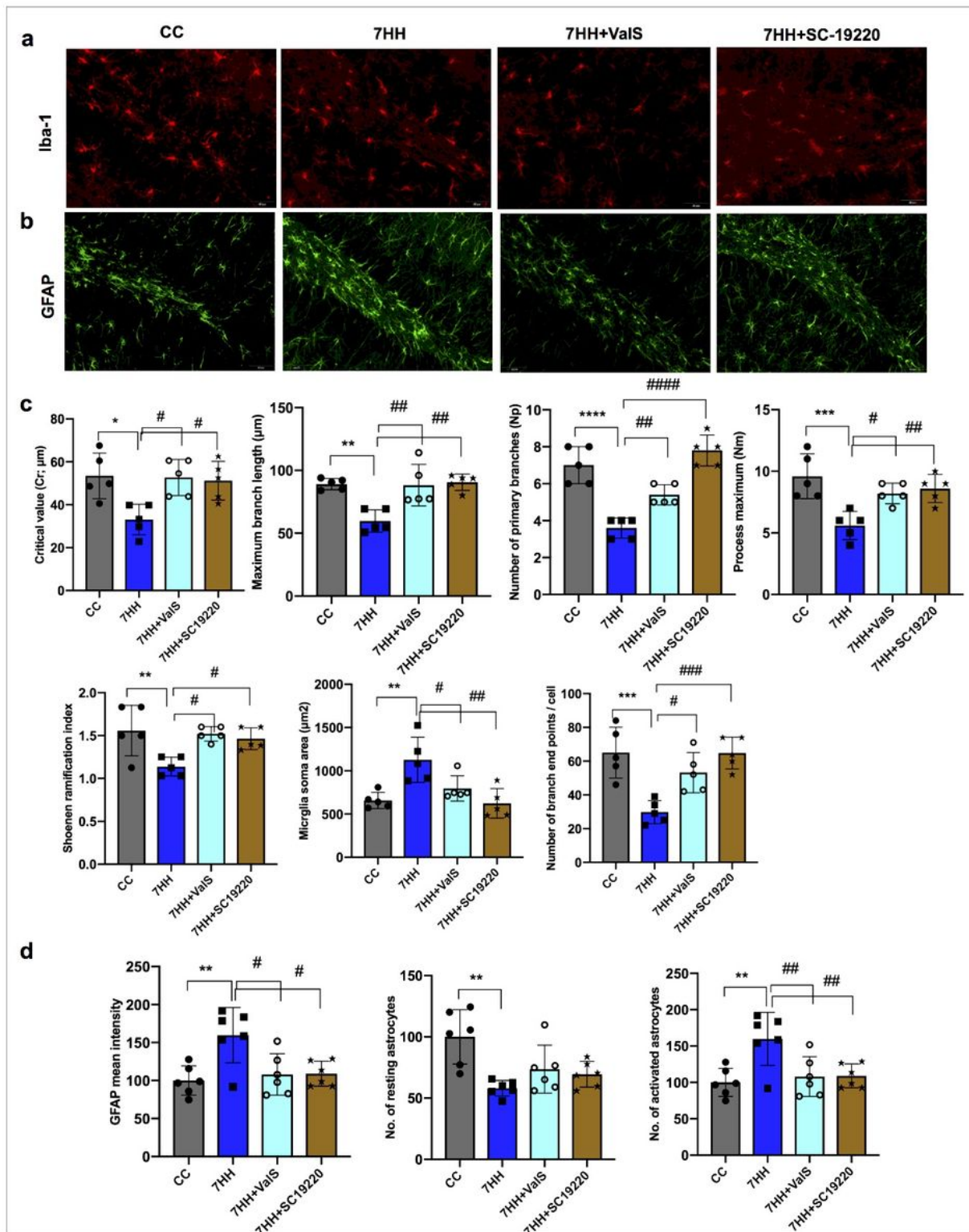
**Figure 11**

Inhibition of COX-1 and its EP1 R during HH stress boosts neuronal cell proliferation and survival in DG. [A] Representative IHC images of BrdU, Ki-67 and [B] DCX hosph labelled cells in the DG region at 7HH exposure and after Valeryl salicyclate and SC19220 treatment.[C] Statistical analysis graph (One way ANOVA, n=6) showing an elevation in BrdU and Ki-67 labelled cells after treatment with ValS and SC-19220 indicating rescue in number of proliferative cells in DG. [D] Elevation in immature neuronal



population labelled with DCX after treatment with ValS and SC19220 at 7HH as compare to only 7HH group. [E] Representative co-labelled IHC images of neurogenesis markers (BrdU+ NeuN+, BrdU+GFAP+, BrdU+DCX+, NeuN +DCX+) showing differential effect on co-labelled cells after treatment with ValS and SC19220 compare to 7HH. [F] Administration of ValS and SC19220 during 7HH boost neuronal proliferating cells indicated by BrdU+SOX+ population but no difference in BrdU+GFAP+ cells. [G] Elevation in surviving neurons after treatment with ValS and SC19220 indicated by BrdU+DCX+ and DCX+NeuN+ in DG as compare to 7HH exposed rats. Scale bars: 50  $\mu$ m, data displayed as mean  $\pm$  SEM. \*p < 0.05, \*\*p<0.01, \*\*\*p<0.001 versus control, # values are in comparison to 7HH group.





**Figure 12**

Activation of glial cells reverted with pharmacological blockade of COX-1 and EP1 R. [A] Representative fluorescence images of Iba-1 stained microglia and [B] GFAP stained astrocytes in DG after Vals and SC19220 administration during 7HH. [C] Sholl and skeleton analysis. It revealed that 7HH exposure altered microglia morphology and treatment with Vals and SC19220 rescued it. [D] Statistical analysis

graph indicate that in DG after ValS and SC19220 inhibition decreased mean intensity and the number of activated astrocytes. No difference on resting astrocyte observed.

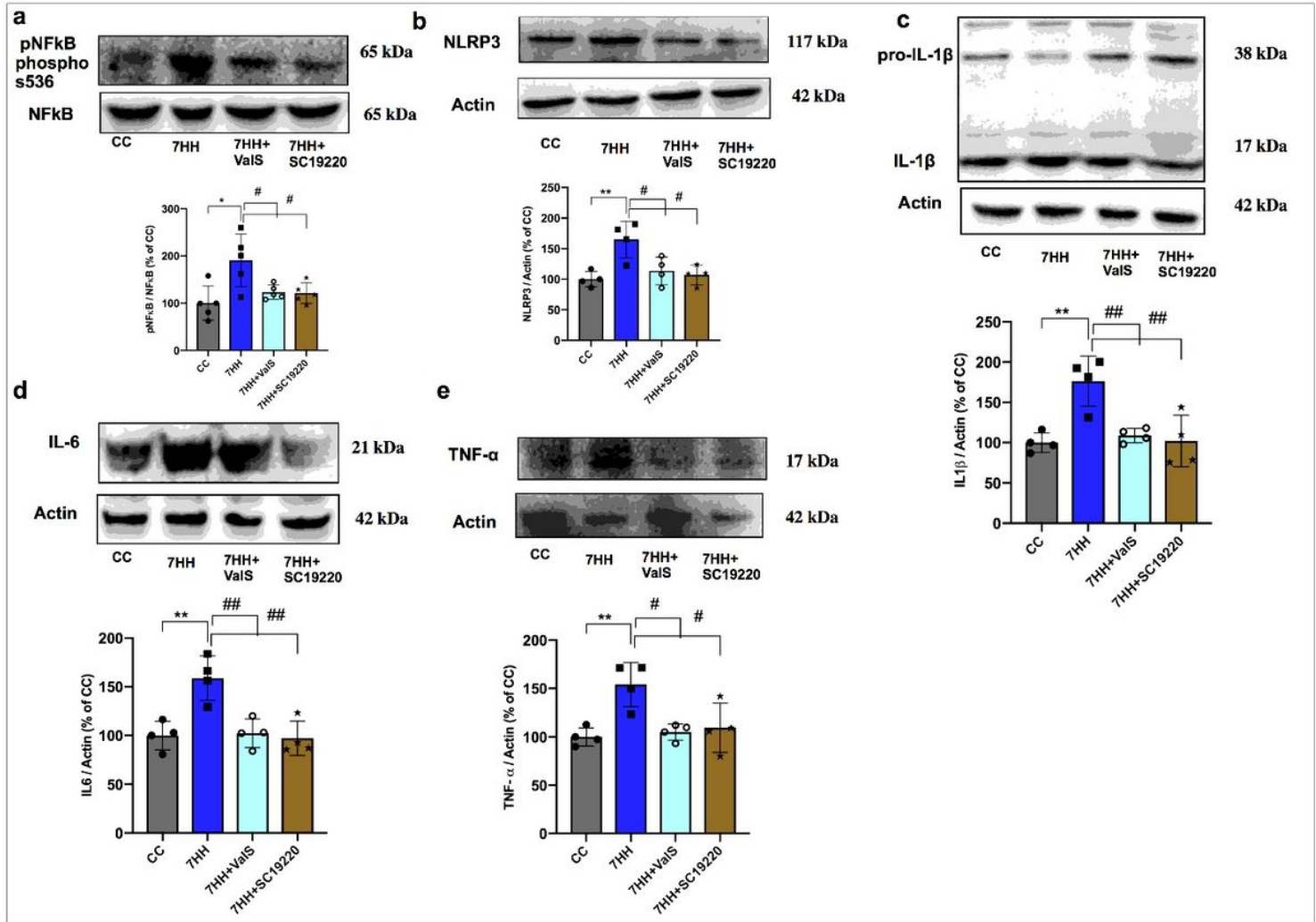


Figure 13

Activation of NLRP3/NFκB and associated neuro-inflammation mitigated after pharmacological blockade of COX-1 and EP1 R. [A] Representative immunoblots of pNFκB and ANOVA analysis revealed reduction in the phosphorylation of NFκB after ValS and SC19220 administration during 7HH compare to 7HH. [B] Representative immunoblots and ANOVA analysis showed induction in NLRP3 expression at 7HH in hippocampal lysate, reduced after 7HH+ValS and SC19220 administration during HH compare to only HH group.[C] Representative immunoblots and statistical analysis revealed decrease in expression of IL1β during HH upon administration of COX-1 inhibitor and EP1 antagonist as compare to 7HH group. [D] Representative immunoblots and statistical analysis showing reduction in expression of IL-6 and [E] TNF-α during HH upon administration of COX-1 inhibitor and EP1 antagonist.

## Supplementary Files

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