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ETV5 is a Putative Differentially Expressed Transcription Factor of Interleukin 1β Under Neuroinflammatory Conditions in Murine Cerebellum

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ABSTRACT

Neuroinflammation is the underlying cause of a number of neurodegenerative disorders. While the current understanding of its molecular mechanisms is continually increasing, the identification and consequently the roles of transcription factors (TFs) in the respective molecular events under neuroinflammatory conditions in distinct brain regions is still lacking. To address the said gap in knowledge, first we have identified the TFs specific to IL-1β a key player in mediating inflammatory process using online databases. Next, we identified the interacting partners of the significantly differentially expressed TFs out of the selected ones, and the mRNA expression changes were studied for both the differentially expressed TF along with its interacting partners. Finally, we explored the expression of these genes from some online available RNA-seq datasets for brain tissue, neurons, microglia and astrocytes. The results showed significant upregulation of Etv5 TF in male mouse brain hippocampus along with a significant downregulation of three of its predicted interacting partners, i.e., Cop1, Det1 and Bcl6b. Besides presenting new targets for neuroinflammation studies, the discrepancies of expression between different brain regions as well as among the genders suggest that more data with respect to intricate brain region resolution along with single cell data could reflect subtle changes in brain microenvironments and individual cells, which can lead to a more precise pathological understanding of the neuroinflammatory process.

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ARS conceptualization, methodology, funding acquisition, project administration, resources, supervision, writing (review and editing). AT-AK conceptualization, investigation, methodology, writing (original draft). Both the authors have read and approved the final submission.

Key words
Neuroinflammation, Neurodegeneration, Transcription factors, Gene expression analysis, RT-PCR, RNA-seq analysis

INTRODUCTION

The inflammation of brain and spinal cord, together constituting our central nervous system (CNS), is known as neuroinflammation. Being implicated in a variety of somatic and neurological illnesses, the most common cues of its initiation known till date are toxicity to various metabolites, traumatic brain and spinal cord injuries, viral and/or bacterial toxicity, and autoimmune mediated neuroinflammation (Lyman *et al*., 2014; Feinstein *et al*., 2016). Apart from being central to the mutual pathology of a number of acute and chronic neurological disorders, neuroinflammation has also been found to be a major cause and aggravating factor for the development of neurodegenerative diseases. Moreover, its role in the early development of some chronic conditions, e.g., dementia, has also been reported (Lyman *et al*., 2014; Kwon and Koh, 2020; DiSabato *et al*., 2016).

Being pleiotropic mediators and effectors of cells' and tissues' inflammatory processes and of various autoimmune disorders, interleukin-1 α (IL-1α) and interleukin-1 β (IL-1β) are archetypal proinflammatory cytokines. Of these, IL-1β also performs crucial homeostatic functions in a healthy organism. These include the regulation of body temperature, sleep, and feeding (Dinarello, 1996, 2004). Consequently, its overproduction is associated with various pathophysiological anomalies such as inflammatory bowel disease, rheumatoid arthritis, osteoarthritis, vascular diseases, neuropathy, Alzheimer's disease and multiple sclerosis (Braddock and Quinn, 2004).

Some of the common documented events during the process of neuroinflammation include: Increased activation of brain's resident immune cells (glial cells), altered permeability of blood brain barrier (BBB), leukocyte invasion, and increased concentration of the

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pro-inflammatory cytokines (Rejo *et al*., 2008; Guzyman-Martinez, 2019). IL-1 being upregulated in several of the neurodegenerative disorders such as Parkinson's disease (PD) (Leal *et al*., 2013; Stojakovic *et al*., 2017), Alzheimer's disease (Cacabelos *et al*., 1991, 1994), multiple sclerosis (Lin and Edelson, 2017) and amyotrophic lateral sclerosis (Meissner *et al*., 2010), also makes IL-1β a key player in the process of neuroinflammation too, along with other inflammatory processes of the body. The pathways activated by overproduction of IL-1β include NFkB-dependent transcription of some other pro-inflammatory cytokines such as TNF-α, interferons and IL-6; activation of neutrophil recruiting chemokines; and stimulation of synaptophysin and Tau proteins' production in neurons (Acuner-Ozbabacan *et al*., 2014; Fan *et al*., 2007; Pyrillou *et al*., 2020).

The regulatory elements of *IL-1B* include a TATA box within the promoter, a CRE (cAMP responsive element), an AP-1 (activator protein 1) promoter site, an SP-1/PU.1 (the ETS family of transcription factor) binding site, and an NF-κB (nuclear factor kappa B) binding site (Tjitro *et al*., 2018; Perez *et al*., 1999; Lahiri *et al*., 2002; Shirakawa *et al*., 1993). The induction of *IL-1B* gene expression can be brought about via various stimuli including both microbial and non-microbial types. The initial translation product is a propeptide (31 kD), the pro-IL-1β, which is cleaved to the mature and biologically active form of IL-1β by the action of cysteine protease caspase-1 (Zepter *et al*., 1997; Herzog *et al*., 2005; Eder, 2009).

The presence of both neuroinflammation and tissue damage in post mortem brain tissues makes it difficult to determine whether upregulation of *IL-1B* is caused by, or a consequence of neuroinflammation (Hewett *et al*., 2012). This also urged the need to explore *IL-1B* along with its potential mediators to get a better understanding of its precise role in the process of neuroinflammation. This study was therefore aimed at identification of the transcription factors (TF) specific to *IL-1B* promoter/enhancer region and then study their differential expression under neuroinflammatory conditions using in vivo LPS mouse models.

MATERIALS AND METHODS

Identification and Screening of TFs for the promoter/enhancer region of IL-1B

Various online available databases were utilized for the identification of the promoter region of *Il-1B* gene. These include GeneCards (https://www.genecards.org/), the eukaryotic promoter database (https://epd.epfl.ch//index.php), and super-enhancer database (http://www.licpathway.net/sedb/). These databases also identified the TFs having the binding sites for the promoter of *Il-1B* and among them, only those TFs were preferred having the highest scores from the online databases. The resulting TFs were further screened on the basis of their tissue specific high or low expression profiles so as to select only those relevant to the tissue being used in the study i.e., brain.

Neuroinflammation murine models

Male and female BALB/c mice, approximately 6 weeks of age, were housed in groups of 4 in cages containing rodent bedding. Food and water were available *ad libitum* and temperature was maintained at 25°C. All experiments were carried out on mice divided into two groups, experimental drug group and vehicle control group receiving physiological saline injections only.

BALB/c mice 6-8 weeks of age were administered with a single dose of 5 mg/kg of lipopolysaccharide (LPS) solution (Sigma Cat # L2880-25MG) dissolved in 0.9% saline solution via intraperitoneal route. The controls received injections of saline solution only. The sacrifice of animals was carried out after 24 h of LPS treatment.

Animal killing and dissections

The mice were sacrificed by cervical dislocation. Briefly, the backside of a knife was placed on the dorsal side of the neck at the junction between skull and the vertebrae. Then a little pressure was applied through the knife while pulling the animal from its tail so as to dislocate the head from the rest of the body in a single jerk to minimize animal discomfort. The bioethical clearance certificate was obtained from the Bioethics Committee of the School of Biological Sciences, University of the Punjab.

The brains were further dissected to get two regions i.e., hippocampus and cerebellum. These two regions are shown in the Supplementary Figure S1 which represents the regional map of murine brain. The protocol for dissection of these regions was followed from Li (2011). The cerebellum is located on the dorsal side of the brain and on the lower side as can be seen in the Figure 1. First, the portion of the brain containing the cerebellum was separated from the cerebral cortices by making a vertical cut. Then the tissue underneath the cerebellum consisting of midbrain, pons and medulla was separated from it very carefully with the help of fine curved forceps.

The dissection of hippocampi is a bit difficult than the cerebellum as they are located on the inside of the cerebral cortices and are covered with tissue. Therefore, the dissection procedure of hippocampus is explained a little more as in the Figure 1B, C since the crescent shaped hippocampi are placed on the dorsal side of the brain covered with a fatty tissue the removal of which makes the hippocampus visible.

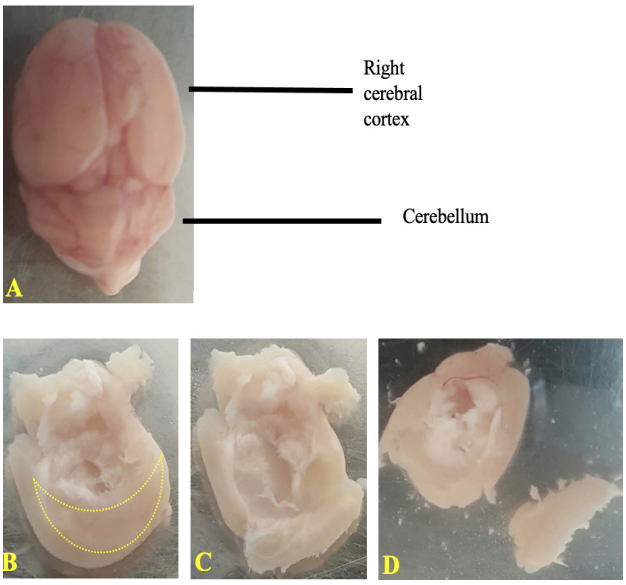

Fig. 1. Dissection of murine brain to separate out hippocampus. A, Dorsal view of the mouse brain. On the lower side is the cerebellum and on the upper side are the cerebral cortices. Each cerebral cortex contains hippocampus embedded in its ventral inner side. B, right cerebral cortex is placed on its dorsal side with the crescent shaped hippocampus clearly visible. C, hippocampus flipped inside out; and D, hippocampus separated from the rest of the tissue.

qRT PCR of IL-1β TFs

For total cell RNA extraction, the tissues were homogenized in TRIzol reagent via trituration and total RNA was extracted according to the manufacturer's protocol. The RNA was used for complementary cDNA synthesis after DNase treatment using Revert Aid First Strand cDNA synthesis kit (Thermofisher Scientific, Cat # K1622).

Extracted RNA was quantified using pico200 spectrophotometer at $A_{260/280}$. For DNase treatment of RNA sample, Invitrogen kit (Cat # 18068015) was used for this procedure as per instructions of the kit. The cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (Cat # K1681).

The quantitative expression analysis of genes was done by real time PCR analysis. Maxima SYBR Green/ ROX qPCR master mix (2X; Thermofisher Scientific, Cat # K0222), cDNA, and 5 µM forward and reverse primers' mix were used for RT-PCR (the sequences of the primers are provided in Table I. Two-step thermal cycle protocol with optimized annealing temperatures (Table I) of each primer pair was used for amplification and quantification of amplicons on PikoReal Real-Time PCR system (Thermofischer Scientific). Melting curve data with every 0.2°C increment was taken to determine the uniformity of PCR product. *HMBS* was used as a reference gene in the study. Significance analysis for fold differences of biological replicates was done using t-test for means.

Table I. Sequences of primers used for RT-PCR along with their optimized annealing temperatures.

Gene name	Primer	Sequence (5'-3')	Optimized primer annealing temperature	Product length (bp)
HMBS	Forward	CACTGGCTTCCCGTTCACTA	58-61	160
	Reverse	GCCAGGGGAGGTGAGAAAC		
ETV5	Forward	CAAGGTCAAGAAAACCCGCC	60	
	Reverse	CGCTGAGGGACCTTAATCGG		70
KLF16	Forward	GTGGCCCTAATCAACACTCCT	60	
	Reverse	AGATGGTGCAGCTACCAAGAC		186
COP1	Forward	ATGCAGCCATGAATGTGTGT	59	233
	Reverse	TGTCCACTTGCTGTTTGCTC		
DET1	Forward	GTCTTGTACCCGTGGTGCTT	60	172
	Reverse	AAAACCAGGACGCCTACCTT		
GDNF	Forward	GCCAGCCAGAGAGAAAACAC	59	234
	Reverse	AAGGCATTAGCTTTGGAGCA		
BCL6B	Forward	ACAGCTGGGTTTGAATGTCC	60	246
	Reverse	AAGGCAGCCCTCTTTTCTTC		
DUSP6	Forward	GAATAATCCGGCGAGAAACA	60	168
	Reverse	AATGAAGGTGCCCAGTTTTG		

Cross-examination of differentially expressed genes via expression atlas

The genes, for which mRNA expression was significantly differential, were further cross examined with respect to their protein expression at basal level using Expression Atlas database (Expression Atlas, 2020). This database is maintained by the European Bioinformatics Institute that is a hub of information for RNA and protein expression for different studies, organisms, and experimental conditions. Since mRNA of *Etv5* was being differentially expressed in the cerebellum of both the genders, its basal level protein was checked using this database.

Functional protein-protein association networks

For studying the functional protein association networks of Etv5, STRINGS (Search Tool for Retrieval of Interacting Genes and protein, Version 11.0, https://string-db.org/), BioGRID (https://thebiogrid.org/), Database of Interacting Proteins (DIP, https://dip.doe-mbi.ucla.edu/dip/Main.cgi) and IntAct (https://www.ebi.ac.uk/intact/home) databases were used (Szklarczyk *et al*., 2018) to find not only the established biological interacting partners from experiments and curated databases but also the predicted interactions from gene neighborhood, fusions, co-occurrence, co-expression, homology and text-mining techniques.

The re-analysis of RNA-seq datasets taken from Gene Expression Omnibus (GEO) database was done (Khan *et al*., 2022). In short, fastq files of LPS versus control treated mouse brain (accession number GSE153369), neurons, astrocytes and microglia (accession number GSE75246) were analysed using different packages from R Bioconductor such as limma, edgeR, gplots, org.Hs.eg.db, RcolorBrewer and Glimma (Warnes *et al*., 2005; McCarthy *et al*., 2012; Ritchie *et al*., 2015; Su *et al*., 2017). and the average expression along their p values were noted for significance analyses.

RESULTS

Putative TFs of IL-1B

The online search resulted in the identification of a number of TFs interacting with IL-1B gene regulatory elements. Further sorting of these TFs on the basis of their differential expression in different tissues resulted in the identification of *Klf16* and *Etv5* exhibiting the highest expression in brain as compared to other tissues (Supplementary Table SI).

mRNA expression analysis of TFs under neuroinflammatory conditions

mRNA expression analysis revealed that *Klf16* did not show any significant difference in expression in both the brain regions of male and female mice (Fig. 2). However, in case of *Etv5*, significant ($p < 0.005$) upregulation of 1.25 folds was observed only in male hippocampus (Fig. 3A). Quite interestingly, its expression was found to be downregulated in male cerebellum (Fig. 3B) as well as in female hippocampus (Fig. 3C) and cerebellum (Fig. 3D), although these fold differences were found to be non-significant.

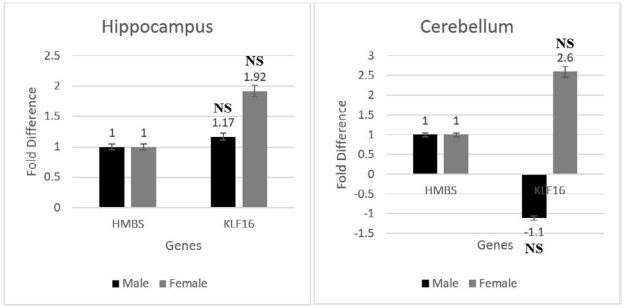

Fig. 2. mRNA expression of the transcription factor Klf16 represented in terms of fold difference up/ down regulation in hippocampus and cerebellum. NS - ($p > 0.05$ = non-significant)

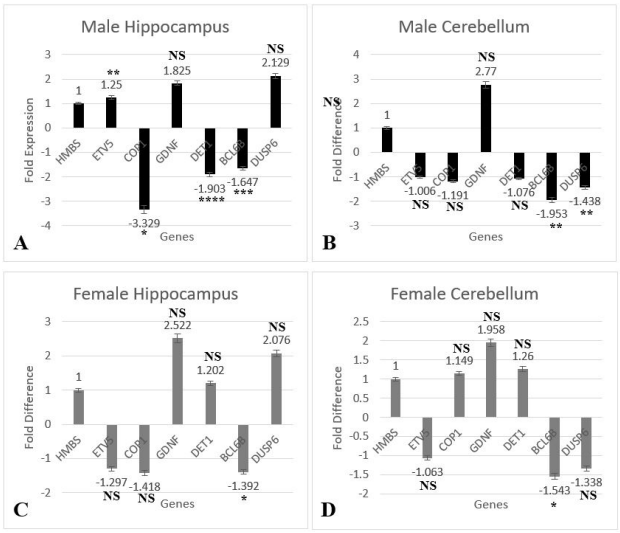

Fig. 3. mRNA expression of the transcription factor Etv5 along with its interacting partners Cop1, GDNF, Det1, Bcl6b, and Dusp6, represented in terms of fold difference up/down regulation. A, male hippocampus; B, male cerebellum; C, female hippocampus; and D, female cerebellum. * ($p < 0.05$), ** ($p < 0.005$), *** ($p < 0.0005$), **** ($p < 0.00005$), and NS ($p > 0.05$ = non-significant).

After getting significant expression difference in *Etv5* transcript, we further searched for the interacting protein

partners of *Etv5* of both human and mouse origin of which the mRNA expression of *Cop1*, *GDNF*, *Det1*, *Bcl6b* and *Dusp6* was further analyzed using RT-PCR. The protein partners for mouse and human *Etv5* protein from STRING database are given in Figure 4 in the form of node diagrams. Supplementary Table SII shows the interacting protein partners of *Etv5* from all the databases for mouse and human, respectively.

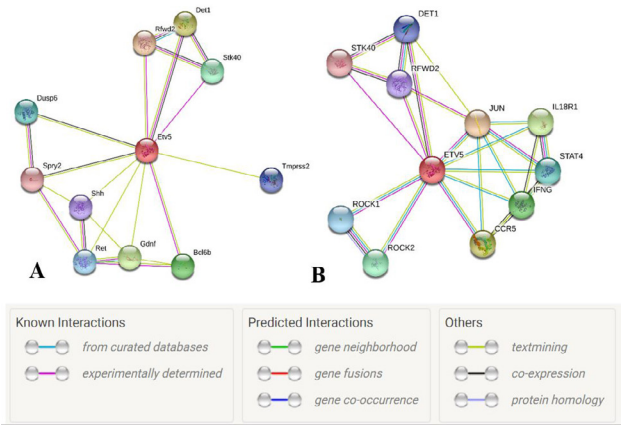

Fig. 4. Node graph of the interacting partners of Etv5. A, *Mus musculus* and B, *Homo sapiens*, with proteins represented by network nodes. The node in red color is the query protein while other nodes are the proteins having first shell of interaction with the query protein (STRINGS, ETV5 *Homo sapiens*).

In line with the significant upregulation of *Etv5* gene, three of its interacting partners, i.e., *Cop1*, *Det1* and *Bcl6b* showed significant downregulation, while non-significant upregulation was observed for *GDNF* and *Dusp6* in male hippocampus. In male cerebellum, significant downregulation of *Bcl6b* and *Dusp6* was seen. In female mice, only *Bcl6b* was found to be showing significant downregulation in both the hippocampus and cerebellum.

When the protein expression of Etv5 was checked as a validation to see its baseline conditions under normal conditions via Human Protein Atlas project (Expression Atlas, 2020), it was found that in the cerebellum of both male and female subjects, its expression was moderate (Fig. 5).

Expression of ETV5 from RNA seq data sets

When the expression of *Etv5* and its selected interacting partners was checked from RNA-seq data sets for brain tissue, neurons, microglia and astrocytes, only *Det1* significant difference in average expression with a value of -1.729 ($p < 0.005$) (Table II).

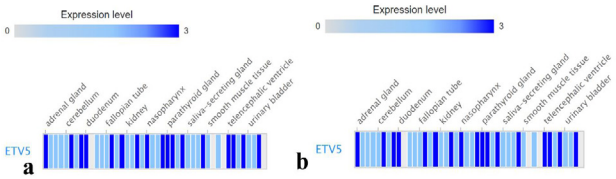

Fig. 5. Protein expression levels of Etv5 in different tissues of human subjects. A, male; and B, female.

Table II. Average expression of Etv5 from LPS treated neuroinflammation RNA-seq datasets. * ($p < 0.05$), ** ($p < 0.005$), NS ($p > 0.05$ = non-significant).

Genes	Average expression			
	Brain	Neurons	Microglia	Astrocytes
Etv5	-0.424^{ns}	6.352^{ns}	N/A	N/A
Cop1	N/A	0.73^{ns}	N/A	-1.310^{ns}
GDNF	-0.913^{ns}	-0.619^{ns}	N/A	N/A
Det1	N/A	N/A	N/A	-1.729**
Bcl6b	0.524^{NS}	0.351^{NS}	N/A	N/A
Dusp6	-0.160^{ns}	0.624	N/A	N/A

DISCUSSION

This study was focused on the identification of *IL-1B* specific transcription factors of brain as IL-1β serves a pivotal role in the mediation of inflammatory responses. Consistent with the significant differential expression of *Etv5* mRNA only in male hippocampus, most of its interacting partners (*Cop1*, *Det1* and *Bcl6b*) also showed significant differential expression (downregulation) only in male hippocampus. An interesting difference to note was that while *Etv5* was being upregulated, the interacting partners were being downregulated in male hippocampi. In the male cerebellum, the only genes significantly differentially expressed were *Bcl6b* and *Dusp6* (downregulation), and in female hippocampi and cerebellum only *Bcl6b* showed significant downregulation. Further exploration of the genes under study from the RNA-seq data sets of LPS treated brain tissue as well as from neurons, microglia and astrocytes revealed significant downregulation of *Det1* in astrocytes (Table II). Among all the genes, *Bcl6b* was found to be common among the two brain regions in both the genders, all exhibiting downregulation of it. Also, the downregulation of *Det1* in male hippocampus was corroborated with its down regulation in astrocytic RNA-seq data.

Being a member of the PEA3 family of TFs, Etv5 binds to a conserved DNA motif having sequence GGAA/T (Oh

et al., 2010). Some of the important known roles of Etv5 include: Coordination of limb development; regulation of epithelial-to-mesenchymal transition in some cancer types; control of gene expression in spermatogonial stem cells; inhibition of sonic hedgehog gene expression; and positive regulation of some miRNAs important in spermatogonial stem cells renewal (Zhang *et al*., 2009; 2010; Wu *et al*., 2011; Niu *et al*., 2011). Besides these, although still poorly understood, the roles of Etv5 in immune responses include an increased interferon gamma (IFN-γ) production from T_H1 cells (Thieu *et al*., 2008; Ouyang *et al*., 1999), increased IL-17 production in T_H17 cells (thus playing a role in allergic inflammation development) (Pham *et al*., 2014), and suppression of the activation of adipose tissue resident macrophages (Hu *et al*., 2021). Since activation of these macrophages contributes in the development of chronic inflammation, and Etv5 being identified as a major suppressor of the process, the upregulation of *Etv5* transcript in male hippocampus in the current study presents an interesting target for future studies of *Etv5* on brain resident macrophages i.e., microglia. Corroborated with the previous studies indicating a regulatory role of Etv5 on transcriptional activation of IL-10, IL-17 and IL-9 expression (Pham *et al*., 2014; Koh *et al*., 2016, 2017), the precise mechanism of its role in macrophage activation regulation is yet to be unveiled.

We observed a downregulation of *Cop1* gene in hippocampi of both the genders and in male cerebellum although significant results were those of male hippocampus only. Previous studies have found a neuroprotective role of *Cop1* in neurodegenerative disorders (Gorantla *et al*., 2007, 2008). Corroborating the neuroprotective role, recent knockout studies of Cop1 have shown an increased pro-inflammatory response in brain resident macrophages, i.e., microglial cells (Ndojaa *et al*., 2020). Since Det1 is a partner of Cop1 E3 ubiquitin ligase complex, its downregulation is in concert with *Cop1* downregulation (which has been found to be almost double in magnitude to that of *Det1* (Fig. 3B). Previous roles of Det1 have been found in the mediation of cellular differentiation and proliferation in multicellular organisms (Nassrallah *et al*., 2018), however, no known roles could be found of Det1 in the process of neuroinflammation. Similarly, although previous roles of Bcl6b and Dusp6 have been found in the regulation of inflammatory responses (Bunting and Melnick, 2013) and endothelial inflammation (Hsu *et al*., 2018), respectively, their precise roles in the process of neuroinflammation are yet to be studied. These findings present important genes for further detailed studies with respect to the modulation of neuroinflammatory process in hippocampus tissue. Also, the brain region along with gender differences are also evident from the current study highlighting the need to focus on individual brain regions of each gender.

CONCLUSIONS AND RECOMMENDATIONS

In the current study, we have presented the expression profiles *IL-1B* associated transcription factor *Etv5* along with some of its interacting partners as molecular targets of neuroinflammatory process. Also, the current study presents the dissection of these TFs with respect to not only the two important brain regions hippocampus and cerebellum due to their functional implications in memory and motor function, respectively, but also with respect to gender differences. Moreover, the identification of significantly differentially expressed genes present interesting target genes for further studies on neuroinflammation. Furthermore, future research on the extensive molecular characterization of all brain regions as well as individual cell types could provide a complete landscape not only for the recognition of each of these brain regions and cells in the process of neuroinflammation, but also would guide the development of more targeted therapeutic interventions.

ACKNOWLEDGEMENT

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DECLARATIONS

Ethical statement

The study was approved by the Institutional Review Board (IRB) and Institutional Animal Care and Use Committee (IACUC) of University of the Punjab

Data availability statement

All the data has been included in this article.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220612100622

Statement of conflict of interest

The authors have declared no conflict of interest.

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