Altered neurogenic pathways in experimental rat models of ischemic cerebral stroke:

A transcriptome based meta-analysis

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1) **Abstract**

Objectives

Promoting neurogenesis mediated recovery is one of the most sought after strategies in recovery after cerebral stroke. In this paper we elucidate how neurogenesis related genes are altered in the early stroke environment, to hint at potential pathways for therapeutic recovery.

Materials and Methods

Around 97 microarray datasets derived from stroke affected rat brains were collected from NCBI-GEO. Datasets were normalized and subjected to meta-analysis in Network Analyst to identify differentially expressed genes. Gene enrichment analyses were carried out using GSEA, and WebGestalt, and results were visualized using Cytoscape Enrichment mapping.

Results

Nearly 939 differentially expressing genes were identified in the cerebral stroke group. Among them 30 neurogenesis related genes were identified through enrichment mapping analysis, and 35 genes through Protein- Protein Interaction analysis. Highest upregulated neurogenesis genes were found to be TSPO, GFAP, VIM, and TGFB1. Highest Downregulated neurogenesis genes were found to be THY1, NR1D1, CDK5, STX1B, and NOG.

Conclusions

Through this study, we have identified that during the acute time frame after stroke, majority of the neurogenesis genes related to neural proliferation and neural differentiation are downregulated, while the majority of the genes related to neuronal migration were upregulated. Single or combined therapeutic approach against the identified dysregulated genes could greatly aid neural restoration and functional recovery during the postischemic stage.

Keywords

Stroke, MCAo, Transcriptome, Meta-analysis, Microarray, Neurogenesis

2) Introduction

Ischemic stroke is caused by an abrupt disruption in the blood flow and impairments in vascular compartments of the brain. The onset of ischemic stroke leads to the deprivation of oxygen and glucose in brain cells, especially neurons, as they have very poor energy reserves. The pathophysiological events following this blockage ultimately leads to neuronal loss in the infarct regions leaving the peri-infarct region vulnerable to necrotic and apoptotic cell death followed by fibrotic formations [1, 2]. As the majority of strokes occur due to abnormalities of the middle cerebral artery, the striatum appears to be highly vulnerable to ischemia among other brain regions. Hence, the prominent neuronal loss in the ischemic striatum leaves the patient with a major motor deficit or to a paralysed state [3]. Recombinant tissue plasminogen activator (rTPA) has been the effective management therapy available for recovery after ischemic stroke, however with a therapeutic window of fewer than 4.5 hours from the onset. This leaves no actual solution as a treatment option for stroke beyond the golden hours. Hence, establishment of valid treatment against MCAo stroke has been an important area in preclinical research , and it needs ample research to establish more appropriate therapeutic targets for the fruitful outcome.

Adult Neurogenesis is the innate ability of the brain to continuously produce new neuronal cells even till a later stage of life. Adult Neurogenesis occurs predominantly in two major stem cell niches of the brain, namely subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the subventricular zone (SVZ) present along the lateral ventricles of the brain. Experimental paradigms targeting the regulation of neurogenesis to promote neural

regeneration as a treatment option against stroke is an ongoing scientific task for the past two decades. Despite the accumulation of reports, regenerative strategies against stroke are very feeble due to the lack of complete understanding in the critical aspects of signalling mechanisms.

With the recent scientific advancement in omics approaches, powerful tools like microarray analysis, RNA-seq and other Seq techniques have become easily accessible to the research community. The data from these experiments, though different in study design, but enables cross-comparison, provided appropriate control measures were taken. Though there is a huge accumulation of MCAo stroke gene expression data, the experimental designs in each study tend to focus on only a few specific scientific problems, it is still challenging to extract the maximal information on brain regeneration from those data. To address these issues, the establishment of a meta-analysis that integrates and inter-validates quantitative findings of multiple independent datasets from publicly available microarray data in databases including the Gene Expression Omnibus (GEO, [HTTP:// ncbi.nlm.nih.gov/geo](http://ncbi.nlm.nih.gov/geo)) would be highly instrumental, as it can offer increase statistical accuracy due to augmented sample size, control quality for between-study variation, and overcoming the bias of individual studies. The results from such an integrated meta-analysis experiment could help in further identification of potential biomarkers and druggable targets which could be further translated into diagnostic and treatment applications. Hence, this meta-analysis attempts to establish a potential list of differentially expressed genes, which could be further analysed to identify dysregulated signalling pathways that determine the regulation of adult neurogenesis from the available microarray data from rat stroke brain.

3) Materials and Methods

3.1) Collection of microarray data

A computerised search was carried out in NCBI GEO against the terms “MCAO” and “stroke” with the organism set as *Rattus norvegicus*. The microarray datasets of experimental cerebral stroke, and studies with similar experimental conditions were shortlisted. Inclusion criteria considered for the study have been supplied in the table-1. Details about the collected datasets are listed in table-2. Here, 97 samples (54 MCAo brain samples vs 43 sham control brain samples) were selected from 10 microarray datasets. An overview of the meta-analysis conducted in this study is shown in Figure-1. The studies collected utilized a range of microarray platforms like Agilent, Affymetrix, and Illumina. Throughout the study, only the ipsilateral cortex of the rat brain was considered.

3.2) Data processing

Series matrix files were downloaded from the appropriate datasets listed in NCBI and processed by uploading to multiple gene expression table analysis options in Network analyst 3.0. (<https://www.networkanalyst.ca/>) [4, 5]. For non-compatible datasets, the datasets were processed using SOURCE batch search [6], where the probes from the array chip design were mapped into the appropriate ENTREZ gene ID. The probes that did not match with an Entrez id were not included. Each dataset was individually validated and uploaded to the network analyst software. The study batch effect among different datasets was adjusted using the Combat option [7].

3.3) Preparation of differentially expressed genes list

The datasets were normalized by variance stabilizing normalization (VSN) followed by quantile normalization to minimize between slide variations [8]. Then meta-analysis [9] was

carried out by combining p-values using Fisher's method [10] with a significance set at 0.01. The resulting output produced a ranked list of differentially expressed genes. This ranked gene list was further used for pathway enrichment analysis.

3.4) Pathway enrichment analysis

The ranked gene list containing upregulated and downregulated genes, and the GO: BP(Gene Ontology: BioProcess) rat GMT file was uploaded to GSEA software for enrichment analysis [11]. The resulting GSEA ranked file was uploaded to the following software modules for various data analyses.

3.4.1) Enrichment map in Cytoscape

To explore the differentially expressed (DE) genes between sham and MCAo groups, the GSEA ranked file was uploaded to the enrichment map application in Cytoscape. Q- value was adjusted to 0.01 and edge cutoff was increased to 0.75. Further, the layout was adjusted to have a defined data and the resulting pathways were annotated. Nodes of interest were selected to observe the list of altered gene lists under the specific gene ontology node. These node-specific gene expression data were also analysed using the Genemania plugin.

3.4.2) WebGestalt

WebGestalt is an online-based enrichment analysis software, useful in analyzing clusters of hits in enriched metabolic and signalling pathways (like KEGG and GO) [12]. Gene set enrichment analysis generated multiple highly significant clusters that showed a common trend as to their function.

3.4.3) Network analyst

Two kinds of analyses were carried out using the DE candidate genes list in the network visual analysis module. Opting for the Transcription Factor (TF)-miRNA interactome analysis reveals non-coding miRNA interaction with the significant genes uploaded. Uploading the data to protein-protein interaction analysis helped in obtaining a string database based interactome map [13].

3.5) Venn Diagram Analysis

The differentially expressed gene lists and the highest interacting proteins obtained from the aforementioned analyses were then subjected to venn diagram analysis with previously available gene ontologies related to neurogenesis. The ontology libraries chosen for analyses were i) MANGO (The Mammalian Adult Neurogenesis Gene Ontology), ii) neuroblast proliferation GO:0007405, iii) neuron migration (GO:0001764) iv) neuron differentiation (GO:0030182)

4) Results

4.1) 939 Differentially expressed genes have been identified in MCAo stroke

An overview of the meta-analysis conducted in this study is shown in Figure-1. The effect of normalization on the datasets had been illustrated in figure-2 exhibiting the difference between pre-normalized and post normalized datasets. Post normalization of the intensities of the achieved datasets was equally distributed amongst the samples well within the median levels. Normalization helps in ensuring minimal experimental variations between the microarray datasets. The normalized datasets were subjected to meta-analysis to identify the differentially expressed genes (DE genes) between MCAo and sham rat brain samples. Fisher's test with $P=0.01$ set during the meta-analysis resulted in the identification of 939 DE

genes between the groups. The top 50 upregulated, and top 50 downregulated genes in MCAo animals were listed in tables 2 and 3 respectively. Since the current study focuses keenly on identifying the dysregulated genes related to neurogenesis, the DE gene rank list was applied to Gene Set Enrichment Analysis and the enriched gene data was utilized for different visualization and analysis purposes.

4.2) 35 Neurogenesis related genes have been identified through PPI analysis

Continuation of the analysis module in network analyst through network visual analytics-enabled two types of analysis. One being the transcription factor (TF)- miRNA co-regulatory network analysis (Figure-3), and the second being generic protein-protein interaction analysis (Figure-4). TF-miRNA interactome analysis revealed that TFs like Neurod1, Vimentin, Aquaporin4 were found to be interacting with miRNAs predominantly. These TFs are also crucial during the phases of adult neurogenesis [14-16]. Protein-protein Interaction (PPI) network analysis was also carried out to understand the relation between DE genes based on the intermediate genes of the network. The “zero-order” PPI network contained 163 nodes with 255 interacting edges among them. The most highly interacting node was identified to be Akt1 with betweenness centrality = 5808.71, degree = 22 and Ptk2b With betweenness centrality = 3865.3, degree = 3. A supplementary table with the top 10 interacting nodes is attached (Table-5). In relation to genes related to neurogenesis, around 35 interacting genes were identified by comparing the nodes with GO: Neurogenesis (GO:0022008). The interacting genes related to GO: Neurogenesis were listed in supplementary data (Table-6). Among the proteins related to neurogenesis, the highest interacting among them is found to be Akt1- a known promoter of neurogenesis, Stat3- a known promoter of neuroblast migration, Mtor- a known regulator of neurogenesis, Casp3, Myc- a known promoter of neural differentiation.

4.3) Enrichment analysis using WebGestalt

Moreover, to increase an understanding of the biological roles of DE genes in post MCAo conditions, gene ontology analysis and enrichment analysis were performed in Webgestalt to classify the DE genes into specific readable clusters based on their biological processes, cellular components, and molecular functions during the post MCAo conditions. Gene ontology revealed that biological regulation and localization were predominated with more than 50 % of the DE genes, whereas cell proliferation accounted for less than 15 % of the DE genes. Most of the DE genes were localized to membrane, and nucleus, and fewer in ECM, and ribosomes. Molecular functions related to protein binding were more enriched when compared to oxygen binding and other interactions were least enriched (Fig-5-B). The directed acyclic graph was also constructed to visualize the interaction of enriched gene ontology as schematically depicted in Fig. 5-C. The Kegg based molecular function analysis revealed significant downregulation in nodes like regeneration, immune effector process, while also showing significant upregulation of nodes like regulation of trans-synaptic signalling and glutamate receptor signalling pathway among others (Fig-5-B). Elucidation of the regeneration node in molecular function analysis revealed that neurogenic genes like GFAP, TGFB1, PCNA, JAG1 were significantly upregulated in the MCAo stroke brain.

4.4) Enrichment Mapping using Cytoscape

Cytoscape Enrichment map analysis of the GSEA enriched gene list revealed that among various dysregulated pathways, the neurogenesis pathway is one of the significant among them as it passed q value of 0.01 (Fig-6). It depicted that the genes responsible for negative regulation of neurogenesis had been significantly upregulated, which leads to the fact that as whole neurogenic process was impaired in the MCAo group. Genes under the node were

displayed in table 7 along with their fold-change score (CombinedTstat), where lower the value corresponds to higher the expression in the MCAo group. The majority of the shortlisted genes have a relation with neurogenesis pathways (Fig-7). Their relevance to different stages of neurogenesis has been discussed ahead.

5) Discussion

5.1) Differentially expressed (DE) Genes in MCAo rat brain when compared with sham

MCAo affected rat brain has an infarct region that encompasses dead cells as a result of the occlusion and the subsequent pathological effects [17]. Several attempts have been made on the development of a treatment strategy to restore lost neurons after stroke. Among the strategies, one of the most capable approaches is the stimulation of neurogenesis to alleviate the neuronal loss sustained in the brain during and after the stroke [18]. Hence in this article, we carry out the identification of dysregulated neurogenic pathways created in post stroke conditions, as this will help in gaining clinically useful insights into a potential therapeutic or diagnostic application. The workflow comprised computation analysis to identify the DE gene list followed by enrichment analysis and subsequent efficient graphical summary for exploratory visualization.

Through a meta-analysis of the datasets, we identified that 939 genes were differentially expressed in the MCAo group in comparison with the sham group. Among them, 369 genes were upregulated and 570 genes were downregulated, both of them significantly with a minimum combined P-value of 5.1732E-6 (Fisher's 1% test). PIK3R2 was identified to be the highest downregulated gene in this meta-analysis, followed by PCSK2, B4GALNT1, CX3CL1, SLC6A1. The significantly upregulated genes are identified as LGALS3, TSPO, TIMP1, CD14, HSPB1. TF- miRNA interactome analysis revealed that highest interacting

transcription factors to be Tagln (Expression: -91.4), Vim (Expression: -158.62), Vsnl1 (Expression: 113.4), Itgb1 (Expression: -109.38), Neurod1 (Expression: 86.53). The highest interacting miRNA was found to be rno-mir-124-3p, rno-mir-290, rno-mir-29b-3p respectively. Through WebGestalt based enrichment analysis, neurogenic genes like GFAP, TGFB1, PCNA, JAG1 were identified to be less enriched in the MCAo rat brain. From these candidates, we could sense a trend that the majority of these genes have a say in the process of neurogenesis as cross-verified using MANGO [19]. In enrichment analysis using Cytoscape, neurogenesis related pathways were chosen. Nodes under negative regulation of neurogenesis were found to be significantly upregulated. Within this node, the differentially altered genes were identified. Significantly downregulated genes were found to be THY1 (Thy-1 Cell Surface Antigen), NR1D1 (Nuclear Receptor Subfamily 1 Group D Member 1), CDK5 (Cyclin-Dependent Kinase 5), STX1B (Syntaxin 1B), NOG (Noggin). Significantly upregulated genes under the neurogenesis node were found to be TSPO (Translocator Protein), GFAP (Glial Fibrillary Acidic Protein), VIM (vimentin), TGFB1 (Transforming Growth Factor Beta 1), STAT3 (Signal Transducer And Activator Of Transcription 3). The results revealed that a significant number of genes and pathways related to neurogenesis were altered during the acute phase after stroke. It is well studied that excitotoxicity caused by stroke leads to a major depletion of neurons in the striatal region [20], but their effect on the neurogenic niche present in the subventricular zone is very feebly understood [21]. This meta-analysis sheds light on the unknown by comparing how the genes and pathways regulating the neurogenic cells in the SVZ region are dysregulated in early post-stroke conditions.

Since the process of SVZ neurogenesis generally comprises the stages- proliferation, migration, differentiation, and maturation, [22] we will individually discuss how the genes regulating each of this phase were found to be altered in this study and the same has been

portrayed in the figure 7. To facilitate the discussion, Venn diagram analysis of 939 DE genes with appropriate Gene ontologies as mentioned in methodology was carried out and only the overlapping DE genes had been further discussed here.

5.2) SVZ- neuroblast Proliferation

Neuroblast proliferation plays a major role in the production of new neurons as well as the replenishment of the neurogenic niche cells. Any deficiencies to proliferation will lead to a decrease in neuroblast production and subsequent neurogenic niche depletion [23]. These consequences could severely decline the neuroregeneration potential of the brain after stroke. Particularly in this meta-analysis CX3CL1, TGFB1, PTN genes were found to be significantly dysregulated among genes related to neural proliferation. CX3CL1 gene (C-X3-C Motif Chemokine Ligand 1), codes for fractalkine, whose receptors are present in microglia and is crucial for their migration [24]. This gene is significantly downregulated with a combined Tscore of 120.76 in MCAo animals. Being a transmembrane cytokine, CX3CL1 plays a role in communication between the microglia and neuron through its interaction with the CX3CL1 receptor [25]. Microglia plays a crucial role in the survival and proliferation of neural precursor cells [26]. Also, when cx3cl1 is cleaved by α -, β -, and γ -secretase, they create a cx3cl1-ICD which gets translocated into the nucleus to induce survival mode in cells [27]. Fan et al have previously reported that in 5xFAD Tg mice with overexpression of the cx3cl1-ct, a significant reduction in amyloid deposition and reduced neuronal loss, attributed especially to the increased neuroblast proliferation [27]. In post-stroke conditions, decreased cx3cl1 expression could hinder signalling with microglia in addition to disruption of cell survival and neuronal proliferation [28]. TGFB1 (Transforming Growth Factor Beta 1) (CombinedTscore= -155.89), is significantly upregulated in post-stroke conditions. Wachs et al have shown that the addition of TGFb1 to the adult neural

stem and progenitor cell cultures (ANPs) resulted in a significant decrease in proliferation i.e. 25% in one-week treatment [29]. Thus TGF- β 1 limits the potential of adult neural stem and progenitor cells to proliferate without altering the capacity of self-renewal or the fate of differentiation. PTN (Pleiotrophin) (CombinedTscore= 65.16) gene codes for pleiotrophin, is significantly downregulated in post-stroke conditions. PTN knockout mice (PTN^{-/-}) display increased neuronal stem cell proliferation in the adult mouse cerebral cortex, and exogenous PTN decreases neuronal stem cell proliferation and facilitates cell differentiation [30]. Downregulation of CX3CL1 and upregulation of TGF β 1 hinders neural proliferation in post-stroke conditions while downregulation of PTN facilitates a supporting action for neural proliferation.

5.3) Neuroblast Migration

After the proliferation of neuroblasts, in the normal brain, they migrate towards the olfactory bulb via the rostral migratory stream (RMS) [22]. However, the previous studies have extensively shown that in conditions like stroke, epilepsy, and other early stages of neurodegenerative diseases, these neuroblasts divert from RMS and invade sites of neuronal damage in and around striatal regions [18]. This happens innately in an attempt to replenish the damages sustained. However, this diverted migration only happens for a short window of time. During the short window of time, the certain proteins that promote neuroblast migration are expressed at the site of damage and beyond this window, their expression is diluted [31]. Any altered expression of genes correlated to neuroblast migration was identified in this meta-analysis, found to be PLAA (Phospholipase A2 Activating Protein), GNRH1(gonadotropin-releasing gonadotropin-releasing hormone 1), and STAT3(signal transducer and activator of transcription 3). PLAA has functions in degradation targeting ubiquitous proteins via the ubiquitin-proteasome network. Due to disrupted post-endocytic

trafficking to the lysosome, PLAA KO mice have shown to accumulate dysfunctional VLDLR (Very low-density lipid receptor) in Purkinje cells, which results in disrupted purkinje cell migration as well as their differentiation [32]. In meta-analysis, Plaa(Phospholipase A2 Activating Protein) (CombinedTscore= -74.158), is significantly upregulated in post stroke condition. Higher levels of PLAA expression in post-stroke conditions could lead to improved levels of ubiquitin-protein degradation and could lead to lesser accumulation of disruptive proteins like vldlr which have direct implications in affecting neuroblast migration [33]. To accompany this VLDLR (CombinedTscore= 42.394) expression is downregulated in post-stroke conditions. Larco et al showed that GnRH-(1-5) prevents cellular migration of GN11 cells (GnRH-secreting neuronal cell line) by inhibiting STAT3 signals [34]. STAT3 modulation acts as a mechanism by which extracellular surroundings may control the GnRH neuron migration rate [35]. Meta-analysis revealed significant downregulation of GNRH1 (CombinedTscore= 39.72) and very significant upregulation of STAT3(CombinedTscore= -144.69), both attributing to improvement in the chances of neuroblast migration in the post-stroke brain. In the meta-analysis, significantly altered genes related to neuroblast migration have all cumulatively resulted in promotion of the neuronal migration process. Various other research has shown disruption in slit/robo proteins in post-stroke conditions to be a reason for poor migration after stroke [36]. This meta-analysis provides a new perspective on this matter.

5.4) Neuroblast Differentiation and Maturation

Post arrival to the site of damage, it is important for the neuroblast to differentiate into neurons to complement for any functional recovery. Through meta-analysis 13 significantly altered genes related to neuronal differentiation were identified, of which the downregulated genes were NEUROD1(CombinedTscore= 78.801), FEZ1 (CombinedTscore= 78.226),

SOCS2(CombinedTscore= 75.286), CNTN4 (CombinedTscore= 58.293), GDF11(CombinedTscore= 57.759), CNTN2 (CombinedTscore= 53.043), MTOR (CombinedTscore= 43.593), SH3GL3(CombinedTscore= 43.274), MAP1B(CombinedTscore= 37.003), and upregulated genes are CALR(CombinedTscore= -71.614), JAG1(CombinedTscore= -68.159), BMP2(CombinedTscore= -57.386), NGFR(CombinedTscore= -49.317). NEUROD1 is one of the well-studied factors in neurogenesis and has been frequently targeted as an option for recovery in neurodegenerative diseases. Gao et al has validated that NEUROD1 ablation in SVZ neurospheres and in-vivo models had resulted in disruption of the survival and differentiation of the newborn neurons [37]. In addition, NeuroD1 specifically binds regulatory elements of neuronal genes, which are silenced by epigenetic pathways in the development, thus proving their importance in neuroblast differentiation [38]. Kang et al had exhibited through shRNA based FEZ1 silencing studies in an in-vivo model that FEZ1 acts synergistically with DISC1 to control the dendritic development of newborn neurons [39]. CNTN2 is a cell-adhesion molecule that is regularly considered as a marker for mature neurons and it is also majorly expressed in regenerating neurons [40]. mTOR signalling is important for neural stem cell maintenance and differentiation and for brain growth [41]. Romine et al have demonstrated that restoration of the mTOR pathway in the ageing brain reestablished neurogenesis [42]. SH3GL3 is a crucial positive regulator of neural differentiation and it interacts with Huntingtin, which has been known to transcriptionally upregulate BDNF expression [43]. Mature neurons consistently express MAP1B and it is a crucial regulator of the structural components of the cell. MAP1B KO resulted in an increased number of excitatory immature symmetrical neurons with altered pre-synaptic modifications [44]. Downregulation of these positive regulators of neural differentiation will severely affect the chances of differentiation in the early phases of post-stroke conditions.

Shih et al showed that the CALR(Calreticulin) deficiency substantially decreased the neuronal differentiation caused by NGF [45], similarly CRT over-expression enhanced neuronal differentiation through simultaneous activation of an ERK-dependent MAPK pathway [46]. However, Dedhar et al have shown that Calreticulin can suppress transcriptional activities of the retinoic acid receptor in vivo, as well as neuronal differentiation caused by retinoic acid [47]. Meta-analyses have revealed that CALR is up-regulated and because of its contrasting roles in the aspect of neural differentiation, a detailed study is needed with respect to SVZ neurogenesis. Nyfeler et al have demonstrated that JAG1 (Jagged1) treatment of NSCs in vitro, substituted for EGF and identified to be a key regulator of NSC- self-renewal and differentiation [48]. However, when astrocytes are co-cultured with neurons, they inhibit neuronal differentiation by endocytosis of Jagged1 through notch pathway [49]. Thus, positive regulation of CALR and JAG1 could have a negative say in the neural differentiation in post-stroke conditions. Yan et al have demonstrated that BMP2 facilitated the differentiation of NSCs into dopaminergic neurons through the involvement of miR-145 and Nurr1 mechanisms [50], thus showing they have a positive say in neuronal differentiation. NGFR (Nerve growth factor receptor) serves as a common receptor for a number of growth factors like BDNF, NGF among others [51] These trophic factors play a crucial role in neural differentiation and the meta-analysis revealing an upregulation of NGFR that would result in an increase in chances of neural differentiation.

6) Conclusion

In conclusion, this stringently concorded meta-analysis of differential transcriptomes between sham and MCAo rats helped in the identification of dysregulated genes and pathways in various crucial phases of neurogenesis during early post-stroke conditions. This work acts as a reference for future studies, through which the findings may be validated. Thus, the

findings of this study may provide fundamental clues into the pathophysiology of neurogenesis in the early phase after stroke, which could be translated into a therapeutic approach in future.

Conflict of interest:

The authors declare that they have no conflict of interest.

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Contributions:

Conceptualization: SAR, AM; data acquisition: SAR; analysis and interpretation of data: SAR, GE, DG, SKJ, MK, AM; writing—original draft preparation: SAR; writing—review and editing: SAR, SKJ, MK, AM; critical revision of the manuscript for intellectual content: SKJ, MK, AM; supervision: AM

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Table-1: Inclusion Criteria Considered For the Study

Inclusion Criteria Considered For the Study	
Species	Rattus norvegicus
Strain	Sprague-Dawley, Wistar
Sex	Male
Age	3 Months - 20 Months (Majority of the sample are in the adult age group ie above 6 months)
MCAo Occlusion period	60 mins, 90 mins, 120 mins, Permanent
Sample collection period after MCAo	3hrs to 7 days (Majority of the sample were collected within the first 3 days)
Any model comprising pre-treatment before MCAo was not considered for the study.	

Table-2: Summary of transcriptome data used in this study

Datasets	No.Of Samples		Platform	References
	Sham	MCAO		
GSE97537	5	7	GPL1355 [Rat230_2] Affymetrix Rat Genome 230 2.0 Array	Unpublished Submission
GSE78731	5	5	GPL15084 Agilent-028279 SurePrint G3 Rat GE 8x60K Microarray (Probe Name version)	(52)
GSE52001	3	3	GPL14746 Agilent-028282 Whole Rat Genome Microarray 4x44K v3 (Probe Name version)	(53)
GSE61616	5	5	GPL1355 [Rat230_2] Affymetrix Rat Genome 230 2.0 Array	(54)
GSE33725	6	6	GPL7294 Agilent-014879 Whole Rat Genome Microarray 4x44K G4131F (Probe Name version)	(55)
GSE17929	2	4	GPL85 [RG_U34A] Affymetrix Rat Genome U34 Array /// GPL341 [RAE230A] Affymetrix Rat Expression 230A Array	(56)
GSE36010	8	8	GPL7294 Agilent-014879 Whole Rat Genome Microarray 4x44K G4131F (Probe Name version)	(57)
GSE23651	8	8	GPL6101 Illumina ratRef-12 v1.0 expression beadchip	Unpublished Submission
GSE46267	1	8	GPL6101 Illumina ratRef-12 v1.0 expression beadchip	(58)
Total Samples	43	54		

Table-3: List of top 50 Upregulated genes in stroke brain identified through transcriptome meta-analysis

Gene	Name	Entrez id	CombinedTstat
LGALS3	galectin 3	83781	-217.89
TSPO	translocator protein	24230	-206.96
TIMP1	TIMP metalloproteinase inhibitor 1	116510	-196.16
CD14	CD14 molecule [Source:RGD Symbol;Acc:620588]	60350	-194.96
HSPB1	heat shock protein family B (small) member 1	24471	-191.16
GFAP	glial fibrillary acidic protein]	In multiple Geneids	-187.73
CD63	Cd63 molecule	29186	-186.66
C1QC	complement C1q C chain	362634	-181.31
HMOX1	heme oxygenase 1	24451	-180.39
RIPK3	receptor-interacting serine-threonine kinase 3	246240	-178.3
CCL2	C-C motif chemokine ligand 2	24770	-172.5
ARPC1B	actin related protein 2/3 complex, subunit 1B	54227	-168.72
ATF3	activating transcription factor 3	25389	-168.66
S100A4	S100 calcium-binding protein A4	In multiple Geneids	-168.12
LITAF	lipopolysaccharide-induced TNF factor	In multiple Geneids	-165.64
ANXA1	annexin A1	In multiple Geneids	-164.9
HCK	HCK proto-oncogene, Src family tyrosine kinase	25734	-164.47
TNFRSF1A	TNF receptor superfamily member 1A	25625	-163.26
IFITM3	interferon induced transmembrane protein 3	361673	-162.77
ICAM1	intercellular adhesion molecule 1	25464	-160.78
PTPN6	protein tyrosine phosphatase, non-receptor type 6	116689	-160.7
C1S	complement C1s	192262	-159.03
VIM	vimentin	81818	-158.62
TBXAS1	thromboxane A synthase 1	24886	-157.81
GADD45A	growth arrest and DNA-damage-inducible, alpha	25112	-157.48
TGFB1	transforming growth factor, beta 1	59086	-155.89
PSMB9	proteasome 20S subunit beta 9	24967	-153.44
CAPG	capping actin protein, gelsolin like	297339	-153.19
PLA2G4A	phospholipase A2 group IVA	In multiple Geneids	-152.19
IER3	immediate early response 3	294235	-149.84
CD48	Cd48 molecule	245962	-149.09
GRN	granulin precursor	29143	-148.19
CASP1	caspase 1	25166	-147.52

LY86	lymphocyte antigen 86	291359	-146.6
CTSL	cathepsin L	25697	-146.41
MYC	MYC proto-oncogene, bHLH transcription factor	24577	-146.14
STAT3	signal transducer and activator of transcription 3	25125	-144.69
PSME2	proteasome activator subunit 2	29614	-144.02
ANXA3	annexin A3	25291	-140.37
MGP	matrix Gla protein	25333	-140.35
PCNA	proliferating cell nuclear antigen	25737	-138.9
DYNLT1	dynein light chain Tctex-type 1	83462	-137.9
IL18	interleukin 18	29197	-137.5
P2RY6	pyrimidinergic receptor P2Y6	117264	-136.78
CTSC	cathepsin C	25423	-136.49
LGALS3BP	galectin 3 binding protein	245955	-132.14
FGR	FGR proto-oncogene, Src family tyrosine kinase	79113	-130.06
P4HB	prolyl 4-hydroxylase subunit beta	25506	-129.66
RBP1	retinol binding protein 1	25056	-129.14
CTSB	cathepsin B	64529	-128.67

Table-4: List of top 50 downregulated genes in stroke brain identified through transcriptome meta-analysis

Gene	Name	Entrez id	CombinedTstat
PIK3R2	phosphoinositide-3-kinase regulatory subunit 2	29741	127.2
PCSK2	proprotein convertase subtilisin/kexin type 2	25121	121.37
B4GALNT1	beta-1,4-N-acetyl-galactosaminyl transferase 1	64828	121.04
CX3CL1	C-X3-C motif chemokine ligand 1	89808	120.76
SLC6A1	solute carrier family 6 member 1	79212	118.59
ATP2B2	ATPase plasma membrane Ca ²⁺ transporting 2	24215	118.25
GRIN1	glutamate ionotropic receptor NMDA type subunit 1	24408	117.46
THY1	Thy-1 cell surface antigen	24832	117.15
KCNV1	potassium voltage-gated channel modifier subfamily V member 1	60326	116.7
SCN1B	sodium voltage-gated channel beta subunit 1	29686	115.96
NEU2	neuraminidase 2	29204	115.39
LIN7B	lin-7 homolog B, crumbs cell polarity complex component	60377	114.26
NPY5R	neuropeptide Y receptor Y5	25340	113.95
ST3GAL2	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	64442	113.65
VSNL1	visinin-like 1	24877	113.4
ATP1A1	ATPase Na ⁺ /K ⁺ transporting subunit alpha 1	24211	112.52
CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	287005	111.85
CLEC11A	C-type lectin domain containing 11A	29313	111.07
PTK2B	protein tyrosine kinase 2 beta	50646	111.03
PDE1B	phosphodiesterase 1B	29691	110.69
DLX5	distal-less homeobox 5	25431	110.59
B3GAT1	beta-1,3-glucuronyltransferase 1	117108	110.16
PLCB1	phospholipase C beta 1	24654	108.99
VAMP1	vesicle-associated membrane protein 1	25624	108.88
CHRM4	cholinergic receptor, muscarinic 4	25111	108.53
DHCR7	7-dehydrocholesterol reductase	64191	107.78
GRIA3	glutamate ionotropic receptor AMPA type subunit 3	29628	106.39
SHISA4	shisa family member 4	360848	106.14
PDE2A	phosphodiesterase 2A	81743	105.91
PRKAR2B	protein kinase cAMP-dependent type II regulatory subunit beta	24679	105.86
SLC4A3	solute carrier family 4 member 3	24781	105.47
NRGN	neurogranin]	64356	105.07
DBN1	drebrin 1	81653	104.96

NPTN	neuroplastin	56064	104.93
MCF2L	MCF.2 cell line derived transforming sequence-like	117020	104.78
GRIN2A	glutamate ionotropic receptor NMDA type subunit 2A	24409	103.02
KCNJ4	potassium inwardly-rectifying channel, subfamily J, member 4	116649	102.97
KALRN	kalirin, RhoGEF kinase	84009	102.46
SYT2	synaptotagmin 2	24805	102.26
GFRA2	GDNF family receptor alpha 2	In multiple Geneids	102.18
OPCML	opioid binding protein/cell adhesion molecule-like	116597	101.75
KCNAB2	potassium voltage-gated channel subfamily A regulatory beta subunit 2	29738	101.74
FGF14	fibroblast growth factor 14	63851	100.31
NEFM	neurofilament medium	24588	99.746
TRIM54	tripartite motif-containing 54	362708	99.65
STXBP1	syntaxin binding protein 1	25558	99.618
AKAP5	A-kinase anchoring protein 5	171026	98.686
ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca ²⁺ transporting 2	29693	98.431
NR4A1	nuclear receptor subfamily 4, group A, member 1	79240	97.132
RAB3A	RAB3A, member RAS oncogene family	25531	96.975

Table-5: Top 10 Interacting Nodes in PPI Analysis

Label	Degree	Betweenness	Expression
Akt1	22	5808.71	54.118
Cdk1	11	1780.5	-106.67
Pcna	10	896.87	-138.9
Nfkb1	9	1095.09	-111.02
Pik3r2	8	2668.38	127.2
Gna11	8	1679.93	92.205
Stat3	8	1270.92	-144.69
Syk	8	504.98	-83.095
Actn2	7	3294	84.253
Hck	7	3016.68	-164.47

Table-6: Interacting proteins related to GO: Neurogenesis

S.No	Label	Degree	Betweenness	Expression
1	Akt1	22	5808.71	54.118
2	Stat3	8	1270.92	-144.69
3	Mtor	7	1183.27	49.327
4	Casp3	6	1507.09	-83.013
5	Myc	6	1086.18	-146.14
6	Grin2a	5	3768	103.02
7	Itgb1	5	2510	-109.38
8	Calr	5	1398.5	-63.295
9	Tgfb1	5	502.61	-155.89
10	Ywhae	4	1146.1	43.157
11	Ngfr	4	448.79	42.502
12	Klf4	4	444.21	-58.029
13	Grin1	4	16	117.46
14	Ptk2b	3	3865.3	111.03
15	Kalrn	3	593.51	102.46
16	Rab11a	3	237.59	-70.132
17	Egr2	3	236.47	69.087
18	Arf6	3	155.84	-87.126
19	Col3a1	2	819.5	-102.35
20	Rab8a	2	799.01	-54.952
21	Lamc1	2	161	-102.14
22	Adm	2	161	-116.72
23	Hspb1	2	161	-191.16
24	Pak1	2	29.21	94.689
25	Tgfb2	2	0.5	-41.91
26	Pld2	2	0	42.035
27	Ctnn	1	0	42.753
28	Ifngr1	1	0	-119.15
29	Nr4a2	1	0	50.835
30	Rab3a	1	0	96.975
31	Prkcz	1	0	59.732
32	Nr1d1	1	0	95.173
33	Csfl	1	0	-71.804
34	Vim	1	0	-158.62
35	Wee1	1	0	58.245

Table-7: majorly altered genes related to neurogenesis obtained from cytoscape enrichment map

Gene	Ranks
THY1	117.15
NR1D1	95.17
CDK5	82.8
STX1B	76.62
NOG	74.74
CNTN4	70.94
PTN	65.16
EPHA7	63.97
CNTN2	60.96
GDF11	57.76
GNRH1	39.72
PAFAH1B1	-38.55
SYT4	-39.08
FKBP4	-44.99
CALR	-63.3
IDH2	-67.54
CD24	-81.23
JAG1	-82.33
PMP22	-83.38
ID1	-86.77
ARF6	-87.13
CD38	-95.75
COL3A1	-102.35
LGALS1	-120.2
DYNLT1	-137.9
STAT3	-144.69
TGFB1	-155.89
VIM	-158.62
GFAP	-187.73
TSPO	-206.96

FIGURE LEGENDS

Figure-1: Overview of the enrichment analysis process

Fig-1: Synopsis of meta-analysis conducted in this study. 10 microarray datasets comprising 97 total samples were chosen from NCBI GEO webpage. Datasets were annotated with Entrez id to facilitate compatibility in network analyst 3.0 software. After validation the data were uploaded to Network analyst software, where the samples were normalized and subjected to meta-analysis, thus obtaining the resulting Ranked Differentially expressed gene list comprising 939 genes. The differentially expressed genes between sham and MCAo stroke affected animals were subjected to enrichment analysis through Cytoscape, WebGestalt and string analysis.

Figure-2: Normalization of Datasets

Fig-2: Normalization of transcriptome data. A) Total intensity distribution among microarray datasets before and after normalization is shown. B) Variation in intensity values from each sample before and after normalization is shown.

Figure-3: TF- miRNA interactome

Fig-3: TF-miRNA interactome graph. The diagram represents the interaction between miRNA and transcription factors of DE genes where nearest represent those with higher interaction score.

Figure 4: Venn diagram expressing overlapping between PPI list and GO:BP Neurogenesis lists

Figure-5: Gene Ontology Classification, Pathway enrichment analysis- Clusters of highly significant pathways based on KEGG Database, Acyclic Graph of enriched processes based on GO Biological Processes

Fig-5: WebGestalt outputs. A) Pathway enrichment analysis of DE genes between MCAo and Sham animals, resulting in multiple clusters of significantly altered pathways based on KEGG database. B) Gene ontology classification. Each bar represents an ontology category and the height signifies the number of DE genes observed under each category. C) Branching graph depicts functional interaction between various GO categories, where the steel blue colored categories are positively related while the deep orange colored categories are negatively related.

Figure-6- Cytoscape enrichment data

Fig-6: Enrichment map from Cytoscape. The graphic depicts interaction between various enriched nodes(Dots). Lines connecting the nodes are called edge. Blue nodes depict more enrichment in MCAo group while the red nodes depict more enrichment in Sham group.

Figure-7: Graphical Representation of the altered neurogenic genes in post stroke environment

Figure-7: Coronal section of the adult rat brain is shown in the upper left. SVZ region comprises Ependymal cells (Pink) lining along the ventricles, with Type B1 stem cells(Green) adhering to them. Type B1 cells give rise to Type-C Transit amplifying Cells(Yellow). During proliferation Type-C cells rapidly divide to form Type-A neuroblasts(Light orange). These neuroblasts migrate along the blood vessels (Red) to reach striatum after stroke. Once reaching the site, the neuroblasts differentiate into immature neurons(violet) and give rise to arborizations. They turn into functional neurons after maturation. The table depicts the genes that upregulate or downregulate the different phases of neurogenesis. The green upward arrows represent genes found to be upregulated in MCAo animals in the meta-analysis study, while the red downward arrow represents genes found to be downregulated in MCAo animals in the meta-analysis study.

ACUTE POST-STROKE ENVIRONMENT

Dysregulations to
Neurogenesis Genes
Largely Unexplored

**DATA
collection**

**DATA
Processing**

**DATA
Enrichment
& Analysis**

Fig-1

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Fig-2

A

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Before Normalization

After Normalization

B

Before Normalization

After Normalization

Fig-3

Fig-4

Fig-5

Fig-6

Negative Regulation of Neurogenesis

Fig.7