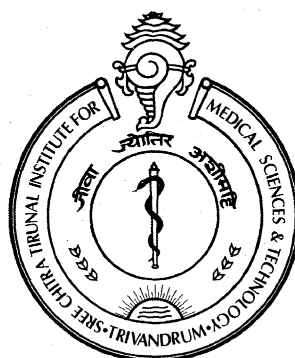
UNDERSTANDING THE JERKY GENE AND ITS INVOLVEMENT IN THE MOLECULAR PATHWAYS ASSOCIATED WITH SEIZURE

SIDDHARTH BANERJEE

PhD THESIS APRIL 2008



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MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM 695 011

UNDERSTANDING THE JERKY GENE AND ITS INVOLVEMENT IN THE MOLECULAR PATHWAYS ASSOCIATED WITH SEIZURE

A THESIS PRESENTED

BY

SIDDHARTH BANERJEE

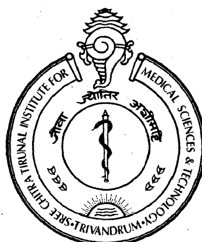
TO

THE DIVISION OF MOLECULAR MEDICINE

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY



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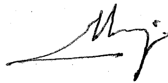
DECLARATION

I, **Siddharth Banerjee,** hereby declare that I had personally carried out the work depicted in the thesis entitled **"Understanding the Jerky gene and its involvement in the molecular pathways associated with seizure"** under the direct supervision of **Dr. Anoopkumar Thekkuveettil**, Scientist E, Division of Molecular Medicine, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help sought and acknowledged.



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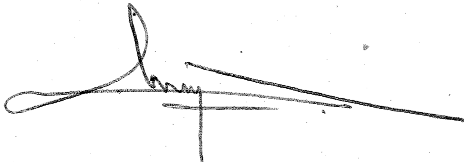

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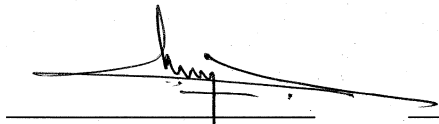

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CONTENTS

Page

Acknowledgements i

List of figures ii

List of tables vii

Abbreviations viii

Synopsis xi

Chapter 1 **INTRODUCTION** 1

Chapter 2 **REVIEW OF LITERATURE** 6

2.1. **Epilepsy: disorder of neuronal system** 6

2.2. **Animal models for epilepsy** 7

2.3. **Gene expression variation during epilepsy** 9

2.3.1 Seizure-induced gene expression 9

2.3.2. Global expression profiling in seizure 10

2.3.3. Proteomic perspective of gene expression 12

2.4. **Post-transcriptional regulation and RNA-binding proteins** 13

2.4.1. Post-transcriptional regulation 13

2.4.2 RNA-binding protein in post-transcriptional regulation 15

2.4.3. RNA-binding proteins in neurons 16

2.4.4. RNA-binding proteins in global gene expression regulation 16

2.5. **Jerky** 18

2.5.1. Jerky gene inactivation caused seizure 18

2.5.2. Nuceic acid-binding by Jerky 19

2.5.3. Localization of Jerky 21

2.5.4. Human Jerky gene and epilepsy 22

2.5.5. Human Jerky gene and single nucleotide polymorphism 22

Page

Chapter 3 MATERIALS AND METHODS **25**

3.1. Competent cell preparation of XL1 blue *E.coli* strain **25**

3.2. Transformation of $CaCl_2$-competent cells **26**

3.3. XL1 blue electrocompetent cell preparation **26**

3.4. Electroporation of XL1 blue electrocompetent cells **27**

3.5. Plasmid isolation-Alkaline lysis method (Minipreparation) **28**

3.6. Plasmid isolation-Alkaline lysis method (Maxiperparation) **30**

3.7. Genomic DNA isolation from blood **31**

3.8. Agarose gel electrophoresis **32**

3.9. RNA isolation from tissue by guanidium thiocyanate-phenol extraction method **33**

3.10. RNA isolation from tissue using TRI reagent method **35**

3.11. RNA electrophoresis on formaldehyde-agarose gel **35**

3.12. SDS-PAGE for protein electrophoresis **37**

3.13. Silver-staining of protein on SDS-PAGE **38**

3.14. Silver-staining of DNA on polyacrylamide gel **40**

3.15. Seizure induction in rat and mice **41**

3.16. Microarray analysis and PANTHER gene expression analysis **42**

3.17. Cloning and constructs **42**

3.17.1. Cloning of Jerky **42**

3.17.2. Cloning of Jerky-pulldown genes **44**

3.17.3. RNA constructs of pulldown genes **44**

3.17.4. Cloning of synaptotagmin 1, 4 and 10 .... **44**

3.17.5. RNA constructs for synaptotagmin 1, 4 and 10 .... **46**

3.17.6. Constructs for transcribing smaller RNAs of Syt 1 and Syt 10 .... **47**

3.17.7. Construct for *in vitro* translation of Nedd4 .... **48**

3.18. Expression of GST-Jerky recombinant protein **48**

Page

3.18.1 Purification of GST-Jerky recombinant protein 50

3.18.2. Micrococcal nuclease treatment of purified protein 50

3.19. RNA-pulldown assay 50

3.20. 5'-End-labeling of primer 52

3.21. Labeling of parital cDNA from pulldown RNA 52

3.22. Differential display-RT PCR 53

3.23. DNA elution from polyacrylamide gel 55

3.24. *In vitro* transcription and labeling of RNA 55

3.25. Electrophoretic mobility shift assay 56

3.26. Slot blot filtering binding assay 57

3.27. *In vitro* translation assay 58

3.28. Recruitment of human subjects for Jerky gene profiling 59

3.29. Amplification of human jerky gene 60

3.30. Multiple restriction site-single strand conformational polymorphism (MRF-SSCP) 61

Chapter 4 RESULT AND DISCUSSION 63

4.1. Seizure-associated gene expression changes 63

4.2. Role of Jerky in translational regulation 75

4.3. Jerky gene variations in human seizure disorder 93

Chapter 5 SUMMARY AND CONCLUSIONS 103

Chapter 6 REFERENCES 109

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LIST OF FIGURES

Figure No.	Figure Caption	Page No.
Figure 1.	Time course of biochemical, anatomical and functional changes after seizure (adapted from Cole, *et al.*, 2002).	**9**
Figure 2.	Schematic representation of synaptic reorganization of epileptic hippocampus. Recurrent excitatory networks formed by reorganized mossy fibres in the epileptic dentate gyrus (adapted from McNamara, 1999).	**10**
Figure 3.	Post-transcriptional regulation of mRNA within the neuron. Processed mRNAs form translationally inactive ribonucleoprotein complexes and transported to distal dendrites where local protein synthesis takes place in response to specific stimuli (adapted from Bramham and Wells, 2007).	**14**
Figure 4.	Ribonucleoprotein complex formation represents the nodes of information accumulation and transfer. RNA-binding proteins bind to mature mRNAs and determine the spatial and temporal fate of translation.	**15**
Figure 5.	Volcano Plot depicting the global gene expression. Each coordinate represents a gene probe plotted against its corresponding log2 of fold change against its p-value. Coordinates with $p \leq 0.05$ and atleast ±2 fold change are marked red.	**64**
Figure 6.	Gene expression variation within 4 h of pilocarpine-induced seizure. **A** represents the number of genes that underwent upregulation by certain fold change. **B** classifies the upregulated genes based on its biological process.	**65**
Figure 7.	Gene expression at SP and RP in select biological processes. Expression levels of a selected number of genes within each process are represented as fold change in graphical and tabular format.	**71**
Figure 8.	Cloning of Jerky in to pTZ57R/T. Lanes M and U indicate the molecular weight marker and the undigested form of the pTZ-Jerky plasmid, respectively. Lane 1 is the 1688 bp amplified product of Jerky; Lanes 2, 3, 4, 5 and 6 represent the restriction-digested pTZ-Jerky with Bgl1, EcoR1, Sma1, Pst1 and Apa1 enzymes respectively. Molecular weight of the expected products from pTZ-Jerky digestion and its corresponding lane are indicated in the table.	**76**

Figure No.	Figure Caption	Page No.

Figure 9. Cloning of Jerky into pGEX-4T-1 and Expression of GST-Jerky. Lanes M and U indicate the molecular weight marker and the undigested form of the pGEX-Jerky plasmid, respectively. In **A,** endfilled Jerky DNA is ligated at Sma1 site of pGEX. Lane 1- Klenow endfilled Jerky DNA, lane 2 and 3 represent the undigested and Sma1 linearized pGEX-4T-1 plasmid. **B** confirms the pGEX-Jerky clone by restriction digestion with EcoR1 (lane 4), Bgl1 (lane 5), Pst1 (lane 6), BamH1-Xho1 (lane 7) and Sma1-EcoR1 (lane 8). Molecular weight of the expected products from pGEX-Jerky digestion and its corresponding lane are indicated in the table. **C** shows the expression of GST and GST-Jerky protein expression at 1 h, 2 h and 3 h after IPTG induction. The 87 kDa purified GST-Jerky is also run in the last lane. Cloning of Jerky in the Sma1 site of pGEX-4T-1 is depicted in the plasmid map.. **77**

Figure 10. Pulldown RNA using GST-Jerky protein. **A** represents the total RNA from control and epileptic hippocampus used for pulldown experiment. **B** represents the labeled cDNAs of pulldown RNA with 'no-protein' bead complex (lane1) and GST-Jerky anchored bead complex (lane 2). **C** shows the differential display gel on total RNA as well as pulldown RNA. Lanes 1 & 2 are differential display of control and epileptic total RNA, respectively, using primer pairs HT11A-LMM5. Lanes 3 & 4 are the differential display on epileptic total RNA and pulldown RNA, respectively, using primer pairs HT11A-MOB5. **D** shows the differential display gels from which bands from pulldown lanes were eluted, amplified and cloned for sequencing. Lanes 1, 2, 3, 6 and 7 were differential display with primer pairs HT11A-LMM5, whereas lanes 4, 5, 8 and 9 were differential display with primer pairs HT11A-MOB5. Lanes 1 & 4 and 6 & 8 were amplified from total RNA of epileptic and control respectively. Lanes 2 & 5 and 3, 7 & 9 were amplified from pulldown RNA of epileptic and control respectively. Arrow-marked bands in the pulldown lanes were sliced out for elution.. **78**

Figure 11. Reamplification and Cloning of jerky pulldown genes. Lane 1, 2, 3, 4 are reamplified DNA of Nedd4, ATP6, ND4 and Aip2. Lanes 5, 8, 9 correspond to undigested forms of pTZ-Nedd4, pTZ-ND4 and pTZ-ATP6. Lane 6, 7, 10 correspond to EcoR1-Sal1 digested forms of pTZ-Nedd4, pTZ-ND4 and pTZ-ATP6, confirming the cloned insert. **79**

Figure 12. Cloning of Syt 1, Syt 4 and Syt 10. Lanes M and U indicate the molecular weight marker and the undigested form of the respective plasmids. Amplified products of coding region of Syt 1 (lane 1), Syt 4

Figure No.	Figure Caption	Page No.

(lane 3) and Syt 10 (lane 5) were cloned in to pTZ57R/T vector. Clones pTZ-Syt 1, pTZ-Syt 4 and pTZ-Syt 10 were confirmed by restriction digestion using Rsa1 (lane 2), Xba1 (lane 4) and BamH1 (lane 6) respectively. The pTZ-Syt clones were digested with Sal1-EcoR1 to release the cloned inserts of Syt1 (lane 8), Syt 4 (lane 10) and Syt 10 (lane 12), to be subcloned in to pGEM-T Easy vector at Sal1-EcoR1 sites. Digestion with Sal1-EcoR1 was carried out to confirm the clones pGEMT-Syt 1 (lane 14), pGEMT-Syt 4 (lane 16) and pGEMT-Syt 10 (lane 18). Lanes 7, 9, 11, 13, 15, 17 represent the undigested plasmids of pTZ-Syt 1, pTZ-Syt 4, pTZ-Syt 10, pGEMT-Syt 1, pGEMT-Syt 4 and pGEMT-Syt 10. Molecular weight of the expected products from digestions of Syt clones and their corresponding lane are indicated in the table. **81**

Figure 13. Linearized vectors with insert in T7 orientation were used for *in vitro* transcription. Lanes 1, 4, 5, 7, 9 and 11 represent the undigested plasmids of pGEMT-ATP6, pGEMT-ND4, pGEMT-Syt 1, pGEMT-Syt 4, pTZ-Nedd4 and pGEMT-Syt10. Linearized products of pGEMT-ATP6 (lane2), pGEMT-ND4 (lane 3), pGEMT-Syt 1 (lane 6), pGEMT-Syt 4 (lane 8), pTZ-Nedd4 (lane 10) and pGEMT-Syt10 (lane 12) are used for *in vitro* transcription. Molecular weight of the linearized product of clones used for *in vitro* transcription and its corresponding lane are indicated in the table. **82**

Figure 14. Mobility shift assay with recombinant jerky protein (n = 3 to 6). Lanes 1, 2, 3 in Cntrl 1 represent Nedd4 RNA alone, Nedd4 RNA+ 0 h bacterial lysate, Nedd4 RNA+purified GSTJerky protein. Lanes 4, 5, 6 in Cntrl 2 represent Syt10 RNA alone, Syt10 RNA+purified GST-Jerky protein, Syt10 RNA+purified GST protein. Lanes 8, 10, 12, 14, 16 and 18 represent RNA alone of ATP6, ND4, Nedd4, Syt 1, Syt 4 and Syt 10. Lanes 7, 9, 11, 13, 15 and 17 represent GST-Jerky protein RNP with transcripts of ATP6, ND4, Nedd4, Syt 1, Syt 4 and Syt 10. .... **84**

Figure 15. Competitive mobility shift assay. Labeled probes were competed with its own unlabeled transcripts for binding to GST-Jerky protein (n = 2). ND4 labeled RNA was competed against 50-150% identical cold RNA. Syt 1 and Syt 10 were competed against 25-100% of its own cold transcripts. Labeled Syt 4 RNA was competed against 100-400% of cold RNA. **84**

Figure 16. Slot blot assay to measure binding affinity of GST-Jerky towards various gene transcripts (n = 3 to 6). Binding of gradient concentration of recombinant jerky protein to constant amount of labeled gene transcripts

Figure No. **Figure Caption** **Page No.**

of Nedd4, ATP6, ND4, Syt 1, Syt 4 and Syt 10 was analyzed for respective binding kinetics. **85**

Figure 17. Kinetics of binding of GST-Jerky protein to different target transcripts. Nedd4, ATP6, ND4, Syt 1 and Syt 10 have similar binding affinity towards GST-Jerky protein. Syt 4 has marginally higher affinity towards jerky protein. Values of each coordinate on the graph are the average of three to six experiments. **86**

Figure 18. Constructs for smaller RNAs from Syt 1 and Syt 10 coding sequence. **A** Different constructs used in the study for Syt I and Syt 10. **B** Syt 1 smaller constructs. pTZ-Syt $1^{356-758}$ and pTZ-Syt $1^{356-1268}$ were confirmed by Sal1-EcoR1 digestion in lane 2 (2834 bp, 459 bp) and 4 (5 kb, 983 bp) respectively. Xba1-linearised pTZ-Syt $1^{356-758}$ (lane 8, 3293 bp) and pTZ-Syt $1^{356-1268}$ (lane 6, 3817 bp) were used for in vitro transcription. Lanes 1 & 7 and 3 & 5 are undigested forms of pTZ-Syt $1^{356-758}$ and pTZ-Syt $1^{356-1268}$. pGEMT-Syt $1^{781-1268}$ was confirmed by Sal1-EcoR1 (lane 10, 2977 bp, 557 bp) digestion and linearized with Sal1 (lane12, 3534 bp) for use in in vitro transcription. Lanes 9 & 11 are undigested forms of pGEMT-Syt $1^{781-1268}$. **C** Syt 10 smaller constructs. pGEMT-Syt 10 was digested with EcoRV (lane 1, 4320 bp,252 bp) and used for transcription for Syt 10^{1-1059}. pBS-Syt 10 clone was initially digested with BamH1 digestion (lane 2) and then partially digested with EcoRV (lanes a-e). The 475 bp Syt$10^{1060-1533}$ partially digested product was cloned in to BamH1-EcoRV positions of pTZ57R/T. The pTZ- Syt$10^{1060-1533}$ was confirmed by BamH1-EcoRV (lane 3, 2882 bp, 252 bp, 223 bp) and Sal1-EcoR1 (lane 4, 2834 bp, 523 bp) digestion. The Syt$10^{1060-1533}$ was subcloned into pGEM-T Easy vector and confirmed by Sal1-EcoR1 (lane 5, 2977 bp, 523 bp) digestion. PGEMT- Syt$10^{1060-1533}$ was linearized by Sal1 (lane 6, 3500 bp) to be used for *in vitro* transcription. **87**

Figure 19. Slot blot analysis of smaller RNA constructs with GST-Jerky protein (n = 3 to 4). From the signal on the nylon membrane, the lanes A, B, C, D denote transcripts of Syt 1, Syt $1^{356-1268}$, Syt $1^{356-758}$ and Syt $1^{781-1268}$. Lanes E, F, G denote transcripts of Syt 10, Syt10^{1-1059} and Syt$10^{1060-1533}$. **88**

Figure 20. Kinetics of binding of GST-Jerky protein to smaller RNA constructs of Syt 1 and Syt 10. GST-Jerky binds to all the transcripts with almost similar affinity. Values of each coordinate on the graph are the average of three to four experiments.. .... **89**

Figure No.	Figure Caption	Page No.
Figure 21.	Subcloning of full coding Nedd4 sequence for *in vitro* translation. **A** pBS(SK)-Nedd4 was confirmed by sequencing as well as restriction digestion. **B** has the Nedd4 insert of 2863 bp was released by Sal1-Not1-Bgl1 digestion (lane 5) and cloned in Sal1-EcoR1 sites of pBS(KS). After cloning of Nedd4 (lane 8) in to Sal1-EcoR1 digested pBS(KS) (lane 7), the clone was confirmed by resriction digestion with EcoRV (lane 9) and EcoR1 (lane 12). Molecular weight of the expected products from digestions of pBS(SK)-Nedd4 and pBS(KS)-Nedd4 clones and its corresponding lane are indicated in the table.	**91**
Figure 22.	*In vitro* translation of Syt 1 and Nedd4 in rabbit reticulocyte lysate system. Syt 1 and Nedd4 translated protein have a molecular size of 47 and 102 kDa. In presence of GST-Jerky protein, the translation is repressed, and denatured protein restores the translation albeit partially. The expression difference between Nedd4+ GST-Jerky and Nedd4+ denatured GST-Jerky is relevant (p=0.01, n=3)	**92**
Figure 23.	Human Jerky gene SNP screening. **A** PCR plan for amplifying the entire jerky gene using four sets of PCR. **B** Genomic DNA isolated from blood samples.**C** Amplified products from four sets of amplification. Lane 1, 2, 3 and 4 are PCR products from set 1, 2, 3 and 4. **D** PCR amplicons were digested with restriction enzymes for MRF-SSCP SNP screening (lanes 5-8). **E** Molecular weight of the expected products from digestion of different jerky amplicons and its corresponding lane are indicated in the table.	**94**
Figure 24.	Representative MRF-SSCP gels of all sets of Jerky PCRs. Lane U denotes the nondenatured sample of that particular set. The ssDNA bands appear in the upper region of the gel.	**95**
Figure 25.	Set 4 samples showing variation on MRF-SSCP (n = 2 to 3) were confirmed by sequencing and the nature of variant was deciphered. Lanes 1, 2, 3 represent three non-epileptic controls; Lanes 4 and 5 are two juvenile epilepsy patients; Lane 6 frontal lobe epilepsy patient; Lanes 7, 8, 9 are three temporal lobe epilepsy patients; Lane 10, 11 and 12 represent patients with Lennox gastaut syndrome, Non-temporal lobe focal epilepsy and primary generalized epilepsy respectively. Banding pattern variation were observed in lanes 4, 5, 6, 9 and 11 and later confirmed by sequencing. Data regarding the lanes showing variations, the corresponding patient's epilepsy type and the nature of variation confirmed by sequencing, are represented in the table.	**96**

LIST OF TABLES

Table No.	Table Caption	Page No.
Table 1.	*In vitro* mRNA targets of Jerky on affymetrix array (adapted from Liu, *et al.*, 2003)). Jerky binds to large subset of mRNA targets with varied functional roles.	**21**
Table 2.	Single Nucleotide Polymorphisms within the Jerky gene listed in the SNP data bank	**23-24**
Table 3.	Top-ranked genes overexpressed during SP	**65**
Table 4.	PANTHER analysis of gene expression variation during SP and RP. overexpressed genes.	**67-69**
Table 5.	Genes identified from GST-Jerky pulldown experiment. Nedd4 and ND4 were amplified from pulldown RNAs of epileptic and control respectively using primer pairs HT11A-LMM5. ATP6 and Aip2 were amplified from pulldown RNAs of epileptic and control respectively using primer pairs HT11A-MOB5.	**80**
Table 6.	Clinical details of the patients checked for SNP within JRK gene. Details such as age of onset, family history, Seizure frequency, pathological features and drug response were collected and tried to correlate with the SNPs detected.	**97-99**

ABBREVIATIONS

Aip2	ADP-ribosylation factor-like 6 interacting protein 2
APS	ammonium persulphate
ARE	AU-rich elements
ATP	adenosine triphosphate
ATP6	ATP synthase 6
BDNF	brain derived neurotrophic factor
bp	base pair
BSA	bovine serum albumin
CA	ammon's horn
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaMKII	calmodulin kinase II
cDNA	complementary DNA
DMS0	dimethyl sulphoxide
DTT	dithiotreitol
EDTA	ethylene diamine tetraacetic acid
EEG	electroencephalogram
EGTA	ethylene glycol tetraacetic acid
ELAV	embryonic lethal abnormal visual protein
FE	focal epilepsy
FLE	frontal lobe epilepsy
FMRP	fragileX mental retardation protein
GABA	gamma aminobutyric acid
GADD45	growth arrest and DNA damage inducible gene 45
GST	glutathione s-transferase
Hsp70	heat shock protein 70

HTH	helix-turn-helix
IL2	interleukin 2
IPTG	isopropyl β–D-1-thiogalactopyranoside
JME	juvenile myoclonic epilepsy
JRK/JH8	human jerky gene
K_2HPO_4	dipotassium hydrogen phosphate
kb	kilo base
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	monopotassium phosphate
LB	lurea Broth
Lys	lysine
Met	methionine
$MgCl_2$	magnesium chloride
MOPS	3-(N-morpholino)-propanesulfonic acid
MRF-SSCP	multiple restriction fragment- single strand conformational polymorphism
mRNA	messenger ribonucleic acid
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
ND4	NADH dehydrogenase, subunit 4
Nedd4	neural precursor cell expressed developmentally downregulated gene 4
PAGE	polyacrylamide gel electrophoresis
PANTHER	protein analysis through evolutionary relationships
RBP	RNA-binding protein
RNA	ribo nucleic acid
DNA	deoxyribo nucleic acid
RNP	ribonucleoprotein
RP	recovery period
rpm	revolutions per minute

SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulphate
SE	status epilepticus
Ser	serine
SLBP	stem-loop binding protein
SNP	single nucleotide polymorphism
SP	seizure period
STAT	signal transducer and activator of transcription
Syt	synaptotagmin
TAE	tris acetate EDTA buffer
TBE	tris borate EDTA buffer
TE	tris-EDTA buffer
TEMED	tetramethylethylenediamine
Thr	threonine
TLE	temporal lobe epilepsy
UTP	uridine triphosphate
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
ZBP1	zipcode-binding protein 1

SYNOPSIS

Seizure is a complex disorder characterized by synchronous firing of a large set of neurons. It is not well understood how the initial onset of seizure induces long term changes in neurons and predispose to recurrent seizures. Seizure-induced gene expression variations and its regulation may have a critical role in making neurons susceptible to hyperactivation.

Chapter 1 introduces the topic on seizure-induced biochemical variations and its impact on seizure susceptibility. Seizure activates intracellular signaling molecules by the heavy influx of Ca^{2+} through NMDA-receptors. Following the kinases activation, various transcription factors are activated leading to upregulated transcription of early genes and various other biochemical pathways. Pilocarpine model for epilepsy represents the ideal model to study gene expression variation in the hippocampus of brain. Akin to neuronal cell loss and mossy fiber sprouting in human epilepsies, which make it susceptible to recurrent seizure, pilocarpine model exhibits spontaneous seizures days to weeks following initial insult. Gene expression variations accompanying seizure-induction has long-lasting consequences, making it prone to recurrent seizure. However, it is not well understood how the transcripts are regulated in the system. The present study aimed to understand the variations in transcript levels after seizure as well as the role of a RNA binding protein, Jerky, in translational regulation of mRNAs. In addition, we also verified the mutations in Jerky gene in epileptic patients.

Chapter 2 reviews the literature relevant to understanding of this research study. Human epilepsies are a group of disorder whose genetic components are different and relate to each specific disorder. Animal models of seizure are used to study the biochemical, anatomical, pathophysiological and genetic aspects of epilepsy. Pilocarpine model is a chemiconvulsant model to study human seizure disorder. Onset of seizure triggers several changes at molecular level especially in gene transcription and translation. In cells, post-transcriptional regulation of the overexpressed transcripts can temporarily uncouple the process of translation and transcription. RNA-binding proteins mediate the post-transcriptional regulatory steps by the formation of translationaly-inactive RNP complexes with the transcripts. Jerky, a neuron specific protein and also a candidate gene for epilepsy, binds with a large subset of RNAs, forming translationaly-inactive RNP complex. Epilepsy has been studied to identify the genetic predisposing factors for the disease development. Even though several SNPs have been identified within the human jerky gene, no jerky gene mutation has been associated with seizure disorder.

Chapter 3 details the techniques employed in attaining the research goal, as under materials and methods. Seizure was induced in healthy male wistar rats by intraperitoneal pilocarpine injection. mRNA from the hippocampus of the 4h (seizure period), 24h (post seizure) and control were isolated using oligo-dT beads. The microarray experiment was conducted using RT-IVT kit and Rat Genome Survey Microarray slide. Data analysis was carried using ‘R’ and ‘Spotfire’. The seizure-upregulated genes were classified based on its biological function using the PANTHER gene expression analysis tool. The gene

expression variations between 4h and 24h (and control) were grouped based on the biological processes.

The complete coding region of mice jerky gene was amplified, cloned and expressed in pGEX-4T1 bacterial expression system. Total RNA from the control and 2h-epileptic hippocampal tissue of male swiss albino mice were isolated by guanidium thiocyanate- phenol extraction method. Jerky-binding RNAs were pulldown using GST-Jerky-anchored glutathione beads and used as templates for the differential display RTPCR using polyT reverse primers and arbitrary forward primers. Selected bands were gel eluted, reamplified, cloned and sequenced.

For the RNA-protein interaction studies, electrophoretic mobility shift assay and slot blot assay were performed with purified GST-Jerky protein and radiolabeled *in vitro* transcribed RNAs of Nedd4, ND4, ATP6 as well as Syt 1, Syt 4 and Syt 10 and smaller transcripts derived from Syt 1 and Syt 10. Competitive mobility shift assay was carried out by competing increasing concentrations of unlabeled transcripts. Binding affinity of jerky protein with different transcripts was reconfirmed by slot blot assay. TnT Rabbit reticulocyte lysate was used for *in vitro* translation of full-coding transcripts of Syt 1 and Nedd4 in presence/absence of purified GST-Jerky protein and denatured GST-Jerky. The translated labeled protein was run on SDS-PAGE and analyzed using phosphorimager.

SNP-analysis of the human jerky gene of 25 epileptic patients and 8 non-epileptic controls were carried out as a pilot study. Using gene-specific primers, the entire jerky gene was amplified from genomic DNA using four sets of PCRs. SNP-screening was performed by multiple restriction fragment single strand conformational polymorphism

(MRF-SSCP) technique. Sequence analysis and alignment was carried out by CLUSTALW algorithm.

Chapter 4 reports the results from this research study and discusses its relevance. Seizure triggered the upregulation of 2953 gene transcripts by over 2-fold. PANTHER analysis classified the upregulated-genes to 222 biological processes. Several biological processes that take place in the aftermath of seizure, such as apoptosis and neurogenesis included genes which overexpressed within hours of seizure-induction. During post seizure (24h) 85 genes were further upregulated than the 4h-seizure period and 859 genes underwent downregulation. However, 68% of the seizure-upregulated genes did not undergo major variations in their transcript levels even after 24h. The results suggested that transcripts of biological processes that take place days to weeks following seizure got transcribed at 4h seizure period and retained even during post seizure. Post-transcriptional regulation mediated by global RNA-binding proteins could be a plausible explanation for regulation of these gene transcripts. We selected Jerky as one of the candidate protein for the post transcriptional regulation of genes.

Recombinant GST-Jerky protein was used for pulldown of RNAs from the hippocampal tissue. Differential display technique estimated that jerky bound with a large subset of total RNAs. Nedd4, ND4 and ATP6 were among the few Jerky-pulldown transcripts identified in this study. RNA-protein interaction between jerky and several transcripts of Nedd4, ATP6, ND4, Syt 1, Syt 4 and Syt 10 were confirmed by mobility shift assays. Synaptotagmin (Syt) isoform gene transcripts were interesting because of its function in vesicular exocytosis and implications of overexpression of Syt 4 and 10

during seizure. The binding affinity of jerky with all the above transcripts as well as smaller RNA constructs was found to be similar and comparable. Translation efficiency of Syt 1 and Nedd4 using rabbit reticulocyte lysate showed an 80% reduction in the presence of jerky protein. Denatured jerky protein showed no inhibition of translation of Syt 1. These results suggested that Jerky acts in translational repression of most of the transcripts.

The preliminary association study carried out by mutation screening within the human jerky gene of epileptics and non-epileptics revealed SNPs within the 3'-UTR of 5 epileptic patients. The variance in 3'UTR may affect the RNA localization as a prelude to altered jerky protein distribution in the neuron. This, however, needs to be verified in a large cohort of study subjects.

Chapter 5 summarizes the entire work and its relevance to seizure. Seizure triggers the expression of several genes within wide variety of biological processes. Even though many of the transcripts (32%) were downregulated during post seizure period, 68% of the genes remained constant even after 24 hours post-seizure period. Surprisingly many of these transcripts belong to pathways like apoptosis, neurogenesis etc which sets in days or weeks after the seizure and believed to be among the reasons for seizure recurrence. We hypothesized that Jerky along with other global RNA-binding proteins bind with large subset of the overexpressed transcripts making them translationaly inactive until required for further translation. The RNA binding ability for jerky was found to be non-specific; affinities towards short non-coding RNAs were similar and comparable with full length coding RNAs. Binding of jerky to its target transcripts

repressed translation, indicating that jerky may have a critical function in translation regulation. In addition, polymorphisms exclusively observed in epileptic patients within the human jerky gene 3'-UTR suggest that the jerky RNA localization may be altered in neurons, affecting the distribution of jerky protein within the neurons.

Chapter 1

INTRODUCTION

Epilepsy consists of more than 40 clinical syndromes affecting 50 million people worldwide (Jacobs, *et al.*, 2001) and characterized by recurrent unprovoked seizure. A seizure is best defined as 'a brief change in behavior caused by the disordered, synchronous and rhythmic firing of populations of neurons in the central nervous system' (McNamara, 1999). More than a dozen genes have been identified that influence the risk for rare forms of epilepsies with Mendelian mode of inheritance, which account for 0.1% of patients afflicted with epilepsy (Noebels, 2001; Puranam and McNamara, 1999). However, in most of the common epilepsies, the etiology is a manifestation of complex interaction between multiple genes and environmental factors (Risch and Merikangas, 1996). Susceptibility to develop epilepsy is due to the summative effects of many genes and environmental factors and when it exceeds the seizure threshold, seizure manifests (Ottman, 2005).

Progressive biochemical, anatomical and physiological changes in the neuronal network accompany epileptogenesis leading up to recurrent seizures. Although it is known that the cascade of dynamic biological events alters the balance between excitation and inhibition in neural networks (Clark and Wilson, 1999), the molecular

mechanisms underlying epilepsy pathogenesis is vaguely understood. The transiently heavy influx of Ca^{2+} into the neurons activates intracellular signaling molecules which triggers transcription factors leading to upregulated transcription of early genes and various other biochemical pathways culminating in selective neuronal cell death and rewiring of surviving neurons (McNamara, 1999). In spite of the wide acceptance regarding the effect of neuronal cell death and neuronal plasticity in seizure development, the molecular mechanisms underlying the long-term neuronal plasticity and cell death remain poorly understood.

Numerous studies on animal models of seizure recapitulating human seizure disorder in animals, have not only provided a list of candidate genes for epilepsies (Puranam and McNamara, 1999), but also aided in providing insights into the progressive changes in the molecular and cellular level following *status epilepticus* (SE) (Dudek, *et al.*, 2002), making it susceptible to unprovoked recurrent seizure. Many of the pathophysiological changes observed in epileptic human brain tissue have been shown to be present in pilocarpine-induced epileptic rats, including hippocampal sclerosis, mossy fibre sprouting, neuronal hyperexcitability etc (Brooks-Kayal, *et al.*, 1998; Leite JP, 1990; Mello, *et al.*, 1993). Studies that monitored global gene expression pattern in animal models suggest that alterations in gene expression might be necessary to drive events leading to the development of spontaneous seizures during epileptogenesis as well as during epilepsy (Lukasiuk and Pitkanen, 2004).

Till recently, molecular analyses of epilepsy-induced hippocampal plasticity have focused on individual candidate genes with known functions for specific pathogenetic

aspects (Ben-Ari, 2001; Coulter, 2001). Overwhelming data from global gene expression profile studies provide clues on specific biological processes and biochemical pathways such as signal transduction, regulation of transcription, protein synthesis and degradation, basic metabolism, structural proteins and receptors during critical phases of seizure development (Lukasiuk and Pitkanen, 2004). Although early transcriptional alterations may be the first steps that lead to susceptibility to recurrent seizure (Weiser, *et al.,* 1993), earlier studies provide less information on the global expression profile when the animal is undergoing *status epilepticus.* One of the aims of the present study was to evaluate the expression profile during early stages of epileptogenesis in pilocarpine model for epilepsy.

In a broadened perspective, the overexpression of several genes in the wake of seizure, may have limited correlation at the level of proteome. Although mRNA concentrations are widely used as a surrogate for protein abundances, studies comparing mRNA and protein expression on a global scale indicate that mRNA levels only partly correlate with the corresponding protein concentrations (Greenbaum, *et al.*, 2003; Greenbaum, *et al.*, 2002; Tian, *et al.*, 2004). Regulation at the post-transcriptional level by a complex network of RNA-binding proteins ensures the temporal and spatial distribution of transcripts as well as the differential recruitment of mRNA species to the ribosome for protein synthesis, which results in a lack of correlation between the relative amounts of mRNA and the amount of the encoded protein. It is unlikely that every mRNA transcript has its own unique binding protein because tens of thousands of cell proteins would have to be dedicated to controlling posttranscriptional gene expression

and it is possible that structurally/functionally related mRNA subsets may interact with common RNA-binding proteins. RNA-binding proteins that bind to nearly all mRNAs in a global scale, does so, in a sequence independent manner (Keene, 2001). Subsequent to the seizure-induced overexpression of thousands of genes with no sequence similarity and engaged in several biological functions, the present study probed into the role of a RNA-binding protein, Jerky, in post-transcriptional regulation of translation of several transcripts.

Inactivation of the Jerky gene in animal resulted in hyperexcitability and increased susceptibility to seizure, therefore identified as a candidate gene for epilepsy (Toth, *et al.*, 1995). Jerky protein has a neuron-specific expression (Liu, *et al.*, 2002) and by virtue of a N-terminal helix-turn-helix motif, jerky protein binds to large subset of RNAs (Liu, *et al.*, 2003). Identification of transcripts bound by the jerky protein belonged to several functional classes and no sequence similarity. All these observations from earlier studies factored towards trying to elucidate the role of jerky protein in post-transcriptional regulation.

Another interesting aspect of jerky gene was that it is one among the recently evolved functional gene from a transposonal element (Lander, *et al.*, 2001). Higher incidence of single nucleotide polymorphisms (SNP) within the jerky gene in SNP database is in stark contrast to the lack of mutations identified within the jerky gene, situated at 8q24 loci, associated with any human seizure disorder. Epilepsy has long been studied to identify the genetic factors predisposing towards seizure susceptibility, and chromosomal loci, 8q24, which has shown linkage to childhood absence epilepsy (CAE)

and idiopathic generalized epilepsy (Morita, *et al.*, 1998). In the absence of any comprehensive data for variations within the human JRK gene associated with seizure phenotypes, the present study tried to screen human jerky gene from patients with different seizure disorders and check for possible association.

Chapter 2

REVIEW OF LITERATURE

2.1. EPILEPSY: DISORDER OF NEURONAL SYSTEM

Epilepsy is heterogeneous neurological disorder, incorporating numerous epilepsy syndromes with different etiologies, affecting 1-2% of the population worldwide (Annegers, *et al.*, 1982; Jacobs, *et al.*, 2001). The single feature that is common to each of the syndromes is the persistent increase in neuronal excitability that is occasionally and unpredictably expressed as seizure (McNamara, 1999). A seizure is described as 'a brief change in behavior caused by the disordered, synchronous and rhythmic firing of populations of neurons in the central nervous system' (McNamara, 1999).

Over the past two decades there has been a better understanding the molecular pathogenesis of human epilepsies. Progress has been made in identifying mutations in genes encoding ion channel subunits that influence the risk for rare forms of epilepsies with Mendelian mode of inheritance, which account for a small proportion (0.1%) of patients afflicted with epilepsy (Puranam and McNamara, 1999).

Majority of all epilepsies are genetically complex - multiple genes and environmental factors contribute to their etiology (Risch and Merikangas, 1996). A list of candidate genes is emerging from mutagenesis experiments on animals, give insights into

human epilepsies and an understanding of the molecular pathology arising from complex cascade of cellular events accompanying hyperexcitability (Jacobs, *et al.*, 2001; Puranam and McNamara, 1999). Development of animal models provides a powerful tool for elucidating molecular mechanisms that underlie epilepsy pathogenesis, and possibly a novel therapeutic strategy.

2.2. ANIMAL MODELS FOR EPILEPSY

Many features of human seizure disorder can be recapitulated in animal models (Cortez, *et al.*, 2006; Stafstrom and Sutula, 2005; Yang and Frankel, 2004). For example, mutations in genes coding for ion channels results in recurrent seizures in animal models, similar to human epilepsy where single ion channel gene mutations can precipitate the disease (Noebels, 2001). Atleast four of the 17 single loci mutations in mice resulted in epilepsy as a prominent phenotype (Noebels, 1986; Noebels, 2001), exhibit features characteristic of absence seizures- tottering, stargazer, mocha and lethargic. Mutant mice models of cystatin B recreated features of Unverricht-Lundborg syndrome and was correlated clinically in human as well (Pennacchio, *et al.*, 1996). Similarly, mutations in UBE3a and GABAR-β3 in mice resulted in human equivalence of Angelman syndrome (DeLorey, *et al.*, 1998; Jiang, *et al.*, 1998). Studies using knockout models have provided additional candidate genes implicated in seizure phenotype, which encode for ion channels, neurotransmitters, receptors, transporters and many others (Puranam and McNamara, 1999). These results suggested that alterations in the critical pathways like ion channels or neurotransmitter receptors could alter the neuronal function

and lead to seizure phenotype. While the loss of gene function is the primary cause for seizures in these models, it is difficult to decipher the differential contributions of multiple proteins and the biochemical pathways in the onset of seizure as well as in recurrent seizures in such models.

Unlike rare monogenic epilepsies, in most of the complex partial epileptic patients, a transient episode of *status epilepticus* (SE) or febrile seizures during childhood can induce multiple structural and functional alterations that after a latency period result in a chronic epileptic condition (**Figure 1**) (Weiser, *et al.,* 1993). Experimental *status epilepticus* is known to induce death of susceptible neurons and lead to development of chronic epilepsy (Ben-Ari, 1985). The progressive changes in the molecular and cellular level following *status epilepticus* (Ben-Ari, 2001; Dudek, *et al.*, 2002; Mello, *et al.*, 1993), making it susceptible to unprovoked recurrent seizure is best studied in chemoconvulsant models such as kainate model (Nadler, 1981) and pilocarpine model (Turski, *et al.*, 1983), as well as electroconvulsant model such as kindling model (Goddard, *et al.*, 1969; McNamara, *et al.*, 1985).

Local or systemic administration of epileptogenic compounds in rodents leads to pattern of repetitive limbic seizures and *status epilepticus*, which can last for several hours (Cavalheiro, *et al.*, 1982; Leite, *et al.*, 1990; Turski, *et al.*, 1983). Variable latent period follows *status epilepticus* and precedes the chronic phase, which is characterized by the occurrence of spontaneous limbic seizures. Many of the pathophysiological changes observed in epileptic human brain tissue have been shown to be present in pilocarpine-induced epileptic rats, including hippocampal sclerosis, mossy fibre

sprouting, neuronal hyperexcitability, altered GABA receptor functioning and interictal spike patterns on EEG (Brooks-Kayal, *et al.*, 1998; Leite, *et al.*, 1990; Mello, *et al.*, 1993). Pilocarpine model, with its similarities to human epilepsy provides a relevant model to investigate the molecular mechanisms underlying the transition from a normal to an epileptic brain following *status epilepticus*.

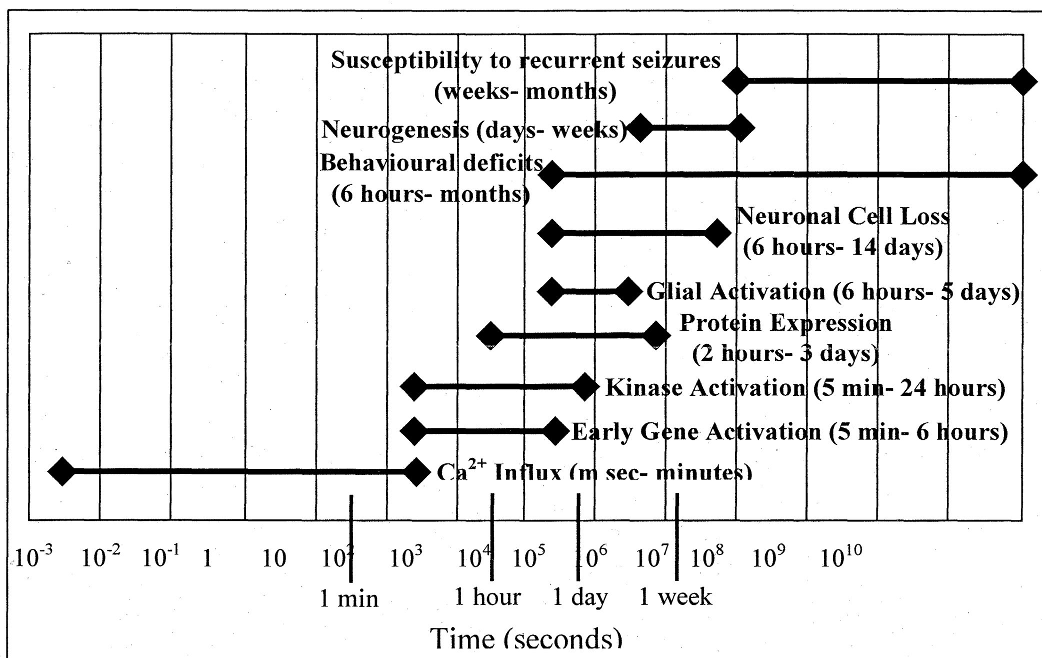


Figure 1: Time course of biochemical, anatomical and functional changes after seizure (adapted from Cole, *et al.*, 2000).

2.3. GENE EXPRESSION VARIATION DURING EPILEPSY

2.3.1. SEIZURE-INDUCED GENE EXPRESSION

Molecular analyses of epilepsy-induced hippocampal plasticity have largely focused on individual candidate genes, with particular emphasis on genes with known functions for specific pathogenetic aspects (Ben-Ari, 2001; Coulter, 2001). It is now

virtually axiomatic that seizures can cause alterations in gene expression (Gall, *et a*

1991; Morgan, *et al.*, 1987; Newton, *et al.*, 2003). Seizure induced by various metho

has shown to induce *c-fos*, other immediate early genes and genes encoding grow

hormone and other peptides (Ernfors, *et al.*, 1991; Morgan, *et al.*, 1987; Saffen, *et a*

1988; Sonnenberg, *et al.*, 1989). Although much is known about the changes in neuron

circuit in epileptic brain (**Figure 2**) the underlying molecular mechanism of the

changes and their role in the development of epilepsy remain poorly understood. Sever

studies that monitored global gene expression pattern in animal models suggest th

alterations in gene expression might be necessary to drive events leading to t

development of spontaneous seizures during epileptogenesis as well as during epilep

(Lukasiuk and Pitkanen, 2004).

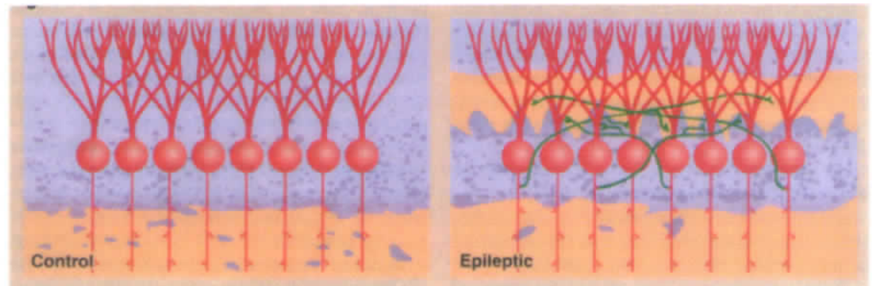


Figure 2: Schematic representation of synaptic reorganization of epileptic hippocampu Recurrent excitatory networks formed by reorganized mossy fibres in the epileptic dentate gyr (adapted from McNamara, 1999).

2.3.2. GLOBAL EXPRESSION PROFILING IN SEIZURE

Gene expression profile in the parietal cortex one day after kainic acid-induc

SE revealed 187 upregulated genes belonged to variety of functional classes, includi

stress-related proteins, proteases, cytoskeletal proteins, receptors and proteins involved in immune responses (Tang, *et al.*, 2002). In another gene expression study, carried out in the rat hippocampus and temporal lobe at 1 day, 4 days and 14 days of amygdala stimulation, several of the differentially expressed genes were associated with neuronal plasticity, gliosis and inflammation, protein synthesis and degradation and in signal transduction (Lukasiuk, *et al.*, 2003). Meanwhile, the pattern of gene expression in the dentate gyrus of the hippocampus in pilocarpine rat model 14 days post-SE represented a wide range of functions with a high number of genes involved in response to injury and cell survival. The authors also found that 37 genes were coregulated during epileptogenesis and development (Elliott, *et al.*, 2003). Most recently, gene expression in the CA3 and entorhinal cortex region of hippocampus was studied at 1 day, 7 days and 100 days in electrically induced SE rat models (Gorter, *et al.*, 2006). The immune response was the most prominent process that was upregulated, while GABA receptor subunits were persistently downregulated. Expression profiling performed on biopsy samples from intractable temporal lobe epilepsy (TLE) patients show differentially expressed genes in biological functions such as basic metabolism, transcription regulation, protein synthesis and degradation, neuronal signaling and synaptic plasticity, gliosis and immune response (Arion, *et al.*, 2006; Becker, *et al.*, 2002; de Lanerolle and Lee, 2005; Jamali, *et al.*, 2006; Ozbas-Gerceker, *et al.*, 2006). For obvious reasons, the resected tissue from patients undergoing surgery for intractable TLE does not allow the detection of the molecular changes occurring during the early phases in pathological cascade that lead to hippocampal sclerosis. The incongruent data on gene expression

variations from various studies originates from the animal models as well as, from human samples (with heterogeneity in age, sex, etiology), the analytical method used (different microarray platforms or SAGE), the selection of different epileptogenic brain regions and difference in the temporal points selected for expression analysis) resulted in a debate on validity of these observed changes. However, the basic pattern emerging from all the expression studies on seizure reveals genes performing wide range of molecular functions including signal transduction, regulation of transcription, protein synthesis and degradation, basic metabolism, structural proteins and receptors (Lukasiuk and Pitkanen, 2004).

Although early transcriptional alterations may be the first steps that lead to susceptibility to recurrent seizure, earlier studies provide less information on the global expression profile when the animal is undergoing *status epilepticus* or even during early stages of epileptogenesis. The gene expression variation accompanying first seizure insult could lead to long term changes in pilocarpine model making it prone to recurrent spontaneous seizures (Weiser, *et al.,* 1993).

2.3.3. PROTEOMIC PERSPECTIVE OF GENE EXPRESSION

It is tacitly assumed that altered mRNA levels predict similar changes in functional protein expression. Although mRNA concentrations are widely used as a surrogate for protein abundances, studies comparing mRNA and protein expression on a global scale indicate that mRNA levels only partly correlate with the corresponding protein concentrations (Greenbaum, *et al.*, 2003; Greenbaum, *et al.*, 2002; Gygi, *et al.*, 1999; Tian, *et al.*, 2004). The expression levels of some proteins and their corresponding

mRNA can vary as much as 30-fold (Griffin, *et al.*, 2002; Ideker, *et al.*, 2001). Such a dynamic covariation between gene expression and proteome is exemplified by regulatory events at post-transcriptional level. Whether the information in a mRNA is translated immediately, stored for later use, or routed to other locations, is dependent on post-transcriptional regulatory steps.

2.4. POST-TRANSCRIPTIONAL REGULATION AND RNA-BINDING PROTEINS

2.4.1. POST-TRANSCRIPTIONAL REGULATION

Post-transcriptional regulation acts on mature mRNAs by stabilizing/degradation of RNA, targeting the mRNAs to distal processes and by translational regulation of the transcript (Halbeisen, *et al.*, 2008). In neurons, mRNAs are transported over long distances in a microtubule-dependent manner in the form of large granules consisting of RNA-binding proteins, ribosomes and translation factors (**Figure 3**) (Hirokawa, 2006; Kiebler and Bassell, 2006). To ensure a highly restricted protein distribution, the mRNA translation is repressed during transport and then activated upon arrival at its destination. The dependence of translation on proper mRNA localization would prevent the synthesis of proteins that are either en route or mislocalized (Pfeiffer and Huber, 2006). Translational regulation concerns the differential recruitment of mRNA species to the ribosome for protein synthesis, which results in a lack of correlation between the relative amounts of mRNA and the amount of the encoded protein (Gebauer and Hentze, 2004). Activity-dependent translation of localized mRNAs likely occurs via combination of mechanisms in neurons, including activation of the general protein synthetic machinery

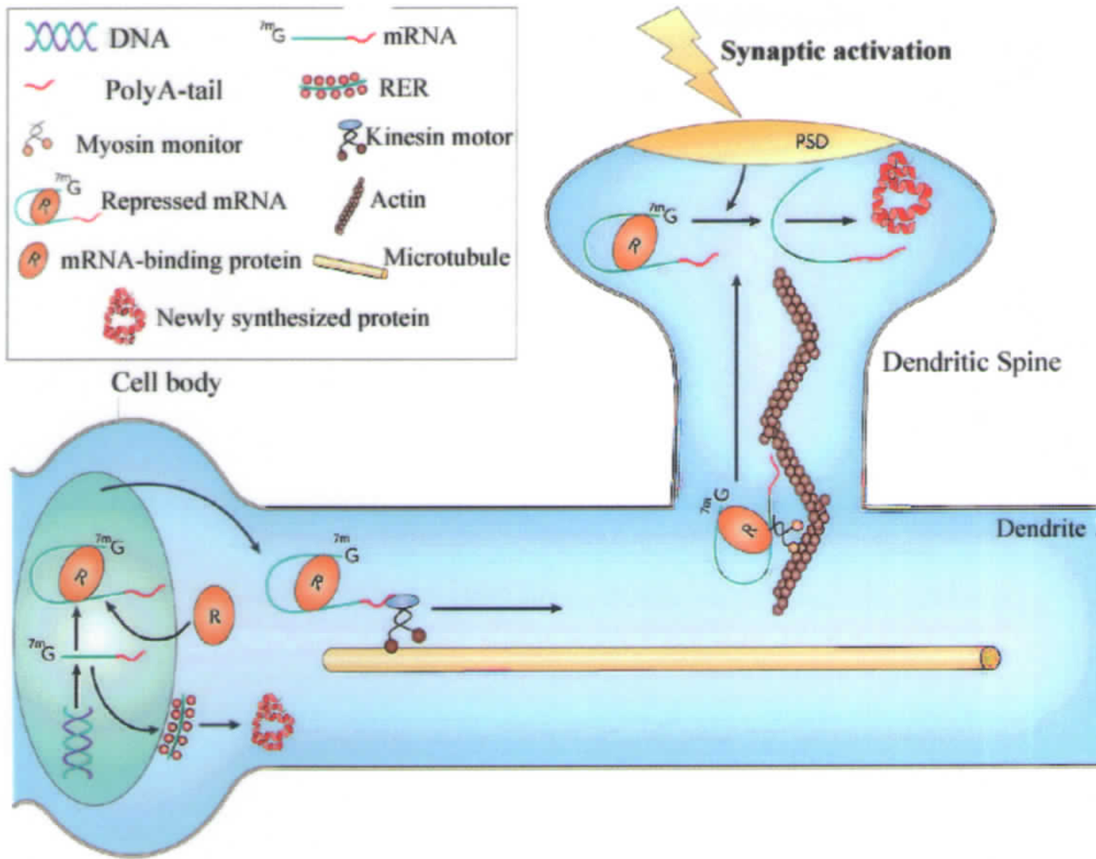


Figure 3: Post-transcriptional regulation of mRNA within the neuron. Processed mRNAs for translationally inactive ribonucleoprotein complexes and transported to distal dendrites whe local protein synthesis takes place in response to specific stimuli (adapted from Bramham an Wells, 2007).

in dendrites and release from repression of specific mRNAs in response to specif stimuli (Grossman, *et al.*, 2006). The abundance of each mRNA in the cell is determin not only by the rate at which it is produced, but also by its rate of degradation (Hargrov and Schmidt, 1989). mRNA decay mostly involves combinatorial interactions of RNA

binding protein (RBP) enabling stimulus-dependent decay programs through the integration of diverse signals (Halbeisen, *et al.*, 2008; Hollien and Weissman, 2006).

2.4.2. RNA-BINDING PROTEIN IN POST-TRANSCRIPTIONAL REGULATION

Assembly of mRNAs into ribonucleoprotein particles helps in sequestering of gene transcripts destined for post-transcriptional regulation. Messenger ribonucleoprotein complexes (RNP) consisting of RNA-binding proteins (RBP) and related mRNAs likely represent nodes of information transfer and accumulation (Keene, 2001)(**Figure 4**).

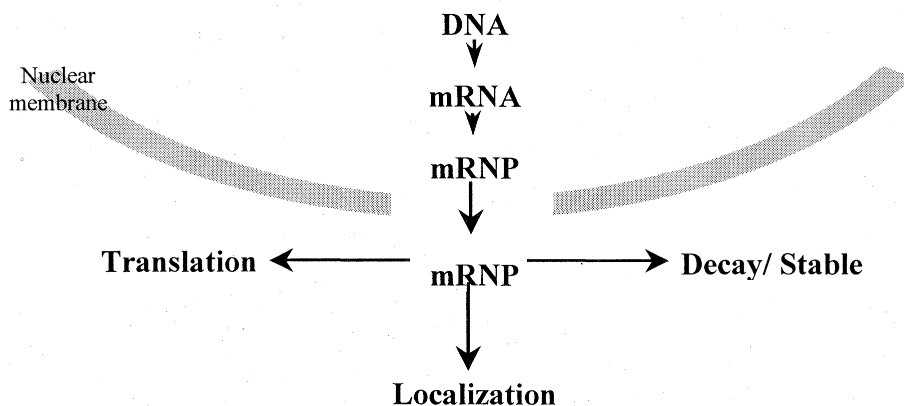


Figure 4: Ribonucleoprotein complex formation represents the nodes of information accumulation and transfer. RNA-binding proteins bind to mature mRNAs and determine the spatial and temporal fate of translation

RBPs are at the core of post-transcriptional regulation, involved in nuclear and cytoplasmic RNA processing (Dreyfuss, *et al.*, 2002). The formation of RNP complex brings about temporal uncoupling of the process of transcription and translation, and holds the RNAs in a translationally inactive state until destined for translation/degradation. The importance of post-transcriptional processing in neuronal

cell gene regulation is underscored by functional examples of specific RBPs (Agnes and Perron, 2004; Perrone-Bizzozero and Bolognani, 2002).

2.4.3. RNA-BINDING PROTEINS IN NEURONS

ELAV proteins bind selectively with AU-rich elements (ARE) in 3'-UTR of various mRNAs (Gao and Keene, 1996) and exert a positive regulatory function on gene expression through posttranscriptional stabilization (Ford, *et al.*, 1999; Jain, *et al.*, 1997) and possible enhanced translation (Antic, *et al.*, 1999). Fragile X mental retardation protein (FMRP) is a RNA-binding protein that may suppress translation of its mRNA targets as well as act as a switch to stimulate protein synthesis in response to extracellular signals (Todd, *et al.*, 2003; Weiler, *et al.*, 2004). In *Drosophila*, Staufen is thought to promote translation of properly localized mRNAs and to repress translation of target mRNAs that are mislocalized (Dubnau, *et al.*, 2003). Zipcode-binding protein 1 (ZBP1) binds to 54-nucleotide'zipcode' in 3'-UTR of β-actin mRNA and playas role in both transport to dendrites and translational repression (Huttelmaier, *et al.*, 2005).

2.4.4. RNA-BINDING PROTEINS IN GLOBAL GENE EXPRESSION REGULATION

Emerging evidences from a recent study found 380 putative RBPs in the mouse genome, 221 of which were detected in brain, and 16 of which showed neuronal-specific expression (McKee, *et al.*, 2005). It is not likely that every mRNA transcript has its own unique binding protein because tens of thousands of cell proteins would have to be dedicated to controlling posttranscriptional gene expression and it is possible that

structurally/functionally related mRNA subsets may interact with common RBP. RNA-binding proteins that bind to nearly all mRNAs in a global scale, does so, in a sequence independent manner (Keene, 2001). Global RBPs localized both in the nucleus and cytoplasm can ensure sequestration of large subset of messages away from ribosomal assembly located within the cell body and keep in a translationally dormant state until destined for translation.

Other than FMRP, Staufen and ELAV, other global RBPs that mediate translational repression/enhance include SLBP, TIAR and Csx1. SLBP protein can identify and bind to 16-nucleotide stem-loops in the 3'-UTR of histone mRNAs and coordinately regulate them by translational repression (Sanchez and Marzluff, 2002; Whitfield, *et al.*, 2004). TIAR is a RBP that that inhibits protein synthesis transiently in response to short wavelength UV irradiation. It binds to 3'-UTR of mRNAs encoding translation factors such as eIF4A, eIF4B, eIF4E and c-Myc and potentially suppress their translation (Mazan-Mamczarz, *et al.*, 2006). Csx1 is a global RBP that brings about translational repression of several transcripts in response to oxidative stress (Rodriguez-Gabriel, *et al.*, 2003). In all these cases, the translational repression resulting from the interaction of global RBPs with its target mRNAs is activated by way of cellular stimuli.

Similarly, differentially expressed transcripts during seizure may partake in post-transcriptional regulation, by forming RNP complexes in the brain. We hypothesize the role of Jerky, a RNA-binding protein implicated as a candidate gene for epilepsy, in

forming RNP complex with most RNAs and possibly repress the translation of its target mRNAs.

2.5. JERKY

2.5.1. JERKY GENE INACTIVATION CAUSED SEIZURE

Phenotypically, mice in which the jerky gene was inactivated led to recurrent limbic seizure with no detectable brain abnormalities or neurological symptoms other than seizures (Toth, *et al.*, 1995). Mice with transgenic insertion of SV40 Tag at the jerky loci were created by Toth et al (Toth, *et al.*, 1995). Progenies of the transgenic mice showed handling-induced seizure. Both hemizygotes and homozygotes displayed seizures, indicating that the mutation is dominant. Seizure behavior was reminiscent of idiopathic generalized epilepsies in human, with sequential seizure behavior of myoclonic seizure (characterized by head rearing combined with single jerks of the body), clonic seizures (characterized by rapidly repetitive jerks involving the whole body and falling) and tonic seizure (slow hind limb extension). EEG recordings from the neocortex and hippocampus indicated large amplitude interictal-like spikes in the dentate gyrus and spike-and-wave patterns in the neocortex, which was considerably higher than in nontransgenic animals. cfos immunoreactivity was observed in the granular cells of dentate gyrus, CA pyramidal cells and amygdala after 2 h of handling-induced seizure in jerky hemizygotes, indicating neuronal activation after seizure. The only apparent consequence of jerky deficit in jerky hemizygotes is hyperexcitability and lower threshold for induced-seizure (Donovan, *et al.*, 1997).

2.5.2. NUCLEIC ACID-BINDING BY JERKY

Jerky protein displays homology to a number of nuclear regulatory proteins including centromere binding protein B (CENP-B), POGO-R11, RAG3 and PDC2, suggesting jerky might have DNA-binding property (Toth, *et al.*, 1995). The N-terminal 168 residues of Jerky were deemed necessary and sufficient for its DNA as well as RNA binding property (Liu, *et al.*, 2003). Structural homology and sequence similarity of Jerky and CENP-B at N-terminal revealed that Jerky might have two Helix-turn-helix (HTH) motifs at its N-terminus. The HTH motif, contains three helical regions that are folded into a compact globular structure, with the helices 1 and 2 lying parallel to each other and across the third helix (Banerjee-Basu and Baxevanis, 2001). Jerky bound to fragmented genomic DNA as well as mRNA, but the interaction with DNA was less robust than with mRNAs. On sucrose gradient centrifugation of cerbral cortiex extract, Jerky was found to cosediment with 80S monosomes, indicating that jerky comigrates with translationally inactive mRNP complex (Liu, *et al.*, 2002). Jerky bound with synthetic RNA homopolymers, and mRNAs directly with physiologically relevant binding affinity. A ribonomic estimation of the subset of mRNAs that binds with Jerky was determined by microarray analysis of Jerky-selected brain mRNAs. Affymetrix MGU74Av2 oligonucleotide microarray probed with input mRNAs revealed a total of 1603 genes (12.85%) hybridized with jerky-selected mRNAs (Liu, *et al.*, 2003). The target gene transcripts belonged to several functional clusters (**Table 1**).

Rank	Target gene product	Biological function
1	Prostaglandin D2 synthase	
4	Fas death domain-associtaed protein	Cellular stress-related,apoptosis-associated protein
9	Ribosomal protein, large, P1	Ribosomal protein
10	Centrin 3	
11	Aldehyde dehydrogenase 2	
12	α_1- tubulin	Cytoskeletal protein
13	S100 calcium binding protein A13	
15	Cytochrome c oxidase, subunit VIIc	Cellular stress-related,apoptosis-associated protein
17	GAPDH	
19	Ribosomal protein L27a	Ribosomal protein
21	Ribosomal protein L44	Ribosomal protein
23	NADH dehydrogenase	
24	Ubiquitin B	
25	α_2- tubulin	Cytoskeletal protein
26	Ribosomal protein S11	Ribosomal protein
28	α_6- tubulin	Cytoskeletal protein
30	Ribosomal protein S28	
31	Creatine kinase, brain	
33	SOD-1, soluble	Cellular stress-related,apoptosis-associated protein
36	ATP synthase, H^+ transporting	
38	Metallothionein-1 activator	
40	Ribosomal protein L26	Ribosomal protein
42	Purkinje cell protein 4	
43	Ribosomal protein L7	Ribosomal protein
45	Parvalbumin	
47	Split hand/foot deleted gene 1	
51	Ribosomal protein L13	Ribosomal protein
53	Retinol binding protein 1, cellular	
54	H3 histone, family 3A	
55	Mago-nashi homolog	
57	ATP synthase, H^+ transporting	
58	Ribosomal protein L30	Ribosomal protein
60	Succinate coenzyme A ligase	
65	Calmodulin 2	
66	Peptidyly prolyl isomerase A	
67	Cytochrome c oxidase, subunit Vb	Cellular stress-related,apoptosis-associated protein
68	Fatty acid binding protein 5	
70	Spermidine/spermine transferase	
73	Retinoblastome-binding protein 9	
75	Ribosomal protein S12	Ribosomal protein
78	H3 histone, family 2	

Table 1: *In vitro* mRNA targets of Jerky on affymetrix array (adapted from Liu, *et al.*, 2003)). Jerky binds to large subset of mRNA targets with varied functional roles. (contd-)

79	Apolipoprotein E	
82	β-actin, cytoplasmic	Cytoskeletal protein
85	Mitochondrial ribosomal protein L3	Ribosomal protein
86	Ribosomal protein S19	Ribosomal protein
88	Synapsin 1	
89	Actin-related protein 2/3 complex	Cytoskeletal protein
91	Lysophospholipase 1	
92	Cytochrome c oxidase, subunit XVII	Cellular stress-related,apoptosis-associated protein
95	Metallothionein 1	
97	Cytochrome c oxidase, subunit Via	Cellular stress-related,apoptosis-associated protein
99	Cytochrome c somatic	Cellular stress-related,apoptosis-associated protein
100	Melanocortin 5 receptor	
102	Dynein, cytoplasmic, light chain 1	Cytoskeletal protein
107	Metallothionein 3	
109	Periplakin	
110	Mitochondrial ribosomal protein L13	Ribosomal protein
113	Ribosomal protein S7	Ribosomal protein
114	Procollagen, type XI, alpha 2	

Table 1: *In vitro* mRNA targets of Jerky on affymetrix array (adapted from Liu, *et al.*, 2003)). Jerky binds to large subset of mRNA targets with varied functional roles.

2.5.3. LOCALIZATION OF JERKY

Jerky mRNA was found to be ubiquitously expressed throughout the central nervous system including the frontal cortex, brain stem, hippocampus, thalamus and olfactory bulb. Jerky transcript was also observed in all adult peripheral tissues, with maximum expression seen in the testis (Donovan, *et al.*, 1997). However, western blot revealed a brain-specific expression of jerky protein in both nuclear and cytoplasmic fraction. The 62 kDa jerky protein is prominent in the cytoplasmic fraction than in the nuclear fraction (Liu, *et al.*, 2002). Jerky was also immunolocalized to neuronal cells in a rat hippocampal culture.

2.5.4. HUMAN JERKY GENE AND EPILEPSY

The human homologue of jerky, JRK/JH8, was mapped to 8q24 chromosomal loci and evinced keen interest by virtue of the loci linked to idiopathic epilepsies including childhood absence epilepsy (Morita, *et al.*, 1998). Mutational analyses within the coding region of JRK/JH8 in two CAE families identified seven nucleotide changes, two of which lead to amino acid substitutions. However, these changes did not cosegregate with the disease phenotype (Morita, *et al.*, 1999). A *de novo* nonconservative mutation to a potential glycosylation site in the human homologue of jerky (JRK/JH8) in an epileptic patient was reported; however a pathogenic role for this mutation in seizures has not been established (Moore, *et al.*, 2001).

2.5.5. HUMAN JERKY GENE AND SINGLE NUCLEOTIDE POLYMORPHISM

It is posited that most complex epilepsies are influenced by the effect of variation at several or multiple genes, in the form of common single nucleotide polymorphisms (SNPs), wherein each gene contributes a small or modest effect to the epilepsy phenotype, and by itself is insufficient to cause epilepsy. The updated human Jerky sequence submitted in public database (NM_003724.2) has 36 single nucleotide polymorphisms (SNP) within its gene (**Table 2**) (www.ncbi.nlm.nih.gov/SNP/snp_ref .cgi?locusId=8629&chooseRs=all). Inspite of the high density of variations observed within the human jerky gene the variations have not been associated with any seizure disorder. The collated polymorphisms in the 5' and 3'- UTR as well as coding regions of

JRK gene in the epileptic patients needs to be systematically evaluated of their potential functional consequences.

Region	Contig position	mRNA position	dbSNP clusterID	Function	dbSNP allele	Protein residue	Codon position	Aminoacid position
3' near gene	56888111		rs4736363	3' near gene	A/C			
	56888112		rs4736364	3' near gene	C/T			
	56888196		rs4736365	3' near gene	A/G			
	56888412		rs4736366	3' near gene	C/T			
	56888434		rs12675227	3' near gene	C/T			
	56888476		rs4736367	3' near gene	A/G			
	56888682		rs3735994	3' near gene	C/T			
	56889485		rs3808497	3' near gene	C/G			
	56889500		rs2976401	3' near gene	C/G			
	56889610		rs2976400	3' near gene	C/T			
	56889652		rs3824208	3' near gene	C/G			
	56889881		rs3839865	3' near gene	-/C			
3' UTR	56890331	3596	rs11781073	3' UTR	G/T			
	56890364	3563	rs2978971	3' UTR	A/G			
	56890436	3491	rs11781103	3' UTR	C/G			
	56890705	3222	rs3735995	3' UTR	A/G			
	56890876	3051	rs750529	3' UTR	C/G			
	56890930	2997	rs750530	3' UTR	A/G			
ORF	56891920	2007	rs2978972	synonymous	T	Cys	3	519
				contig reference	C	Cys	3	519
	56891967	1960	rs35283248	missense	A	Ser	1	504
				contig reference	G	Gly	1	504
	56891980	1947	rs35419434	synonymous	T	Cys	3	499
				contig reference	C	Cys	3	499
	56892049	1878	rs2976399	synonymous	A	Ala	3	476
				contig reference	G	Ala	3	476
	56892051	1876	rs17846351	missense	A	Thr	1	476
				contig reference	G	Ala	1	476
	56892068	1859	rs3735999	missense	G	Arg	2	470
				contig reference	A	Gln	2	470
	56892093	1834	rs10591060	synonymous	-		1	462
				contig reference	TG		1	462
	56892364	1563	rs3802234	synonymous	T	Asn	3	371
				contig reference	C	Asn	3	371
	56892415	1512	rs754957	synonymous	C	His	3	354
				contig reference	T	His	3	354
	56892700	1227	rs3802232	synonymous	C	Asn	3	259
				contig reference	T	Asn	3	259
	56892755	1172	rs35436748	missense	T	Leu	2	241
				contig reference	C	Pro	2	241
	56893096	831	rs34800931	synonymous	A	Glu	3	127
				contig reference	G	Glu	3	127
	56893270	657	rs2978973	synonymous	C	Ser	3	69

				contig reference	G	Ser	3	69
	56893388	539	rs34288113	missense	T	Met	2	30
				contig reference	C	Thr	2	30
5' UTR	56893751	176	rs6997771	5' UTR	G/T			
	56894079		rs13255558	5' near gene	C/T			
5' near gene	56894214		rs11780221	5' near gene	A/T			
	56894354		rs11785012	5' near gene	A/G			

Table 2: Single Nucleotide Polymorphisms within the Jerky gene listed in the SNP data bank

Chapter 3

MATERIALS AND METHODS

3.1. COMPETENT CELL PREPARATION FOR XL1 BLUE *E.COLI* STRAIN

<u>XL1 blue *Escherechia coli* strain</u>

Tetracycline resistant, blue-white selection, high competency *E.coli* strain.

F' [lacIq lacZΔM15 proAB+ Tn10(Tetr)] endA1 supE44 gyrA96 hsdR17 lac recA1 relA1 thi (Genes listed signify mutant alleles)

<u>Tetracycline</u>

Stock solution - 5 mg/ml in ethanol

Working concentration - 50 µg/ml in LB media

All steps were carried out at 4°C unless otherwise specified. Single colony of XL1 blue was inoculated in 5.0 ml of Lurea Bertani (LB) media containing 50 µg/ml tetracycline and cultured overnight at 37°C. 2.0 ml of overnight culture was inoculated in 100 ml LB media and cultured at 37°C until OD_{600} reached 0.3. The bacterial culture was placed in ice for 10 min and spun down at 1100 g for 10 min at 4°C. The supernatant was drained out. The pellet was gently resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$. The resuspended cells are centrifuged at 1100 g for 10 min at 4°C and the supernatant was decanted. The pellet was resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$. 140 µl of DMSO

was added and mixed by swirling, before keeping in ice for 15 min. Once more, 140 μl DMSO was added to the suspension and kept in ice for 10 min. From the final resuspension, 200 μl was aliquotted in pre-chilled microfuge tubes and stored at -80°C.

3.2. TRANSFORMATION OF $CaCl_2$-COMPETENT CELLS

X-gal (5-bromo-4-chloro-3-indolyl-β -D-galactoside)

Stock solution - 50 mg/ml in dimethylformamide

Working concentration - 167 μl of stock solution for 100 ml of media

IPTG (isopropylthio-β-D-galactoside)

Stock solution - 1.0 M (2.38 g in 10 ml of distilled water)

Working concentration - 33.3 μl of stock solution for 100 ml of media

The frozen competent cells were transferred in 50 μl aliquottes to sterile pre-chilled microfuge tubes and kept in ice. ~50 ng of DNA in 1 to 2 μl volume was added to the competent cells and mixed by gentle tapping and stored on ice for 20 min. The tubes were transferred to 42°C for 90 s and rapidly chilled in ice for 2 min. 950 μl of LB media was added to the transformed cells and incubated at 37°C for 1.5 h in rotary shaker. 100 μl to 200 μl of the transformation mix was plated on LB agar containing 50 μg/ml of ampicillin. For blue-white selection of clones with insert, the media was added with appropriate amounts of X-gal and IPTG.

3.3. XL1 BLUE ELECTROCOMPETENT CELL PREPARATION

10% Glycerol

50 ml of glycerol was mixed with 450 ml of deionised water and sterilized by autoclaving at 10psi for 10 min; stored at 4°C.

All steps were carried out in ice except otherwise specified. Single colony of XL1 blue was inoculated in 5.0 ml of lurea bertani (LB) media containing 50 µg/ml tetracycline and cultured overnight at 37°C. 2.5 ml of overnight culture was inoculated in 250 ml LB media and cultured at 37°C until spectrophotometric OD_{600} reached 0.5 - 0.7. The bacterial culture was placed in ice for 20 min and spun down at 4000 g for 15 min at 4°C. The supernatant was completely drained out. The pellet was gently resuspended in 250 ml of ice-cold 10% glycerol using cut-tips. The resuspended cells were centrifuged at 4000 g for 15 min at 4°C and the supernatant drained completely. The cells were resuspended in 125 ml of ice-cold 10% glycerol and centrifuged at 4000 g for 15 min at 4°C. After draining away the supernatant, the pellet was resuspended in 10 ml of ice-cold 10% glycerol and again spun at 4000 g for 15 min at 4°C. The resultant cell pellet obtained after complete draining the supernatant, was resuspended in 500 µl ice-cold 10% glycerol. From this final resuspension, 40 µl was aliquotted in pre-chilled microfuge tubes and stored at -80°C.

3.4. ELECTROPORATION OF XL1 BLUE ELECTROCOMPETENT CELLS

The elecetrocompetent cells were placed in ice for chilling alongwith the electroporation cuvette. The ligation mixture containing the clone to be transformed was diluted 10 times and 2 µl of the diluted ligation product was added to the competent cells for incubation in ice for 1 min. The competent cell mix was transferred to the bottom of the cuvette in between the electrodes. Pre-standardized pulse electric discharge was

applied on the cells using BioRad micropulser. Immediately after the pulse, the cells were added with 1 ml LB media and gently but rapidly mixed and transferred into a microfuge tube. The transformation mix was incubated at 37°C for 1 h in rotary shaker before plating on LB agar plates containing ampicillin, X-gal and IPTG.

3.5. PLASMID ISOLATION- ALKALINE LYSIS METHOD (MINIPREPARATION)

STE solution

10 mM Tris-Cl (pH 8.0)

0.1M NaCl

1mM EDTA (pH 8.0)

GTE solution

50 mM Glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Alkaline lysis solution II

0.2 N NaOH

1% SDS

Alkaline lysis solution III

3 M potassium acetate

11.5% glacial acetic acid

Tris- saturated Phenol

Phenol equilibrated in 0.1 M Tris-Cl (pH 8.0) until pH is >7.8; stored in dark bottles for periods up to 1 month.

Chloroform-Isoamyl alcohol

Chloroform and isoamyl alcohol mixed in 24:1 v/v.

Tris EDTA (TE) buffer

10 mM Tris-Cl (pH 7.4)
1 mM EDTA (pH 8.0)

Single bacterial colony was inoculated in 5.0 ml sterile Lurea Bertani media (LB media) containing ampicillin at a concentration of 50 µg/ml and grown overnight at 37°C with vigorous shaking in an environmental shaker. 1.5 ml of the overnight culture was pelleted down in a microfuge tube at 10,000 rpm for 30 s at 4°C. The supernatant was decanted completely. The pellet was resuspended in 375 µl STE buffer by vortexing. The resuspended cells were centrifuged at 10,000 rpm for 30 s at 4°C, and the supernatant was discarded. The bacterial pellet was resuspended by vigourous vortexing in 100 µl ice-cold GTE buffer. 200 µl of freshly prepared alkaline lysis solution-II was added to the bacterial resuspension and mixed by flicking the tube 5-10 times and placed in ice for 5 min. 150 µl of alkaline lysis solution-III was added and mixed by gently inverting the tube several times. The tube was placed in ice for 5 min before centrifuging at 10,000 rpm for 5 min at 4°C. The supernatant was pipetted into a fresh microfuge tube, added equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and vortex-mixed for 30 s. The emulsion was centrifuged at 10,000 rpm for 5 min at 4°C. The upper colorless aqueous phase was pipetted into a new microfuge tube, added equal volume of chloroform-isoamyl alcohol (24:1) and mixed by vigorous vortexing for 30 s. The aqueous phase was separated by centrifugation at 10,000 rpm for 5 min at 4°C and pipetted into a fresh tube. The plasmid-DNA was precipitated from the aqueous layer by adding equal volume of isopropanol and incubation at -80°C for 30 min. The DNA was

pelleted down at 10,000 rpm for 10 min at 4°C and the supernatant completely drained out. The pellet was washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 10 min at 4°C. The DNA pellet was air-dried for 15 min and resuspended in 50 μl TE buffer.

3.6. PLASMID ISOLATION- ALKALINE LYSIS METHOD (MAXIPREPARATION)

Single bacterial colony was inoculated in 50 ml sterile Lurea Bertani media (LB media) containing ampicillin (50 μg/ml) and grown overnight at 37°C with vigorous shaking in an environmental shaker. The cells were pelleted at 4100 rpm for 15 min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 20 ml ice-cold STE by vigorous vortexing. The cells were again pelleted down at 6000 rpm for 15 min at 4°C and supernatant discarded. The bacterial pellet was resuspended in 1.8 ml GTE solution by vortexing as well as swirling. 4 ml of freshly prepared alkaline lysis solution-II was added and mixed by gentle inversion and keep at room temperature for 10 min. 2 ml of alkaline solution-III was added and swirled several times and kept in ice for 10 min. The bacterial lysate was centrifuged at 11,000 rpm for 30 min at 4°C and the supernatant was filtered through 4-ply gauze and collected in a 50 ml centrifuge tube. DNA was precipitated by adding 0.6 V of isopropanol to the supernatant at room temperature for 10 min and centrifuging at 8000 rpm for 15 min at room temperature. 1 ml of 70% ethanol was added to the pellet and centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was dissolved in 300 μl TE buffer and transferred into microfuge tube.

The plasmid DNA was extracted with phenol following the small scale plasmid isolation procedure, The DNA pellet was air-dried for 15 min and resuspended in 250 µl TE buffer.

3.7. GENOMIC DNA ISOLATION FROM BLOOD

RBC Lysis buffer

10 mM Tris-Cl (pH 8.0)
0.1 M EDTA (pH 8.0)
0.5% SDS
20 µg/ml DNase-free RNaseA. (RNaseA is added to the buffer just before use)

Proteinase K

Stock solution - 14.8 mg/ml in sterile distilled water
Working concentration - 215 µg/ml or 7.2 µl of stock for 500 µl

Tris- saturated Phenol

Chloroform-Isoamyl alcohol

2 ml of venous blood sample was collected in EDTA-containing sterile vacutubes and stored at 4°C. 0.5 ml of blood was mixed with 1X PBS in equal volume and centrifuged at 3500 g for 15 min at 25°C. The supernatant was discarded. The thick pellet was resuspended in RBC-Lysis buffer and incubated at 37°C for 1 h. 7.2 µl of proteinase –K stock was added to the mixture and incubated at 55°C for overnight in rotary shaker. The lysis-mixture was cooled to room temperature and mixed with tris-saturated phenol in equal volume; invert-mixed several times to emulsify and incubated at 25°C for 40 min. The aqueous layer was separated from phenolic layer by centrifugation at 5000 g for

15 min and collected in a fresh tube. It was mixed with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and incubated at room temperature for 10 min. The solution was centrifuged at 5000 g for 15 min and collected the upper aqueous phase in fresh tube. It was homogeneously mixed with equal volume of chloroform-isoamyl alcohol (24:1) and kept at room temperature for 15 min. The solution was spun at 5000 g for 15 min and the upper aqueous layer was collected in a fresh tube. The genomic DNA was precipitated from the aqueous phase by adding equal volume of isopropanol and one-fifth volume of 10 M ammonium acetate and keeping at –80°C for 1 h. The DNA was pelleted at 13,000 g for 30 min. The pellet was washed with 500 µl of 70% ethanol and centrifuged at 13,000 g for 30 min. The DNA pellet was air-dried for 15 min and dissolved in 50 µl sterile distilled water.

3.8. AGAROSE GEL ELECTROPHORESIS

<u>1X TAE buffer</u>

40 mM Tris-acetate
1 mM EDTA

<u>6X gel loading buffer</u>

0.09% bromophenol blue
0.09% xylene cyanol FF
60% glycerol
60 mM EDTA

The casting tray and the combs are thoroughly cleaned and the casting tray sealed. Appropriate amounts of agarose was added to 1X TAE buffer to prepare 0.7% / 1% / 2%

gels. The agarose was melted in microwave and allowed to cool to approximately 60°C before spiking it with 2 µl of 10 mg/ml ethidium bromide. The molten agarose was poured carefully into the casting tray and allowed to cool. The comb was removed and the gel placed immersed in 1X TAE running buffer. DNA samples was added with 0.2 volume of 6X gel-loading buffer and loaded into the well. The electrophoresis carried out at 5-20 V/cm.

3.9. RNA ISOLATION FROM TISSUE BY GUANIDIUM THIOCYANATE-PHENOL EXTRACTION METHOD

Solution D

4 M Guanidium thiocyanate

25 mM sodium citrate

0.5% (w/v) sodium lauryl sarcosinate

0.1 M β-mercaptoethanol (add just before use)

10X DNase I buffer

100 mM Tris-Cl (pH 7.6)

25 mM $MgCl_2$

5 mM $CaCl_2$

2 M Sodium citrate (pH 4.0)

588.2 g of sodium citrate tribasic dihydrate was dissolved in 1000 ml of RNase-free deionised water; adjust pH and sterilized by autoclaving.

Water-saturated phenol

Chloroform-Isoamyl alcohol (49:1)

All experimental steps were carried out at 4°C unless otherwise mentioned. The microfuge tubes, pipette tips, reagents, solutions and water were ensured for RNase-free.

~100 mg of the brain tissue was homogenized in 1 ml of solution D using a tissue homogenizer. It was added with equal volume of water-saturated phenol and mixed by gentle inversion. 50 μl of 2 M sodium acetate was added to it and mixed by gentle inversion. 100 μl of chloroform-isoamyl alcohol (49:1) was finally added and mixed by gentle inversion. The tube was kept in ice for 15 min before centrifugation at 10,000 g for 20 min at 4°C and the aqueous phase was collected in a fresh tube. The RNA was precipitated by adding equal volume of isopropanol and keeping at –80°C for 45 min. The RNA was pelleted down at 10,000 g for 30 min at 4°C and RNA resuspended in 300 μl of solution D. The resuspended RNA was reprecipitated by adding one volume of isopropanol and keeping at –80°C for 45 min. The pellet was spun down at 10,000 g for 20 min at 4°C and washed with 500 μl of 75% ethanol. It was centrifuged at 10,000 g for 20 min at 4°C and the pellet air-dried for 15-20 min. The RNA was dissolved in 50 μl of RNase-free deionised water at 65°C for 10 min and stored at –80°C until experimentation.

DNase treatment of RNA: 50 μl of RNA was added with 2 U of DNase I in presence of 5.6 μl of 10X DNase I buffer and incubated at 37°C for 20 min. The reaction was extracted with one volume of phenol-chloroform-isoamyl alcohol, followed by one volume chloroform-isoamyl alcohol. The RNA was precipitated with one volume of isopropanol and 75% ethanol washed. Finally, RNA was resuspended in 50 μl of deionised water.

3.10. RNA ISOLATION FROM TISSUE USING TRI REAGENT METHOD

All experimental steps were carried out at 4°C unless otherwise mentioned. The microfuge tubes, pipette tips, reagents, solutions and water were ensured for RNase-free. 50-100 mg of brain tissue was homogenized in 1 ml of TRI reagent and spun down at 12,000 g for 10 min at 4°C to remove insoluble materials. The supernatant was collected in fresh tube and kept at room temperature for 5 min. 200 μl of chloroform was added to the homogenate and vigorously mixed for 15 s before centrifuging at 12,000 g for 15 min at 4°C. The upper colorless aqueous phase was added with 0.1 volume of isopropanol and incubated at room temperature for 5 min. The supernatant was collected in a fresh tube after centrifuging at 12,000 g for 10 min at 4°C. The RNA was precipitated by adding 440 μl of isopropanol and incubating at room temperature for 10 min. The RNA was pelleted at 12,000 g for 10 min at 4°C; washed in 75% ethanol and centrifuged at 12,000 g for 5 min at 4°C. The pellet was air-dried for 10 min and dissolved in 25 μl sterile deionised water at 60°C for 10 min. The RNA was stored at -80°C.

3.11. RNA ELECTROPHORESIS ON FORMALDEHYDE-AGAROSE GEL

10X MOPS buffer

0.2 M MOPS [3-(N-morpholino)-propanesulfonic acid] (pH 7.0)
20 mM sodium acetate
10 mM EDTA (pH 8.0)
Sterilize by autoclaving and store in dark bottle.

Formaldehyde gel loading buffer

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

50% (v/v) glycerol

1 mM EDTA (pH 8.0)

Formaldehyde (37%, pH >4.0, 12.3 M stock)

Ethidium bromide (200 μg/ml)

Casting the RNA Gel: The electrophoresis apparatus and glasswares used for electrophoresis were thoroughly cleaned, treated with 3% hydrogen peroxide and rinsed in RNase-free deionised water. For casting 1% formaldehyde-agarose gel, 1.0 g of agarose was dissolved in 72.25 ml of deionised water and allowed to cool to 60°C. 10 ml of 10X MOPS buffer as well as 17.8 ml of formaldehyde was added and mixed by swirling alongwith the melted agarose. It was poured and allowed to set in the casting tray with the comb assembled. The gel was placed in horizontal gel electrophoresis apparatus with 1X MOPS electrophoresis buffer.

Preparing the RNA sample for gel run: Adjusted the volume of each RNA sample to 4.5 μl with RNase-free deionised water. Then added with 2 μl of 10X MOPS buffer, 3.5 μl of formaldehyde and 10 μl of formamide. The RNA was incubated at 65°C for 10 min and chilled on ice for 2 min. The RNA sample was added with 2 μl of formaldehyde gel loading buffer and 1 μl of ethidium bromide. The RNA is loaded on the formaldehyde-agarose gel and run at 5 V/cm and viewed on UV-transilluminator.

3.12. SDS-PAGE FOR PROTEIN ELECTROPHORESIS

Resolving Gel (12%, 10 ml)

Water	-	3.3 ml
30:0.8% acrylamide-bisacrylamide	-	4.0 ml
1.5M Tris (pH 8.8)	-	2.5 ml
10% SDS	-	0.1 ml
10% APS	-	0.1 ml
TEMED	-	0.004 ml

Stacking Gel (5%, 2 ml)

Water	-	1.4 ml
30:0.8% acrylamide-bisacrylamide	-	0.33 ml
1.0M Tris (pH 6.8)	-	0.25 ml
10% SDS	-	0.02 ml
10% APS	-	0.02 ml
TEMED	-	0.002 ml

3X Gel loading dye

150 mM Tris-Cl (pH 6.8)

300 mM DTT

6% SDS

0.3% Bromophenol blue

30% glycerol

Staining Solution

50% Methanol

10% Glacial acetic acid

0.25% Coomassie brialliant blue R250

Destaining Solution

50% Methanol

10% Glacial acetic acid

The glass plates were assembled and sealed with 0.8 mm spacers on its sides and bottom. The resolving gel was first poured in between the glass plates and overlaid with butanol, until the gel was completely polymerized. The stacking gel was poured over the polymerized resolving gel and the comb placed in between the glass plates, such that the bottom of the well was atleast 1-2 cm above the resolving gel. After the gel was completely polymerized, the comb was removed and the cleaned well. The glass plate was assembled vertically and 1X tris-glycine running buffer was filled in the top- and bottom-tank. Protein samples were added with 0.5 volume of 3X protein loading dye and denatured at 100°C for 10 min; placed in ice for 2 min and loaded into the well. Current was applied at 8V/cm until the dye front entered the resolving gel. Further electrophoresis was carried out at 15 V/cm till dye front reached the bottom of the gel. The gel was stained in staining solution and destained using destaining solution to view the protein bands.

3.13. SILVER STAINING OF PROTEIN ON SDS-PAGE

Fixing solution-1

45% Methanol

12% glacial acetic acid

Fixing solution-2

10% Ethanol

5% glacial acetic acid

Potassium chromate solution

Stock solution - 0.3 M (882 mg in 10 ml water)

Working concentration – 1 ml of stock solution made up to 100 ml with water; 20 μl of nitric acid is also added to working solution.

Impregnation solution

204 mg of silver nitrate (final concentration -1.2 M) in 100 ml of water.

Developer solution

11.87 g of sodium carbonate and 0.2 ml of formaldehyde (37%, 12.3 M stock), made up to 400 ml with water.

Stopper solution

1 % glacial acetic acid (100 ml)

Farmer's Reducer

Sol-1 - 37 mg of potassium ferricyanide in 5 ml of water

Sol-2 - 2.4 g of sodium thiosulphate in 10 ml of water

Mix 1 ml of *sol-1* and 4 ml of *sol-2* and make up to 100 ml with water.

Gloves were used while handling of all solutions. The gel was kept in 100 ml of fixing solution-1 for 30 min with gentle agitation. The gel was transferred into 100 ml of fixing solution-2 for 10 min and replaced thrice. It was transferred into 100 ml of potassium chromate solution for 2-5 min with gentle rocking. The gel was washed with 100 ml water four times for 30 s each. It was kept in impregnation solution for 30 min with gentle rocking. The gel was briefly rinsed twice in 100 ml of developer solution before allowing the bands to develop in 200 ml of developer solution. The staining was terminated by replacing the gel in 200 ml stopper solution. Optionally, unwanted background on the gel was minimized by treating with freshly prepared farmer's reducer followed by thorough washing with water ten times.

3.14. SILVER STAINING OF DNA ON POLYACRYLAMIDE GEL

Fixing Solution

50 ml of glacial acetic acid made up to 500 ml with water

Impregnation Solution

1 g of silver nitrate is added to 1000 ml of water. 1.5 ml of formaldehyde (37%, 12.3 M stock) was added to the impregnation solution just before using.

Developer Solution

30 g of sodium carbonate was dissolved in 1000 ml of water. 2 mg of sodium thiosulphate and 1.5 ml of formaldehyde was added immediate before use. Cooling the solution to 10°C before use helped in reducing background.

Stopper solution

50 ml of glacial acetic acid made up to 500 ml with water

Farmer's Reducer

187 mg of potassium ferricyanide and 2.4 g of sodium thiosulphate was dissolved in 250 ml water. Solution prepared just before use.

Gloves were used while handling of all solutions. The polyacrylamide gel on which the DNA was resolved was fixed in 10% glacial acetic acid for 15 min twice followed by washing in water thrice for 2 min each. The gel was impregnated with silver nitrate solution for 20 min twice before rinsing the gel in water for 5-10 s. The developer solution was poured on to the gel and gently rocked until dark brown bands of the DNA appeared. The developer solution was changed over twice during this step. The action of developer was terminated by adding the stopper solution and keeping for 5 min. The gel was thoroughly washed with water 2-3 times with water to remove traces of any solutions. The gel was then dried on Whatman filter paper-1. Optionally, unwanted

background on the gel was removed by treating with Farmer's reducer for 45 s followed by thorough washing with water for ten times.

3.15. SEIZURE INDUCTION IN RAT AND MICE

Atropine

Stock concentration - 0.65 mg/ml

Working concentration – 0.04 mg/kg bodyweight for rat and mice

Pilocarpine

Rat: 380 mg/kg diluted in 200 μl 0.9% saline

Mice: 200 mg/kg diluted in 100 μl 0.9% saline

Diazepam

Stock concentration - 5 mg/ml

Working concentration- 4 mg/kg bodyweight for rat and mice

Healthy male wistar rats of 180-200 g body weight were subcutaneously injected with Atropine at 0.04 mg/kg. 20 min after atropine administration, intraperitoneal pilocarpine injection was given at 380 mg/kg. Seizure behaviour was monitored for 4 h and 24 h after Pilocarpine-induction. 4 mg/kg diazepam was intraperitoneally injected after 1 h of seizure. Control animal was given atropine injection as well as saline instead of pilocarpine; sacrificed at 4 h. Animal was sacrificed by cervical dislocature and the hippocampus dissected out from the brain immediately after sacrifice.

Healthy male swiss albino mice of 30-35 g body weight were subcutaneously injected with atropine at 0.04 mg/kg. 20 min later, pilocarpine was injected intraperitoneally at 200 mg/kg. Diazepam was administered at 4 mg/kg intraperitoneally

after 30 min of seizure. Animal was sacrificed 2 h post-seizure and hippocampus dissected out immediately from the brain.

3.16. MICROARRAY ANALYSIS AND *PANTHER* GENE EXPRESSION ANALYSIS

mRNA used for the microarray was isolated from the hippocampal tissue of rat using Oligotex mRNA purification kit (Qiagen, Germany). Microarray was performed using the RT-IVT kit and Rat Genome Survey Microarray slide from Applied Biosystem Expression array system (Applied Biosystem, USA), containing targeted probes against 27088 genes (15,990 annotated in Celera database and 10,887 annotated in public database). Microarray hybridization was performed in duplex with 'control' against '4 h', 'control' against '24 h' and '4 h against 24 h'. Data analysis was carried out using 'R' (www.r-project.org) and Spotfire DecisionSite for Microarray Analysis (Spotfire Inc.). From the two independent experiments the average signal intensity of every gene probe ($p \leq 0.05$), was calculated for control, 4 h and 24 h. Those genes were listed which showed atleast two fold increase compared to the control. From the 4 h-upregulated genes, those which underwent 2-fold increase/decrease during 24 h were selected. The classification of the genes based on its biological function was carried out using PANTHER gene expression analysis online tool (www.pantherdb.org).

3.17. CLONING AND CONSTRUCTS

3.17.1 Cloning of Jerky

PCR amplification of the 1688 bp complete coding region of the mice jerky gene, representing 507 to 2177 base position of NM_008415, was carried out using gene

specific primers, JRK-F (5'-GGAATTCCCATGGCCTCCAAGCAGGCTGCA-3') and JRK-R (5'-CGGAATTCGTTGTCACCTGCAGTGGAAGA-3'), in presence of 2 mM $MgCl_2$, 0.4 mM dNTPs and 2 U taq DNA polymerases. PCR amplification conditions used were 94°C for 10 min initial denaturation followed by 30 cycles of 94°C for 1 min, 68°C for 1 min, 72°C for 2 min; final extension was at 72°C for 20 min.

The amplified Jerky gene was cloned in pTZ57R/T vector (pTZ-Jerky) and confirmed by restriction digestion using EcoR1, Pst1, Sma1, Apa1 and Bgl1. For sequencing the cloned insert, the pTZ-Jerky clones in direct as well as in reverse orientation, were digested with Sma1 and the self-ligated so as to have clones, having 913 bp of 5'-end of Jerky ORF in pTZ-5Jerky clone and 775 bp of 3'-end of Jerky ORF in pTZ-3Jerky. The clones were sequenced using both M13 and T7 sequencing primers.

For cloning Jerky into pGEX-4T-1 expression vector, the 4969 bp vector was linearised with Sma1. To avoid self-ligation, it was dephosphorylated by CIP treatment at 37°C for 15 min before terminating the reaction at 55°C for 45 min and addition of 80 mM EDTA and gel eluted. The Jerky DNA was eluted from gel after releasing the fragment by EcoR1 digestion of pTZ-Jerky. The 1679 bp Jerky insert was klenow endfilled at 30°C for 15 min and terminated at 75°C for 10 min and gel eluted. The enfilled Jerky DNA was ligated into Sma1 site of pGEX vector and electroporated in XL1 blue cells (pGEX-Jerky). The clones were confirmed for insert and its orientation by digestion with EcoR1, Pst1, Bgl1 as well as double digestion with BamH1-Xho1 and Sma1-EcoR1.

3.17.2. Cloning of Jerky-pulldown genes

From the differential display polyacrylamide gel, selected bands from the pulldown lane were cut and the DNA eluted out. Using the eluted DNA as template, PCR reamplification was carried out with corresponding differential display primers. The amplified product was cloned into pTZ57R/T vector and sequenced.

3.17.3. RNA constructs of pulldown genes

The transcribed RNA of all the pulldown genes was used for RNA-protein interaction experiments. Among the pulldown genes cloned in pTZ57R/T vector, the 94 bp Nedd4 DNA was cloned (pTZ-Nedd4) in the reverse orientation (HindIII→EcoR1) and was used for in vitro transcription using T7 RNA polymerase after EcoR1-linearisation. The ND4 (152 bp) and ATP6 (157 bp) cloned in direct orientation (EcoR1→HindIII) in pTZ57R/T (pTZ-ND4 and pTZ-ATP6) were released by Sal1-EcoR1 double digestion and cloned in pGEM-T Easy vector (3015 bp) at Sal1-EcoR1 site (pGEMT-ND4 and pGEMT-ATP6), so as to transcribe with T7 RNA polymerase.

3.17.4. Cloning of Synaptotagmin 1, 4 and 10

Synaptotagmin 1 (Syt 1) cDNA was prepared by reverse transcription of total RNA isolated from normal rat hippocampus using Syt1primer2 (5'-AAAGGCTTCGTTTTCCCTTTAC-3'). The RNA template and the primer were denatured at 70°C for 5 min and kept in ice for 1 min. The mixture containing 4 µl of 5X RT reaction buffer and 0.5 mM dNTPs components were meanwhile incubated at 37°C for 5 min and added to the template RNA. 100 U of M-MuLV reverse transcriptase enzyme was added to the reaction and kept at 42°C for 60 min and the reaction

inactivated at 70°C for 10 min. The cDNA was used for PCR amplification using primers, Syt1primer1 (5'-TGAACCAAAAATGGTGAGTGC-3') and Syt1primer2 in presence of 2 mM $MgCl_2$, 0.4 mM dNTPs and 2 U taq DNA polymerases. Following initial denaturation at 94°C for 10 min and 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min the final extension at 72°C for 15 min was carried out.

Synaptotagmin 4 (Syt 4) was reverse transcribed from 6 h- epileptic rat hippocampal total RNA using 1 µl of Syt4 reverse primer (5'-GCTAACCATCACAGAGCAT-3') in a final reaction of 20 µl containing 1X RT reaction buffer, 0.5 mM dNTPs and 100 U M-MuLV reverse transcriptase. The primer and the RNA template was denatured at 70°C for 1 min and kept in ice for 2 min. The other components including buffer, dNTP and enzyme were added to the denatured template and reverse transcribed at 37°C for 60 min and reaction inactivated at 70°C for 10 min. The cDNA thus obtained was used for PCR amplification using Syt 4 forward primer (5'-ACATGGCTCCTATCACCACC-3') and Syt 4 reverse primer in presence of 2 mM $MgCl_2$, 0.4 mM dNTPs and 2 U taq DNA polymerases. PCR amplification conditions used was 94°C for 10 min initial denaturation followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; final extension was at 72°C for 15 min.

Synaptotagmin 10 (Syt 10) was reverse transcribed from 24 h- epileptic hippocampal total RNA isolated using Syt 10 reverse primer (5'-TTATGGTGTGGACGGTGG-3'). The RNA template and the primer were denatured at 70°C for 5 min and kept in ice for 1 min. The mixture containing 4 µl of 5X RT reaction

buffer and 0.5 mM dNTPs components were meanwhile incubated at 37°C for 5 min and added to the template RNA. 100 U of M-MuLV reverse transcriptase enzyme was added to the reaction and kept at 42°C for 60 min and the reaction inactivated at 70°C for 10 min. The cDNA was used for PCR amplification using primers, Syt10 forward primer (5'-CAAGATGAGTTTCCGCAAGG-3') and Syt10 reverse primer in presence of 2 mM $MgCl_2$, 0.4 mM dNTPs and 2 U taq DNA polymerases. Following initial denaturation at 94°C for 10 min and 30 cycles of touchdown amplification at 94°C for 30 s, 65-50°C for 30 s, 72°C for 2 min and amplification with 10 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; final extension was at 72°C for 15 min.

The PCR products of Syt 1, Syt 4 and Syt 10 were cloned into pTZ57R/T vector in the direct orientation (pTZ-Syt 1, pTZ-Syt 4 and pTZ-Syt 10).

3.17.5. RNA constructs for Synaptotagmin 1, 4 and 10

Direct clones of pTZ-Syt 1, pTZ-Syt 4 and pTZ-Syt 10 were digested with EcoR1-Sal1 to release the cloned inserts. The fragments of Syt 1 (1320 bp), Syt 4 (1306 bp) and Syt 10 (1626 bp) were cloned into Sal1-EcoR1 site of pGEM-T Easy vector in T7 orientation. The clone was confirmed by EcoR1-Sal1 digestion to release their respective inserts. Clones pGEMT-Syt 1, pGEMT-Syt 4 and pGEMT-Syt 10 were Sal1-linearised and used for in vitro transcription. The EcoR-Sal1 fragment of Syt 10 was also cloned into pBS at their respective sites.

The pGEMT- Syt 1 clone was also used for *in vitro* translation using rabbit reticulocyte lysate.

3.17.6. Constructs for transcribing smaller RNAs of Syt 1 and Syt 10

Subregions within Syt 1 ORF were separately amplified and cloned. Following primers were used for the purpose:

Syt1primer2 : 5'-AAAGGCTTCGTTTTCCCTTTAC-3'

Syt1primer3 : 5'-TGGATGACGATGCTGAAACC-3'

Syt1primer4 : 5'-GGCCAAAATCCACGGTGTTC-3'

Syt1primer5 : 5'-ATCTCCAGAGCGCTGAGAAAG-3'

Syt $1^{356 \text{ to } 758}$ was amplified using Syt1primer3 and Syt1primer4. Syt $1^{781\text{-}1268}$ was amplified using Syt1primer5 and Syt1primer2. Syt $1^{356\text{-}1268}$ was amplified using Syt1primer3 and Syt1primer2. The amplified products were cloned into pTZ57R/T. Reverse clones of pTZ-Syt $1^{356\text{-}758}$ and pTZ-Syt $1^{356\text{-}1268}$ were Xba1-linearised and used for *in vitro* transcription using T7 RNA polymerase. The Syt$1^{781\text{-}1268}$ was released from the forward clone pTZ-Syt $1^{781\text{-}1268}$ by EcoR1-Sal1 digestion and subcloned into pGEM-T Easy vector at Sal1-EcoR1 site in T7 orientation. The clone was Sal1-linearised and used for in vitro transcription with T7 RNA polymerase.

Similarly, transcripts representing subregions of Syt 10 ORF were also synthesized. Syt $10^{1\text{-}1059}$ RNA were transcribed from EcoRV-linearised pGEMT-Syt 10. The Syt $10^{1060\text{-}1533}$ was obtained by EcoRV-partial digestion of BamH1-digested pBS-Syt 10 and cloning the 475 bp fragment in pTZ57R vector at BamH1-EcoRV site and subcloning into pGEM-T at Sal1-EcoR1 sites. The clone was Sal1-linearised for in vitro transcription using T7 RNA polymerase.

3.17.7. Construct for *in vitro* translation of Nedd4

pBS(SK+)-Nedd4 clone in the T3 orientation was originally gifted by Sharad Kumar's group. The clone was however confirmed by sequencing and by restriction digestions with EcoR1, EcoR1-Pst1, BamH1-HindIII and Sal1-Not1. The insert cloned into Sma1 site of the vector represents 200 to 2998 base positions of NM_10890 containing the complete Nedd4 ORF as well as 23 bases in 5'-UTR and 127 bases in 3'-UTR. To clone the Nedd4 insert into pBS(KS+), the pBS(SK+)-Nedd4 clone was digested with Sal1-Not1 and the 2863 bp fragment was ligated into Sal1-Not1 sites of pBS(KS+). The orientation of the gene was confirmed by EcoR1 and EcorV digestion

3.18. EXPRESSION OF GST-JERKY RECOMBINANT PROTEIN

Phosphate buffered saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

Sterilise by autoclaving at 15 psi for 20 min and store in 4C

Lysozyme

Stock solution - 10 mg/ml

Working concentration- 1 µg/µl of culture

DNase I

Stock solution - 10 µg/ml

Working concentration – 30 µl of stock solution in 1 ml bacterial laysate

Glutathione Elution buffer

50 mM Tris-Cl (pH 8.0)

10 mM glutathione

Micrococcal nuclease

Stock concentration: 15 U/µl in 20 mM HEPES-KOH, 50 mM NaCl, 50% Glycerol

Working concentration: 200 U/ml in presence of 1 mM $CaCl_2$

pGEX-Jerky BL21 single colony was inoculated in 5 ml LB media containing 50µg/ml ampicillin and grown for overnight at 37°C in environmental shaker. From the overnight culture, 1 ml was inoculated in 50 ml of LB ampicillin media and grown at 37°C with rotary shaking until OD_{600} reached 0.5. 1 ml of the bacterial culture was pipetted out as the 0 h culture. The bacterial culture was added with 1 mM IPTG and grown at 25°C for 3 h. 1 ml each of the bacterial culture was pipetted out at 1st h, 2nd h and 3rd h. The culture was placed on ice for 20 min and pelleted down at 12,000 rpm for 3 min at 4°C. The pellet was washed twice in 10 ml of ice-cold PBS and pelleted at 12,000 rpm for 3 min at 4°C. The bacterial pellet was resuspended in 1 ml of ice-cold PBS and added with 50 µl of protease inhibitor cocktail, 100 µl of lysozyme to final concentration of 1 mg/ml and kept in ice for 30 min. The cells were lysed by ten cycles of freeze-thaw lysis in liquid nitrogen for 20 s and 37°C water bath for 1 min. The lysate was centrifuged at maximum speed for 20 min at 4°C and the clear supernatant is carefully transferred into fresh tube. Optionally, the lysate was added with 30 ng of DNaseI to reduce the viscosity. The GST-Jerky protein is purified from the soluble lysate fraction by affinity chromatography.

3.18.1. Purification of GST-Jerky recombinant protein

Microspin GST purification column were used to purify the recombinant protein from the soluble lysate as prescribed in the manual. The column buffer was drained by spinning at 3000 rpm for 1 min. 600 μl bacterial soluble lysate was added to the column after capping the bottom of column, mixed by gentle inversion for 10 min and spun at 3000 rpm for 1 min after uncapping the bottom. The column was washed twice with 400 μl of PBS and centrifuged at 3000 rpm for 1 min. The GST-tagged protein was eluted by adding 100 μl glutathione elution buffer into the column and incubating for 10 min. The eluate was centrifuged out at 3000 rpm for 1 min. The elution step was repeated twice. The eluted protein is added with 50 μl of protease inhibitor cocktail and stored at 4°C.

3.18.2. Micrococcal nuclease treatment of purified protein

Microspin-column purified protein was treated with 200 U/ml of micrococcal nuclease in presence of 10 mM Tris-Cl (pH 8.8) and 1mM $CaCl_2$ at 37°C for 30 min. The reaction was terminated by adding 5 mM EDTA (pH 7.0).

3.19. RNA-PULLDOWN ASSAY

Protein-binding buffer (PB buffer)

15 mM sodium phosphate

0.15 M sodium chloride (pH 7.4)

RNA-binding buffer (RB buffer)

25mM Tris-Cl (pH 7.5)

200mM KCl

1mM DTT

0.05% Tween20

1mM $MgCl_2$

1mM EGTA

40μg/ml BSA

***Activation of Glutathione CL-Agarose beads*:** Activation of beads was carried out by pelleting 50 μl of the beads at 5000 g for 3 min at 4°C. The beads were resuspended in 50 μl of protein-binding buffer (PB buffer) and centrifuged at 5000 g for 3 min at 4°C. The step was repeated five times and finally the beads were resuspended in 50 μl of PB buffer.

Protein Anchoring on Beads: 5 μg of micrococcal nuclease-treated purified GST-Jerky protein in 50 μl was incubated with 50 μl of the activated glutathione beads in presence of 1% TritonX 100 at 4°C for 1 h. The bead-protein complex was washed with 250 μl of ice-cold PB buffer five times and centrifuged at 5000 g for 3 min at 4°C. The beads were finally resuspended in and mixed occasionally for 15 min at 4°C and centrifuged at 5000 g for 3 min at 4°C; this resuspension step was carried out twice and the complex finally resuspended in 400 μl of RNA-binding buffer (RB buffer) Activated beads without anchored protein were used as negative control.

***RNA pulldown using bead-protein complex*:** 5 μg of total RNA was added to the complex in 400 μl RNA-binding buffer (RB buffer) containing 40 μg/ml of yeast tRNA. The complex was mixed occasionally for 30 min at 4°C; washed five times with 800 μl prechilled RB buffer; and resuspended in 350 μl of RB buffer. The bead complex was extracted with equal volume of 50:49:1 water-saturated phenol:chloroform:isoamyl

alcohol followed by 49:1 chloroform:isoamyl alcohol extraction. The aqueous layer was added with 2 volume of ethanol and 20 μg glycogen and the RNA precipitated over night at -80°C. The RNA was pelleted down at maximum speed for 10 min at 4°C and dissolved in 10 μl water.

3.20. 5'-END-LABELING OF PRIMER

10.5 pmol of primer

T4 Polynucleotide kinase (10 U/μl)

10X PNK buffer A (for forward reaction)

500 mM Tris-Cl (pH 7.6)

100 mM $MgCl_2$

50 mM DTT

1 mM spermidine

1 mM EDTA

γ-P^{32}-ATP (specific activity: >3000 Ci/mmol)

Forward reaction catalyzes the transfer of the terminal (γ) phosphate of ATP to the 5' termini of the DNA/RNA and even oligonucleotides. 25 μl labeling reaction is set up with 10.5 pmol of the primer, 10 U of T4 polynucleotide kinase, 75 μCi of γ-P^{32}-ATP in presence of 1X PNK buffer A at 37°C for 30 min. The reaction is stopped at 95°C for 5 min.

3.21. LABELING OF PARTIAL cDNA FROM PULLDOWN RNA

M-MuLV Reverse transcriptase (200 U/μl)

Taq DNA polymerase (1 U/μl)

Primers

HT11G - 5'-AAGCTTTTTTTTTTTG-3'

HT11C - 5'-AAGCTTTTTTTTTTTC-3'

HT11A - 5'-AAGCTTTTTTTTTTTA-3'

5X RT reaction buffer

250 mM Tris-Cl (pH 8.3)

250 mM KCl

20 mM $MgCl_2$

50 mM DTT

RNA pulled down with jerky protein-bead complex and no-protein bead complex were reverse transcribed with 0.2 μl each of labeled polyT primers HT11A, HT11C and HT11G in a final reaction of 20 μl containing 1X RT reaction buffer, 0.5 mM dNTPs and 100 U M-MuLV reverse transcriptase. The primers and the RNA template were denatured at 70°C for 1 min and kept in ice for 2 min. The buffer, dNTP components and enzyme were added to the denatured template and reverse transcribed at 37°C for 60 min and reaction inactivated at 70°C for 10 min. 12 μl of labeled cDNA was resolved on 8% urea-polyacrylamide denaturing gel at 10 W.

3.22. DIFFERENTIAL DISPLAY-RT PCR

M-MuLV Reverse transcriptase (200 U/μl)

Taq DNA polymerase (1 U/μl)

Primers

HT11A - 5'-AAGCTTTTTTTTTTTA-3'

LMM-5 - 5'-AAGCTTATCGCCT-3'

MOB-5 - 5'-AAGCTTGATTGCC-3'

5X RT reaction buffer

250 mM Tris-Cl (pH 8.3)

250 mM KCl

20 mM $MgCl_2$

50 mM DTT

<u>10X PCR buffer</u>

100 mM Tris-Cl (pH 8.8)

500 mM KCl

0.8% Nonidet P40

<u>6X Gel loading buffer</u>

0.09% bromophenol blue

0.09% xylene cyanol

60% glycerol

60 mM EDTA

<u>6% PAGE</u> (30 ml)

38:2 acrylamide-bis Acrylaminde	- 4.5 ml
5X TBE	- 6.0 ml
10% APS	- 210 μl
TEMED	- 18 μl
Water	- make up to 30 ml

Equal amounts of pulldown RNA/ total RNAs were reverse transcribed with 1 μl of unlabeled polyT primer HT11A, in a final reaction of 20 μl containing 1X RT reaction buffer, 0.5 mM dNTPs and 100 U M-MuLV reverse transcriptase. The primer and the RNA template was denatured at 70°C for 1 min and kept in ice for 2 min. The other components including buffer, dNTP and enzyme were added to the denatured template and reverse transcribed at 37°C for 60 min and reaction inactivated at 70°C for 10 min. 0.5 μl of the cDNA thus generated, was PCR amplified with P^{32}-labeled polyT primer

HT11A, and arbitrary forward primers, MOB5 or LMM5, in presence of 0.1 mM dNTP, 2 mM $MgCl_2$ and 2 U Taq polymerase. PCR amplification conditions used was 94°C for 4 min initial denaturation followed by 40 cycles of 94°C for 30 s, 40°C for 2 min, 72°C for 30 s; final extension was at 72°C for 15 min. The labeled PCR products were made up to 1X of gel loading buffer prior to electrophoresis on Sequi-Gen GT electrophoresis apparatus (Biorad). The 0.2 mm thick and 45 cm long 6% nondenaturing PAGE gel was prerun at 40 W till temperature of the gel reached 45°C; the samples were run at constant 40 W and 45°C. The gel was vacuum-dried on Watman-1 filter paper and autoradiographically analyzed.

3.23. DNA ELUTION FROM POLYACRYLAMIDE GEL

2X PCR buffer

20 mM Tris-Cl (pH 8.8)
100 mM KCl
0.16% Nonidet P40

The band of interest was cut out from the dried gel and soaked in 20 μl of 2X PCR buffer on a microscope slide to peel and remove the paper debris from the gel. The gel was added with 30 μl of 2X PCR buffer and incubated at room temperature for 5 min. The buffer was completely removed by aspiration. The gel was placed in a microfuge tube and incubated at 94°C for 90 min in presence of 50 μl of 2X PCR buffer. The tube was spun at 13,000 rpm for 2 min and the supernatant collected for PCR re-amplification.

3.24. *IN VITRO* TRANSCRIPTION & LABELING OF RNA

5X Transcription buffer

200 mM Tris-Cl (pH 7.9)

30 mM $MgCl_2$

50 mM NaCl

10 mM Spermidine

<u>T7 RNA polymerase</u> (20 U/μl)

<u>RNase inhibitor</u> (40 U/μl)

<u>BSA</u> (20 mg/ml)

<u>α-P^{32} UTP</u> (specific activity- 3500 Ci/mmol)

0.2 pmol of linearized DNA template was used for *in vitro* transcription using 20 U of T7 RNA polymerase in a final volume of 20 μl containing 0.5 mM of rNTP mix, 2 mg of BSA, 1X transcription buffer, 40 U RNase inhibitor and 50 μCi of α-P^{32} UTP. The transcription was carried out at 37°C for 2 h. Final extension of the transcribed product was carried out by adding 2 μl of 0.5 mM UTP to the transcription reaction and incubating at 37°C for 1 h. The DNA template was degraded by adding 5 U of RNase-free DNase I in presence of 1X DNase buffer and incubating at 37°C for 30 min. RNA was purified by using Eppendoff Perfect RNA mini kit according to the manual, and finally eluted in 50 μl water. Alternatively, unlabeled *in vitro* transcribed RNA was purified by phenol-chloroform extraction and precipitated with 1 V ethanol and 2M ammonium acetate, before dissolving the air-dried pellet in 50 μl water.

3.25. ELECTROPHORETIC MOBILITY SHIFT ASSAY

<u>2X binding buffer</u>

40 mM Tris-Cl (pH 7.5)

100 mM KCl

10 mM $MgCl_2$

2 mM DTT

20% Glycerol

200 μg/ml BSA

6% PAGE

29:1 acrylamide-*bis* acrylamide	- 2.0 ml
10X TBE buffer	- 1.0 ml
10% APS	- 70 μl
TEMED	- 6 μl
Water	- make up to 10 ml

P^{32}-labeled *in vitro* transcribed RNA was briefly denatured at 95°C for 10 min and chilled on ice for 2 min, spun down and incubated with definite amounts of purified recombinant protein in 1X binding buffer at 37°C for 30 min. This reaction mixture was mixed with gel loading buffer and loaded on 6% PAGE. The gel was pre-run at 100 V for 1 h and the sample-run at 200 V (20 V/cm) till bromophenol blue dye reached ¾ of the gel. The signal was detected by autoradiography and analyzed by phosphorimager.

Competitive mobility shift assay: Constant amount of P^{32}-labeled *in vitro* transcribed RNA was competed with 50%, 100%, 200% and 400% of unlabeled cold transcripts for binding to purified protein. Hence, transcripts, both hot and cold, were heat-denatured and bound to purified protein in 1X binding buffer at 37°C for 30 min. The reaction was resolved on non-denaturing PAGE and analyzed by autoradiography.

3.26. SLOT BLOT FILTER BINDING ASSAY

2X binding buffer

40 mM Tris-Cl (pH 7.5)

100 mM KCl

10 mM $MgCl_2$

2 mM DTT

20% Glycerol

200 μg/ml BSA

P^{32}-labeled *in vitro* transcribed RNA was briefly denatured at 95°C for 10 min and chilled on ice for 2 min, spun down and incubated with serial dilutions of purified GST-Jerky in 1X binding buffer at 37°C for 30 min. The protein dilutions ranged from 100 ng (1.1 pmol) to 10 μg (110.05 pmol) of purified GST-Jerky as well as no-protein control. The binding reaction was added with 1X binding buffer to a final volume of 50 μl and then filtered through a sandwich of nitrocellulose membrane (Hybond C-Extra, Amersham BioSciences) on top and Nylon membrane (Hybond N+, Amersham BioSciences) on the bottom using a slot blot manifold (PR648, Amersham Biosciences) under constant vacu-pressure of 100 mBar. The manifold was washed sequentially with 1 ml followed by 100 μl of 1X binding buffer. The membrane was never allowed to dry until washing step was over. The apparatus was dismounted and the membranes dried in vacuum for 10 min without heating. The bound and unbound radioactivity on the nitrocellulose and nylon filters respectively, were measured by phosphorimager analyzer.

3.27. *IN VITRO* TRANSLATION ASSAY

TNT Quick coupled Transcription/Translation Rabbit reticulocyte lysate system

L-S^{35} Methionine (specific activity-1000 mCi/mmol)

1X Loading buffer

50 mM Tris (pH 6.8)

100 mM DTT

2% SDS

10% Glycerol

0.1% bromophenol blue

Fixing Solution

50% Methanol

10% glacial acetic acid

TNT Quick coupled Transcription/Translation rabbit reticulocyte lysate system was used for *in vitro* translation assay. Purified GST-Jerky protein was dialysed against 50 mM Tris (pH 8.0) and the plasmid DNA template was RNase-treated and gel-eluted for use in translation reaction. A 10 μl translation reaction was set up with 775 ng of the plasmid DNA, 6.5 μl RRL, 7.5 μCi L-S^{35} Methionine and 0.25 μl PCR enhancer in presence/ absence of 100 ng of protein as well as heat-denatured protein. The translation reaction was incubated at 30°C for 90 min and terminated by keeping in ice. 5 μl of the translated product was added with 20 μl of 1X loading buffer, denatured at 70°C for 10 min and 12.5 μl loaded on 12% SDS-PAGE. After electrophoresis, the gel was fixed in fixing solution for 30 min; dried and exposed on IP plate for phophorimager analysis.

3.28. RECRUITMENT OF HUMAN SUBJECTS FOR JERKY GENE PROFILING

Institutional ethics committee approval was obtained for recruitment of human subjects and collection of blood and tissue sample. Recruitment of patients was carried out at the Epilepsy Clinic of Sree Chitra Tirunal Institute with help of in-house clinicians. The patient was made aware of the aim and consequences of the intended study and an informed consent obtained from all voluntary participants. Blood samples collected were

immediately coded alphanumerically to mask the identity of the patient throughout the course of research. Twenty five unrelated patients with epilepsy were randomly recruited, including ten mTLE patients who underwent therapeutic surgery for drug-resistant mesial temporal lobe epilepsy, ten juvenile myoclonic epilepsy (JME) patients, two frontal lobe epilepsy (FLE), one patient each with non-temporal focal epilepsy (FE), Lennox Gastaut syndrome and primary generalized epilepsy. Eight non-epileptic case-controls with no known seizure-history in their family were included in the study.

3.29. AMPLIFICATION OF HUMAN JERKY GENE

Primers

Set1-F - 5'-TGTGCCTGGAGATACCATTGTG-3'

Set1-R - 5'-GCTGACTGCTTTTCACTGGATG-3'

Set2-F - 5'-TCCAGTGAAAAGCAGTCAGCCG-3'

Set2-R - 5'-AAAGCGCAGGACTGCGTCAAAG-3'

Set3-F - 5'-CTTTGACGCAGTCCTGCGCTT-3'

Set3-R - 5'-AGGGTCCGTGTCCTCTATCCG-3'

Set4-F - 5'-TAGAGGACACGGACCCTGTGTG-3'

Set4-R - 5'-ACAAATGGGAACTGGGACTGTCG-3'

10X PCR buffer

100 mM Tris-Cl (pH 8.8)

500 mM KCl

0.8% Nonidet P40

25 mM $MgCl_2$

10 mM dNTP

Entire human JRK gene of 3974 bp was amplified from gDNA using four sets of PCRs: first set PCR amplified the region 1- 920 using primers Set1-F and Set1-R; second

set PCR amplified the region 901- 1950 using primers Set2-F and Set2-R; third set PCR amplified the region 1929- 2930 using primers Set3-F and Set3-R; fourth set PCR amplified the region 2914- 3974 using primers Set4-F and Set4-R. PCR was carried out in presence of 0.4 mM dNTPs, 2mM $MgCl_2$ and 2U taq DNA polymerase. PCR amplification conditions used for the Set1, Set3 and Set4 was 94°C for 10 min initial denaturation followed by 40 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 1 min; final extension was at 72C for 10 min. PCR amplification conditions used for the Set2 was 94°C for 10 min initial denaturation followed by 40 cycles of 94°C for 1 min, 72°C for 2 min; final extension was at 72°C for 10 min. Optionally, the products were cloned into pTZ57R/T vector and sequenced.

3.30. MULTIPLE RESTRICTION FRAGMENT- SINGLE STRAND CONFORMATIONAL POLYMORPHISM (MRF-SSCP)

9:1 loading buffer (2X)

90% formamide
10% 1X TBE
0.25% bromophenol blue
0.25% xylene cyanol FF

6X loading dye solution

60% Glycerol
60 mM EDTA
0.09% bromophenol blue
0.09% xylene cyanol FF

7% PAGE (30 ml)

39:1 Acrylamide: bis-acrylamide	- 5.25 ml
10X TBE	- 3.0 ml

10% APS	- 210 µl
TEMED	- 18 µl
Water	- make up to 30 ml

The PCR product from different sets of human JRK gene was restriction digested to smaller fragments. Likewise, each amplified DNA was digested with 5- 10 U of restriction enzyme at ambient temperature for 2 h: set 1 amplicons were digested with Hinf1; set 2 with Sma1; set 3 with Alu1; and set 4 with EcoR1 + Pst1. The digested PCR product was mixed with equal volume of loading buffer, heat denatured at 99°C for 5 min, chilled on ice for 2 min and loaded on PAGE. Nondenatured sample was prepared by adding 6X loading dye to the digested amplicons and run alongwith the denatured samples. The PAGE was prerun at constant 20 W at 45°C for one hour before the sample gel run was carried out at 15 W at 45°C. The gel was silver stained to visualize the bands.

Chapter 4

RESULTS AND DISCUSSION

4.1. SEIZURE-ASSOCIATED GENE EXPRESSION CHANGES

Gene expression variations accompanying seizure development makes neurons susceptible to recurrent seizures (Gall, *et al.*, 1991; Morgan, *et al.*, 1987; Newton, *et al.*, 2003). The emerging scenario from several global gene expression profiling on different models reveal genes performing wide range of molecular functions including signal transduction, regulation of transcription, protein synthesis and degradation, basic metabolism, structural proteins and receptors (Lukasiuk and Pitkanen, 2004). Although early transcriptional events are the preamble to susceptibility to recurrent seizure, less information is available on gene expression profile during early stages of epileptogenesis.

A well-studied chemoconvulsant model, pilocarpine model of seizure in rat, was used for checking the global gene expression pattern within 4 h and 24 h of seizure induction. Within 10 to 15 minutes post-induction, seizure-associated behavioral changes were observed in the animal- akinesia, facial automatism, limbic seizures consisting of forelimb clonus, salivation and masticatory movements appeared sequentially and progressively developed to myoclonic jerks, clonic convulsions and tonic seizures. The *status epilepticus* phase prolonged for several hours and gradually receded by 24 h after

pilocarpine injection. The mRNA isolated from the hippocampal tissue of 4 h and 24 h post-induction (representing the 'seizure-period' (SP) and 'recovery-period' (RP) respectively) were analyzed for global gene expression pattern using Rat Genome Survey Microarray slide (Applied Biosystem). Observations from two independent experiments were used to calculate the average fold change (FC) at SP and RP. In view of the enormity of the data, only those gene probes were selected whose mean signal intensity corresponded to p=0.05 (**Figure 5**).

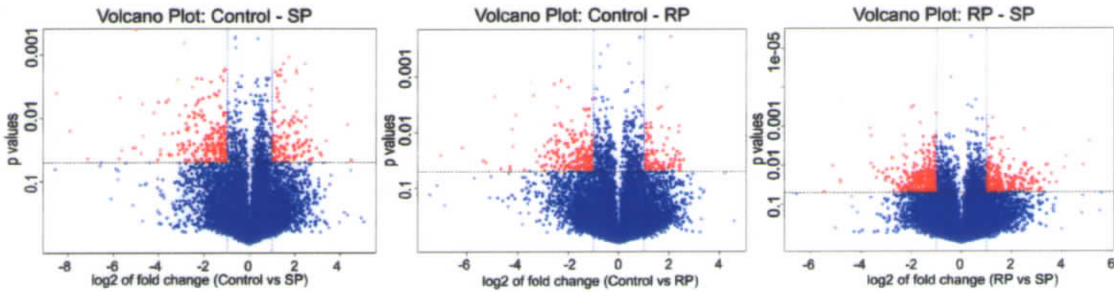


Figure 5: Volcano Plot depicting the global gene expression. Each coordinate represents a gene probe plotted against its corresponding log2 of fold change against its p-value. Coordinates with p=0.05 and atleast ±2 fold change are marked red.

During SP, 2953 genes underwent at least 2-fold upregulation. Most of the over expressed genes displayed 2 to 5 fold increase in their transcript level (2733 genes), however 12 genes showed over 100-fold increase within 4 h of seizure, for example, hsp70 transcript levels shot up even as high as 500-fold (**Figure 6A and Table 3**). Going beyond the identification of series of individual genes that showed a changed expression associated with *status epilepticus*, the SP-overexpressed genes were classified on the

Rank	GeneID	Gene	Function	SP- fold change
1	rCG38317	Hsp70-1a, 1b, NM_212504.1 Heat shock protein 70 kDa	Chaperone	509.82
2	rCG34044	CCl3, NM_013025.2 Chemokine ligand 3	Cytokines	446.60
5	rCG20438	Atf3, NM_012912.1 Activating transcription factor 3	Transcription factor	159.00
6	rCG20898	cfos transcription factor	Transcription factor	141.66
8	rCG30111	Tubulin alpha chain	Cytoskeleton	128.70
10	rCG33135	CCl2, NM_031530.1 Chemokine ligand 2	Cytokines	109.48
11	rCG30202	Olr1, NM_133306.1 Oxidised low density lipoprotein (lectin-like)receptor-1	Intracellular protein receptor	107.30
15	rCG54735	Ugt2a1, NM_022228.1 UDP glycosyl transferase 2 polypeptide A1	Carbohydrate metabolism	85.91
19	rCG442239	GADD45, Growth arrest and DNA damage protein 45	Apoptosis	72.62
21	rCG21815	Hsp27 protein 1, NM_031970.1 Heat shock protein 27 kDa	Chaperone	61.24
22	rCG47671.1	Nxf, NM_153626.1 PAS-type transcription actor	Transcription factor	60.44
24	rCG60600	Egr2, NM_053633.1 Early growth response 2	Transcription regulation	56.19
25	rCG56196	Egr4, NM_019137.1 Early growth response 4	Transcription regulation	52.97
29	rCG28944	Cyr61, NM_031327.2 Cysteine-rich protein 61	Growth factor	35.73

Table 3: Top-ranked genes overexpressed during SP.

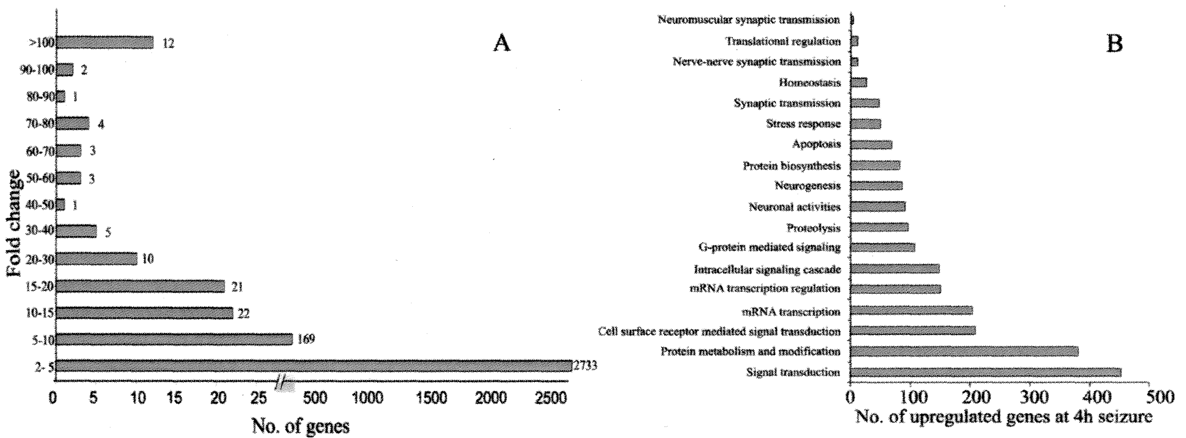


Figure 6: Gene expression variation within 4 h of pilocarpine-induced seizure. **A** represents the number of genes that underwent upregulation by certain fold change. **B** classifies the upregulated genes based on its biological process.

basis of their involvement in biological processes using the online PANTHER analysis (www.pantherdb.org) (Thomas, *et al.*, 2003; Thomas, *et al.*, 2006). PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system is a unique resource that classifies genes by their functions, using published scientific experimental evidence and evolutionary relationships to predict function even in the absence of direct experimental evidence. Among the 222 biological processes to which these upregulated genes belong, maximum number of genes that underwent expression variation in processes such as signal transduction, protein metabolism and modification, Nucleoside, nucleotide and nucleic acid metabolism, and cell surface receptor mediated signal transduction (**Figure 6B & Table 4**).

Seizure being a signal transduction-intensive event and involving extensive activities within the neuronal system brings about marked upregulation of the genes involved in signal transduction, neuronal activity and signaling cascade. Genes involved in transcriptional and translational regulations as well as protein synthesis machinery are also overexpressed to synchronize the many-fold upregulation of several genes. Significantly, the transcript levels of genes involved in stress response, apoptosis and neurogenesis were seen expressed within 4 h of seizure. Spontaneous recurrent seizure manifests as a result of neuronal death and neuronal rewiring, taking place within several hours to days following initial insult (Cole, *et al.*, 2002). Genes coding for such temporally subsequent processes of apoptosis and neurogenesis, getting expressed during *status epilepticus* suggest that the system not only synthesizes mRNA during seizure, but also maintains the transcripts for prolonged period until required for translation.

Biological Function	SP ↑	RP ↑	RP ↓
Signal transduction	452	23	154
Protein metabolism and modification	380	9	83
Nucleoside, nucleotide and nucleic acid metabolism	360	9	81
Developmental processes	293	7	89
Cell surface receptor mediated signal transduction	208	12	74
mRNA transcription	203	5	54
Immunity and defense	191	20	52
Cell communication	156	8	55
mRNA transcription regulation	150	4	42
Intracellular signaling cascade	148	2	39
Protein modification	146	6	41
Transport	133	3	46
Cell proliferation and differentiation	127	6	51
Cell structure and motility	126	6	34
Intracellular protein traffic	114	2	39
Cell cycle	111	7	39
G-protein mediated signaling	107	4	38
Ectoderm development	101	2	31
Protein phosphorylation	96	4	30
Proteolysis	95	3	29
Neuronal activities	90		42
Neurogenesis	85	1	27
Protein biosynthesis	82		4
Mesoderm development	78	3	22
Lipid, fatty acid and steroid metabolism	71	2	18
Oncogenesis	70	4	15
Apoptosis	68	6	20
Ion transport	68	1	28
Ligand-mediated signaling	67	7	26
Cell cycle control	66	4	24
Cell structure	66	2	15
Other metabolism	65	1	18
Cation transport	52		
Cell adhesion	51	3	23
Stress response	49	5	11
Cell motility	48	5	15
Sensory perception	48	1	17
Synaptic transmission	47		21
Pre-mRNA processing	41	1	5
General vesicle transport	40	1	14
Carbohydrate metabolism	39	2	10
Protein folding	39		8
Receptor protein tyrosine kinase signaling pathway	37		14
Cytokine and chemokine mediated signaling pathway	35	7	10
MAPKKK cascade	34		11
Cell adhesion-mediated signaling	33		11
Other intracellular signaling cascade	32		9
mRNA splicing	31	1	5
Endocytosis	30	1	12
Muscle contraction	30		14
DNA metabolism	28	1	4
T-cell mediated immunity	28	2	10
Electron transport	26		2
Gametogenesis	26	1	8
Chromatin packaging and remodeling	25		9
Homeostasis	25	3	8
Calcium mediated signaling	24		12
B-cell- and antibody-mediated immunity	22		9
Protein targeting and localization	22	1	7
Mitosis	21	1	9
Other receptor mediated signaling pathway	21	1	5
Steroid metabolism	21		3
Vision	21		8
Oncogene	20	1	5
Amino acid metabolism	19		3
Embryogenesis	19	1	7
Inhibition of apoptosis	19	2	10
Muscle development	19		5
Coenzyme and prosthetic group metabolism	18		4
Other immune and defense	18	3	3
Other neuronal activity	18		
Exocytosis	17		6
Lipid and fatty acid transport	17		5
Other developmental process	17	1	3
Blood circulation and gas exchange	16	1	10

Table 4: PANTHER analysis of gene expression variation during SP and RP overexpressed genes (contd...)

Biological Function	SP ↑	RP ↑	RP ↓
Chemosensory perception	16	1	5
Cytokine/chemokine mediated immunity	16	6	4
Olfaction	16	1	5
Skeletal development	16		6
DNA repair	15	1	2
Induction of apoptosis	15	2	3
JNK cascade	15		6
Macrophage-mediated immunity	15	2	6
Neurotransmitter release	15		6
Receptor mediated endocytosis	15	1	5
Spermatogenesis and motility	15		6
Protein glycosylation	14		5
Small molecule transport	14	1	3
Angiogenesis	12	1	3
JAK-STAT cascade	12	3	2
Miscellaneous	12		3
NF-kappaB cascade	12	1	4
Other transport	12		3
Protein complex assembly	12		4
Protein targeting	12		2
Regulation of vasoconstriction, dilation	12	1	7
Anion transport	11	1	4
Blood clotting	11	2	2
Cholesterol metabolism	11		2
DNA replication	11		2
Fatty acid metabolism	11		4
Granulocyte-mediated immunity	11	1	5
Nerve-nerve synaptic transmission	11		8
Nuclear transport	11		1
Other polysaccharide metabolism	11		5
rRNA metabolism	11		1
Translational regulation	11		1
Tumor suppressor	11	1	1
Other oncogenesis	10	1	2
Extracellular matrix protein-mediated signaling	9	1	2
mRNA transcription elongation	9		
Phosphate metabolism	9		5
Phospholipid metabolism	9		2
Purine metabolism	9		3
Sulfur metabolism	9		3
Heart development	8		1
Hematopoiesis	8	1	
Lipid metabolism	8		3
Natural killer cell mediated immunity	8		1
Other carbon metabolism	8		3
Other homeostasis activities	8	2	1
RNA catabolism	8	1	
Steroid hormone-mediated signaling	8		1
Chromosome segregation	7	1	2
Cytokinesis	7		3
Extracellular transport and import	7		3
Meiosis	7		1
Other signal transduction	7		
Oxidative phosphorylation	7		
Protein acetylation	7		1
Receptor protein serine/threonine kinase signaling pathway	7	1	2
Regulated exocytosis	7		1
tRNA metabolism	7		
Amino acid activation	6		
Amino acid transport	6		
General mRNA transcription activities	6		1
Lipid and fatty acid binding	6		1
mRNA transcription initiation	6		
Other intracellular protein traffic	6		3
Other lipid, fatty acid and steroid metabolism	6		2
Other protein metabolism	6	1	1
Reverse transcription	6		4
Vitamin/cofactor transport	6		1
Anterior/posterior patterning	5		
Calcium ion homeostasis	5		3
Detoxification	5		2
DNA recombination	5		
Glucose homeostasis	5	1	
Hearing	5		3
Interferon-mediated immunity	5	1	2

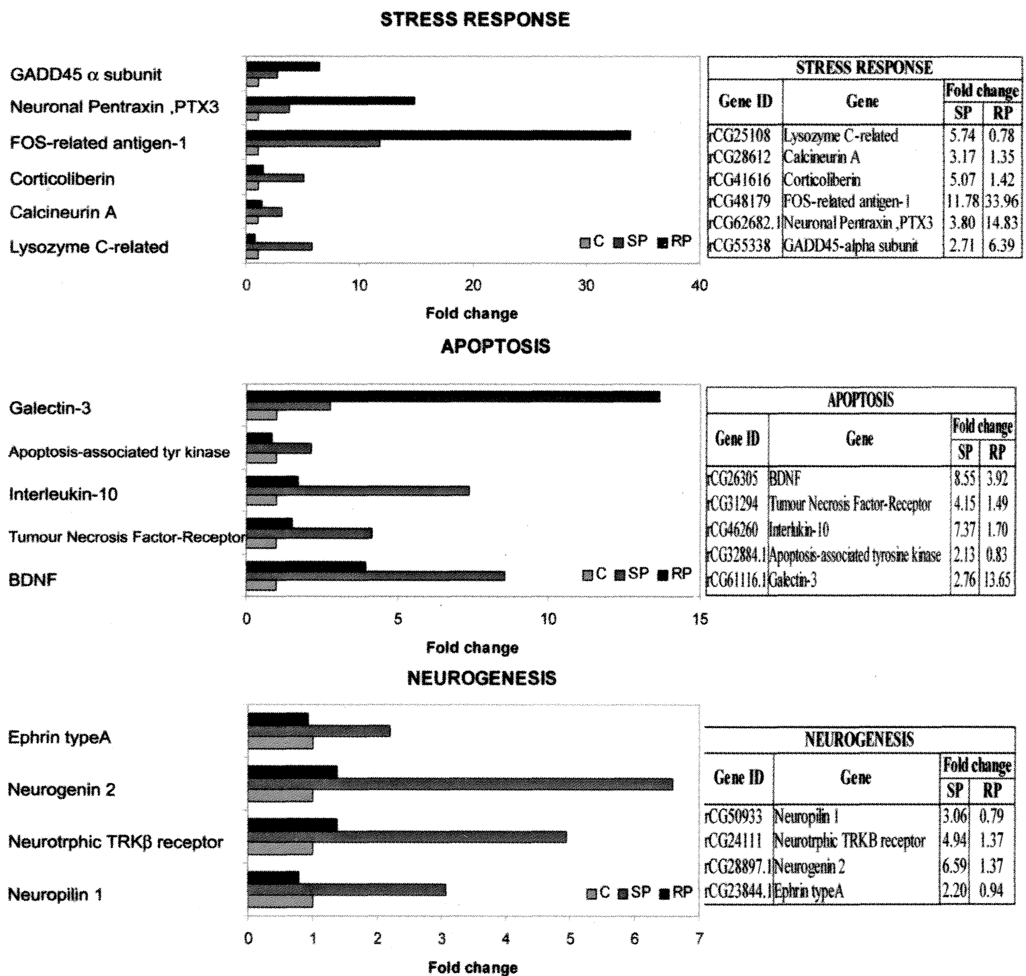
able 4: PANTHER analysis of gene expression variation during SP and RP overexpressed genes (contd...)

Biological Function	SP ↑	RP ↑	RP ↓
Oogenesis	5	1	1
Porphyrin metabolism	5		2
Pyrimidine metabolism	5		
Regulation of lipid, fatty acid and steroid metabolism	5	2	1
Carbohydrate transport	4	1	
Complement-mediated immunity	4		
Lactation, mammary development	4		2
Nucleoside, nucleotide and nucleic acid transport	4		
Other amino acid metabolism	4		1
Other protein targeting and localization	4	1	1
Pain sensation	4		2
Regulation of carbohydrate metabolism	4		2
Segment specification	4		
Steroid hormone metabolism	4		1
Apoptotic processes	3		
Coenzyme metabolism	3		1
Fertilization	3		1
Metabolism of cyclic nucleotides	3		
Monosaccharide metabolism	3		2
Neuromuscular synaptic transmission	3		1
Other carbohydrate metabolism	3		
Other nucleoside, nucleotide and nucleic acid metabolism	3		
Phagocytosis	3		1
Protein-lipid modification	3		
Regulation of nucleoside, nucleotide metabolism	3		
Vitamin metabolism	3		
Acyl-CoA metabolism	2	1	
Amino acid biosynthesis	2		1
Amino acid catabolism	2		
Antioxidation and free radical removal	2		1
Asymmetric protein localization	2		2
Determination of dorsal/ventral axis	2		1
DNA degradation	2		
Endoderm development	2		1
Fatty acid biosynthesis	2		1
Ferredoxin metabolism	2		
Glycogen metabolism	2		1
Glycolysis	2	1	
MHCI-mediated immunity	2		1
mRNA end-processing and stability	2		
mRNA polyadenylation	2		
Nitrogen metabolism	2		1
Non-vertebrate process	2		
Other sulfur metabolism	2		1
Pterin metabolism	2		
RNA localization	2		
Sulfur redox metabolism	2		1
Action potential propagation	1	1	
Constitutive exocytosis	1		1
Fatty acid beta-oxidation	1		
Gluconeogenesis	1		
Gut mesoderm development	1		
MHCII-mediated immunity	1		
Mitochondrial transport	1		
mRNA capping	1		
mRNA transcription termination	1		
Other blood circulation and gas exchange activity	1		1
Other cell cycle process	1	1	
Other coenzyme and prosthetic group metabolism	1		
Other mRNA transcription	1		
Other nitrogen metabolism	1		
Other pathways of electron transport	1		
Peroxisome transport	1		
Pheromone response	1		
Phosphate transport	1		1
Polyphosphate biosynthesis	1		1
Polyphosphate catabolism	1		1
Protein methylation	1		
Regulation of phosphate metabolism	1		1
Tricarboxylic acid pathway	1		
Vitamin biosynthesis	1		

Table 4: PANTHER analysis of gene expression variation during SP and RP overexpressed genes.

The enhanced transcript levels of 2953 genes during *status epilepticus* was probed for their steady state after the animal had recovered from seizure i.e. 24 h post-induction. It was anticipated that some genes might still be transcribed actively, while some others may get degraded beyond their half-life and some gene transcripts may not undergo considerable variation in their levels after 24 h of seizure. On sorting out the number of genes that underwent either ≥2-fold increase or ≥2-fold decrease during RP, out of the 2953 SP-upregulated genes, 85 genes (3%) were further upregulated during 24 h and 859 genes (29%) downregulated. Meanwhile, mRNAs from 2009 genes (68%) underwent little or no changes from their SP-upregulated levels. To investigate if the altered gene expression profile favors any particular biological process to be either upregulated or downregulated was checked by PANTHER analysis (**Table 4**). No particular biological process was selectively upregulated or downregulated. All biological processes had modest number of genes (20-35%) decreasing by over 2-fold and 1-10% increasing by over 2-fold (**Figure** 7). The rest of genes in all processes (55-70%) showed no significant variation in expression. Out of the 452 SP-upregulated genes involved in signal transduction 154 (34%) were downregulated, 23 (5%) were upregulated and 275 (61%) of them showed little change. Showing similar trend, out of 148 genes in intracellular signaling cascade, 48 (33%) had reduced transcript levels, 2 (1%) had stepped up their transcript level and the rest 98 genes (66%) showed no variation. Seizure had triggered overexpression of 90 genes involved in neuronal activities during SP, 42 (47%) of which were downregulated within 24 h. Instantaneous overexpression of 203

genes in mRNA transcription was observed in SP, however 54 (27%) were downregulated and only 5 (3%) were further upregulated. In processes of stress response and apoptosis, 23-29% genes were downregulated, 9-10% were upregulated and 62-67% remained unchanged. 27 (32%) of the 85 genes involved in neurogenesis were downregulated, 1 (1%) gene was overexpressed and 57 (67%) genes displayed no variation.



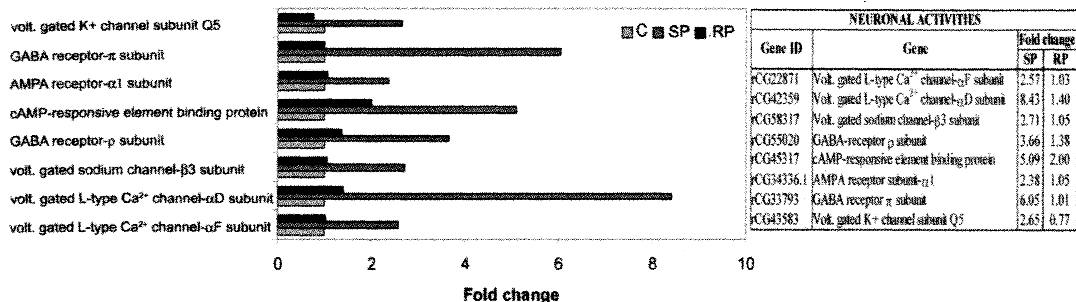
STRESS RESPONSE			
Gene ID	Gene	Fold change	
		SP	RP
rCG25108	Lysozyme C-related	5.74	0.78
rCG28612	Calcineurin A	3.17	1.35
rCG41616	Corticoliberin	5.07	1.42
rCG48179	FOS-related antigen-1	11.78	33.96
rCG62682.1	Neuronal Pentraxin ,PTX3	3.80	14.83
rCG55338	GADD45-alpha subunit	2.71	6.39

APOPTOSIS			
Gene ID	Gene	Fold change	
		SP	RP
rCG26305	BDNF	8.55	3.92
rCG31294	Tumour Necrosis Factor-Receptor	4.15	1.49
rCG46260	Interlukin-10	7.37	1.70
rCG32884.1	Apoptosis-associated tyrosine kinase	2.13	0.83
rCG61116.1	Galectin-3	2.76	13.65

NEUROGENESIS			
Gene ID	Gene	Fold change	
		SP	RP
rCG50933	Neuropilin 1	3.06	0.79
rCG24111	Neurotrphic TRKB receptor	4.94	1.37
rCG28897.1	Neurogenin 2	6.59	1.37
rCG23844.1	Ephrin typeA	2.20	0.94

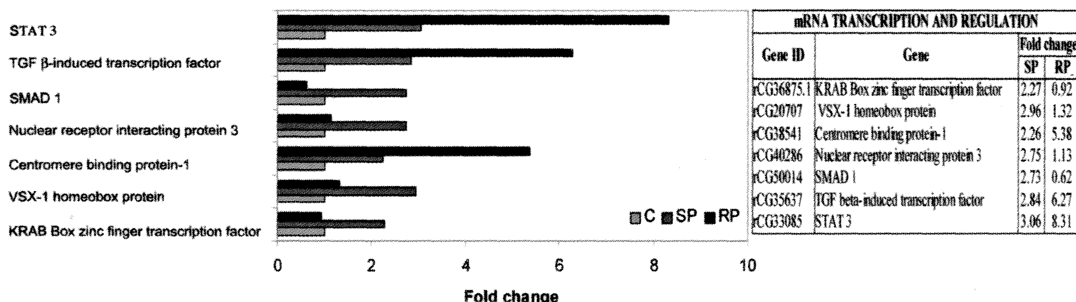
Figure 7: Gene expression at SP and RP in select biological processes. Expression levels of a selected number of genes within each process are represented as fold change in graphical and tabular format. (contd-)

NEURONAL ACTIVITIES



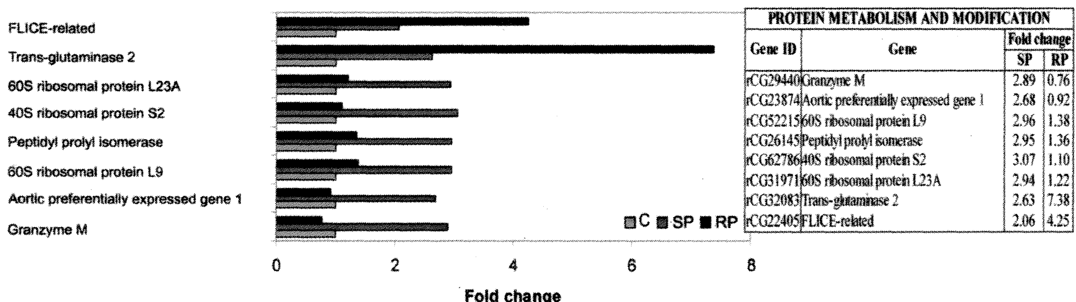
NEURONAL ACTIVITIES			
Gene ID	Gene	Fold change	
		SP	RP
rCG22871	Volt. gated L-type Ca^{2+} channel-αF subunit	2.57	1.03
rCG42359	Volt. gated L-type Ca^{2+} channel-αD subunit	8.43	1.40
rCG58317	Volt. gated sodium channel-β3 subunit	2.71	1.05
rCG55020	GABA-receptor ρ subunit	3.66	1.38
rCG45317	cAMP-responsive element binding protein	5.09	2.00
rCG34336.1	AMPA receptor subunit-α1	2.38	1.05
rCG33793	GABA receptor π subunit	6.05	1.01
rCG43583	Volt. gated K+ channel subunit Q5	2.65	0.77

mRNA TRANSCRIPTION & REGULATION



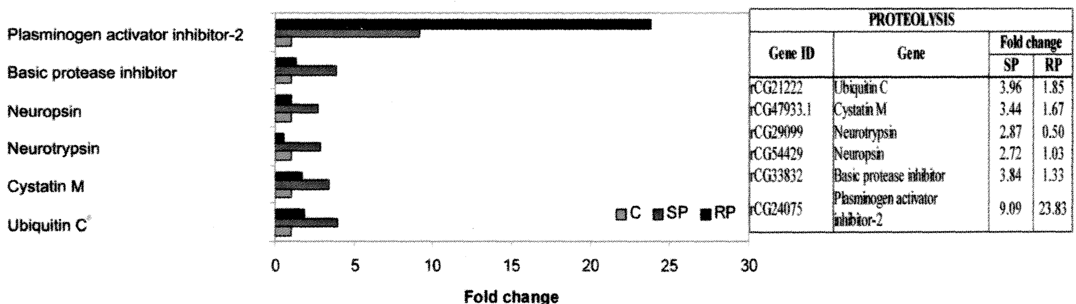
mRNA TRANSCRIPTION AND REGULATION			
Gene ID	Gene	Fold change	
		SP	RP
rCG36875.1	KRAB Box zinc finger transcription factor	2.27	0.92
rCG20707	VSX-1 homeobox protein	2.96	1.32
rCG38541	Centromere binding protein-1	2.26	5.38
rCG40286	Nuclear receptor interacting protein 3	2.75	1.13
rCG50014	SMAD 1	2.73	0.62
rCG35637	TGF beta-induced transcription factor	2.84	6.27
rCG33085	STAT 3	3.06	8.31

PROTEIN METABOLISM & MODIFICATION



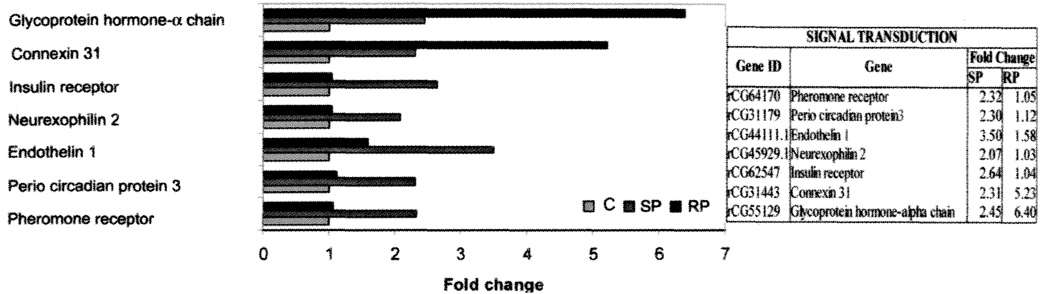
PROTEIN METABOLISM AND MODIFICATION			
Gene ID	Gene	Fold change	
		SP	RP
rCG29440	Granzyme M	2.89	0.76
rCG23874	Aortic preferentially expressed gene 1	2.68	0.92
rCG52215	60S ribosomal protein L9	2.96	1.38
rCG26145	Peptidyl prolyl isomerase	2.95	1.36
rCG62786	40S ribosomal protein S2	3.07	1.10
rCG31971	60S ribosomal protein L23A	2.94	1.22
rCG32083	Trans-glutaminase 2	2.63	7.38
rCG22405	FLICE-related	2.06	4.25

PROTEOLYSIS

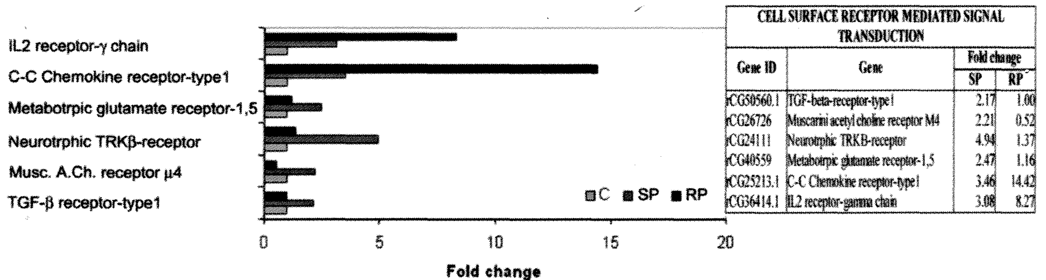


PROTEOLYSIS			
Gene ID	Gene	Fold change	
		SP	RP
rCG21222	Ubiquitin C	3.96	1.85
rCG47933.1	Cystatin M	3.44	1.67
rCG29099	Neurotrypsin	2.87	0.50
rCG54429	Neuropsin	2.72	1.03
rCG33832	Basic protease inhibitor	3.84	1.33
rCG24075	Plasminogen activator inhibitor-2	9.09	23.83

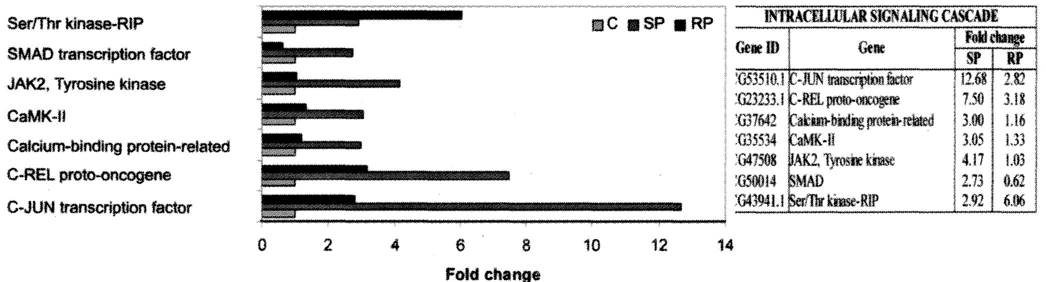
Figure 7: Gene expression at SP and RP in select biological processes. Expression levels of a selected number of genes within each process are represented as fold change in graphical and tabular format. (contd-)



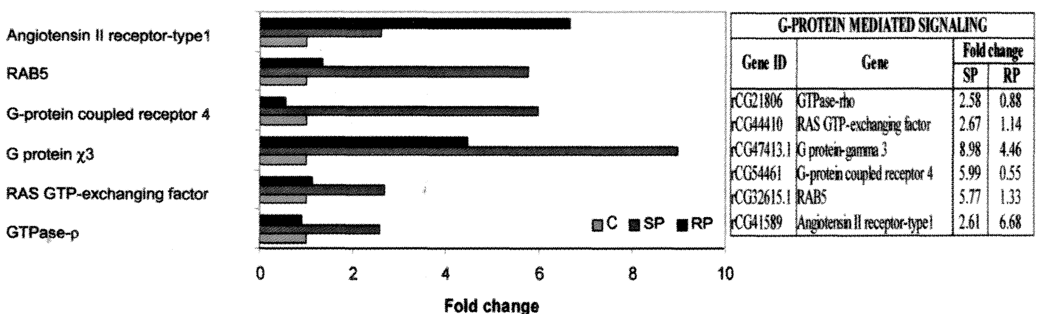
SIGNAL TRANSDUCTION			
Gene ID	Gene	Fold Change	
		SP	RP
rCG64170	Pheromone receptor	2.32	1.05
rCG31179	Perio circadian protein3	2.30	1.12
rCG44111.1	Endothelin 1	3.50	1.58
rCG45929.1	Neurexophilin 2	2.07	1.03
rCG62547	Insulin receptor	2.64	1.04
rCG31443	Connexin 31	2.31	5.23
rCG55129	Glycoprotein hormone-alpha chain	2.45	6.40



CELL SURFACE RECEPTOR MEDIATED SIGNAL TRANSDUCTION			
Gene ID	Gene	Fold change	
		SP	RP
rCG50560.1	TGF-beta-receptor-type1	2.17	1.00
rCG26726	Muscarini acetyl choline receptor M4	2.21	0.52
rCG24111	Neurotrphic TRKB-receptor	4.94	1.37
rCG40559	Metabotrpic glutamate receptor-1,5	2.47	1.16
rCG25213.1	C-C Chemokine receptor-type1	3.46	14.42
rCG36414.1	IL2 receptor-gamma chain	3.08	8.27



INTRACELLULAR SIGNALING CASCADE			
Gene ID	Gene	Fold change	
		SP	RP
:G53510.1	C-JUN transcription factor	12.68	2.82
:G23233.1	C-REL proto-oncogene	7.50	3.18
:G37642	Calcium-binding protein-related	3.00	1.16
:G35534	CaMK-II	3.05	1.33
:G47508	JAK2, Tyrosine kinase	4.17	1.03
:G50014	SMAD	2.73	0.62
:G43941.1	Ser/Thr kinase-RIP	2.92	6.06



G-PROTEIN MEDIATED SIGNALING			
Gene ID	Gene	Fold change	
		SP	RP
rCG21806	GTPase-rho	2.58	0.88
rCG44410	RAS GTP-exchanging factor	2.67	1.14
rCG47413.1	G protein-gamma 3	8.98	4.46
rCG54461	G-protein coupled receptor 4	5.99	0.55
rCG32615.1	RAB5	5.77	1.33
rCG41589	Angiotensin II receptor-type1	2.61	6.68

Figure 7: Gene expression at SP and RP in select biological processes. Expression levels of a selected number of genes within each process are represented as fold change in graphical and tabular format.

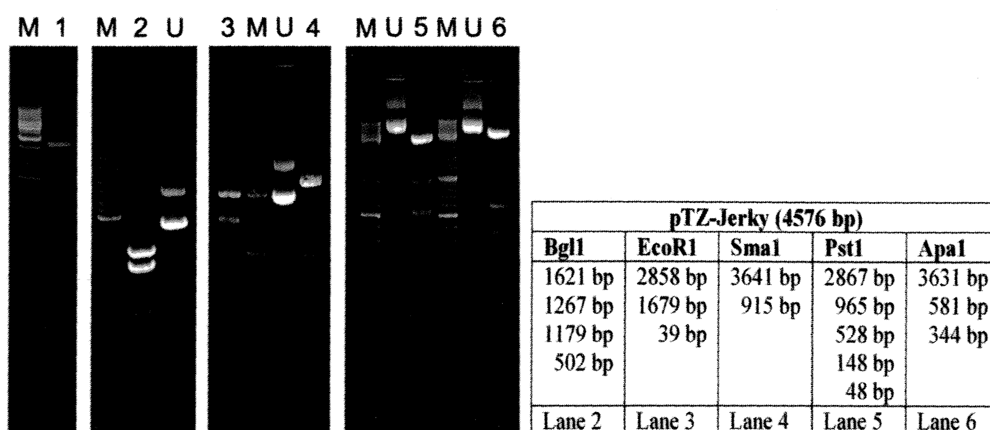
The data suggest that a significant number of genes transcribed during *status epilepticus* are maintained even after seizure had passed, probably to be translated into proteins at a later time point. It is to be mentioned that the microarray technique measures the difference in the relative abundance of cellular mRNAs in the system (and not the newly transcribed mRNAs), and also does not differentiate between translationally active or inactive mRNAs. The transcripts required for pathways like apoptosis and neurogenesis may not be immediately required by the system because those pathways sets in at a later time points in epileptic brain (Cole, *et al.*, 2002).

Correlation of the increased transcription of many genes during SP, to similar increases in respective protein levels is not expected since several studies suggest potential discordance between mRNA and protein levels (Greenbaum, *et al.*, 2003; Greenbaum, *et al.*, 2002; Gygi, *et al.*, 1999; Tian, *et al.*, 2004). More evidence prove mRNAs transcribed in the nuclei are subcellular localized to distal dendrites and their translation tightly regulated (Halbeisen, *et al.*, 2008). Accordingly, there is a temporal uncoupling between the processes of transcription and translation, brought about by post-transcriptional regulation. Role of RNA-binding proteins in bringing about sequestration of mRNAs from the translational machinery was the next objective of this research.

4.2. ROLE OF JERKY IN TRANSLATIONAL REGULATION

Although several RNA-binding proteins (RBP) have been reported for brain-specific expression and neuronal localization (McKee, *et al.*, 2005), the putative RBP that can bind to several target mRNAs from diverse biological processes, must do so in a sequence-independent manner. To ensure that seizure-induced overexpressed transcripts do not come in contact with ribosomal assembly, the RBP must sequester the mature transcripts even before entry into cytoplasm, holding them in a translationally inactive state until destined for translation or degradation. Jerky, a RNA-binding protein and also a candidate gene for epilepsy, was the focus of our attention. Its nucleo-cytoplasmic localization within neuronal cells and ability to bind to large subset of transcripts was sufficient reason to probe Jerky's role in global post-transcriptional regulation.

As the initial step to this direction, we intended to use recombinant GST-Jerky protein and confirm its RNA-binding ability before probing for its role in translational repression. Therefore, the PCR amplicon of the coding region of mice Jerky gene was cloned into pTZ57R/T and confirmed by restriction digestion using EcoR1, Pst1, Sma1, Apa1 and Bgl1 (**Figure 8**). Having further confirmed the cloned Jerky DNA by sequencing, the Jerky DNA was cloned into pGEX-4T-1 bacterial expression vector at Sma1 site in-frame to the GST-tag sequence in the vector. The Jerky DNA released by EcoR1 digestion of pTZ-Jerky, was klenow endfilled and ligated into Sma1 site of pGEX-4T-1 (**Figure 9A**). The pGEX-Jerky clone was confirmed by EcoR1, Bgl1, Pst1



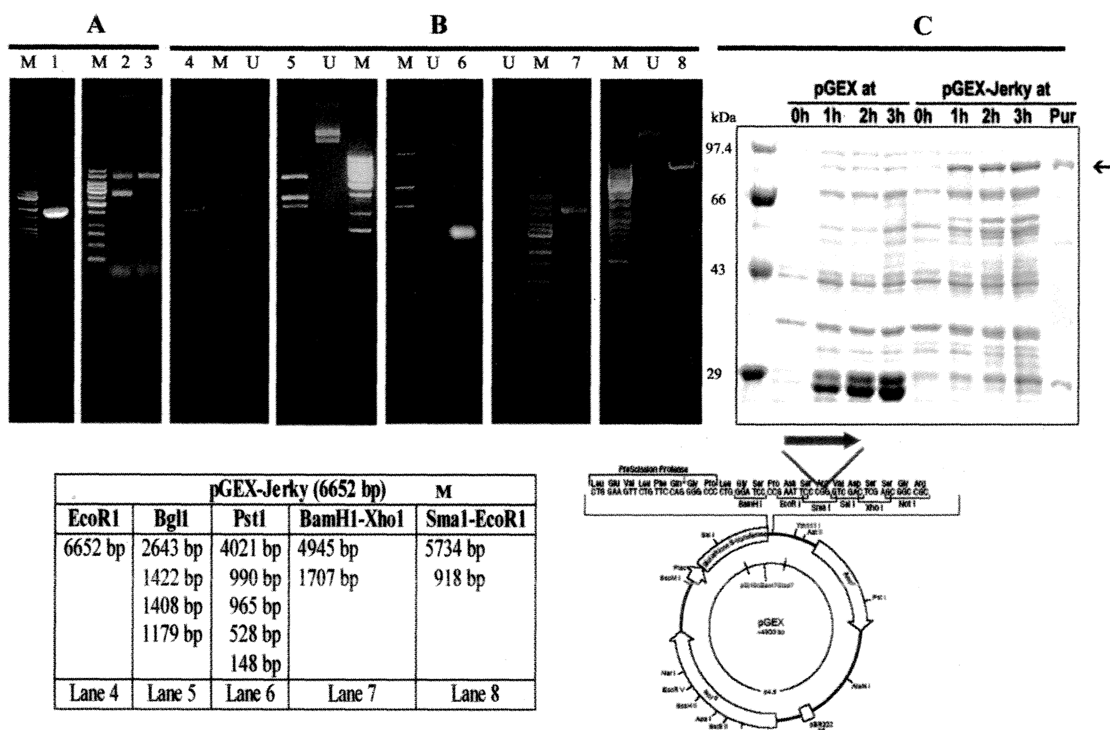
pTZ-Jerky (4576 bp)				
Bgl1	**EcoR1**	**Sma1**	**Pst1**	**Apa1**
1621 bp 1267 bp 1179 bp 502 bp	2858 bp 1679 bp 39 bp	3641 bp 915 bp	2867 bp 965 bp 528 bp 148 bp 48 bp	3631 bp 581 bp 344 bp
Lane 2	Lane 3	Lane 4	Lane 5	Lane 6

Figure 8: Cloning of Jerky into pTZ57R/T. Lanes M and U indicate the molecular weight marker and the undigested form of the pTZ-Jerky plasmid, respectively. Lane 1 is the 1688 bp amplified product of Jerky; Lanes 2, 3, 4, 5 and 6 represent the restriction-digested pTZ-Jerky with Bgl1, EcoR1, Sma1, Pst1 and Apa1 enzymes respectively. Molecular weight of the expected products from pTZ-Jerky digestion and its corresponding lane are indicated in the table.

digestions as well as by double digestion using BamH1-Xho1 and Sma1-EcoR1 (**Figure 9B**). The pGEX-Jerky plasmid was transformed into *E.coli* BL21 cells and the expression of 87 kDa GST-Jerky protein induced by IPTG induction (**Figure 9C**). The recombinant protein was purified and micrococcal nuclease-treated to remove any bacterial RNA/DNA contamination interfering with RNA-pulldown assay. The purified GST-Jerky was used for RNA-pulldown assay on total RNA from control and 2 h-epileptic mice hippocampal tissue.

Pilocarpine-induced seizure in mice elicited expected behavioral changes within 10 minutes of induction. The animal was sacrificed after 2 h of tonic-clonic seizures and

the total RNA isolated from their hippocampus was used for pulldown experiment (**Figure 10A**).



pGEX-Jerky (6652 bp)		M		
EcoR1	**Bgl1**	**Pst1**	**BamH1-Xho1**	**Sma1-EcoR1**
6652 bp	2643 bp 1422 bp 1408 bp 1179 bp	4021 bp 990 bp 965 bp 528 bp 148 bp	4945 bp 1707 bp	5734 bp 918 bp
Lane 4	Lane 5	Lane 6	Lane 7	Lane 8

Figure 9: Cloning of Jerky into pGEX-4T-1 and Expression of GST-Jerky. Lanes M and U indicate the molecular weight marker and the undigested form of the pGEX-Jerky plasmid, respectively. In **A,** endfilled Jerky DNA is ligated at Sma1 site of pGEX. Lane 1- Klenow endfilled Jerky DNA, lane 2 and 3 represent the undigested and Sma1 linearized pGEX-4T-1 plasmid. **B** confirms the pGEX-Jerky clone by restriction digestion with EcoR1 (lane 4), Bgl1 (lane 5), Pst1 (lane 6), BamH1-Xho1 (lane 7) and Sma1-EcoR1 (lane 8). Molecular weight of the expected products from pGEX-Jerky digestion and its corresponding lane are indicated in the table. **C** shows the expression of GST and GST-Jerky protein expression at 1 h, 2 h and 3 h after IPTG induction. The 87 kDa purified GST-Jerky is also run in the last lane. Cloning of Jerky in the Sma1 site of pGEX-4T-1 is depicted in the plasmid map.

The GST-Jerky protein anchored on the glutathione agarose beads was incubated with total RNA from control and epileptic tissue. A no-protein bead complex was used as

negative control, to rule out RNA-binding to the complex other than GST-Jerky. Using P^{32}–labeled polyT primers HT11C, HT11A and HT11G, the pulldown RNA was reverse transcribed and the labeled cDNAs resolved on denaturing urea-polyacrylamide gel, to ensure that pulldown of RNA using recombinant GST-Jerky protein is possible (**Figure 10B**).

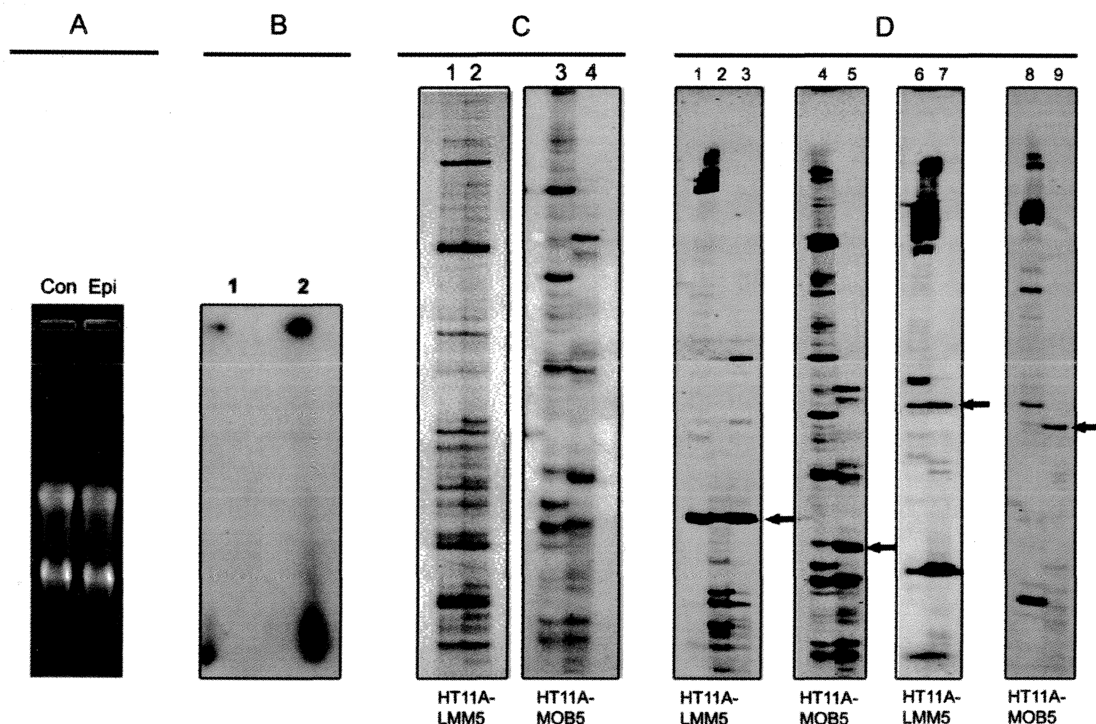


Figure 10: Pulldown RNA using GST-Jerky protein. **A** represents the total RNA from control and epileptic hippocampus used for pulldown experiment. **B** represents the labeled cDNAs of pulldown RNA with 'no-protein' bead complex (lane1) and GST-Jerky anchored bead complex (lane 2). **C** shows the differential display gel on total RNA as well as pulldown RNA. Lanes 1 & 2 are differential display of control and epileptic total RNA, respectively, using primer pairs HT11A-LMM5. Lanes 3 & 4 are the differential display on epileptic total RNA and pulldown RNA, respectively, using primer pairs HT11A-MOB5. **D** shows the differential display gels from which bands from pulldown lanes were eluted, amplified and cloned for sequencing. Lanes 1, 2, 3, 6 and 7 were differential display with primer pairs HT11A-LMM5, whereas lanes 4, 5, 8 and 9

were differential display with primer pairs HT11A-MOB5. Lanes 1 & 4 and 6 & 8 were amplified from total RNA of epileptic and control respectively. Lanes 2 & 5 and 3, 7 & 9 were amplified from pulldown RNA of epileptic and control respectively. Arrow-marked bands in the pulldown lanes were sliced out for elution.

Having observed that GST-Jerky protein binds with a large subset of total RNA, identification of the gene transcripts with affinity for GST-Jerky protein was checked. The pulldown RNA as well as the total RNA were reverse transcribed using HT11A and the cDNAs used for differential display PCR using LMM5 or MOB5 forward primers and labeled HT11A reverse primer. The labeled products were resolved on polyacrylamide gel and autoradiographically analyzed **(Figure 10C)**. Among the many bands observed on the pulldown lane, few were selected for identification **(Figure 10D)**.

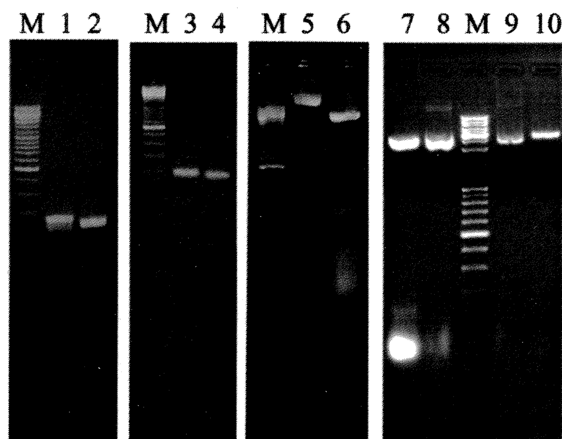
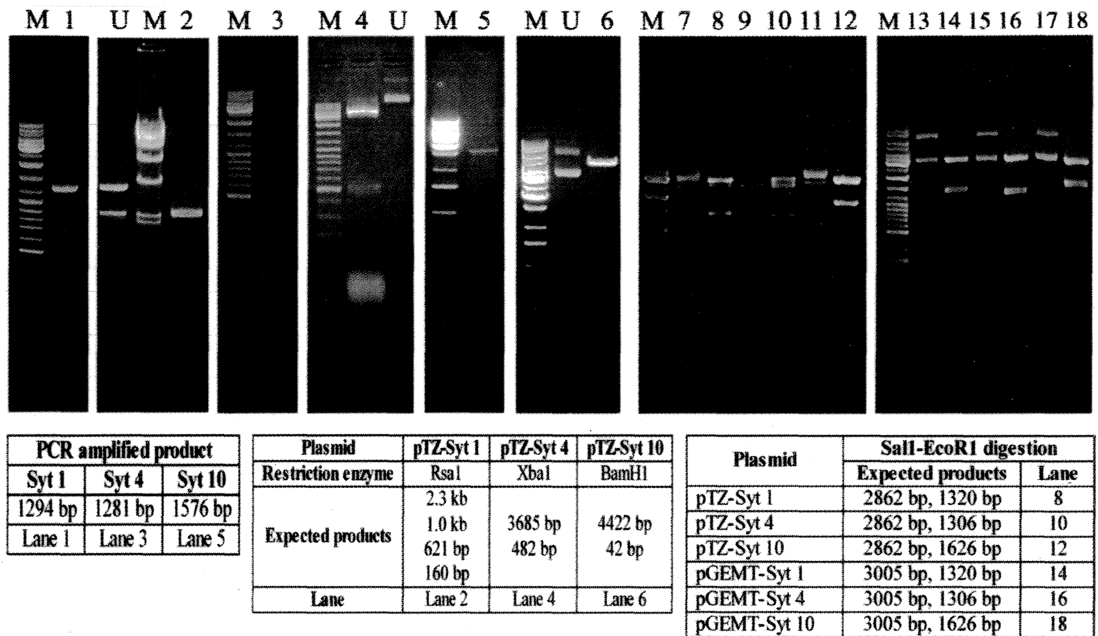


Figure 11: Reamplification and Cloning of jerky pulldown genes. Lane 1, 2, 3, 4 are reamplified DNA of Nedd4, ATP6, ND4 and Aip2. Lanes 5, 8, 9 correspond to undigested forms of pTZ-Nedd4, pTZ-ND4 and pTZ-ATP6. Lane 6, 7, 10 correspond to EcoR1-Sal1 digested forms of pTZ-Nedd4, pTZ-ND4 and pTZ-ATP6, confirming the cloned insert.

The DNA was eluted from the gel, reamplified using corresponding primers and cloned into pTZ57R/T vector for sequence analysis (**Figure 11**). Few of the GST-Jerky pulldown gene transcripts identified were Nedd4, ATP6, ND4 and Aip2 (**Table 5**). Nedd4 is an ubiquitin ligase proposed to regulate cell surface levels of several ion channels, receptors and transporters involved in regulating neuronal excitability, including voltage-gated sodium channels (Fotia, *et al.*, 2004). More recently, Nedd4-2, has been identified as a candidate gene for epileptic photosensitivity (Dibbens, *et al.*, 2007). ND4 and ATP6 are part of the complex 1 and 5 respectively, in mitochondrial oxidative phosphorylation pathway for ATP synthesis (Nakamura, *et al.*, 1993; Nijtmans, *et al.*, 2001). There was neither sequence homology nor any common RNA-binding domain/motif within the pulldown genes, indicative of sequence-independent interaction of GST-Jerky protein with its target transcripts.

Gene & Accession No	**Gene Description**	**Mice Chromosomal loci**	**Gene Function**	**Identity**
Nedd4 BC007184	Neural precursor cell expressed developmentally downregulated gene 4	Chr 9	Protein modification, Ubiquitin cycle	100%
Mt-ATP6 BC012020	Mitochondrial ATP synthase 6	Mitochondrial DNA	H+ transporter activity	99%
Mt-ND4 BC020382	Mitochondrial NADH dehydrogenase, subunit 4	Mitochondrial DNA	Oxidative phosphorylation, ubiquinone biosynthesis	97%
Aip2 BC060501	ADP-ribosylation factor-like 6 interacting protein 2	Chr 17	GTP-binding, GTPase activity	69%

Table 5: Genes identified from GST-Jerky pulldown experiment. Nedd4 and ND4 were amplified from pulldown RNAs of epileptic and control respectively using primer pairs HT11A-LMM5. ATP6 and Aip2 were amplified from pulldown RNAs of epileptic and control respectively using primer pairs HT11A-MOB5.

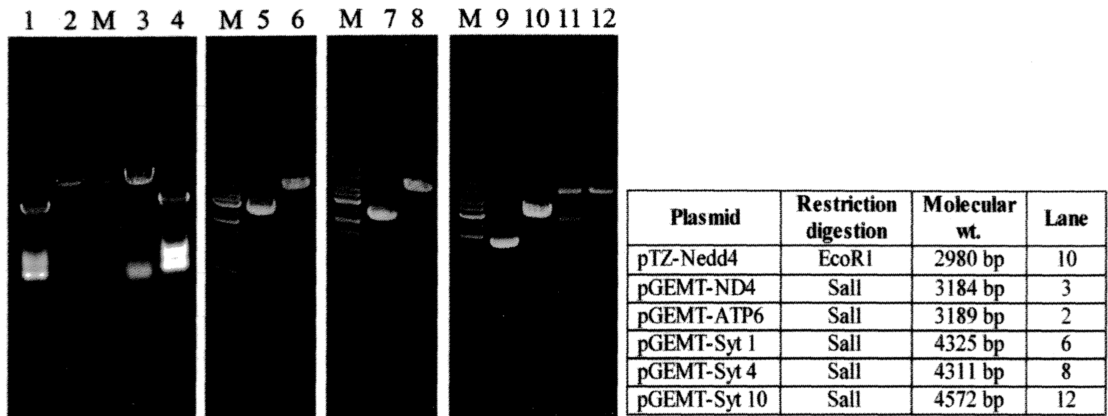


PCR amplified product		
Syt 1	Syt 4	Syt 10
1294 bp	1281 bp	1576 bp
Lane 1	Lane 3	Lane 5

Plasmid	pTZ-Syt 1	pTZ-Syt 4	pTZ-Syt 10
Restriction enzyme	Rsa1	Xba1	BamH1
Expected products	2.3 kb 1.0 kb 621 bp 160 bp	3685 bp 482 bp	4422 bp 42 bp
Lane	Lane 2	Lane 4	Lane 6

Plasmid	Sal1-EcoR1 digestion	
	Expected products	Lane
pTZ-Syt 1	2862 bp, 1320 bp	8
pTZ-Syt 4	2862 bp, 1306 bp	10
pTZ-Syt 10	2862 bp, 1626 bp	12
pGEMT-Syt 1	3005 bp, 1320 bp	14
pGEMT-Syt 4	3005 bp, 1306 bp	16
pGEMT-Syt 10	3005 bp, 1626 bp	18

Figure 12: Cloning of Syt 1, Syt 4 and Syt 10. Lanes M and U indicate the molecular weight marker and the undigested form of the respective plasmids. Amplified products of coding region of Syt 1 (lane 1), Syt 4 (lane 3) and Syt 10 (lane 5) were cloned into pTZ57R/T vector. Clones pTZ-Syt 1, pTZ-Syt 4 and pTZ-Syt 10 were confirmed by restriction digestion using Rsa1 (lane 2), Xba1 (lane 4) and BamH1 (lane 6) respectively. The pTZ-Syt clones were digested with Sal1-EcoR1 to release the cloned inserts of Syt1 (lane 8), Syt 4 (lane 10) and Syt 10 (lane 12), to be subcloned into pGEM-T Easy vector at Sal1-EcoR1 sites. Digestion with Sal1-EcoR1 was carried out to confirm the clones pGEMT-Syt 1 (lane 14), pGEMT-Syt 4 (lane 16) and pGEMT-Syt 10 (lane 18). Lanes 7, 9, 11, 13, 15, 17 represent the undigested plasmids of pTZ-Syt 1, pTZ-Syt 4, pTZ-Syt 10, pGEMT-Syt 1, pGEMT-Syt 4 and pGEMT-Syt 10. Molecular weight of the expected products from digestions of Syt clones and their corresponding lane are indicated in the table.



Plasmid	Restriction digestion	Molecular wt.	Lane
pTZ-Nedd4	EcoR1	2980 bp	10
pGEMT-ND4	SalI	3184 bp	3
pGEMT-ATP6	SalI	3189 bp	2
pGEMT-Syt 1	SalI	4325 bp	6
pGEMT-Syt 4	SalI	4311 bp	8
pGEMT-Syt 10	SalI	4572 bp	12

Figure 13: Linearized vectors with insert in T7 orientation were used for *in vitro* transcription. Lanes 1, 4, 5, 7, 9 and 11 represent the undigested plasmids of pGEMT-ATP6, pGEMT-ND4, pGEMT-Syt 1, pGEMT-Syt 4, pTZ-Nedd4 and pGEMT-Syt10. Linearized products of pGEMT-ATP6 (lane2), pGEMT-ND4 (lane 3), pGEMT-Syt 1 (lane 6), pGEMT-Syt 4 (lane 8), pTZ-Nedd4 (lane 10) and pGEMT-Syt10 (lane 12) are used for *in vitro* transcription. Lane M denotes the molecular weight marker. Molecular weight of the linearized product of clones used for *in vitro* transcription and its corresponding lane are indicated in the table.

Further confirmation on the sequence-independent interaction of Jerky was performed on labeled transcripts coding for Syt 1, Syt 4 and Syt 10. These isoforms of synaptotagmin (Syt) have similar domain architecture, but differ in there physiological expression (Sudhof, 2002). Syts are synaptic vesicle proteins, which act as Ca^{2+}-sensors facilitating rapid exocytosis of synaptic vesicles during high local concentrations of Ca^{2+} following an action potential, thereby triggering membrane fusion (Brose, *et al.*, 1992). While Syt 1 is ubiquitously expressed and involved in vesicular exocytosis, Syt 4 and Syt 10 display seizure-induced overexpression (Babity, *et al.*, 1997; Vician, *et al.*, 1995).

Accordingly, the coding region of Syt 1 was amplified from normal rat hippocampal RNA, whereas, Syt 4 and Syt 10 coding region was amplified from epileptic

hippocampal RNA. The PCR products were cloned in pTZ57R/T and confirmed by restriction digestion. The Syt 1, 4 and 10 were subcloned into Sal1-EcoR1 sites of pGEM-T Easy vector in T7 orientation (**Figure 12**). Linearized plasmids with the cloned insert in the T7 orientation were used for *in vitro* transcription and labeling of gene transcripts (**Figure 13**).

The P^{32}- labeled transcripts were purified and used for binding with purified GST-Jerky protein. Purified GST protein as well as 0 h-bacterial lysate (non-induced) were used as internal controls for the bacterial system expressed recombinant GST-Jerky protein used for binding studies. The RNA-protein complex was resolved through polyacrylamide gel and analyzed autoradiographically. In the event of formation of stable ribonucleoprotein complex (RNP), it exhibits retarded mobility and is visualized as a band on the gel. The recombinant jerky protein formed RNP complex with all the transcripts including Nedd4, ATP6, ND4 as well as Syt 1, Syt 4 and Syt 10 (**Figure 14**). Competitive mobility shift assay was also carried out on ND4, Syt 1, Syt 4 and Syt 10, wherein the labeled gene transcripts were competed against gradiently increasing concentrations of unlabeled cold transcripts (**Figure 15**). With increasing concentration of cold transcripts, the signal intensity from the hot transcripts gradually diminished, indicating binding of jerky proteinto the transcript.

Apparently, Jerky recombinant protein can bind to almost all transcripts in a sequence-independent manner. Physiological relevance of RNA-binding proteins is

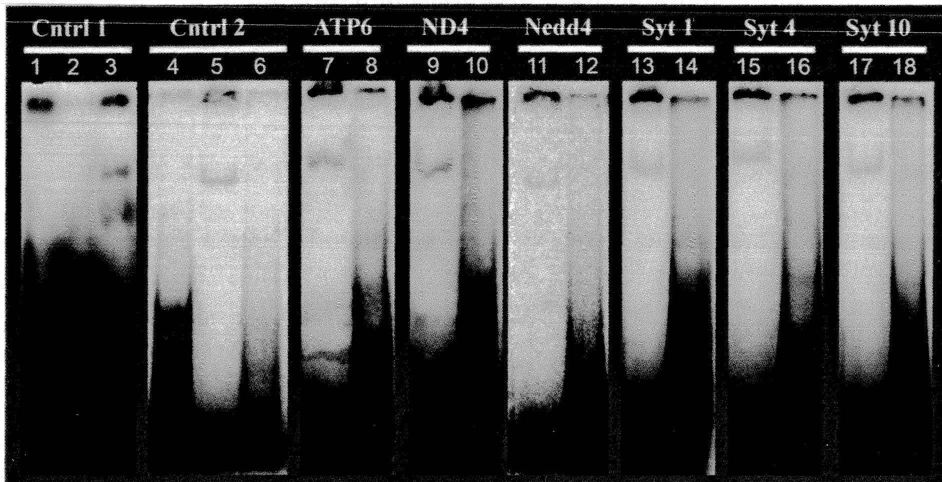


Figure 14: Mobility shift assay with recombinant jerky protein (n = 3 to 6). Lanes 1, 2, 3 in Cntrl 1 represent Nedd4 RNA alone, Nedd4 RNA+ 0 h bacterial lysate, Nedd4 RNA+purified GSTJerky protein. Lanes 4, 5, 6 in Cntrl 2 represent Syt10 RNA alone, Syt10 RNA+purified GST-Jerky protein, Syt10 RNA+purified GST protein. Lanes 8, 10, 12, 14, 16 and 18 represent RNA alone of ATP6, ND4, Nedd4, Syt 1, Syt 4 and Syt 10. Lanes 7, 9, 11, 13, 15 and 17 represent GST-Jerky protein RNP with transcripts of ATP6, ND4, Nedd4, Syt 1, Syt 4 and Syt 10.

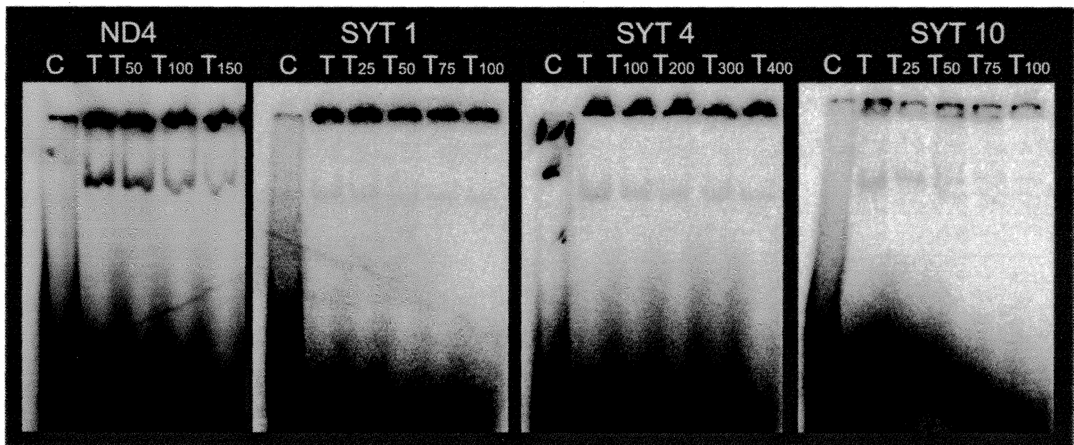


Figure 15: Competitive mobility shift assay. Labeled probes were competed with its own unlabeled transcripts for binding to GST-Jerky protein (n = 2). ND4 labeled RNA was competed against 50-150% identical cold RNA. Syt 1 and Syt 10 were competed against 25-100% of its own cold transcripts. Labeled Syt 4 RNA was competed against 100-400% of cold RNA.

displayed by the binding affinity for its target transcripts. Earlier reports suggested that Jerky protein might have preferential affinity towards some of the gene transcripts. The binding kinetics of recombinant GST-Jerky protein towards different transcripts was evaluated by slot blot assay, wherein the RNP complex is passed through a manifold of nylon and nitrocellulose membrane. The unbound labeled RNA is trapped on the nylon membrane whereas the bound RNP is trapped on nitrocellulose membrane.

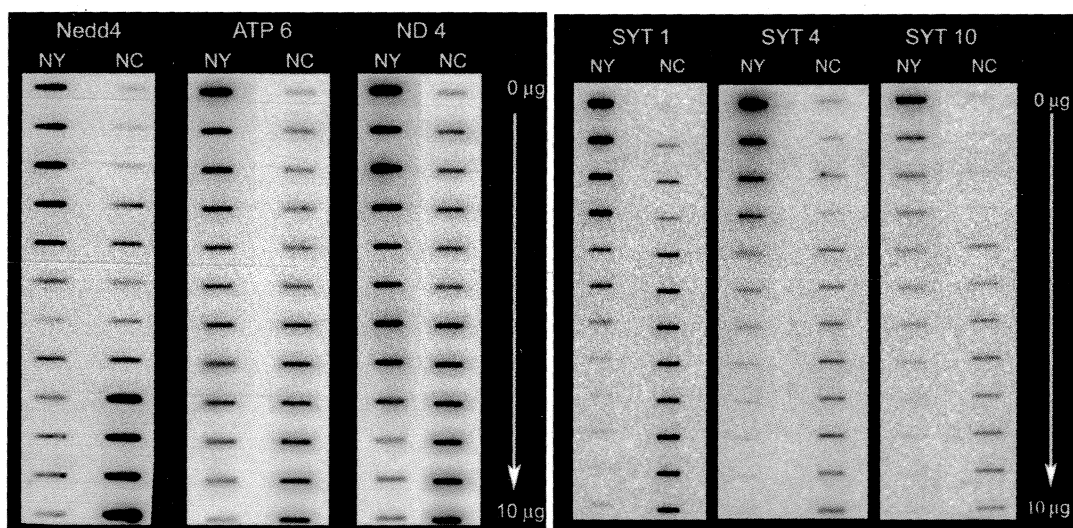


Figure 16: Slot blot assay to measure binding affinity of GST-Jerky towards various gene transcripts (n = 3 to 6). Binding of gradient concentration of recombinant jerky protein to constant amount of labeled gene transcripts of Nedd4, ATP6, ND4, Syt 1, Syt 4 and Syt 10 was analyzed for respective binding kinetics.

Binding reactions were set up containing equal amount of labeled transcript and gradient concentrations of purified GST-Jerky protein (1.1 pmol to 110.05 pmol). Transcripts of Nedd4, ATP6, ND4, Syt 1, Syt 4 and Syt 10 were tried for affinity towards jerky protein (n=3 to 6) (**Figure 16**). Using phosphorimager, the signal intensity for each slot blot was measured from both membranes. The ratio (R) of the intensity from a

sample slot to the 'no-protein' control slot was calculated from the nylon membrane. The logarithm of gradient picomol concentrations of jerky protein used for RNP complex was plotted against its corresponding 1-R. Except Syt 4, all the other transcripts displayed similar binding affinity (**Figure 17**). Syt 4 demonstrated higher affinity towards jerky protein. The recombinant jerky protein has similar affinity towards most transcripts.

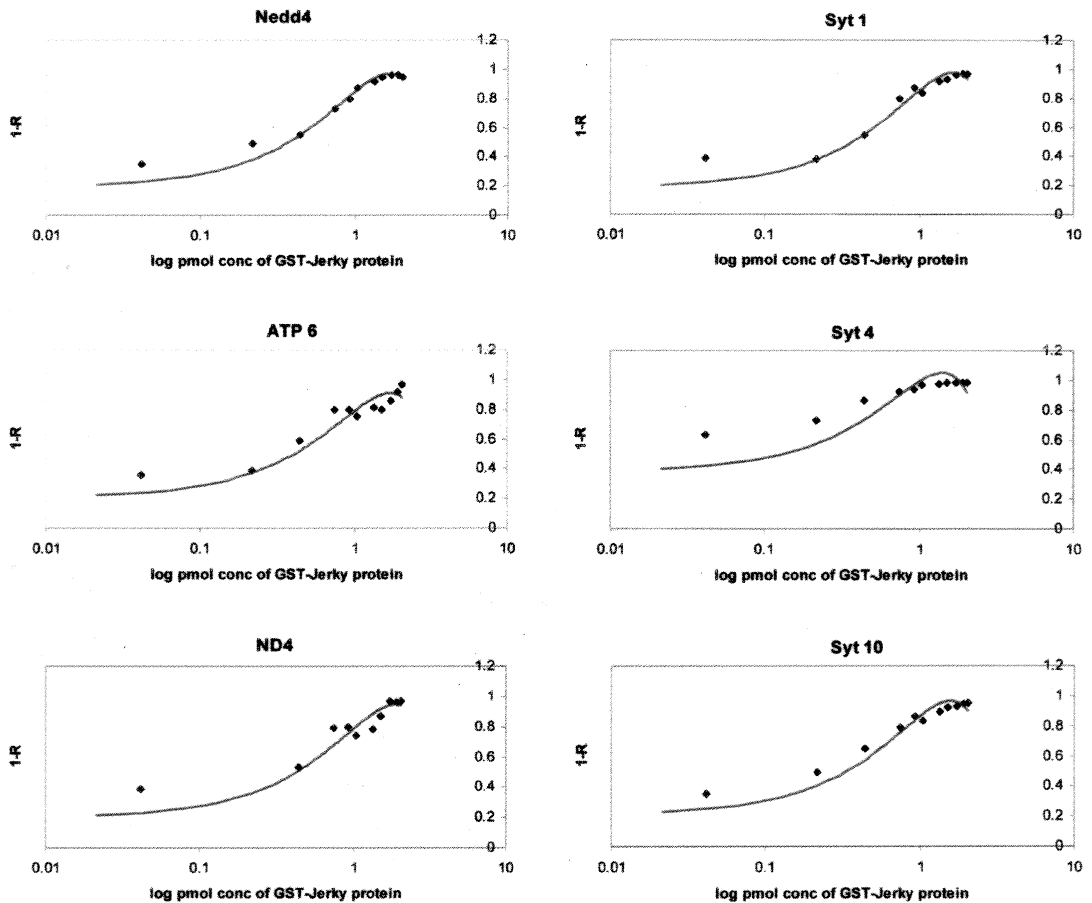


Figure 17: Kinetics of binding of GST-Jerky protein to different target transcripts. Nedd4, ATP6, ND4, Syt 1 and Syt 10 have similar binding affinity towards GST-Jerky protein. Syt 4 has marginally higher affinity towards jerky protein. Values of each coordinate on the graph are the average of three to six experiments.

However, to consider Jerky to be a global RNA-binding protein, it should be able to bind to RNAs not only in a sequence-independent manner, but also in a conformation-independent and size-independent manner. Jerky binding to smaller RNA constructs derived from Syt 1 and Syt 10 that do not code for full-length protein were carried out and checked on slot blot analysis. Smaller constructs of Syt 1 and Syt 10 derived by amplification or restriction digestion were cloned in T7 orientation and linearized for in vitro transcription (**Figure 18**).

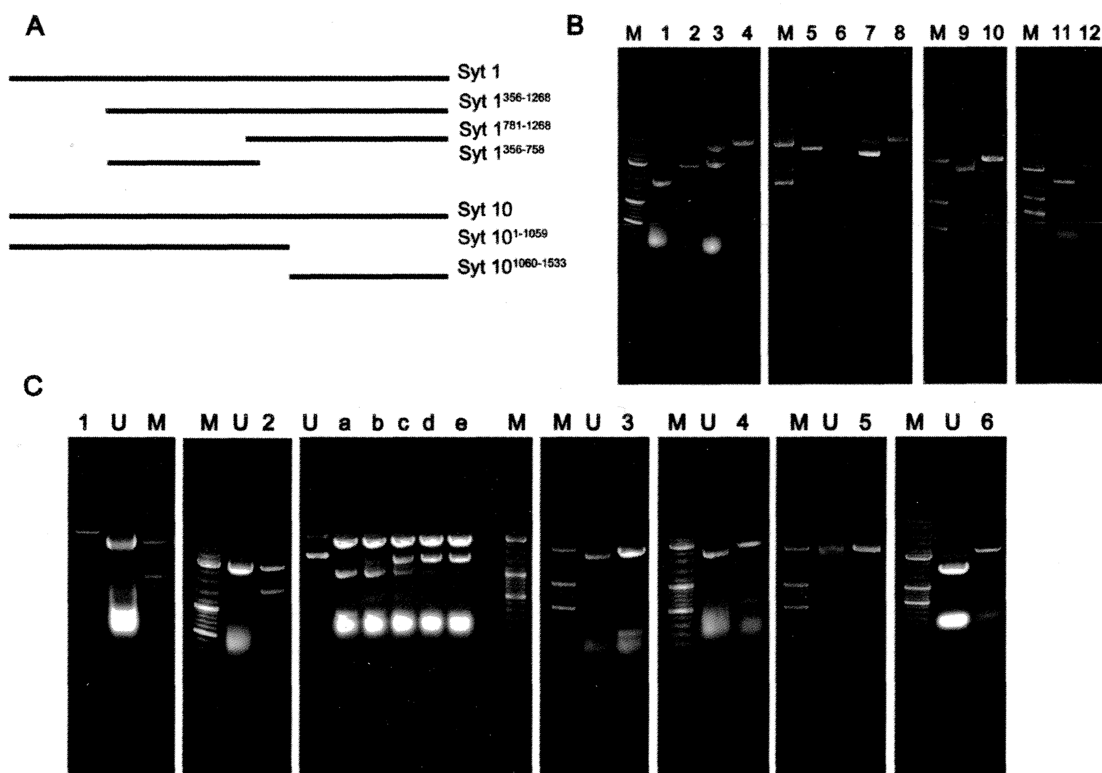


Figure 18: Constructs for smaller RNAs from Syt 1 and Syt 10 coding sequence. **A** Different constructs used in the study for Syt I and Syt 10. **B** Syt 1 smaller constructs. pTZ-Syt $1^{356\text{-}758}$ and pTZ-Syt $1^{356\text{-}1268}$ were confirmed by Sal1-EcoR1 digestion in lane 2 (2834 bp, 459 bp) and 4 (5 kb, 983 bp) respectively. Xba1-linearised pTZ-Syt $1^{356\text{-}758}$ (lane 8, 3293 bp) and pTZ-Syt $1^{356\text{-}1268}$ (lane 6, 3817 bp) were used for in vitro transcription. Lanes 1 & 7 and 3 & 5 are undigested forms

of pTZ-Syt $1^{356-758}$ and pTZ-Syt $1^{356-1268}$. pGEMT-Syt $1^{781-1268}$ was confirmed by Sal1-EcoR1 (lane 10, 2977 bp, 557 bp) digestion and linearized with Sal1 (lane12, 3534 bp) for use in *in vitro* transcription. Lanes 9 & 11 are undigested forms of pGEMT-Syt $1^{781-1268}$. **C** Lanes M and U indicate the molecular weight marker and the undigested form respectively. Syt 10 smaller constructs. pGEMT-Syt 10 was digested with EcoRV (lane 1, 4320 bp,252 bp) and used for transcription for Syt 10^{1-1059}. pBS-Syt 10 clone was initially digested with BamH1 digestion (lane 2) and then partially digested with EcoRV (lanes a-e). The 475 bp Syt$10^{1060-1533}$ partially digested product was cloned into BamH1-EcoRV positions of pTZ57R/T. The pTZ- Syt$10^{1060-1533}$ was confirmed by BamH1-EcoRV (lane 3, 2882 bp, 252 bp, 223 bp) and Sal1-EcoR1 (lane 4, 2834 bp, 523 bp) digestion. The Syt$10^{1060-1533}$ was subcloned into pGEM-T Easy vector and confirmed by Sal1-EcoR1 (lane 5, 2977 bp, 523 bp) digestion. PGEMT- Syt$10^{1060-1533}$ was linearized by Sal1 (lane 6, 3500 bp) to be used for *in vitro* transcription.

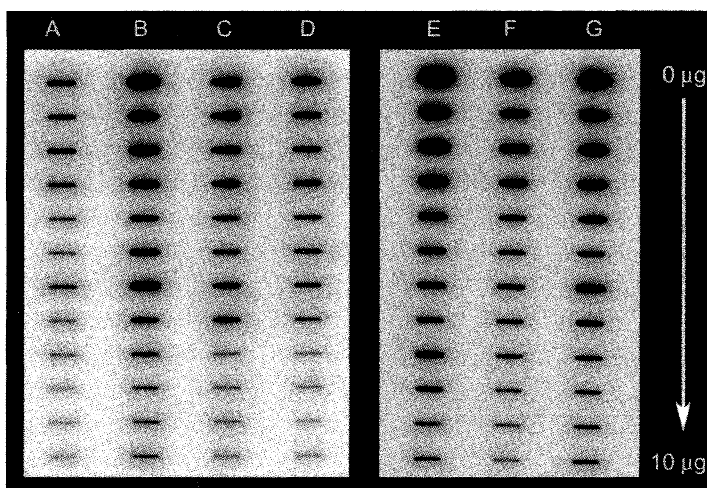


Figure 19: Slot blot analysis of smaller RNA constructs with GST-Jerky protein (n = 3 to 4). From the signal on the nylon membrane, the lanes A, B, C, D denote transcripts of Syt 1, Syt $1^{356-1268}$, Syt $1^{356-758}$ and Syt $1^{781-1268}$. Lanes E, F, G denote transcripts of Syt 10, Syt10^{1-1059} and Syt$10^{1060-1533}$.

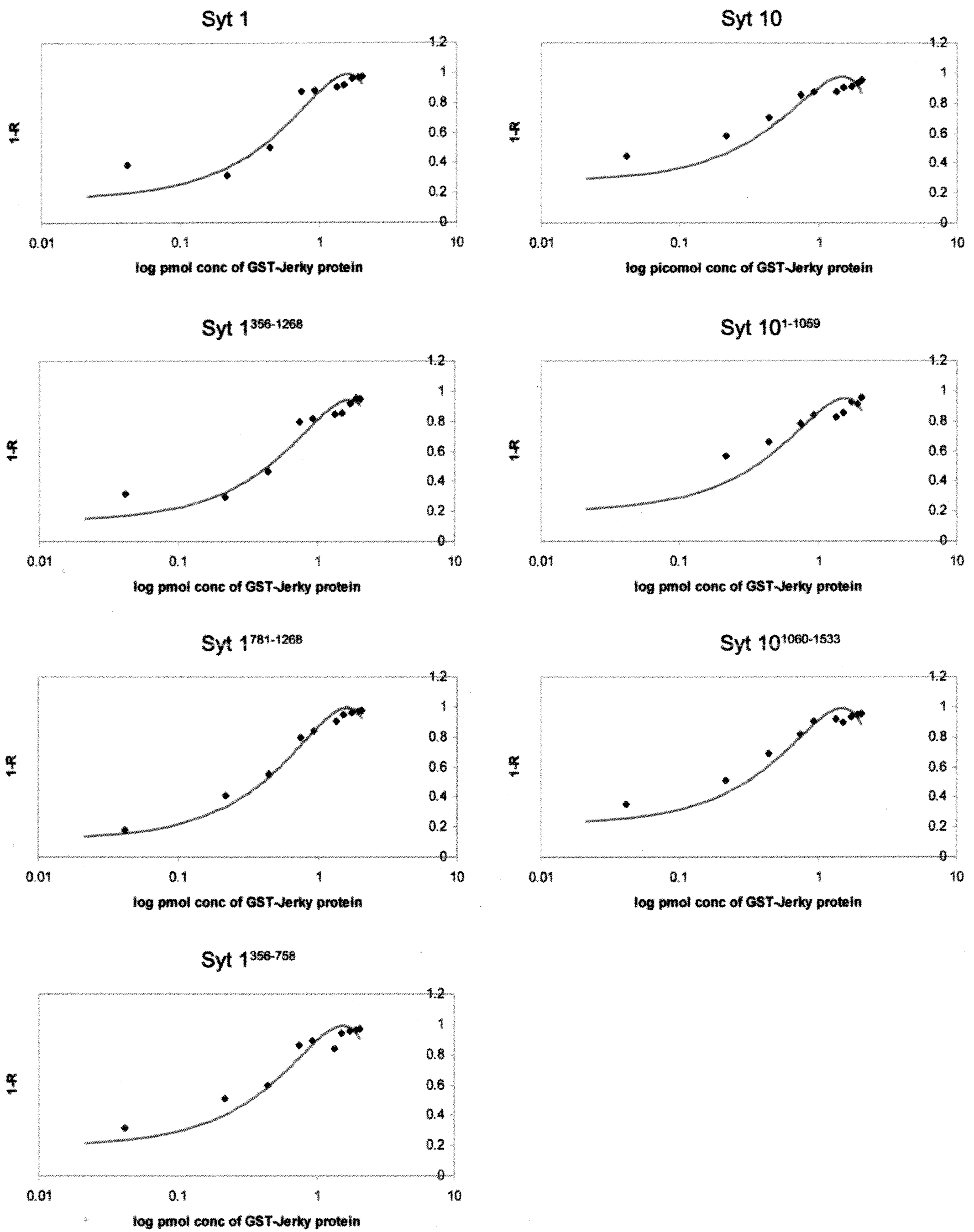
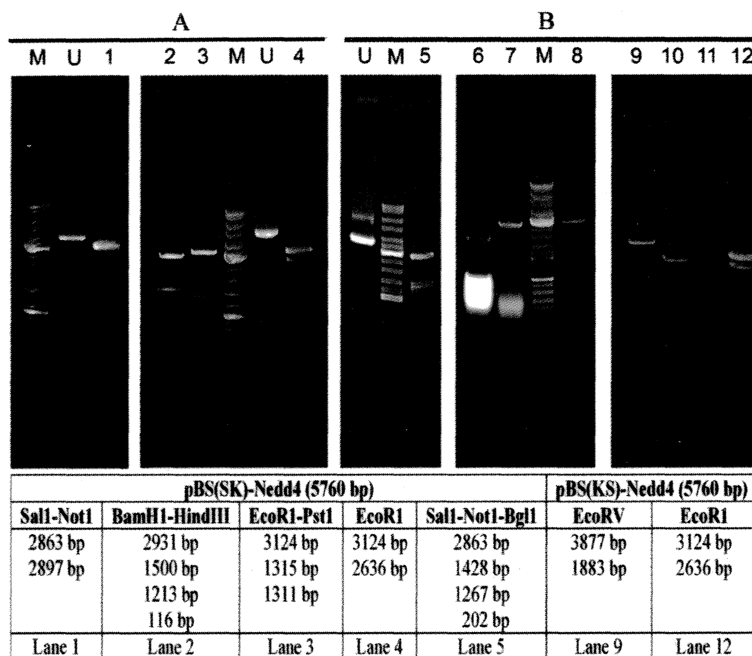


Figure 20: Kinetics of binding of GST-Jerky protein to smaller RNA constructs of Syt 1 and Syt 10. GST-Jerky binds to all the transcripts with almost similar affinity. Values of each coordinate on the graph are the average of three to four experiments.

Binding reaction containing labeled transcripts of these smaller Syt 1 and Syt 10 constructs and GST-Jerky protein was used for slot blot analysis as previously described (n=3) (**Figure 19**). Jerky bound with all the smaller RNAs with very similar binding affinity (**Figure 20**) and comparable to affinities displayed for transcripts such as Nedd4, ATP6 and ND4. The binding of GST-Jerky protein to smaller transcripts confirmed our observation with full-length transcripts, that jerky has non-specific RNA-binding ability and is sequence- independent. The affinity displayed by jerky is biologically relevant and was found to be comparable with most of the transcripts. Having demonstrated and confirmed the RNA-binding property of the recombinant jerky protein, the effect of jerky –binding on translation of these transcripts was checked on *in vitro* translation system such as rabbit reticulocyte lysate.

Plasmid constructs with first methionine coinciding with first codon of the desired protein was used for translating Syt 1 and Nedd4. While pGEMT-Syt 1 was used for Syt1 translation, pBS (KS)-Nedd4 was used for Nedd4 translation (**Figure 21**). The TNT rabbit reticulocyte lysate system provides simultaneous transcription and translation of the protein. The S^{35}-methionine-spiked RRL reaction helps to label and visualize the labeled protein on SDS-PAGE. The effect of translation blockers on translation machinery is well studied in such a system. Purified GST-Jerky protein was added to RRL reaction for translating full coding region peptides of Syt 1 and Nedd4 (**Figure 22**). Other than the ‘no-protein’ control, a heat-denatured GST-Jerky was added to the translation system as a negative control.



pBS(SK)-Nedd4 (5760 bp)					pBS(KS)-Nedd4 (5760 bp)	
Sal1-Not1	BamH1-HindIII	EcoR1-Pst1	EcoR1	Sal1-Not1-Bgl1	EcoRV	EcoR1
2863 bp 2897 bp	2931 bp 1500 bp 1213 bp 116 bp	3124 bp 1315 bp 1311 bp	3124 bp 2636 bp	2863 bp 1428 bp 1267 bp 202 bp	3877 bp 1883 bp	3124 bp 2636 bp
Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 9	Lane 12

Figure 21: Subcloning of full coding Nedd4 sequence for *in vitro* translation. Lanes M and U indicate the molecular weight marker and the undigested form respectively. **A** pBS(SK)-Nedd4 was confirmed by sequencing as well as restriction digestion. **B** has the Nedd4 insert of 2863 bp was released by Sal1-Not1-Bgl1 digestion (lane 5) and cloned in Sal1-EcoR1 sites of pBS(KS). After cloning of Nedd4 (lane 8) into Sal1-EcoR1 digested pBS(KS) (lane 7), the clone was confirmed by resriction digestion with EcoRV (lane 9) and EcoR1 (lane 12). Molecular weight of the expected products from digestions of pBS(SK)-Nedd4 and pBS(KS)-Nedd4 clones and its corresponding lane are indicated in the table.

The signal intensity of the translated product was analyzed by phosphorimager and checked for levels of expression. In presence of GST-Jerky protein, the translation of Syt 1 (n=4) and Nedd4 (n=3) were repressed by 72% and 85%. The translation of Syt 1 was restored back to normal levels on addition of denatured GST-Jerky protein, possibly by the loss of functional RNA-binding property. However, such a complete restoration of translation on addition of denatured protein was not observed in the case of Nedd4. A

partial restoration to 25% of normal expression levels (p=0.01, n=3) may be due to residual binding property of denatured protein to Nedd4 RNA and inhibition of translation.

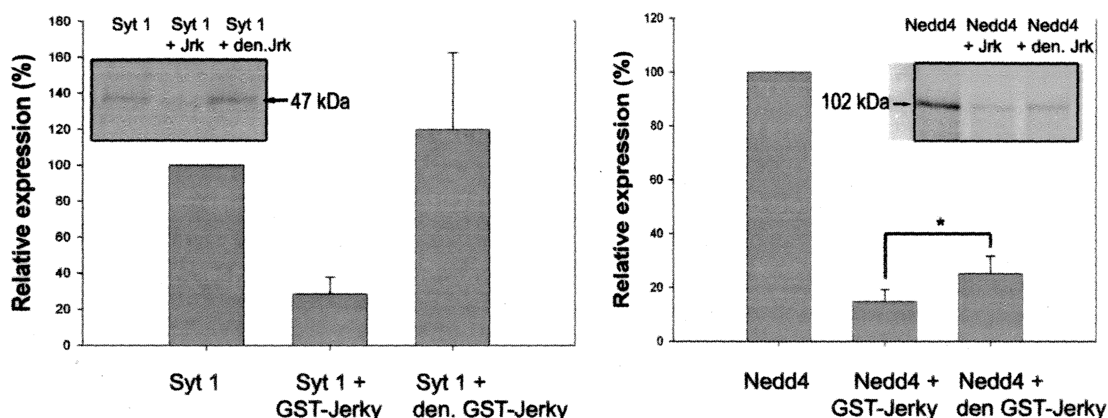
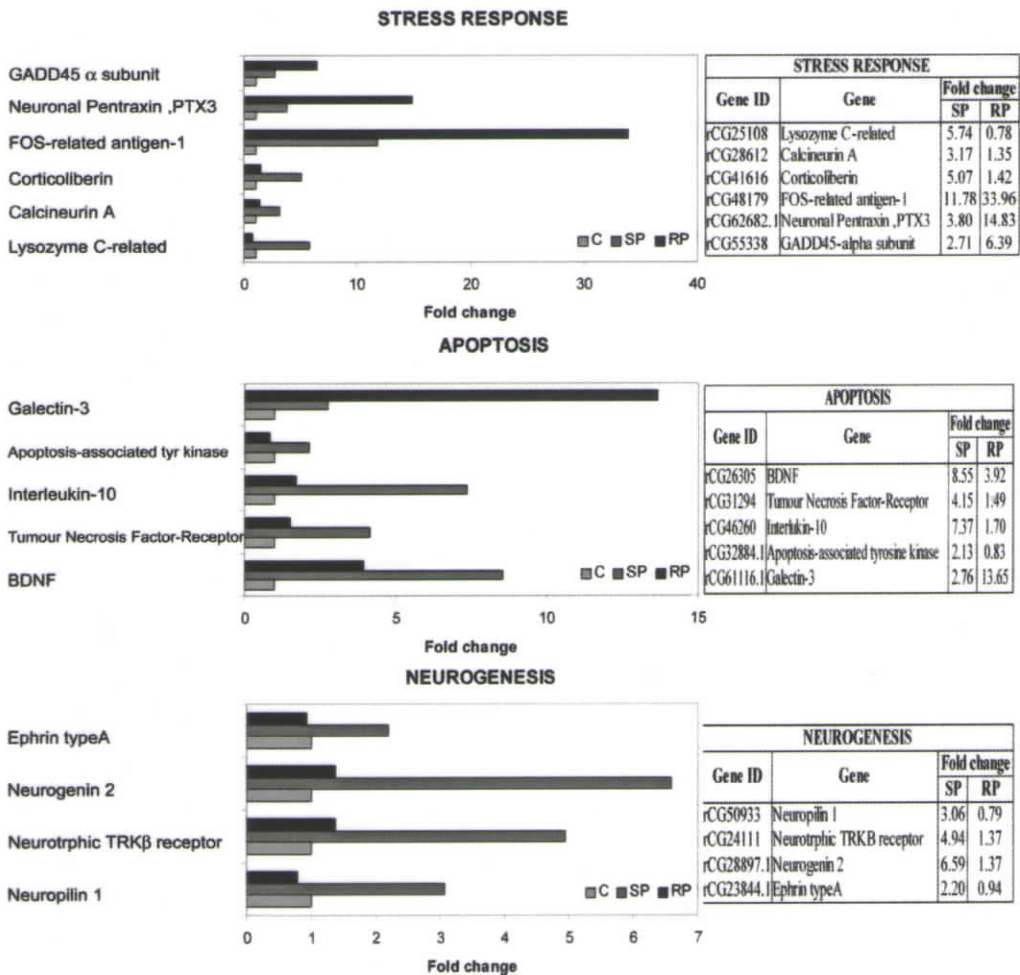


Figure 22: *In vitro* translation of Syt 1 and Nedd4 in rabbit reticulocyte lysate system. Syt 1 and Nedd4 translated protein have a molecular size of 47 and 102 kDa. In presence of GST-Jerky protein, the translation is repressed, and denatured protein restores the translation albeit partially. The expression difference between Nedd4+ GST-Jerky and Nedd4+ denatured GST-Jerky is relevant (p=0.01, n=3)

The above observation sheds new light into the role of Jerky as a translational repressor. Jerky may be forming translationally inactive RNP complexes with a large subset of gene transcripts that may have been drastically upregulated during seizure. Jerky may be a part of a combinatorial assembly of RNA-binding proteins, which destines the overexpressed transcripts for localization or translation or degradation. The exact mechanism of effecting the repression by Jerky protein is yet to be deciphered. In the wake of earlier reports that Jerky gene inactivation leading to seizure, it is speculated

genes in mRNA transcription was observed in SP, however 54 (27%) we downregulated and only 5 (3%) were further upregulated. In processes of stress respon and apoptosis, 23-29% genes were downregulated, 9-10% were upregulated and 62-67 remained unchanged. 27 (32%) of the 85 genes involved in neurogenesis we downregulated, 1 (1%) gene was overexpressed and 57 (67%) genes displayed variation.



STRESS RESPONSE			
Gene ID	Gene	Fold change	
		SP	RP
rCG25108	Lysozyme C-related	5.74	0.78
rCG28612	Calcineurin A	3.17	1.35
rCG41616	Corticoliberin	5.07	1.42
rCG48179	FOS-related antigen-1	11.78	33.96
rCG62682.1	Neuronal Pentraxin ,PTX3	3.80	14.83
rCG55338	GADD45-alpha subunit	2.71	6.39

APOPTOSIS			
Gene ID	Gene	Fold change	
		SP	RP
rCG26305	BDNF	8.55	3.92
rCG31294	Tumour Necrosis Factor-Receptor	4.15	1.49
rCG46260	Interlukin-10	7.37	1.70
rCG32884.1	Apoptosis-associated tyrosine kinase	2.13	0.83
rCG61116.1	Galectin-3	2.76	13.65

NEUROGENESIS			
Gene ID	Gene	Fold change	
		SP	RP
rCG50933	Neuropilin 1	3.06	0.79
rCG24111	Neurotrphic TRKB receptor	4.94	1.37
rCG28897.1	Neurogenin 2	6.59	1.37
rCG23844.1	Ephrin typeA	2.20	0.94

Figure 7: Gene expression at SP and RP in select biological processes. Expression levels of selected number of genes within each process are represented as fold change in graphical ar tabular format. (contd-)

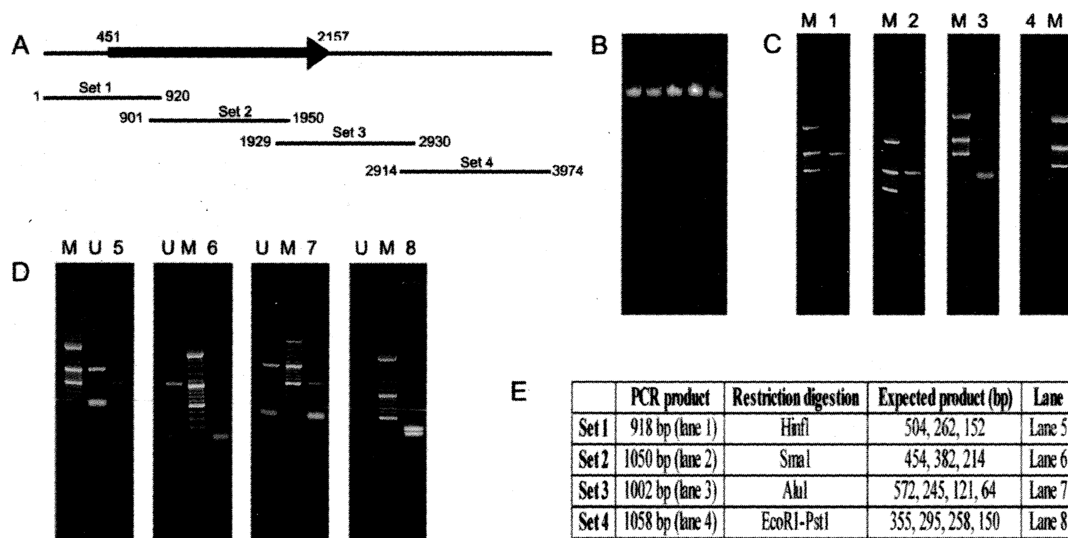
that malfunctioning of Jerky gene can upset the expression as well as temporal and spatial distribution of many target gene products.

4.3. JERKY GENE VARIATIONS IN HUMAN SEIZURE DISORDER

Jerky being a recently evolved gene from tigger transposonal family (Lander, *et al.*, 2001), the gene is undergoing significant molecular evolution as evidenced by the large number of single nucleotide polymorphisms (SNPs) within the human jerky gene (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=8629&chooseRs=all). However, no mutations have been associated with any particular human seizure type. The genetic loci of human jerky gene, 8q24, has shown linkage to childhood absence epilepsy (CAE) and idiopathic generalized epilepsy (Morita, *et al.*, 1998), though never conclusively proven (Morita, *et al.*, 1999). Unlike earlier studies, which scanned for mutations within coding region of human Jerky (JRK) gene, this study screened the entire length of jerky gene including the 5'-UTR, ORF and 3'-UTR to find sequence variations in human subjects with different seizure types.

The study was approved by the institutional ethics committee and blood sample collected from voluntary participants from whom informed consent was obtained. All recruited participants were from similar population strata, confounding misinterpretation of variations observed among case and controls. The samples were alphanumerically coded to mask the identification of patients during research. PCR amplified products of the JRK gene were digested and used for multiple restriction fragment- single strand conformational polymorphism (MRF-SSCP) (**Figure 23**). MRF-SSCP relies on

conformational polymorphism of single stranded DNA with sequence variation and offers advantage of screening a larger amplified region rather than individual smaller amplicons of a large gene in a single reaction by digesting larger DNA into smaller fragments (Lee, *et al.*, 1992; Thongnoppakhun, *et al.*, 2004).



	PCR product	Restriction digestion	Expected product (bp)	Lane
Set 1	918 bp (lane 1)	Hinfl	504, 262, 152	Lane 5
Set 2	1050 bp (lane 2)	Sma1	454, 382, 214	Lane 6
Set 3	1002 bp (lane 3)	Alu1	572, 245, 121, 64	Lane 7
Set 4	1058 bp (lane 4)	EcoR1-Pst1	355, 295, 258, 150	Lane 8

Figure 23: Human Jerky gene SNP screening. **A** PCR plan for amplifying the entire jerky gene using four sets of PCR. **B** Genomic DNA isolated from blood samples.**C** Amplified products from four sets of amplification. Lane 1, 2, 3 and 4 are PCR products from set 1, 2, 3 and 4. **D** PCR amplicons were digested with restriction enzymes for MRF-SSCP SNP screening (lanes 5-8). Lanes M and U indicate the molecular weight marker and the undigested form respectively. **E** Molecular weight of the expected products from digestion of different jerky amplicons and its corresponding lane are indicated in the table.

The digested amplicons of a particular set were denatured and resolved on PAGE under standardized conditions alongwith their respective nondenatured sample, and visualized by silver staining. The discrete banding pattern for each PCR sets was compared among and between the case-samples and the control-samples (**Figure 24**).

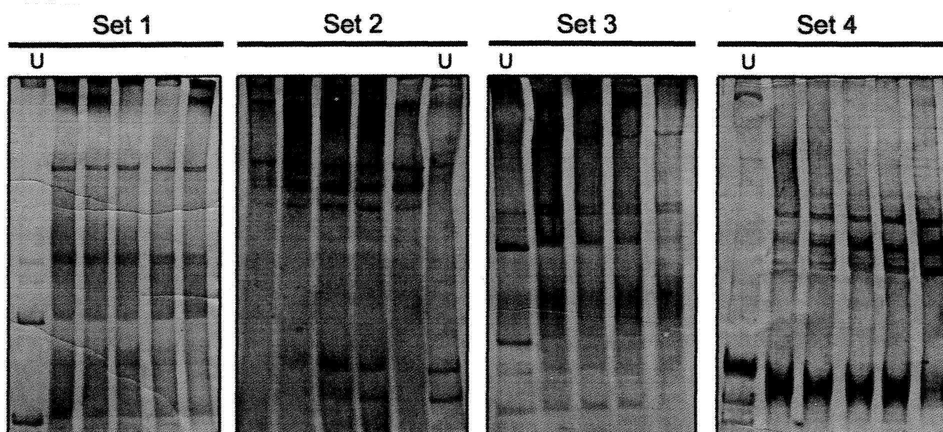
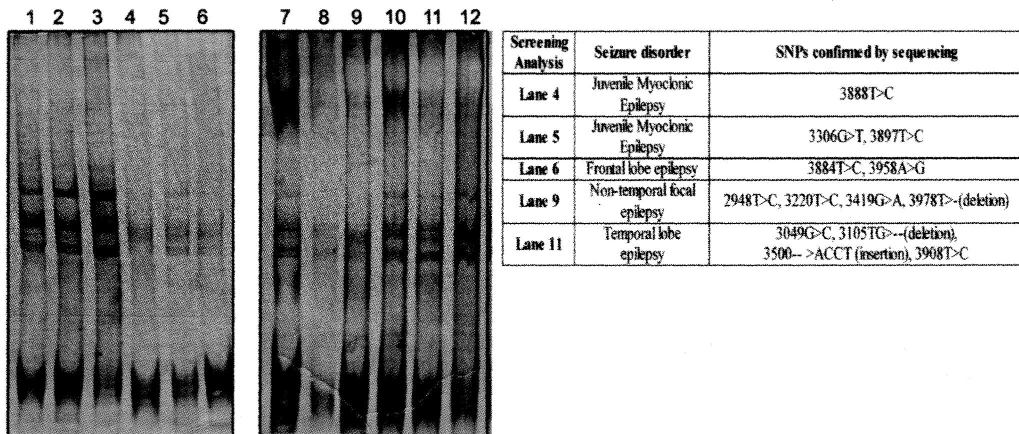


Figure 24: Representative MRF-SSCP gels of all sets of Jerky PCRs. Lane U denotes the nondenatured sample of that particular set. The ssDNA bands appear in the upper region of the gel.

No variation was observed within the set 1, set2 and set 3, which ruled out deleterious SNPs within the coding region of jerky as well as the 5'-UTR. Set 4 mutation screening revealed no variations in any of the non-epileptic controls. However, five epilepsy patients including two juvenile myoclonic epilepsy (JME) patients, one frontal lobe epilepsy (FLE) patient, one non-temporal focal epilepsy patient (FE) and one temporal lobe epilepsy (TLE) patient, showed variation on screening (**Figure 25**). These samples were cloned in pTZ57R/T and sequenced and confirmed for variation. While one of the JME patient has 3888T>C within the 3'-UTR, the other JME patient displayed two SNPs at 3306G>T and 3897T>C. Variations observed in the JME patients didn't cosegregate with the disease phenotype, however occurred in close vicinity within JRK 3'-UTR. The temporal lobe epilepsy patient showed indels (3105TG>-- two base-deletion mutation as well as 3500----/ACCT 4 base-insertion) in addition to base substitutions

3049G>C and 3908T>C. Substitution at 3884T>C and 3958A>G were observed in frontal lobe epilepsy patient. The non-temporal lobe focal epilepsy patient accumulated the SNPs at 2948T>C, 3220T>C, 3419G>A and 3972T>- deletion.



Screening Analysis	Seizure disorder	SNPs confirmed by sequencing
Lane 4	Juvenile Myoclonic Epilepsy	3888T>C
Lane 5	Juvenile Myoclonic Epilepsy	3306G>T, 3897T>C
Lane 6	Frontal lobe epilepsy	3884T>C, 3958A>G
Lane 9	Non-temporal focal epilepsy	2948T>C, 3220T>C, 3419G>A, 3978T>-(deletion)
Lane 11	Temporal lobe epilepsy	3049G>C, 3105TG>--(deletion), 3500-- >ACCT (insertion), 3908T>C

Figure 25: Set 4 samples showing variation on MRF-SSCP (n = 2 to 3) were confirmed by sequencing and the nature of variant was deciphered. Lanes 1, 2, 3 represent three non-epileptic controls; Lanes 4 and 5 are two juvenile epilepsy patients; Lane 6 frontal lobe epilepsy patient; Lanes 7, 8, 9 are three temporal lobe epilepsy patients; Lane 10, 11 and 12 represent patients with Lennox gastaut syndrome, Non-temporal lobe focal epilepsy and primary generalized epilepsy respectively. Banding pattern variation were observed in lanes 4, 5, 6, 9 and 11 and later confirmed by sequencing. Data regarding the lanes showing variations, the corresponding patient's epilepsy type and the nature of variation confirmed by sequencing, are represented in the table.

JRK gene carries neither frameshift nor nonsynonomous SNPs within the protein-coding region. However, the presence of SNPs at the 3'-UTR of JRK gene in JME, FE, TLE and FLE patients is an interesting observation, although none of the SNPs cosegregated with any particular phenotype (**Table 6**).

Patient	Seizure disorder	Age of onset	Family History	Seizure frequency before medication	Pathological features	Response to Drugs	SNP detected
N1	Juvenile Myoclonic Epilepsy	13 years	No			Valproic acid, Tegretal	
N2		15 years	No	Two episodes in two years		Valproic acid	3888T>C
N3		16 years	No	Three episodes in six months		Valproic acid	3306G>T, 3897T>C
N4		18 years	No	Five episodes per month	JME with migraine	Valproic acid	
N7		15 years	No	One episode per month	Nocturnal seizure	Valproic acid, Clobazam	
N11		16 years	No	4-6 per month	JME with psychosis	Valproic acid, Tegretal	
N12		13 years	No	3-6 per month		Valproic acid, Phenytoin	
N13		15 years	No	3-4 per month	Nocturnal seizure	Valproic acid	
N14		15 years	Yes Mother,& Grandmother	2-3 episodes per year		Intractable	
N15			No	2 per year		Valproic acid, Lamotrigine	
N6	Frontal lobe epilepsy	8 years	No	2-10 per day		Intractable; Surgical resectioning carried out	3884T>C, 3958A>G
N4		7 years	No	One episode per month		Clobazam, Carbamazepine	

Table 6: Clinical details of the patients checked for SNP within JRK gene. Details such as age of onset, family history, Seizure frequency, pathological features and drug response were collected and tried to correlate with the SNPs detected. (contd-)

N9	Non-temporal focal epilepsy	10 years	No	Once every 3-6 months		Valproic acid	2948T>C,3220T>C,3419G>A,3978T>-(deletion)
N8	Primary generalized epilepsy	43 years	No	Two episodes of seizure		Phenytoin	
N10	Lennox Gastaut Syndrome	8 months	Yes Elder sister	4-5 per day	Polymorphic seizure, psychomotor retardation	Valproic acid, Clobazam, Lamotrigine	
T16	Temporal lobe epilepsy	14 years	No	5-6 per week	Hippocamapl schlerosis Febrile seizure at 2 ½ years old	Medically refractive and right anterior temporal lobectomy	3049G>C,3105TG>--(deletion) 3500-->ACCT(insertion), 3908T>C
T2		10 years	No	3-4 per month	Hippocamapl schlerosis Febrile seizure at 18 months old	Medically refractive, Right temporal lobectomy	
T3		25 years	No	1-2 per month	Right temporal neocortical cavernoma	Medically refractive, Right temporal neocortical cavernoma lesionectomy	
T4		15 years	No	1-2 per week	Hippocampal schlerosis Febrile seizure at 2 years age	Medically refractive, Left anterior temporal lobectomy with amygdalo hippocampectomy	

Table 6: Clinical details of the patients checked for SNP within JRK gene. Details such as age of onset, family history, Seizure frequency, pathological features and drug response were collected and tried to correlate with the SNPs detected. (contd-)

T8		10 years	No	1-2 per month	Hippocampal schlerosis Febrile seizure at 22 months age	Medically refractive, Right anterior temporal lobectomy with amygdalo hippocampectomy	
T10		5 years	No	5-6 per month	Hippocampal schlerosis Febrile seizure at 18 months age	Medically refractive, Left anterior temporal lobectomy	
T12		1 ½ years	No	Four episodes per month	Hippocamapl schlerosis	Medically refractive, Left anterior temporal lobectomy	
T13		19 years	No		Hippocampal schlerosis Febrile seizure	Medically refractive, Left anterior temporal lobectomy	
T14		10 years	No	1-2 per week	Hippocampal schlerosis	Medically refractive, Right anterior temporal lobectomy	
T15		6 months	No	3-4 per month	Hippocampal schlerosis Febrile seizure at 6months and 1 years of age	Medically refractive, Left anterior temporal lobectomy	

Table 6: Clinical details of the patients checked for SNP within JRK gene. Details such as age of onset, family history, Seizure frequency, pathological features and drug response were collected and tried to correlate with the SNPs detected.

In addition to the SNPs, the presence of 2 bp deletions and 4 bp insertions indicate the JRK 3'-UTR region prone to significant variations. Understandably, these interesting observations warrant a large-scale association study. SNPs within the JRK gene of several epileptic types are in concordance with the shared genetic influence model for complex diseases.

The sequences in the coding region of gene transcripts are tightly conserved since modification in the protein-coding regions of mRNA can have significant functional consequences. Meanwhile, the sequence on the 5'-UTR contains sequences responsible for translation initiation; therefore it has to be more strictly conserved than the 3'-UTR. Evolutionary pressure may thus have taken advantage of the greater degree of freedom of 3'-UTRs to modulate the fate of mRNA molecules (Grzybowska, *et al.*, 2001). It is apparent that the 3'-UTR of RNA can specifically control nuclear export, polyadenylation status, subcellular targeting and the rates of translation and degradation of mRNA (Pesole, *et al.*, 2001). The 3'-UTR may be viewed as a regulatory region that is essential for appropriate expression of many genes. Several human diseases are caused by mutations in 3'-UTR sequences or in trans-acting factors acting at 3'-UTR. For example, defective AU-rich elements (ARE) decay causes stabilization of normally labile mRNAs, resulting in upregulation of their expression and metabolic changes leading to the disease such as mantle cell lymphoma (Rimokh, *et al.*, 1994), chronic arthritis (Kontoyiannis, *et al.*, 1999) and other inflammatory diseases. Myotonic Dystrophy results from inflated number of CTG trinucleotide-repeats in the 3'-UTR of DMPK protein kinase gene (Timchenko, 1999). Stabilization of amyloid precursor protein mRNA is regulated via

hnRNPC binding to its 3'-UTR, which contributes to APP levels in Alzheimer's disease (Rajagopalan, *et al.*, 1998). Translation of mRNAs in neuronal processes can provide a locally renewable source of proteins at sites that may be thousands of micrometers distance from the neuronal cell body. Localized protein synthesis in dendrites is a well-accepted mechanism that can be regulated by neurotransmitters and trophic factors (Steward, 2002). RNAs coding for cytoskeletal proteins (MAP2, Arc), kinases (α-CamKII), Ca^{2+}-binding proteins (dendrin, IP3 receptor typeI) and membrane-bound receptors (glycine receptors, glutamate receptors) have been found localized to distal dendritic regions (Kuhl and Skehel, 1998). Dendritic targeting signals in the 3'-UTR of neuronal RNAs of α-CaMKII, MAP2 and β-actin indicate that sorting of RNA for targeting is mediated by the 3'-UTR (Blichenberg, *et al.*, 1999; Kislauskis, *et al.*, 1994 ; Mayford, *et al.*, 1996). α-CaMKII mRNAs lacking 3'-UTR targeting sequences remained tightly localized in neuronal somata and the levels of α-CaMKII proteins at the postsynaptic densities was significantly reduced than in wildtype mice (Miller, *et al.*, 2002).

The collated variants in the 3'-UTR of JRK identified in the epileptic patients from this study needs to be systematically evaluated of their potential functional consequences. However, we hypothesize that the variations in the 3'-UTR of JRK may affect the localization of jerky transcript and could affect its protein distribution pattern within the neurons. Affected distribution pattern of jerky may result in unequal

translational blockage of mRNAs resulting in biochemical and functional alterations in neurons, which can make it susceptible to hyperactivation.

Chapter 5

SUMMARY AND CONCLUSIONS

The molecular and biochemical basis for epileptogenesis and susceptibility for recurrent seizure has been the focus of epilepsy research for a long time. Effect of gene expression variation accompanying seizure on bringing about long term changes in the neuronal network is understood to be the driving force towards seizure susceptibility. Seizure-associated global gene expression profiling in animal models provide cues to biological processes activated during seizure, however the gene expression variation during initial seizure insult and during early epileptogenesis is not well-studied.

Using pilocarpine model of seizure in rat, the gene expression changes during early phase of epileptogenesis were monitored in this study. The induced animal displayed seizure behavioral pattern reminiscent to tonic-clonic seizure in human and underwent *status epilepticus* for several hours. The global gene expression profile in the hippocampal tissue of pilocarpine-induced seizure rats during *status epilepticus* (seizure period) and during recovery period was monitored using microarray. Following data analysis, list of genes with p-value ≤ 0.05 were calculated for average fold-change at seizure period (SP) and recovery period (RP). The 2953 SP-overexpressed genes

classified on the basis of their involvement in biological processes using PANTER gene expression analysis online tool suggested that seizure triggered substantial overexpression of several genes involved in signal transduction, neuronal activities and signaling cascade. Genes involved in transcriptional and translational regulations as well as protein synthesis machinery were also overexpressed. Significantly, the processes of stress response, apoptosis and neurogenesis which form the keystones in the manifestation of seizure, evidenced overexpression of many genes during status epilepticus, although these processes take place several hours to days following seizure insult. Genes coding for such temporally subsequent processes getting expressed during *status epilepticus* suggest that the system not only responding to the initial events, but also maintains the transcripts for prolonged period until required for translation.

Furthermore, the expression profile of these SP-overexpressed genes were monitored at early stages of epileptogenesis. On sorting the 2953 SP-upregulated genes, 85 genes (3%) were further upregulated ≥2-fold during RP and 859 genes (29%) downregulated ≥2-fold during RP. Rest of the 2009 SP-overexpressed genes (68%) did not undergo significant variations during RP from their base expression level at SP. Notably, none of the biological processes were selectively upregulated or downregulated. But all biological processes had modest number of genes (20-35%) decreasing by over 2-fold and 1-10% increasing by over 2-fold and rest of genes in all processes (55-70%) remained unchanged. The data suggest that a significant number of genes transcribed during *status epilepticus* are maintained even after seizure had passed, probably to be

translated into proteins at a later time point. Likewise, the transcripts required for pathways like apoptosis and neurogenesis overexpressed during seizure, may not be immediately required by the system because those pathways sets in at a later time points in epileptic brain, thereby prevails unchanged until required for translation.

The existence of the overexpressed transcripts in a translationally inactive state temporarily decouples the processes of transcription and translation. The post-transcriptional regulatory steps determines whether the transcribed mRNAs need to be localized to subcellular regions or translated immediately or stored for later use. Role of a RNA-binding protein, which can bring about sequestration of large subset of overexpressed mRNAs was studied.

Jerky, a RNA-binding protein originally identified as a candidate gene for epilepsy and reported to have neuron-specific expression and nucleo-cytoplasmic localization was sufficient reason to probe Jerky's role in global post-transcriptional regulation. Recombinant jerky protein was used to confirm its RNA-binding ability before probing for its role in translational regulation. Jerky binding to large subset of RNA was conspicuous from the RNA-pulldown assay on hippocampal RNA from normal and epileptic mice using the recombinant jerky protein. Among the several transcripts that bound to recombinant jerky protein, few were identified by amplification and sequencing of partial cDNAs generated by differential display RT-PCR technique, to be Nedd4, ATP6, ND4 and Aip2. Sequence comparison between the pulldown-genes revealed neither homology nor distinct RNA-binding domains within these transcripts. Confirmation of the Jerky's sequence-independent RNA-binding property was carried out

by mobility shift assay on in vitro transcribed RNAs of Nedd4, ATP6, ND4, Aip2 as well as Syt 1, Syt 4 and Syt 10. Competitive mobility shift assay performed on gene transcripts reconfirmed the formation of ribonucleoprotein (RNP) complex with that of jerky protein. Since Jerky-RNA interaction was not sequence-dependent, the binding affinity of jerky towards different transcripts was checked by slot blot assay. The kinetics of the binding reactions indicated that jerky had higher affinity towards Syt 4, but similar and comparable affinities with most other transcripts. Moreover, the sequence-independent binding by Jerky to smaller RNAs from sub regions of Syt 1 and Syt 10 with similar affinity as observed with other full-length transcripts, confirmed the non-specific binding nature of jerky protein. Having proven the global RNA-binding property of jerky protein, the role of jerky in translational regulation was studied using rabbit reticulocyte lysate in vitro translation system. Translation efficiency of Syt 1 and Nedd4 showed almost 80% repressed in presence of jerky protein. The translation of Syt 1 was restored back to normal levels on addition of denatured GST-Jerky protein, possibly by the loss of functional RNA-binding property. Similar restoration of translation of Nedd4 was not observed, perhaps due to residual RNA-binding property of denatured protein.

We hypothesize that Jerky may be forming translationally inactive RNP complexes with a large subset of gene transcripts that may have been drastically upregulated during seizure. Jerky may be a part of a combinatorial assembly of RNA-binding proteins, which destines the overexpressed transcripts for localization or translation or degradation. The exact mechanism of effecting the repression by Jerky protein is yet to be deciphered. In the wake of earlier reports that Jerky gene inactivation

leading to seizure, it is speculated that malfunctioning of Jerky gene can upset the expression as well as temporal and spatial distribution of many target gene products, which may trigger seizure pathways.

Although jerk has been suggested as a candidate gene for epilepsy, no mutations have yet been identified within human Jerky gene (JRK). The chromosomal loci of JRK, 8q24, is reported for linkage with childhood absence epilepsy (CAE) and idiopathic generalized epilepsy. Earlier studies attempting to find linkage of sequence variation within coding region of JRK to CAE were inconclusive. SNP database reports large number of polymorphisms within the entire JRK gene. To check on the possibility of identifying sequence variation consistently associated with a particular seizure type, the entire length of JRK gene was amplified using four sets of gene-specific primers, from genomic DNA of patients with different epilepsy types as well as normal controls. This study was approved by the ethics committee and necessary informed consent obtained from voluntary participants. The patients were recruited based on unambiguous clinical diagnosis of particular seizure type. Upon screening of mutations using multiple restriction fragment-single strand conformational polymorphism (MRF-SSCP), sequence variation was detected only in the 3'-UTR of JRK gene of few epileptic patients and subsequently confirmed by sequencing. No similar variation is observed in the JRK gene of normal controls. Although 3'-UTR SNP was noticed in different seizure types, none of the SNPs cosegregated with any particular phenotype. The SNP within the 3'-UTR of JRK identified in the epileptic patients from this study needs to be systematically evaluated for their potential functional consequences and these interesting observations

warrant a large-scale association study. From the study results, it is speculated that the variations in the 3'-UTR of JRK may affect the localization of jerky transcript and could affect its protein distribution pattern within the neurons. Affected distribution pattern of jerky may result in unequal translational blockage of mRNAs resulting in biochemical and functional alterations in neurons, which can make it susceptible to hyperactivation.

The entire study underlines the importance of post-transcriptional regulation of overexpressed gene transcripts during seizure development. Among the several RNA-binding proteins involved in gene expression regulation, the role of jerky in translation regulation possibly in a global-scale is demonstrated. The study also highlights interesting observation of unique SNPs in the 3'-UTR of epileptic patients, possibly having modulatory effect on jerky expression and distribution within the neurons. The regulatory role of jerky in *in vivo* system and isolation of jerky bound RNP complex from brain needs to be further proven. The observation on 3'-UTR justifies large-scale association study on human JRK gene with different seizure types.

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