Title: Deciphering Temporal Gene Expression Dynamics during Epilepsy Development using a Rat Model of Focal Neocortical Epilepsy.

Running title: Temporal changes in Gene Regulation during the Development of Epilepsy.

Authors and Affiliations

Bao-Luen Chang[1,2,3,4]*, Matthew C. Walker[1], Dimitri M. Kullmann[1], Stephanie Schorge[1,5]*

[1] Department of Clinical and Experimental Epilepsy, UCL Queen Square Institute of Neurology, University College London, London, WC1N 3BG, UK

[2] Department of Neurology, Chang Gung Memorial Hospital at Linkou Medical Center, Taoyuan City, 333, Taiwan

[3] School of Medicine, College of Medicine, Chang Gung University, Taoyuan City, Taiwan

[4] Neuroscience Research Center, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan

[5] Department of Neuroscience, Physiology and Pharmacology, University College London, London, WC1E 6BT, UK

*Corresponding author:

Bao-Luen Chang, M.D.,PhD and Stephanie Schorge PhD

Email: m7243@cgmh.org.tw (B.-L. C.); s.schorge@ucl.ac.uk (S.S.)

Department of Neurology, Chang Gung Memorial Hospital-Linkou Medical Center,

No. 5, Fusing St., Gueishan Dist., Taoyuan City 333, Taiwan

Tel.: +(886)-3-328-1200 ext. 8433; Fax: +(886)-3-328-7226

Keywords: Gene expression, Temporal change, Epileptogenesis, Focal neocortical epilepsy, Epilepsy, Stage-dependent.

Number of words in the abstract: 248 words

Number of words in the introduction: 679 words

Number of words in the body of the manuscript: 4000 words

Number of figures: 6

Number of supplemental figures: 3

Number of supplemental tables: 4

Acknowledgements

We thank G. Schiavo (Cancer Research UK) for the gift of tetanus toxin.

Funding

This work was supported by the Medical Research Council, the Royal Society, the Wellcome Trust (WT093205MA), Epilepsy Research UK; the Chang Gung Memorial Hospital, Taiwan (CMRPG3K1021, CMRPG3L0661-2, CMRPG3M1991-2, CMRPG3P0131), and National Science and Technology Council, Taiwan (MOST 108-2314-B-182A-153, MOST 109-2314-B-182A-086, MOST 109-2314-B-182-079, MOST 110-2314-B-182-055).

Conflict of interest statement

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Data Availability

The data that support the findings of this study are made openly and available in this published article and its supplementary information files.

Ethics Approval

All animal experiments were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986, and approved by the University College London ethics committee (Project Licence No: PPL70-7684).

Abstract

Objectives: Epilepsy involves significant changes in neural cells during epileptogenesis. While the molecular mechanism of epileptogenesis remains obscure, changes in gene regulation plays a crucial role in the evolution of epilepsy. This study aimed to compare changes in a subset of specific genes during epilepsy development, focusing on the period after the first spontaneous seizure, to identify critical time windows for targeting different regulators.

Methods: Using a rat model of acquired focal neocortical epilepsy induced by tetanus toxin, we characterised gene expression at acute, subacute and chronic stages (48-72 hours, 2 weeks, and 30 days after first spontaneous seizure, respectively), focusing on genes potentially contribution to epilepsy progression.

Results: We observed dynamic changes in the expression of these gene throughout the period after the first spontaneous seizure. Astrocytic reactions primarily occur early, before epilepsy is well-established. Changes in mammalian target of rapamycin (*Mtor*) and repressor element 1 silencing transcription factor (*Rest*) signalling pathways are highly dynamic and correlated with the progression of epilepsy development. Chemokine C-C-motif ligand (*Ccl2*) is upregulated in the chronic stage indicating activation of the neuroinflammatory pathway. Finally, GABAergic signalling (*Gabra5*) is down-regulated in the late stage after epilepsy is established. Surprisingly, changes in the expression of specific genes are linked to the time since the first seizure, rather than seizure frequency or duration.

Significance: These results suggest that the regulation of specific genes is essentially stage-dependent during the development of epilepsy, highlighting the importance of targeting specific genes at appropriate stages of epilepsy development.

Key bullet points

- Gene expression dynamically changes following the first spontaneous seizure, with distinct regulatory patterns characterized by early astrocytic reactions.
- *mTOR* and *REST* pathways exhibit significant fluctuations as epilepsy progresses, showing significant changes during subacute stages.
- *Ccl2* upregulation in the chronic stage indicates neuroinflammatory pathway activation, while *Gabra5* down-regulation occurs in the late stage, suggesting reduced GABAergic signaling.
- Specific gene expression changes are linked to the time since the first seizure, not seizure frequency or duration.
- Epilepsy development and progression involve stage-specific gene regulation, which is crucial for targeting gene regulation at specific stages for effective epilepsy intervention.

Introduction

Chronic epilepsies comprise more than acute seizures and different epileptogenic mechanisms may be involved in different epileptic seizures and epilepsy syndromes.[1] On the other hand, because different initiating events may lead to similar clinical manifestations (i.e. seizures), it is possible that different epileptic syndromes with diverse epileptogenic mechanisms may share some underlying mechanisms of epilepsy development and progression.

Epileptogenesis is defined as a progressive change from a normal neural network to a hyperexcitable condition that enables the brain to generate spontaneous recurrent seizures. Traditionally, epileptogenesis was considered the process leading to the brain becoming "epileptic" immediately before the first spontaneous seizure.[2] However, it is now understood as a continuously evolving process that extends long beyond the first spontaneous recurrent seizure.[3,4] The emerging concept of epileptogenesis is as a stage which encompasses both the latent period before the first seizure and the period during which initial seizure frequency and severity progressively increases over time.[3-6] The mechanism of epileptogenesis, including epilepsy development and progression, remains obscure but several pathways have been proposed, including dysfunctional or defective ion channels and receptors, immunological and inflammatory pathways, as well as alterations in gene expression.[7,8] The long-term alterations in structure and network excitability are primarily attributed to changes in gene expression that lead to a series of downstream molecular and protein modifications.

Many genes, either by up- or down-regulation, have been proposed to modulate the epileptogenic process in experimental and human studies. Moreover, different stages of the epileptogenic process may involve different genes, as well as individual genes following different expression changes across the time course of epilepsy. For example, *Fos, zif268, Jun, Egr1, Egr4, Homer1, Nurr77* and *Arc* have all been implicated in the early changes of

epileptogenesis.[9,10] *GAD-67, NR1, CaMKII,* and *GluR2* have been shown to be differentially regulated over time and across different areas in tetanus toxin model of motor cortical epilepsy.[11] However, in addition to the early changes, long-term alterations in expression of neurotransmitter receptors may occur in the middle and late phases of epileptogenesis. For instance, repressor element 1-silencing transcription factor (*REST*) and cAMP-responsive element modulator (*CREM*) are transcriptional factors which govern intrinsic homeostasis of neuronal circuits,[12,13] and mammalian target of rapamycin (*mTOR*) is expressed in neurons and astrocytes as a key regulator of translational factors to control protein synthesis related to many functions.[7,14] The overexpression of these genes has been reported in some animal models of epilepsy or in human epileptic tissue.[15] Moreover, dysregulation of ion channels including hyperpolarization-activated cyclic nucleotide-gated channel (*HCN*), GABAergic receptors, and aberrant functions of glial cells have also been identified as potential targets for therapies.[16]

Conflicting findings regarding molecular alterations in epilepsy have been reported in experimental models of epilepsies and some clinical studies. These controversies are likely at least partly because gene expression is dynamically regulated during the development of epilepsy and changes throughout the course of disease. Also, it remains unclear whether specific genes could be common key regulators during epilepsy development. Resolving these questions requires tracking multiple genes through different timepoints correlated with the clinical stages of epilepsy to identify which genes change, and when they might best be targeted.

A vast number of genes are differently regulated in the development of epilepsy. We selected a subset of genes based on different arguments: (1) they have been linked to epileptogenic process; (2) are mechanistically implicated in changes in neuronal function, or (3) have repeatedly been shown to change in epilepsy. In total, we selected 13 candidate genes (Table S1) representing different categories including important transcriptional factors involved in neuronal gene regulation, translational regulators regulating neurodegeneration,

neuron survival, protein synthesis, regulators of synaptic transmission and synaptic plasticity, key synaptic proteins contributing to synaptic vesicle fusion and ultimately impacting synaptic neurotransmitter release, interaction with postsynaptic calmodulin activation, neuronal ion channel genes which play a key role in control of neuronal excitability and rhythmicity, genes supporting inhibitory neurotransmitter signalling, astrocyte function related gene and astrocytic predominantly enzyme, and critical chemokine mediated neuroinflammatory and immune pathways. Here we are interested in elucidating the temporally regulated patterns of the molecular profiles during the development of epilepsy.

Materials and Methods

Experimental design and brain tissue preparation schedule

The tetanus toxin (TeNT) model of focal neocortical epilepsy in rats was used to investigate the gene expression during the established process of epilepsy. Vehicle control and epileptic animals were divided into three timepoints based on time since first spontaneous seizure in epileptic animals (n = 9 in each stage): acute stage (48-72 hours), subacute stage (about 2 weeks), and chronic stage (30 days, Figure 1A). Time matched cortices from vehicle control animals were collected to match timing from epileptic animals (n = 9 in each stage).

Animals

All animal experiments were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986, and approved by the local ethics committee. Adult male Sprague-Dawley rats (6 – 12 weeks old, 260-330 g; Charles River, UK) were housed on a 12-h light/dark cycle (light, 7:00 – 19:00) at a constant temperature (23 ± 1^{o}C) and a humidity of 50 – 60% with free access to food and water. Animals housed individually after surgery.

Surgical procedures

Rats were anaesthetised with isoflurane (2%) and placed in a stereotactic frame (Kopf, USA). Tetanus toxin (15 – 15.6 ng, adjusted for body weight; gift from G. Schiavo, Cancer Research UK) in a volume of 1.0 µl PBS was delivered with digital stereotactic surgery at a rate of 200 nl min^{-1} into layer V of right primary visual cortex (coordinates: 3 mm lateral, 7 mm posterior of bregma, at a depth of 1 mm from pia). After tetanus toxin injection, an electrocorticographic (ECoG) transmitter (A3028E-AA, Open Source Instruments) was implanted subcutaneously for wireless telemetry recordings. The subdural intracranial

recording electrode was positioned in the visual cortex over the tetanus toxin injection site and the reference electrode was implanted in the contralateral frontoparietal cortex. For the rats in the vehicle control group, all the surgical procedures were performed except 1 µl of 0.9% saline was injected instead of tetanus toxin. After surgery, animals were housed in Faraday cages and continuous telemetric ECoG recordings were carried out for the duration of the experiment.

Video-ECoG monitoring, ECoG data acquisition and spontaneous seizures detection

An Internet Protocol (IP) camera time-locked to the wireless ECoG was used to continuously record 24 h/7 days, as previously described.[17] The digitized ECoG was acquired with hardware and software from Open Source Instruments, and was recorded at a sampling rate of 512 Hz, band-pass filtered between 0.3 and 160 Hz, and voltage dynamic range of 20 mV (-13 mV to +7 mV). The ECoG analysis and seizure detection with quantification were visually inspected the entire ECoG dataset by researchers. An ictal episode was defined as an evolution of frequency and amplitude over time with a sudden, repetitive, rhythmic, evolving and stereotypic abnormal electrographic activity with high amplitude (>2 time that of baseline) and a minimum duration of 10 seconds.[18,19]

Immunohistochemistry and cell counts

Animals received tetanus toxin or 0.9% saline co-injected with fluorescent beads (FluoSpheres, 10 µm, yellow/green fluorescent (505/515), Invitrogen) in a final volume of 1 µl into layer V of right visual cortex. One week after injection, brains were collected and sliced at 70 µm and 6 adjacent slices of the peri-injection site were selected as the region of interest for immunochemistry. Staining was performed on free-floating brain sections with the following antibodies: rabbit anti-NeuN (ab177487, Abcam), mouse GFAP (MAB3402, Merk

Millipore), Alexa Fluro 488 donkey anti-rabbit (A-21206, Thermo Fisher Scientific), and Alexa Fluro 555 goat anti-mouse (A-21425, Thermo Fisher Scientific). Images were acquired with Zen 2009 software (Zeiss) on an LSM 710 confocal laser scanning microscope (Zeiss). Both imaging and the subsequent neuron counting were done while blinded to treatment. Volocity 6.0 software was used for colocalization and manual cell counting. A three-dimensional ROI with X: 1000 μm, Y: 1000 μm and Z: approximate 30 μm (10 layers of Z stack) was selected for cell counting (Figure S1). Only clearly visible NeuN and GFAP stained cells confirmed with DAPI staining were assessed.

RNA extraction

A small piece of cortex (3 mm x 3 mm x approximate 1.2 mm thickness of cortex) was microdissected from the area injected epileptic or matched vehicle control animals. RNA was extracted with QIAzol reagent and miRNeasy Mini kit (Qiagen). RNA clean-up was carried out using the RNeasy MinElute Cleanup kit (Qiagen) in a subset of RNA samples until they passed the RNA purity and quality measurement (see supplemental data for detailed methods).

Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in accordance with MIQE guideline.[20] Two-step qRT-PCR was performed and the QuantiTect reverse transcription kit (Qiagen), which includes a genomic DNA-removal step, with 1 μg of RNA from each sample was used according to manufacturer's instructions for reverse transcription. Quantitative PCR was performed by using SYBR green fluorescent staining method with QuantiTect SYBR Green PCR kit (Qiagen) on a Rotor Gene-6000 thermocycler system (Corbett Research Ltd). Each gene was assayed in triplicate and triplicate of non-

template control (NTC) as negative control were run in parallel to the experimental samples in each experiment. Primer design and validation, qRT-PCR data acquisition and analysis carried out as described in detail in supplemental data.

Gene-specific primer sequences are listed in Table S2. Standard curve parameters and PCR reaction efficiency are shown in Table S3.

Statistical analysis

All the data were analysed while researchers were blinded to treatment. The statistical analyses were performed using Prism 6 (GraphPad) or IBM SPSS 24.0.0.0. The D'Agostino-Pearson omnibus normality test was carried out for all data sets. Where appropriate, the statistical significance was assessed using two-tailed unpaired or paired Student's t-test, or two-way ANOVA followed by Tukey or Holm-Sidak post hoc correction for multiple comparisons. The results are presented as mean ± the standard error of the mean (SEM). $P < 0.05$ was considered significant.

Results

Tetanus toxin injection into visual cortex produces a model of focal neocortical epilepsy

We optimized a rat model of acquired occipital cortical epilepsy, as described previously.[17] Microinjection of a single dose tetanus toxin (TeNT) into the visual cortex in rats produced a robust, chronic long-lasting epileptic focus which presented as clear discrete spontaneous seizures (Figure 1B & 1C), characteristic of focal epilepsy in humans. Seizures emerged 3 to 7 days after injection of TeNT without a preceding event of status epilepticus, gradually increased in frequency during the epileptogenic process and reached a frequency plateau approximately 2 weeks after onset. The frequency of seizures decreased thereafter but persisted for at least 5 - 6 weeks. No epileptic discharges or seizures were observed in the vehicle control rats.

The tetanus toxin model of focal neocortical epilepsy does not cause extensive neuronal death

To evaluate whether the TeNT epilepsy model was triggered by toxin induced neuronal death, and to ensure the molecular changes we observed were not caused by cell death or glial proliferation subsequent to the injection of TeNT, immunochemistry was performed in animals 5 days after injection of either TeNT or 0.9% normal saline. NeuN and GFAP cell counting surrounding the site of injection (Figure 2A & 2B) was carried out to calculate the density of neurons and astrocytes. There was no significant difference in the density of neurons between TeNT injected epileptic animals and vehicle control animals (unpaired Student's t test, $p = 0.586$) (Figure 2C, left). This result suggests that TeNT does not lead to significant neuronal loss and confirms that the seizures produced by TeNT are not produced by changes consequent to cell death, which is consistent with other studies.[21,22] Furthermore, GFAP staining showed

no obvious difference in astrocyte activation and proliferation between vehicle control and TeNT injected rats (unpaired Student's t test, $p = 0.064$) (Figure 2B & Figure 2C, right). Minor mechanical tissue disruption caused by the injection needle is inevitable, but this damage was similar in control and TeNT-treated groups. Hence, the mechanical impact of local injection should not interfere with assessment of comparative gene expression between the control and TeNT-treated groups. As a result, the molecular changes during the development of epilepsy identified in this model are likely to mainly result from the epileptogenic process itself instead of from toxin-related neurodegeneration.

Genes are differentially regulated during epilepsy development

To investigate the changing temporal regulation of gene expression throughout the period after first spontaneous seizure, mRNA expression was compared in three different stages during the establishment of epilepsy. As expected, most genes were differently regulated.

In the acute stage (48-72 hours after first seizure), *Gfap*, an astrocyte associated protein, was the only significantly upregulated mRNA ($p = 0.046$, two-way ANOVA) (Figure 3A), while *Bdnf* (brain-derived neurotrophic factor) was the only mRNA significantly down-regulated ($p = 0.035$, two-way ANOVA) (Figure 3B). Several genes were non-significantly increased: *Rest/Nrsf* (repressor element 1-silencing transcription factor/Neuron-restrictive silencer factor), *Mtor* (mammalian target of rapamycin), *Ccl2* (chemokine C-C-motif ligand 2), *Kcna1* (potassium voltage-gated channel subfamily A number 1, Kv1.1), and *Nrgn* (neurogranin) (Figure 3B-E). And the remaining mRNAs were non-significantly decreased: *Adk* (adenosine kinase), *Snap-25* (synaptosomal-associated protein 25), *Crem/IcerII* (cAMP-responsive element modulator), *Gabra5* (Y-aminobutyric acid A receptor, alpha 5), *Hcn1* and *Hcn2* during the acute stage (Figure 3A-E).

During the subacute stage (13-16 days after first seizure), *Gfap*, *Snap-25*, *Rest/Nrsf*, and *Mtor* were all significantly up-regulated (*Gfap*, p = 0.005; Snap-25, p = 0.037; *Rest*, p = 0.001; *Mtor*, p = 0.0003; two-way ANOVA) (Figure 3A-C). No other genes were significantly changed at this timepoint.

By the chronic state (30 days after first seizure), *Ccl2* was strongly up-regulated (p = 0.035, two-way ANOVA) while *Gabra5* was significantly down-regulated (p = 0.024, two-way ANOVA) (Figure 3D). No other genes were significantly changed at this timepoint.

Shared and divergent patterns of gene regulation in the development of epilepsy

Several genes exhibited similar patterns of mRNA expression changes across the three different time points of epilepsy development following the first spontaneous seizure: *Rest/Nrsf*, *Mtor*, *Gfap*, *Snap-25*, *Hcn1* and *Hcn2* all have a similar pattern of regulation during the epileptogenic process, with all showing transient trends upwards during the subacute phase, but dropping back towards control levels as epilepsy becomes established (Figure 4A, Table S4). The changes in mRNA expression of *Mtor*, *Snap-25* and *Hcn1* showed significant increases from acute to subacute stages followed by significantly decreased expression in the chronic stage (Acute vs. Subacute: *Mtor*, p = 0.0023; *Snap-25*, p = 0.0008; *Hcn1*, p = 0.0003; Subacute vs. Chronic: *Mtor*, p < 0.0001; *Snap-25*, p < 0.0001; *Hcn1*, p = 0.0002; two-way ANOVA). While *Rest*, *Gfap* and *Hcn2* were all strongly down-regulated between subacute and chronic periods (*Rest*, p = 0.0006; *Gfap*, p = 0.0103; *Hcn2*, p = 0.0002).

The remaining genes showed variable patterns of change. *Adk*, *Bdnf*, and *Crem* had subtle increases in expression during epilepsy development (Acute vs. Subacute: *Adk*, p = 0.0277; *Bdnf*, p = 0.0087) (Figure 4B, Table S4). While, *Kcna1* and *Nrgn* levels tended to drop (Subacute vs. Chronic: *Kcna1*, p = 0.0238) (Figure 4C, Table S4).

Finally, *Gabra5* and *Ccl2* had mirror images of regulatory patterns (*Ccl2* Subacute vs. Chronic: $p = 0.0028$) (Figure 4D, Table S4).

No apparent correlation between seizure activity and magnitude of change in expression

One potential hypothesis is that during the development of epilepsy seizures drive changes in gene expression, and consequently animals which experience more seizures will have more pronounced changes in mRNA levels. To explore whether the gene expression levels were correlated to seizure activity, we analysed correlations for all selected genes at all stages (Figure S3(A)-(C)). In the acute stage, *Bdnf* and *Gfap* have significant changes in mRNA expression, but the magnitude of change was not obviously correlated with the number of seizures (Figure 5A). We also tested the relationships between *Rest, Mtor, Snap-25, Gfap* which had the most pronounced overexpression in subacute stage, with the number of seizures in each animal experienced during the last week of the subacute phase prior to tissue collection. Again, there were no correlations between the mRNA fold change and the seizure activity during the last week of subacute stage (Figure 5B). For the chronic stage, *Gabra5* and *Ccl2* have significant differences in mRNA expression but no correlations were seen between the change in expression and seizure number during the week prior to tissue collection (Figure 5C). The remaining candidate genes also had no correlations between expression and number of seizures.

It is possible that total time spent in seizures is more important than number of seizures, and so we investigated whether there were any correlations between seizure duration and mRNA expression, however, again no significant associations were observed. These data suggest the selected molecular changes are not driven by seizure frequency or severity, and instead may reflect changes occurring that are not detectable at the EEG level.

***Rest, Mtor* and *Gfap* are regulated as a correlated unit, and *Snap-25* and *Bdnf* are co-regulated**

The distinct patterns of gene regulation suggest that genes respond to different regulatory cues during epilepsy development following the first spontaneous seizure. A cohort of genes, including *Rest, Mtor, Snap-25, Gfap* and *Bdnf*, shared a similar pattern of regulation, with the most pronounced changes occurring during the subacute stage. We validated this pattern using a partial correlation test, controlling for stages of epilepsy progression post-first spontaneous seizure. This confirmed significant correlation in expression of *Rest, Mtor* and *Gfap* (*Rest* vs. *Mtor*: $p < 0.0001$; *Rest* vs. *Gfap*: $p = 0.006$; *Mtor* vs. *Gfap*: $p = 0.016$), and between the expression changes of *Bdnf* and *Snap-25* ($p = 0.01$). In contrast, there were no positive or negative interactions in the comparisons among the remaining genes (Figure 6).

Discussion

Two factors are important for comparing our results to other studies of gene expression during the development of epilepsy. Many studies focus on time since insult, but human clinical studies, seizure onset is potentially more relevant than time from insult, and we have used seizure onset here. Secondly, compared to other commonly used animal models of epilepsy (e.g. kainic acid (K.A.) or pilocarpine models),[23] the epileptogenesis in the TeNT model of focal epilepsy is not triggered by status epilepticus (SE), and is not characterised by extensive cell death. Therefore, changes in gene expression in the TeNT model are mainly attributed to epileptogenic process itself and not to extensive neuronal loss.

Unexpectedly, our data show that the pathological molecular changes of the candidate genes do not correlate to the seizure activity or time spent in seizures. This means that gene regulation during epilepsy development following the first spontaneous seizure may be predominantly disease stage-dependent (or disease phase-dependent) instead of seizure-activity-dependent. This suggests that mechanisms not detected in EEG recordings can drive changes in gene expression associated with epilepsy development and chronic epilepsy. The time-dependent properties of gene regulation can account for, at least in part, the inconsistency in reports assessing different timepoints or models.

For individual genes, our findings offer perspective on what is seen in other studies. For example, increased *Bdnf* mRNA is seen in many *in vitro* experiments and animal models of epilepsy, e.g. K.A., pilocarpine, pentylenetetrazole, kindling, and electroconvulsive shock.[24-26] The up-regulation of *BDNF* is temporary after the onset of seizures and returns to control levels in some published reports,[27-29] but decreased expression of *BDNF* persists in the "undercut" cortical injury model of epilepsy.[30,31] Our data show *Bdnf* mRNA is temporarily down-regulated in the acute phase (48-72 hrs after the onset of first seizure) then return to the control

levels, which is contrary to some studies. A possible explanation for this is that down-regulation of *Bdnf* mRNA is transient, and in models which are associated with neuronal death, the loss of neurons may lead to different regulation of *BDNF* which is implicated in cell death and recovery. Our data suggest that in some forms of epilepsy targeting *BDNF* may not be effective after epilepsy is established.

Similarly changes in *Snap-25* were only seen in the subacute period, and it is not clear whether the transient changes in *Snap-25* are compensatory or pathological, like *BDNF SNAP-25* is not likely to be an effective target after epilepsy is established.

Rest and *Mtor* are co-upregulated during the subacute phase following the first spontaneous seizure but return to near the control levels in the late phase. As with *Snap-25* it is unclear whether *Rest/Nrsf* upregulation is protective or pathological. *REST/NRSF* may have a protective effect by reducing neuronal excitability,[12] and a potential antiepileptic effect of *REST* via repressing *BDNF* expression has also been described.[32] In contrast, the upregulation of *REST/NRSF* may contribute to epileptogenesis by down-regulating *HCN1*.[13,33] Our data suggest interventions targeting *REST/NRSF* would only benefit during the subacute stage following the first spontaneous seizure. The changes in *Rest/Nrsf* regulation are also strongly correlated with those of *Mtor*. A recent study revealed that increased expression of *REST/NRSF* lowers *TSC2* (tuberin) levels in the pheochromocytoma neuronal cell line[34] raising the possibility that changes in *REST/NRSF* can affect expression of the *mTOR* signalling pathway and potentially *mTOR* itself.

Accumulating evidence suggests that *mTOR* dysregulation is not only seen in patients with mTORopathies (e.g. tuberous sclerosis complex and cortical malformations), but it also is associated with many acquired epilepsies including infantile spasms, post-traumatic epilepsy, mTLE, and hypoxia-induced seizures etc..[35,36] Biphasic activation of the *mTOR* pathway

immediately after SE (peak at 3-6 hrs) and a second distinct rise during 5-10 days after SE has been shown in the K.A. model of temporal lobe epilepsy (TLE).[37] We found a striking overexpression of *Mtor* in the subacute stage following the first spontaneous seizure in our non-SE initiated and non-lesional neocortical model of epilepsy, expanding the role of *mTOR* in different epilepsies. Interventions with Rapamycin, an *mTORC1* inhibitor, have been reported to be protective in some models.[37-39] However, while administration of rapamycin does not abolish epileptogenesis, it may still inconsistently suppress seizure activity across different animal models.[40-42] Furthermore, clinical trials in patients with TSC and experimental preclinical studies also showed paradoxical exacerbation of epilepsy with *mTOR* inhibition treatment.[43-45] These inconsistent findings may reflect pro- and anti-epileptic effects at different time points of epilepsy. Our data suggest that the subacute phase rather than late phase of epilepsy development may be a critical time point for treatments targeting *mTOR* expression.

Finally, we find *Gfap* is also co-upregulated in the subacute phase following the first spontaneous seizure. Transient increases of *Gfap* mRNA and protein in the early phase of electrically-induced and PTZ-induced seizures have been reported in literature.[46] We have shown that there is no difference in the minor mechanical tissue disruption between normal saline and TeNT-injected animals, as well as no toxin-induced astrogliosis in the TeNT model of occipital lobe epilepsy. Thus, the changes we see in *Gfap* expression can be considered as astrocytosis in response to the epileptogenic process following the first spontaneous seizure. In this model there was marked reactive overexpression of *Gfap* mRNA predominantly in the acute and subacute stages of epilepsy development with a trend towards returning to control levels later on. This evidence suggests that astrocytes may play a role during the epileptogenic process, but may have relatively less influence after epilepsy has become established. Our data show a strong co-regulatory behaviour among *Rest, Mtor* and *Gfap* during the development of

epilepsy, consistent with a shared co-regulatory network involving *Rest*, *Mtor* and *Gfap* that may play a central role during epilepsy development.

The late phase is most likely of clinical relevance for new treatments and we find two genes with persistent changes in the chronic phase: *Ccl2* and *Gabra5*.

Clinical evidence suggests that *CCL2* (also known as chemokine monocyte chemotactic protein-1, *MCP1*) is overexpressed in epilepsy patients with focal cortical dysplasia,[47] tuberous sclerosis, and TLE.[48-51] Moreover, *CCL2* is upregulated in experimental models, such as pilocarpine[52,53], K.A.[54], and angular bundle stilumation models.[51] We found a significant elevation of *Ccl2* in the chronic stage, only after epilepsy is established. In addition, microglial activation has been shown in the hippocampal tetanus toxin model.[55] This indicates this neuroinflammatory pathway may present an opportunity for treating established epilepsy.

Similarly, the expression of *Gabra5* remained near control levels until the chronic stage and underwent a dramatic decline in the late phase. As the $GABA_A$ α5 subunit or δ subunit is responsible for tonic inhibition,[56] our findings imply that tonic inhibition may be reduced after epilepsy has been established, and represent a therapeutic possibility in established epilepsy.

The neuropathological alterations of neuronal loss and gliosis are absence in the cortex of the TeNT model of focal neocortical epilepsy.[57] However, hippocampal sclerosis can be detected in some patients with chronic extratemporal epilepsy, and hippocampal atrophy has also been observed in focal neocortical epilepsy induced by TeNT injection in the primary motor cortex.[58] It remains unclear whether the remote effect of hippocampal atrophy might influence gene expression changes in the neocortex tissue obtained from neocortical epilepsy. While neuronal loss in hippocampus is considered a potential mechanism in mesial temporal lobe epilepsy, it is less commonly observed in the cortex of neocortical epilepsy. Previous studies from human tissue have further revealed the absence of neuropathological alterations

in the epileptogenic cortex, indicating that chronic seizure activity does not induce cortical tissue damage in focal neocortical dysplasia and cryptogenic neocortical epilepsies.[59]

This study has several limitations. First, the use of Q-PCR to detect a focused set of candidate genes rather than employing broader approaches such as genome-wide sequencing. While Q-PCR allowed us to study specific genes linked to or implicated in the epileptogenic process in detail, it may not capture the full spectrum of gene expression changes occurring during epilepsy development. Sequencing methods, which offer a more comprehensive analysis, could potentially reveal novel therapeutic targets and enable comparisons with large datasets from existing studies. Future research should consider incorporating genome-wide sequencing to provide a broader and more comprehensive understanding of the genetic changes involved in epilepsy. Additionally, the use of only male animals may necessitate replication in female rats to determine whether the changes in gene expression during epilepsy development and progression are consistent across genders. Nevertheless, our findings still highlight a potential new avenue in targeting specific regulators at different clinically relevant time windows.

Conclusions

In summary, our work highlights the importance of targeting different genes at specific time points to modify the progression and treatment of epilepsy. Notably, we find that changes in gene regulation following the first spontaneous seizure do not correlate with seizure activity, suggesting an underlying mechanism that is not detected by EEG is driving these alterations.

Statements & Declarations

Acknowledgements

We thank G. Schiavo (Cancer Research UK) for the gift of tetanus toxin.

This work was supported by the Medical Research Council, the Royal Society, the Wellcome Trust (WT093205MA), Epilepsy Research UK; the Chang Gung Memorial Hospital, Taiwan (CMRPG3K1021, CMRPG3L0661-2, CMRPG3M1991-2, CMRPG3P0131), and National Science and Technology Council, Taiwan (MOST 108-2314-B-182A-153, MOST 109-2314-B-182A-086, MOST 109-2314-B-182-079, MOST 110-2314-B-182-055).

Competing interests

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Author Contributions

Bao-Luen Chang: Conceptualization, Methodology, Experimentation, Data collection, analysis and interpretation, Writing-Original Draft, Writing-Review & Editing, Visualization, Funding acquisition.

Stephanie Schorge: Conceptualization, Resources, Data interpretation, Writing-Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Matthew C. Walker: Conceptualization, Writing-Review & Editing, Supervision.

Dimitri M. Kullmann: Conceptualization, Writing-Review & Editing, Supervision.

ORCID of the authors:

Bao-Luen Chang: ORCID 0000-0002-4547-6155

Stephanie Schorge: ORCID 0000-0003-1541-5148

Matthew C. Walker: ORCID 0000-0002-0812-0352

Dimitri M. Kullmann: ORICD 0000-0001-6696-3545

Data Availability

The data that support the findings of this study are made openly and available in this published article and its supplementary information files.

Ethics Approval

All animal experiments were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986, and approved by the University College London ethics committee (Project Licence No: PPL70-7684).

References

1. Vreugdenhil M, Hack SP, Draguhn A, et al. Tetanus toxin induces long-term changes in excitation and inhibition in the rat hippocampal CA1 area. *Neuroscience* 2002;114:983-994.

2. Sloviter RS, Bumanglag AV. Defining "epileptogenesis" and identifying "antiepileptogenic targets" in animal models of acquired temporal lobe epilepsy is not as simple as it might seem. *Neuropharmacology* 2013;69:3-15.

3. Pitkanen A, Engel J, Jr. Past and present definitions of epileptogenesis and its biomarkers. *Neurotherapeutics* 2014;11:231-241.

4. Sloviter RS. Epileptogenesis meets Occam's Razor. *Curr Opin Pharmacol* 2017;35:105-110.

5. Williams PA, White AM, Clark S, et al. Development of spontaneous recurrent seizures after kainate-induced status epilepticus. *J Neurosci* 2009;29:2103-2112.

6. Kadam SD, White AM, Staley KJ, et al. Continuous electroencephalographic monitoring with radio-telemetry in a rat model of perinatal hypoxia-ischemia reveals progressive post-stroke epilepsy. *J Neurosci* 2010;30:404-415.

7. Goldberg EM, Coulter DA. Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction. *Nat Rev Neurosci* 2013;14:337-349.

8. Loscher W, Klitgaard H, Twyman RE, et al. New avenues for anti-epileptic drug discovery and development. *Nat Rev Drug Discov* 2013;12:757-776.

9. Rakhade SN, Jensen FE. Epileptogenesis in the immature brain: emerging mechanisms. *Nat Rev Neurol* 2009;5:380-391.

10. Liang F, Jones EG. Zif268 and Fos-like immunoreactivity in tetanus toxin-induced epilepsy: reciprocal changes in the epileptic focus and the surround. *Brain Res* 1997;778:281-292.

11. Liang F, Jones EG. Differential and time-dependent changes in gene expression for type II calcium/calmodulin-dependent protein kinase, 67 kDa glutamic acid decarboxylase, and glutamate receptor subunits in tetanus toxin-induced focal epilepsy. *J Neurosci* 1997;17:2168-2180.

12. Pozzi D, Lignani G, Ferrea E, et al. REST/NRSF-mediated intrinsic homeostasis protects neuronal networks from hyperexcitability. *EMBO J* 2013;32:2994-3007.

13. McClelland S, Brennan GP, Dube C, et al. The transcription factor NRSF contributes to epileptogenesis by selective repression of a subset of target genes. *Elife* 2014;3:e01267.

14. Vezzani A. Before epilepsy unfolds: finding the epileptogenesis switch. *Nat Med* 2012;18:1626-1627.

15. Wong M. Mechanisms of epileptogenesis in tuberous sclerosis complex and related malformations of cortical development with abnormal glioneuronal proliferation. *Epilepsia* 2008;49:8-21.

16. Gorter JA, van Vliet EA, Aronica E, et al. Potential new antiepileptogenic targets indicated by microarray analysis in a rat model for temporal lobe epilepsy. *J Neurosci* 2006;26:11083-11110.

17. Chang BL, Leite M, Snowball A, et al. Semiology, clustering, periodicity and natural history of seizures in an experimental occipital cortical epilepsy model. *Dis Model Mech* 2018.

18. Fisher RS, Scharfman HE, Decurtis M. How Can We Identify Ictal and Interictal Abnormal Activity? *Issues in Clinical Epileptology: A View from the Bench* 2014;813:3-23.

19. Pitkanen A, Kharatishvili I, Narkilahti S, et al. Administration of diazepam during status epilepticus reduces development and severity of epilepsy in rat. *Epilepsy Res* 2005;63:27-42.

20. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;55:611-622.

21. Jefferys JG, Evans BJ, Hughes SA, et al. Neuropathology of the chronic epileptic syndrome induced by intrahippocampal tetanus toxin in rat: preservation of pyramidal cells and incidence of dark cells. *Neuropathol Appl Neurobiol* 1992;18:53-70.

22. Jiruska P, Finnerty GT, Powell AD, et al. Epileptic high-frequency network activity in a model of non-lesional temporal lobe epilepsy. *Brain* 2010;133:1380-1390.

23. Reddy DS, Kuruba R. Experimental models of status epilepticus and neuronal injury for evaluation of therapeutic interventions. *Int J Mol Sci* 2013;14:18284-18318.

24. Binder DK. Role of BDNF in Animal Models of Epilepsy. *Encyclopedia of Basic Epilepsy Research, Vols 1-3* 2009:936-941.

25. Binder DK, Croll SD, Gall CM, et al. BDNF and epilepsy: too much of a good thing? *Trends Neurosci* 2001;24:47-53.

26. Liang F, Le LD, Jones EG. Reciprocal up- and down-regulation of BDNF mRNA in tetanus toxin-induced epileptic focus and inhibitory surround in cerebral cortex. *Cereb Cortex* 1998;8:481-491.

27. Chavko M, Nadi NS, Keyser DO. Activation of BDNF mRNA and protein after seizures in hyperbaric oxygen: implications for sensitization to seizures in re-exposures. *Neurochem Res* 2002;27:1649-1653.

28. Gall CM. Seizure-induced changes in neurotrophin expression: implications for epilepsy. *Exp Neurol* 1993;124:150-166.

29. Nawa H, Carnahan J, Gall C. BDNF protein measured by a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels. *Eur J Neurosci* 1995;7:1527-1535.

30. Prince DA, Parada I, Scalise K, et al. Epilepsy following cortical injury: cellular and molecular mechanisms as targets for potential prophylaxis. *Epilepsia* 2009;50 Suppl 2:30-40.

31. Li H, McDonald W, Parada I, et al. Targets for preventing epilepsy following cortical injury. *Neurosci Lett* 2011;497:172-176.

32. Garriga-Canut M, Schoenike B, Qazi R, et al. 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nat Neurosci* 2006;9:1382-1387.

575 33. McClelland S, Flynn C, Dube C, et al. Neuron-restrictive silencer factor-mediated
576 hyperpolarization-activated cyclic nucleotide gated channelopathy in experimental
577 temporal lobe epilepsy. *Ann Neurol* 2011;70:454-464.

578 34. Tomasoni R, Negrini S, Fiordaliso S, et al. A signaling loop of REST, TSC2 and beta-
579 catenin governs proliferation and function of PC12 neural cells. *J Cell Sci* 2011;124:3174-
580 3186.

581 35. Meng XF, Yu JT, Song JH, et al. Role of the mTOR signaling pathway in epilepsy. *J Neurol*
582 *Sci* 2013;332:4-15.

583 36. Ryther RC, Wong M. Mammalian target of rapamycin (mTOR) inhibition: potential for
584 antiseizure, antiepileptogenic, and epileptostatic therapy. *Curr Neurol Neurosci Rep*
585 2012;12:410-418.

586 37. Zeng LH, Rensing NR, Wong M. The mammalian target of rapamycin signaling pathway
587 mediates epileptogenesis in a model of temporal lobe epilepsy. *J Neurosci* 2009;29:6964-
588 6972.

589 38. Talos DM, Sun H, Zhou X, et al. The interaction between early life epilepsy and autistic-
590 like behavioral consequences: a role for the mammalian target of rapamycin (mTOR)
591 pathway. *PLoS One* 2012;7:e35885.

592 39. Huang X, Zhang H, Yang J, et al. Pharmacological inhibition of the mammalian target of
593 rapamycin pathway suppresses acquired epilepsy. *Neurobiol Dis* 2010;40:193-199.

594 40. Buckmaster PS, Lew FH. Rapamycin suppresses mossy fiber sprouting but not seizure
595 frequency in a mouse model of temporal lobe epilepsy. *J Neurosci* 2011;31:2337-2347.

596 41. Sliwa A, Plucinska G, Bednarczyk J, et al. Post-treatment with rapamycin does not prevent
597 epileptogenesis in the amygdala stimulation model of temporal lobe epilepsy. *Neurosci*
598 *Lett* 2012;509:105-109.

599 42. Drion CM, Borm LE, Kooijman L, et al. Effects of rapamycin and curcumin treatment on
600 the development of epilepsy after electrically induced status epilepticus in rats. *Epilepsia*
601 2016;57:688-697.

602 43. French JA, Lawson JA, Yapici Z, et al. Adjunctive everolimus therapy for treatment-
603 resistant focal-onset seizures associated with tuberous sclerosis (EXIST-3): a phase 3,
604 randomised, double-blind, placebo-controlled study. *Lancet* 2016;388:2153-2163.

605 44. Muncy J, Butler IJ, Koenig MK. Rapamycin reduces seizure frequency in tuberous sclerosis
606 complex. *J Child Neurol* 2009;24:477.

607 45. Zeng LH, McDaniel S, Rensing NR, et al. Regulation of cell death and epileptogenesis by
608 the mammalian target of rapamycin (mTOR): a double-edged sword? *Cell Cycle*
609 2010;9:2281-2285.

610 46. Torre ER, Lothman E, Steward O. Glial response to neuronal activity: GFAP-mRNA and
611 protein levels are transiently increased in the hippocampus after seizures. *Brain Res*
612 1993;631:256-264.

613 47. Iyer A, Zurolo E, Spliet WG, et al. Evaluation of the innate and adaptive immunity in type
614 I and type II focal cortical dysplasias. *Epilepsia* 2010;51:1763-1773.

615 48. Wu Y, Wang X, Mo X, et al. Expression of monocyte chemoattractant protein-1 in brain
616 tissue of patients with intractable epilepsy. *Clin Neuropathol* 2008;27:55-63.

617 49. Andjelkovic AV, Pachter JS. Characterization of binding sites for chemokines MCP-1 and
618 MIP-1alpha on human brain microvessels. *J Neurochem* 2000;75:1898-1906.

619 50. Vezzani A, French J, Bartfai T, et al. The role of inflammation in epilepsy. *Nat Rev Neurol*
620 2011;7:31-40.

621 51. Broekaart DWM, Anink JJ, Baayen JC, et al. Activation of the innate immune system is
622 evident throughout epileptogenesis and is associated with blood-brain barrier dysfunction
623 and seizure progression. *Epilepsia* 2018;59:1931-1944.

624 52. Foresti ML, Arisi GM, Katki K, et al. Chemokine CCL2 and its receptor CCR2 are
625 increased in the hippocampus following pilocarpine-induced status epilepticus. *J*
626 *Neuroinflammation* 2009;6:40.

627 53. Xu JH, Long L, Tang YC, et al. CCR3, CCR2A and macrophage inflammatory protein
628 (MIP)-1a, monocyte chemotactic protein-1 (MCP-1) in the mouse hippocampus during
629 and after pilocarpine-induced status epilepticus (PISE). *Neuropathol Appl Neurobiol*
630 2009;35:496-514.

631 54. Bozzi Y, Caleo M. Epilepsy, Seizures, and Inflammation: Role of the C-C Motif Ligand 2
632 Chemokine. *DNA Cell Biol* 2016;35:257-260.

633 55. Shaw JA, Perry VH, Mellanby J. Tetanus toxin-induced seizures cause microglial
634 activation in rat hippocampus. *Neurosci Lett* 1990;120:66-69.

635 56. Bonin RP, Zurek AA, Yu J, et al. Hyperpolarization-activated current (In) is reduced in
636 hippocampal neurons from Gabra5-/- mice. *PLoS One* 2013;8:e58679.

637 57. Mainardi M, Pietrasanta M, Vannini E, et al. Tetanus neurotoxin-induced epilepsy in mouse
638 visual cortex. *Epilepsia* 2012;53:e132-136.

639 58. Otte WM, Bielefeld P, Dijkhuizen RM, et al. Focal neocortical epilepsy affects
640 hippocampal volume, shape, and structural integrity: a longitudinal MRI and
641 immunohistochemistry study in a rat model. *Epilepsia* 2012;53:1264-1273.

642 59. Rossini L, Garbelli R, Gnatkovsky V, et al. Seizure activity per se does not induce tissue
643 damage markers in human neocortical focal epilepsy. *Ann Neurol* 2017;82:331-341.

644

Figure Legends

Figure 1. Experimental design and representative spontaneous focal neocortical seizures

(A) A schematic representation of the overall procedure, showing how brain tissue was obtained from three different stages according to the onset of the first spontaneous seizure in epileptic rats, and their corresponding vehicle control animals. (B) A representative ECoG trace showing an ictal event from a tetanus toxin injected rat, showing a long-lasting seizure and the evoluation of epileptic activity with different frequency, amplitude, and waveforms. Panels 1-4 expand different regions of the event showing the evolving activity. (C) Raster plots of all seizures from the occurrence of the first spontaneous seizure over the entire recording period for individual animals of acute, subacute, and chronic groups (n = 9 animals in each group).

Figure 2. Immunohistochemistry and volumetric cell count around the region of visual cortex injection

(A) A representative immunofluorescence image of a brain slice showing the injection targeting layer V of primary visual cortex and the area (white square) selected for cell counting. (B) Neuronal and astrocytic cell counting around the site of injection and the morphology of astrocytes. There is no obvious tissue disruption or other apparent difference surrounding the injection tract between the 0.9% saline injected vehicle control rats and the TeNT injected epileptic rats. NeuN, a neuronal marker (green), and GFAP, an astrocyte marker (red). (Scale bar: 150 μm). (C) Average density of neurons and astrocytes surrounding the zone of 0.9% saline or TeNT injection. There is no significant difference in the density of both neurons and astrocytes between control and TeNT-treated epileptic animals. Cell counting was carried while blinded to treatment. (n = 9 sections from 3 animals in each group). Data are presented as mean ± SEM.

669

670 **Figure 3. mRNA quanitification of genes of interest in different stages of epilepsy**
671 **development**

672 (A) – (E) These selected candidate genes have been linked to the development of epilepsy in
673 different crucial neuronal functional pathways or have been repeatedly shown to change in
674 epilepsy. (A) *Gfap* is significantly up-regulated during both acute and subacute periods,
675 whereas there is no clear mRNA fold difference compared with control in *Adk*, an astrocyte-
676 specific enzyme across the whole period of epilepsy. Acute: *Gfap*, p = 0.046; *Adk*, p = 0.240.
677 Sub-Acute: *Gfap*, p = 0.005; *Adk*, p = 0.769. Chronic: *Gfap*, p = 0.934; *Adk*, p = 0.99.

678 (B) The SNARE gene, *Snap-25* has strong overexpression in subacute stage only. *Bdnf* is a
679 neurotrophin gene showing significant down-regulation in acute phase followed by a tendency
680 of up-regulation. Acute: *Snap-25*, p = 0.562; *Bdnf*, p = 0.035; *Nrgn*, p = 0.991. Sub-Acute:
681 *Snap-25*, p = 0.037; *Bdnf*, p = 0.974; *Nrgn*, p = 0.819. Chronic: *Snap-25*, p = 0.130; *Bdnf*, p =
682 0.780; *Nrgn*, p = 0.420.

683 (C) The mRNA level of *Crem/IcerII* is mildly down-regulated, whilst *Rest/Nrsf* is up-regulated
684 over the entire period of epileptogenic process and revealing a significant increase of *Rest/Nrsf*
685 in subacute period. *Mtor* which not only contributes to gene transcription but also protein
686 translation, displays remarkable overexpression in subacute stage. Acute: *Crem/IcerII*, p =
687 0.050; *Rest*, p = 0.187; *Mtor*, p = 0.628. Sub-Acute: *Crem/IcerII*, p = 0.092; *Rest*, p = 0.001;
688 *Mtor*, p = 0.0003. Chronic: *Crem/IcerII*, p = 0.310; *Rest/Nrsf*, p = 0.99; *Mtor*, p = 0.279.

689 (D) *Ccl2*, a chemokine gene, exhibits a rise over all stages and is significantly up-regulated in
690 chronic stage only. In contrast, *Gabra5* has obvious hypo-expression in chronic phase. Acute:
691 *Gabra5*, p = 0.121; *Ccl2*, p = 0.949. Sub-Acute: *Gabra5*, p = 0.658; *Ccl2*, p = 0.825. Chronic:
692 *Gabra5*, p = 0.024; *Ccl2*, p = 0.035.

(E) The neuronal ion channel genes, *Kcna1* and *Hcn1* and *Hcn2*, all have a tendency of increased expression in subacute stage, but the changes are mild and variable during the development of epilepsy. Acute: *Kcna1*, p = 0.464; *Hcn1*, p = 0.114; *Hcn2*, p = 0.969. Sub-Acute: *Kcna1*, p = 0.094; *Hcn1*, p = 0.138; *Hcn2*, p = 0.192. Chronic: *Kcna1*, p = 0.959; *Hcn1*, p = 0.085; *Hcn2*, p = 0.055.

In each comparison n = 9 animals in each group; matched measures two-way ANOVA followed by Sidak correction for multiple comparisons, *p < 0.05, **p < 0.01, ***p < 0.001. Data are shown as mean ± SEM.

Figure 4. Patterns of gene regulation during epilepsy development in the TeNT model of epilepsy

(A) – (D) Trends comparing the patterns of gene regulation over time in different stages of the development of epilepsy. The mRNA expression changes are presented as fold difference (FC) and the mRNA value in control group is "1". (A) *Rest, Mtor, Gfap, Snap-25, Hcn1* and *Hcn2* increased expression levels from acute to subacute stages followed by decreased expression from subacute to chronic stages. (B) *Adk, Bdnf* and *Crem* have a mild rising tendency from acute to subacute stages then reach a plateau. (C) *Kcna1* and *Nrgn* have a decline trend from acute to chronic stages. (D) *Gabra5* was slightly down-regulated, whilst *Ccl2* was up-regulated during the all stages and both have an inflection point at subacute stage. Data are shown as mean of mRNA fold change (FC).

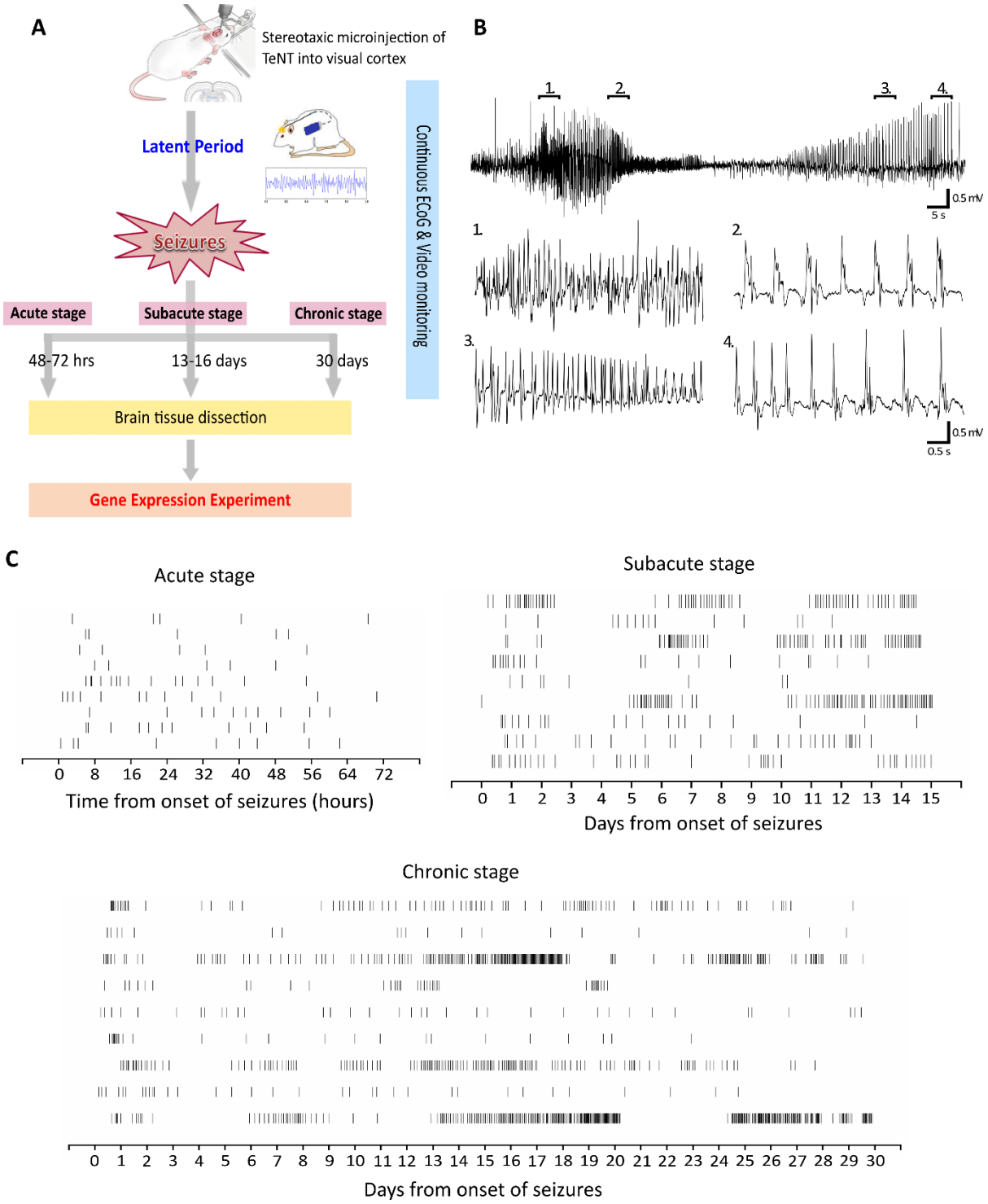
Figure 5. Correlation between expression of selected genes and seizure activity

(A) The correlation coefficient analysis between mRNA fold changes and the log (number of total seizures (sz) + 1) for *Bdnf* and *Gfap* in acute stage. (B) The relationships of mRNA expression for *Rest, Mtor, Snap-25, Gfap* and the log (number of sz during the last week of subacute stage + 1). (C) Plot of *Gabra5* and *Ccl2* gene expression and the log (number of sz in the last week of chronic period +1). (n = 9 animals in each group; Pearson correlation coefficients).

Figure 6. The relationships between expression of different genes

The gene-gene correlations showing that there was a significant positive correlation in mRNA expression of *Rest* vs. *Mtor* ($p < 0.0001$), *Rest* vs. *Gfap* ($p = 0.006$), *Mtor* vs. *Gfap* ($p = 0.016$), and *Snap-25* vs. *Bdnf* ($p = 0.01$). (n = 27 animals; Partial correlations and stages of epilepsy development as a control variable followed by False Discovery Rate correction with Benjamini, Krieger and Yekutieli method for multiple comparisons).

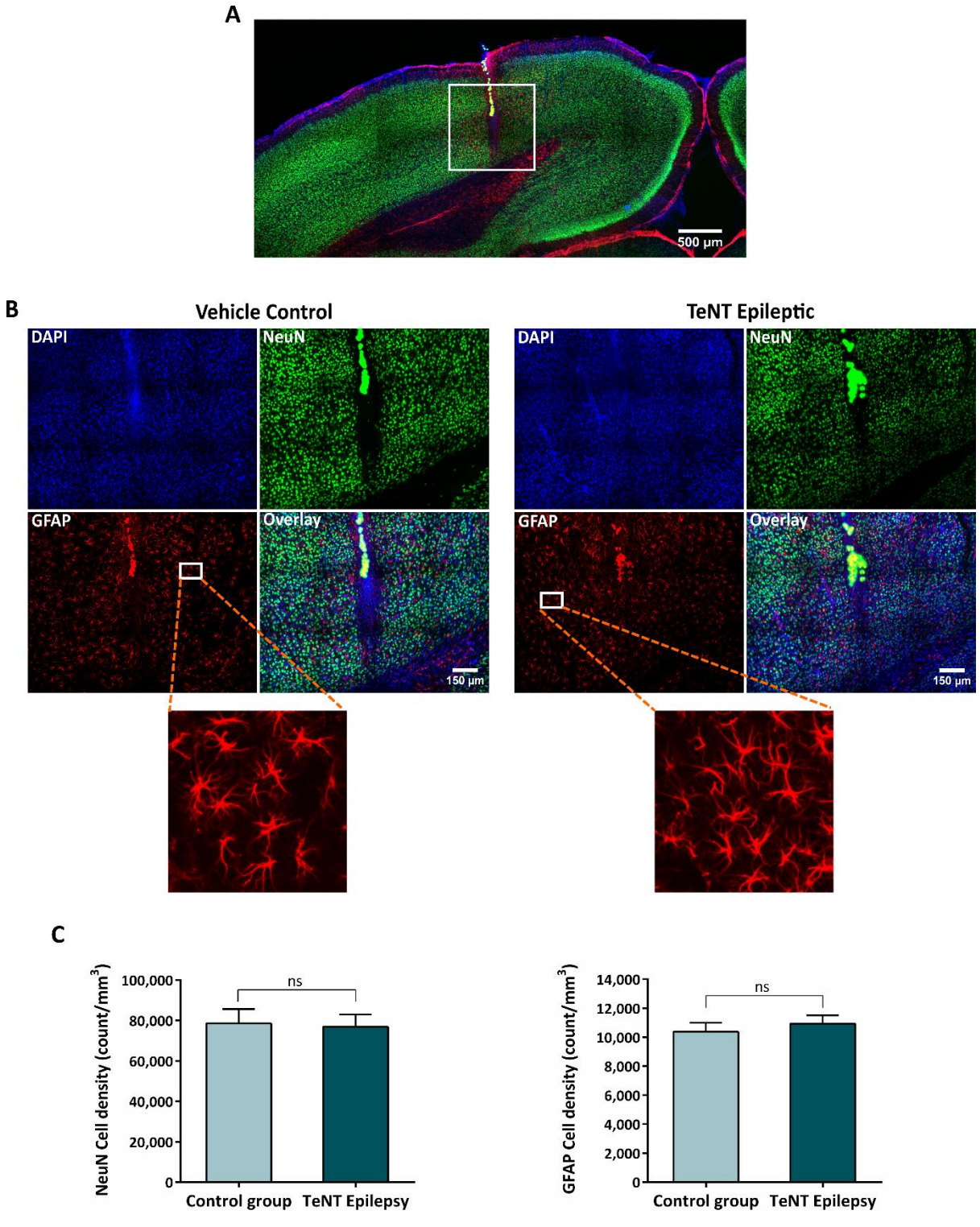
729 Fig 1



730

731

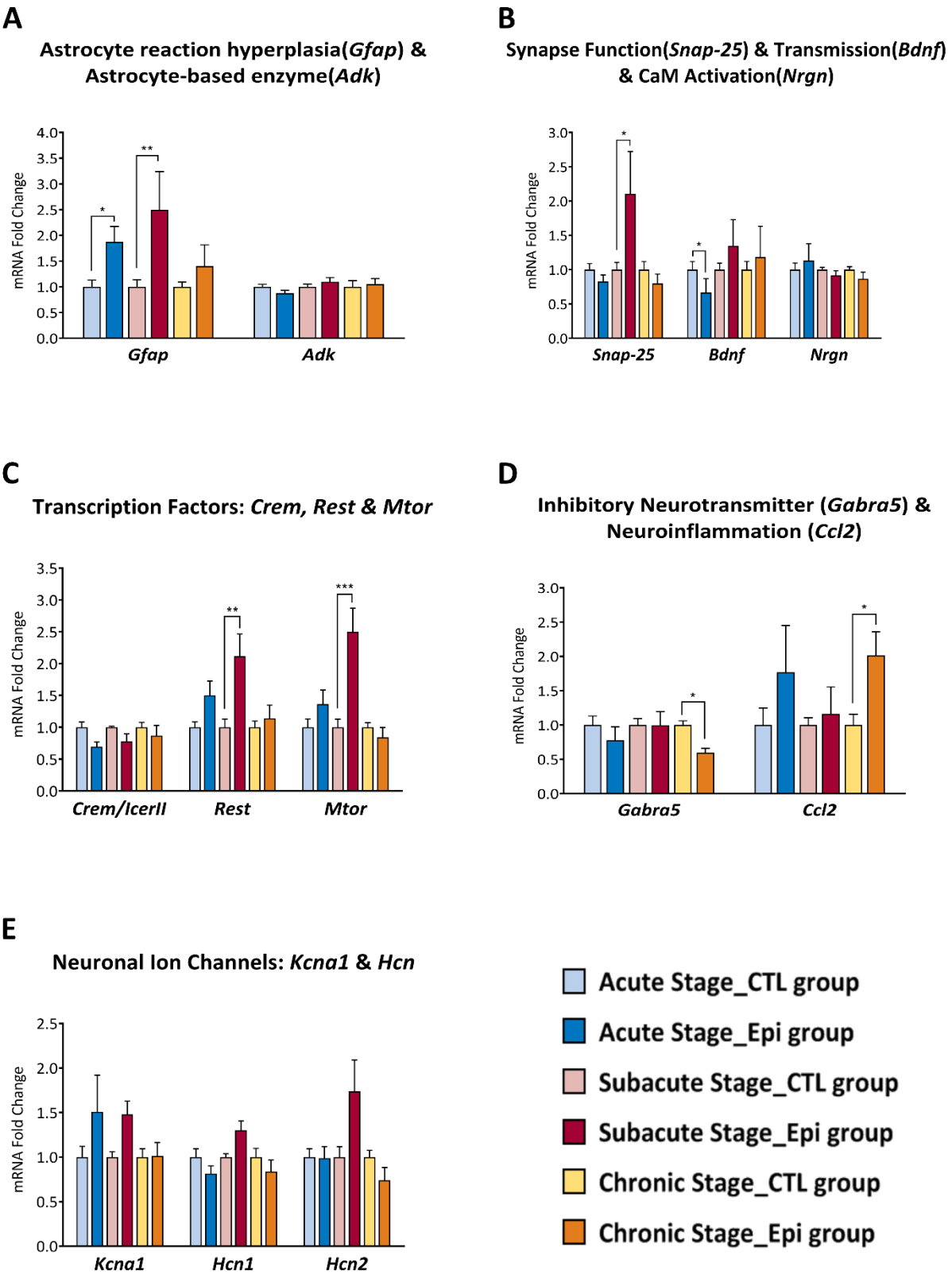
732 Fig 2



733

734

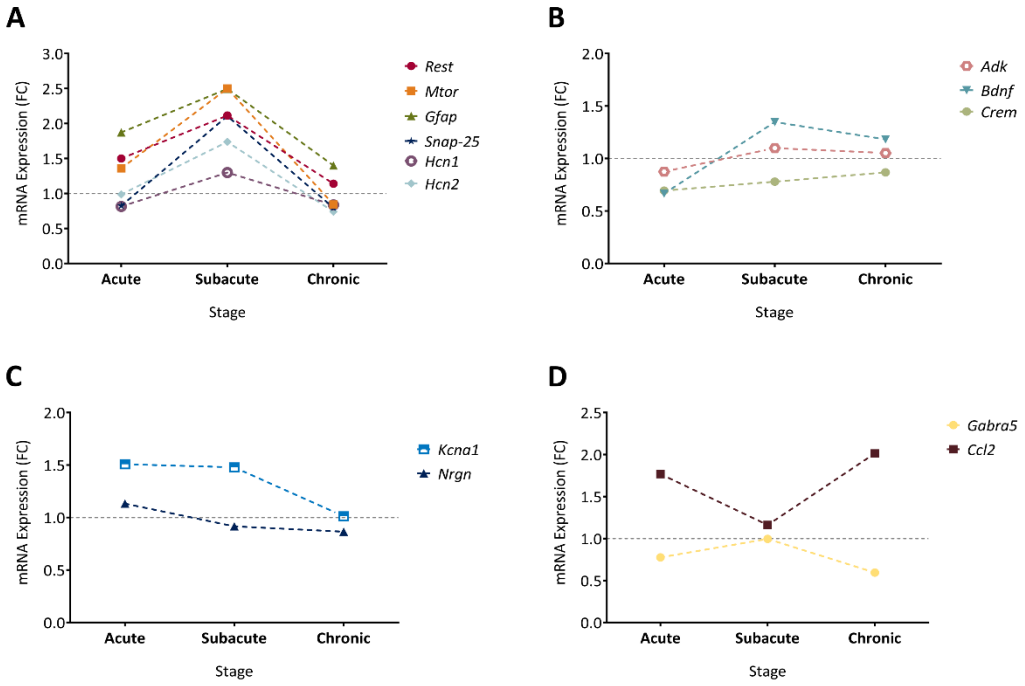
735 Fig 3



736

737

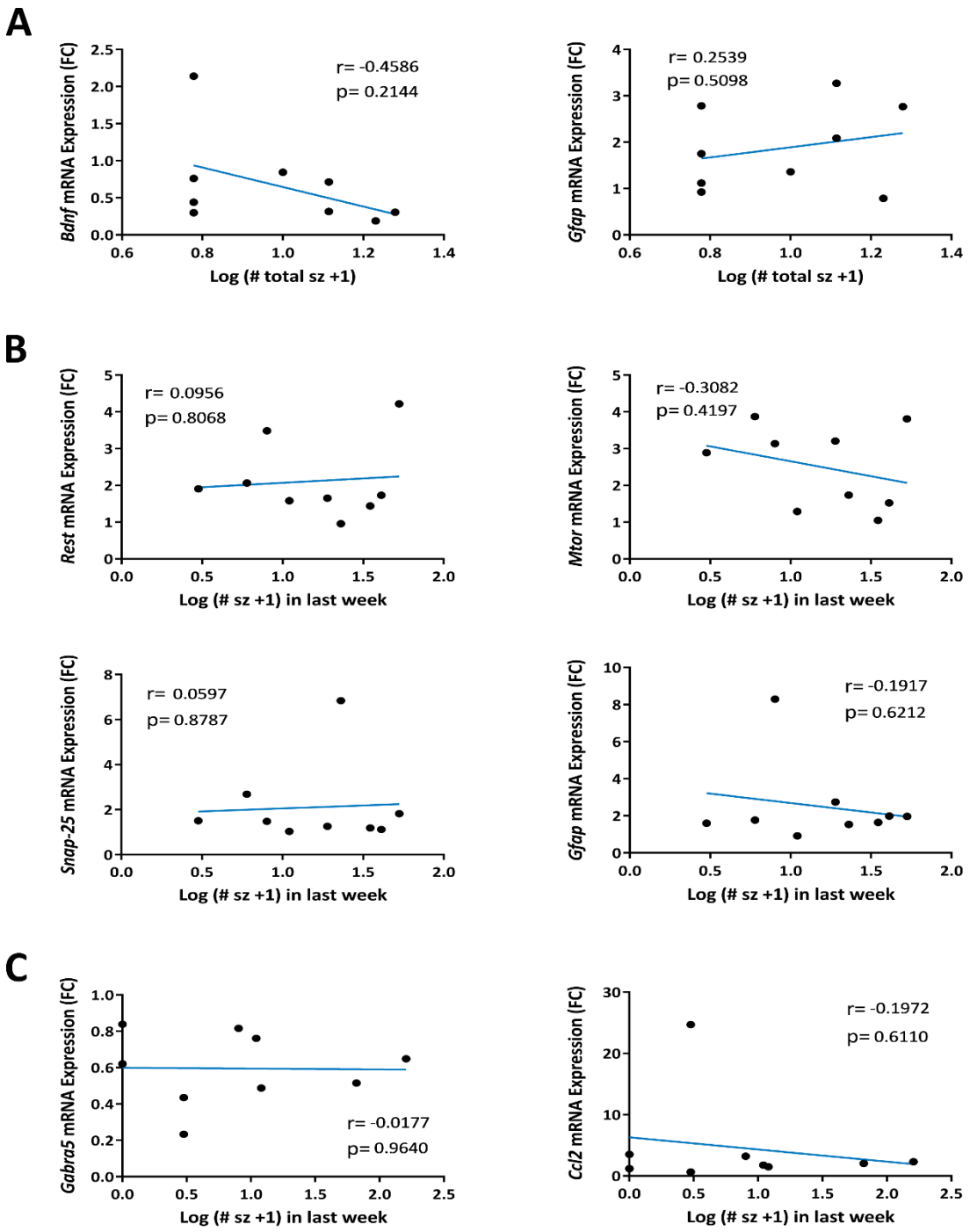
738 Fig 4



739

740

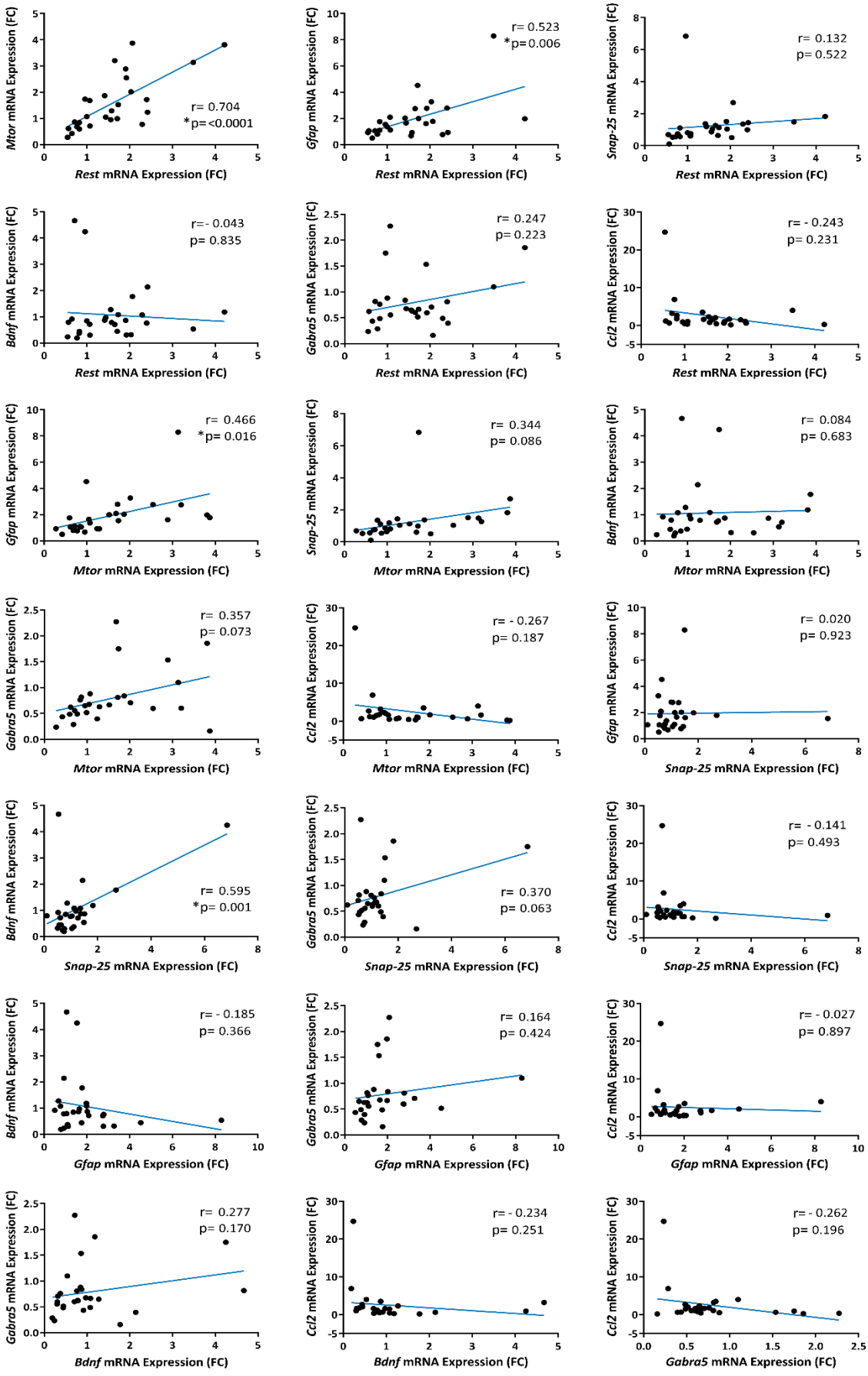
741 Fig 5



742

743

744 Fig 6


Mtor mRNA Expression (FC)
Rest mRNA Expression (FC)
r= 0.704
*p=<0.0001
Gfap mRNA Expression (FC)
r= 0.523
*p=0.006
Snap-25 mRNA Expression (FC)
r= 0.132
p= 0.522
Bdnf mRNA Expression (FC)
r=- 0.043
p= 0.835
Gabra5 mRNA Expression (FC)
r= 0.247
p= 0.223
Ccl2 mRNA Expression (FC)
r= - 0.243
p= 0.231
r= 0.466
*p= 0.016
r= 0.344
p= 0.086
r= 0.084
p= 0.683
r= 0.357
p= 0.073
r= - 0.267
p= 0.187
r= 0.020
p= 0.923
r= 0.595
*p= 0.001
r= 0.370
p= 0.063
r= - 0.141
p= 0.493
r= - 0.185
p= 0.366
r= 0.164
p= 0.424
r= - 0.027
p= 0.897
r= 0.277
p= 0.170
r= - 0.234
p= 0.251
r= - 0.262
p= 0.196

745