BRAFV600E Expression in Mouse Neuroglial Progenitors Increase Neuronal Excitability, cause appearance of balloon-like cells, Neuronal Mislocalization, and Inflammatory Immune Response

Roman Goz
University of Connecticut - Storrs, roman.goz@uconn.edu

Follow this and additional works at: https://opencommons.uconn.edu/dissertations

Recommended Citation
https://opencommons.uconn.edu/dissertations/1771
BRAFV600E Expression in Mouse Neuroglial Progenitors Increase Neuronal Excitability, cause appearance of balloon-like cells, Neuronal Mislocalization, and Inflammatory Immune Response

Roman Goz, PhD
University of Connecticut, 2018

Abstract

Low-grade neuroepithelial tumors (LNETs) are the most frequent tumors in patients undergoing resective surgery for epilepsy treatment. BRAFV600E represent the most frequent mutation in LNETs. In the RAS-RAF-ERK (MAPK) pathway activation of receptor tyrosine kinase leads to activation of BRAF protein. By mimicking constitutive activation of the kinase domain in RAS-independent manner BRAFV600E represents much higher activating substrate of MAPK pathway than BRAF wild type. Increased activation of MAPK leads to alterations of cell cycle progression, cell growth, proliferation, and cell fate. It also influences cortical neuron migration. Introducing human BRAFV600E into neuroglial progenitors in mouse cortex on embryonic day 14 to 15 induced astrogliosis. It also disrupted cellular morphology, causing appearance of balloon-like cells, disrupted neuronal migration to the upper cortical layers, altered tissue-wide gene expression, and induced neuronal hyperexcitability. Hyperexcitability was evident as higher action potential (AP) frequencies in response to depolarizing current injections in whole-cell current-clamp configuration of the patch-clamp technique; more depolarized by 7-10 mV resting membrane potential (RMP) and increased by about 100 MΩ input resistance (Rin). Single APs showed more hyperpolarized voltage threshold in BRAFV600E expressing neurons and those neurons had lower minimal required current (rheobase) to fire APs. Increased initial deflection in response to the hyperpolarizing current pulses - SAG and a rebound excitation at the
end of those pulses were observed, which was found to result from increase in the hyperpolarization activated conductance (Ih current). In whole-cell voltage-clamp configuration BRAFV600E neurons have increased Ih compared to the control conditions. Additionally, sustained, tetraethylammonium sensitive voltage activated potassium currents were decreased in BRAFV600E neurons. Activation of potassium currents with retigabine decreased action potential firing frequency. Recording of action potential dependent spontaneous post-synaptic currents (sPSCs) showed increased frequency in BRAFV600E neurons, those sPSCs frequencies were higher in untransfected neighbors. Video and Electrocttogicographic recording in the freely moving mice showed spontaneous epileptiform activity with behavioral manifestations. The findings indicate that BRAFV600E mutations are sufficient to partially capture the pathophysiology of LNETs. The conclusion has implications to the possible courses of, and therapeutic targets for LNET associated epilepsy.

**Keywords**

Low-grade neuroepithelial tumors, BRAFV600E, balloon cells, Focal Cortical Dysplasia, Gangliogliomas, Dysembryoplastic Neuroepithelial Tumors, MAPK, mTOR, PiggyBac.
BRAFV600E Expression in Mouse Neuroglial Progenitors Increase Neuronal Excitability, cause appearance of balloon-like cells, Neuronal Mislocalization, and Inflammatory Immune Response.

Roman Goz

B.Sc. Bar-Ilan University, Israel, 2005
M.Sc. Hebrew University of Jerusalem, Israel, 2011

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut

2018
Approval page

Doctor of Philosophy Dissertation

BRAFV600E Expression in Mouse Neuroglial Progenitors Increase Neuronal Excitability, cause appearance of balloon-like cells, Neuronal Mislocalization, and Inflammatory Immune Response

Presented by

Roman Goz B.Sc., M.Sc.

Major Advisor

Joseph J. LoTurco

Associate Advisor

Anastasios V. Tzingounis

Associate Advisor

Daniel K. Mulkey

Associate Advisor

Alexander C. Jackson

Associate Advisor

Harvey A. Swadlow

University of Connecticut

2018
Acknowledgements

Special thanks to my family, my mom, who supported me all my life, my late brother, my sister and my niece.

I’m grateful to Dr. Anastasios Tzingounis for listening to whatever crazy and wrong Ideas I had. Thanks to my PI Dr. Joseph LoTurco for teaching me how to do science. Thanks to all who doubted me for making me increase the scientific rigor and effort in my work and ridding me of the psychological burden of increasing self-doubt. All the surrounding skepticism caused me to rethink each and every experiment, and test it twice before presenting the results.

Gratus sum omnibus qui diligunt me, et in meam sententiam.

Alii - habent sua quaestiones, mordere me ...
# Table of Contents

## Summary ............................................................................................................................ 1

## Chapter 1 Introduction ........................................................................................................ 5

1.1 Low grade epilepsy associated neuroepithelial tumors and MCDs ...................... 6

1.2 Genetic etiology .............................................................................................................. 9

**PI3K-AKT3-MTOR PATHWAY IS UPREGULATED in MCDs AND LNETs** .......................... 13

**RAS-RAF-ERK PATHWAY IS UPREGULATED in LNETs** .................................................. 16

Raf oncogenes and proteins function in disease .................................................................. 19

Raf gene family proteins and molecular interactions ......................................................... 20

Function of Raf proteins through mouse models .............................................................. 23

**Other pathways, genes and chromosomal alterations in LNETs** ................................. 25

1.3 Excitability mechanisms contributing to epileptogenesis in MCDs and LNETs. ... 29

**Proliferation, migration, differentiation and cell growth** ............................................... 29

1.4 Histopathological features and their hyperexcitability in MCDs and LNETs .... 31

**Dysmorphic cytomegalic neurons** .................................................................................. 31

**Immature misoriented neurons** ..................................................................................... 33

**Balloon cells** ................................................................................................................... 34

1.5 Changes of intrinsic neuronal excitability mechanisms in MCDs ....................... 35

**Passive properties changed in MCDs** .......................................................................... 36

**Active properties changed in MCDs** .......................................................................... 37

1.6 Changes of synaptic excitability in MCDs and LNETs ........................................... 39

**Role of GABA receptors in MCDs** .............................................................................. 39

**Role of glutamate receptors in MCDs and LNETs** ....................................................... 42

1.7 Non-neuronal network hyperexcitability mechanisms in MCDs and LNETs .... 46

**Astrocytes – normal physiological function** ................................................................. 46

**Astrocytes activation in MCDs and LNETs** ................................................................. 49

**Microglia – normal physiological function** .................................................................... 52

**Microglia activation in MCDs and LNETs** .................................................................... 53

## Chapter 2 Research Objectives and Approach ................................................................. 56

## Chapter 3 Results ................................................................................................................ 60

Goal-1 **Cytoarchitectural and morphological alterations in murine cortex of BRAFV600E transfected animals** ................................................................. 60

Goal-2 **RNA sequencing of transfected with GLAST+ BRAFV600E, BRAFWT, CONTROL-FP BRAINS TISSUE** .............................................................................. 66

Goal-3 **BRAFV600E transfected neurons are hyperexcitable in ex-vivo** ...................... 71

BRAFV600E decreases delayed rectifier potassium currents ........................................ 77

BRAFV600E increases Ih current ...................................................................................... 80

BRAFV600E increases frequency of excitatory post-synaptic events ................................ 85

**Unsupervised hierarchical cluster analysis indicates BRAFV600E transformation of pyramidal neuron cell type physiology.** ........................................ 88

**Other somatic mutations associated with focal cortical dysplasias cause changes in electrophysiological properties distinctly different from BRAFV600E** ........................................ 90

## Chapter 4 Caveats and Future Experiments ..................................................................... 94
CHAPTER 5 CONCLUSION ........................................................................................................95

Table of Figures

Figure 1 - Molecular Pathways in MCDs and LNETs ................................................................12
Figure 2 - RAF protein and gene structure ...........................................................................21
Figure 3 - Dysmorphogenesis, migrational delay, astrogenesis and neurogenesis in BRAFV600E brains ........................................................................62
....................................................................................................................................................62
Figure 4 - Nestin+ BRAFV600E large cells remained closer to the lateral ventricle .......64
Figure 5 - Increased activation of astrocytes in BRAFV600E transfected brains .............65
Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade ...............................................................68
Figure 7 - BRAFV600E neurons are hyperexcitable ..............................................................73
Figure 8 - Singe AP and passive electrophysiological properties ........................................75
Figure 9 - BRAFV600E decrease sustained potassium currents .........................................78
Figure 10 - \( I_h \) is increased in BRAFV600E neurons ............................................................82
Figure 11 - ZD7288 decrease SAG and rebound excitation ..................................................84
Figure 12 - sPSCs frequencies are increased in BRAFV600E neurons, and in their untransfected neighbors .................................................................86
Figure 13 - Unsupervised hierarchical clustering of the electrophysiological properties showed segregation of BRAFV600E neurons from other conditions .................................................................................89
Figure 14 - Different somatic mutations in MTOR and MAPK pathways have different effect on neuronal excitability .................................................................91
Figure 15 - sPSCs frequencies are increased in Nestin+ BRAFV600E neurons compared to Glast+ BRAFV600E neurons ........................................93
Figure S1 ........................................................................................................................................98
Figure S2 Action Potential voltage threshold correlation to RMP ........................................99
Figure S3 Miniature post-synaptic currents .........................................................................100

Tables

Table 1 - G O T E R M biological protein production pathways enrichment in Glast+ BRAFV600E compared to control-FP and to Glast+ BRAFwt from 402 upregulated genes at \( p < 0.01 \) ........................................70
Table 2 - G O T E R M Biological protein production pathways enrichment in Glast+ BRAFV600E compared to Glast+ control-FP and to Glast+ BRAFwt from 262 downregulated genes at \( p < 0.01 \) ........................................70
Table 3 - Sustained K+ current averages of maximal values and their current density ..........79
Table 4 – TEA sustained K+ current averages of maximal values and their current density ........80
Table 5 - \( I_h \) peak and current density .................................................................................83
Acronyms glossary

4EBP1 - Eukaryotic translation initiation factor 4E-Binding Protein 1
AEDs – Anti-Epileptic Drugs
AG - Angiocentric Gliomas
AKT – AK mouse strain Thymoma
ALDH1L1 - Aldehyde Dehydrogenase 1 Family Member L1
AMOG – Adhesion Molecule On Glia
AMPK - Adenosine-Monophosphate-activated Protein Kinase
AQP4 – Aquaporin 4
AP – Action Potential
ARAF – A variant of Rapidly Accelerated Fibrosarcoma
BRAF – B variant of Rapidly Accelerated Fibrosarcoma
C1q - Complement C1q
C3 – Complement C3
CCL2 - C-C Motif Chemokine Ligand 2
CDC – Centers for Disease Control and Prevention
CDK5 - Cyclin-Dependent Kinase-5
CLCN6 - Chloride Voltage-Gated Channel 6
CR - Conserved Domains
CRD – Cysteine Rich Domain
CRAF – C variant of Rapidly Accelerated Fibrosarcoma
CREB - CAMP Response Element Binding protein
Cx3cr1 - C-X3-C Motif Chemokine Receptor 1
CXCL10 - C-X-C Motif Chemokine Ligand 10

d-OT - diffuse oligodendrogial tumors

DA - Diffuse Astrocytoma

dab1 – disabled 1

DAP12 - DNAX Activating Protein of 12KDa

DNETs - Dysembryoplastic NeuroEpithelial Tumors

DUSPs - Dual Specificity Phosphatases

EAAT1 – Excitatory Amino Acid Transporter 1

ECoG – ElectroCorticoGraphic

EdU - 5-Ethynyl-2’-deoxyUridine

EGFP – Enhanced Green Fluorescent Protein

eLF4G – eukaryotic translation initiation factor 4G

ERK – Extracellular signal-Regulated Kinase

ERM - Ezrin, Radixin and Moesin

EWSR1 - EWS RNA Binding Protein 1

FAM131B - FAmily with Sequence Similarity 131 Member B

FCD – Focal Cortical Dysplasia

FDG-PET - Fluorine-18-Fluorodeoxyglucose Positron Emission Tomography

FGF - Fibroblast Growth Factor

FGFRs – Fibroblast Growth Factor Receptor 1

FOXO - FOrkhead boX O pathway

FZR1 - FiZzy and cell division cycle 20 Related 1

GSK - Glycogen Synthase Kinase

GABA – Gamma Amino Butyric Acid
GAD - Glutamate Decarboxylase
GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase
GAT1 – GABA Transporter 1
GE - Ganglionic Eminence
GEF - Guanine nucleotide Exchange Factor
GFAP – Glial Fibrillary Acidic Protein
GG – GanglioGlioma
GLAST – GLutamate Aspartate Transporter 1
GNAI1 - G Protein Subunit Alpha I1
GNET – Glio-NeuroEpithelial Tumor
GPCR - G-Protein Coupled Receptors
Grb2 - Growth factor receptor-bound protein 2
H3F3A - H3 Histone Family Member 3A
HCA – Hierarchical Cluster Analysis
HCR - hybridization-chain-reaction
HLA-DR - Human Leukocyte Antigen antigen D Related
HME – HemiMegalEncephaly
HMGB1 - High Mobility Group Box 1
HS – Hippocampal Sclerosis
IGF – Insulin-like Growth Factor
IGF1R – Insulin Growth Factor Receptor 1
IHC - ImmunoHistoChemistry
IL-1β – InterLeukin 1 β
ILAE – International League Against Epilepsy

IUE – *In-Utero* Electroporation

KCC2 - potassium-chloride co-transporter

Kir4.1 – Inwardly Rectifying K⁺ channel 4.1

LEATs – Low-grade Epilepsy Associated Tumors see LNETs

LNETs – Low-grade NeuroEpithelial Tumors associated with epilepsy

MACF1 - Microtubule-Actin Crosslinking Factor 1

mAHP – median AfterHyperPolarization

MAPK – Mitogen Activated Protein Kinase

MCDs – Malformations of Cortical Development

ME - Megalencephaly

MEK – Mitogen activated protein kinase Kinase

MHC – Major Histocompatibility Complex

MKRN1 - Makorin Ring Finger Protein 1

Mn-SOD – Mitochondrial Superoxide Dismutase

Mnk - MAPK Signal Integrating Kinases

MP – Membrane Potential

mRFP – monomeric Red Fluorescent Protein

MRI – Magnetic Resonance Imaging

MTLE - Medial Temporal Lobe Epilepsy

mTOR – mechanistic Target Of Rapamycin

MYB - MYB Proto-Oncogene, Transcription Factor
MYBL1 - MYB Proto-Oncogene Like 1
NCS - Neuronal Stem Cells
NMDA - N-Methyl-D-Aspartate
NKCC1 - sodium-potassium co-transporter 1
NTRK2 - NeuroTrophic Receptor Tyrosine Kinase 2
QKI - Quaking Homolog, KH Domain RNA Binding
qRT-PCR – quantitative RT-PCR
P70S6K - P70 Ribosomal S6 Kinase
P90RSK1 - P90 Ribosomal Six Kinase-1
PA - Pilocytic Astrocytoma
PATZ1 - POZ/BTB And AT Hook Containing Zink Finger 1
PCA – Principal Component Analysis
PCR – Polymerase Chain Reaction
PCR-HRM - PCR-high resolution melting
PDGFR α - Platelet-Derived Growth Factor Receptor α
PDK –Pyruvate Dehydrogenase Kinase
PGNET – papillary GNET
PIK3CA - Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit α
PIP2 - phosphorylates phosphatidylinositol-4,5-biphosphate
PIP3 - phosphatidylinositol-3,4,5-triphosphate
PTEN - Phosphatase and Tensin Homolog
PV - Parvalbumin
PXA – Pilocytic XanthoAstrocytoma
RAGE - Receptor for Advanced Glycation End products
RAPTOR – Regulatory Associated Protein Of mTOR

RAS – RAt Sarcoma protein

RBD - RAS-GTP Binding Domain

REDD1 - REgulation of DNA Damage response 1

RGNT - Rosette forming Glio-Neuronal Tumor

RICCTOR - Rapamycin-Insensitive Companion of mammalian Target Of Rapamycin

Rin – input Resistance

RMP – Resting Membrane Potential

RNF130 - Ring Finger Protein 130

RT-PCR – Reverse Transcription PCR

RTK – Receptor Tyrosine Kinase

S6 – Ribosomal Protein S6

S6K – Ribosomal Protein S6 Kinase

S100β - S100 Calcium Binding Protein B

SAPK/JNK – Stress Activated Protein Kinase/Jun-N-terminal protein Kinase

sIPSCs – spontaneous Inhibitory Post-Synaptic Currents

SIRPα - Signal Regulatory Protein α

SLMAP - SarcoLeMa Associated Protein

SOS - Son Of Sevenless – a guanine nucleotide exchange factor

SRGAP3 - SLIT-ROBO Rho GTPase Activating Protein 3

SSCP - Single-Strand Conformation Polymorphism

sPSCs – spontaneous Post-Synaptic Currents

TGF - Transforming Growth Factor
TLR - Toll Like Receptor
TNFα - Tumor Necrosis Factor α
TRKB – Tyrosine Receptor Kinase B
TSC - Tuberous Sclerosis Complex
VGAT – Vesicular GABA Transporter
VZ - Ventricular Zone
WES - Whole-Exome-Sequencing
WGS – Whole-Genome-Sequencing

*. Small p before the protein acronym means - phosphoactivated
Epilepsy affects about 50 million people worldwide. Epilepsy patients with drug refractory recurrent seizures and disrupted brain structure on MRI are referred to resective surgery to remove epileptic foci (Blumcke et al., 2016). The second most common finding in epilepsy surgery patients, after focal cortical dysplasia (FCD) in children and young adults and after hippocampal sclerosis (HS) in adult patients are LNETs. LNETs are also the most frequent tumors found in resective surgery for epilepsy treatment patients. Ganglioglioma and DNETs represent the most frequent entities of this group. Together with malformations of cortical development (MCDs) like FCD, tuberous sclerosis (TSC) syndrome, and different types of brain growth disorders LNETs represent a group without known molecular mechanisms that cause ictogenesis and subsequent epileptogenesis (Barkovich et al., 2015; Crino, 2011). Disruption of cortical cytoarchitecture, cellular dysmorphogenesis and seizures that occur with different characteristic pathologic severity in all those disorders may share a common molecular pathway, overactivation of which may shift neuronal network together with astroglial components towards seizure prone states. Small groups of hyperexcitable neurons may be sufficient in such conditions to induce ictogenesis, and, permissive tissue environments may promote recurrent seizures, or epilepsy.

BRAFV600E mutations are the most frequent mutations in LNETs (Koelsche et al., 2013; Schindler et al., 2011). It is also one of the most studied mutations in solid cancers, and is found in papillary thyroid, colorectal and melanoma (Davies et al., 2002). I introduced human BRAFV600E by in-utero electroporation in a subset of cortical cells to develop a mouse model of LNETs. The results show a different histopathology, resembling pilocytic astrocytoma and
ganglioglioma along with disruption in cortical cytoarchitecture, delayed neuronal migration, and increased astrogliosis.

In Chapter 1 I describe current definition of low-grade neuroepithelial tumors, their history and genetic etiology and relationship to specifically MCDs. I discuss the common molecular pathways shared between MCDs and LNETs. Further discussion is concentrated on BRAF protein and its’ function in the RTK-RAS-RAF-MEK-ERK pathway. Other pathways, mutations in which were found in LNETs are partially covered. Following subchapters I discuss possible hyperexcitability mechanisms and present findings from previous studies on different histopathological features and their presumed contributions to network hyperexcitability, glutamate ionotropic and metabotropic receptor alterations in MCDs and LNETs, and GABAergic receptor alterations. Microglial and astroglial mechanisms role in epileptogenesis in MCDs and LNETs are also discussed.

In Chapter 2 I present the research objectives and the methodological approach.

In Chapter 3 I present the results on alterations in cortical cytoarchitecture in BRAFV600E transfected animals versus control-Fluorophore (control-FP) transfected and BRAF wild type (BRAFwt). I show that expression of BRAFV600E has different effects on cells’ fate depending on the driver promoter used to label different neuroglial progenitor populations. Using Immunohistochemistry together with RNA-sequencing I show that BRAFV600E expression in mouse cortex activates astrocytes and induces expression of genes related to the inflammatory innate immune response, and genes including classic complement pathway. I show that BRAFV600E expressing neurons display hyperexcitability in whole-cell patch-clamp ex-vivo recording in coronal slices of somatosensory cortex. Expression of BRAF wild type did not
cause hyperexcitability suggesting BRAFV600E mutation induces effects beyond overexpression of BRAF.

Alteration of ion channels may be a possible mechanism of hyperexcitability. In whole-cell current clamp BRAFV600E neurons reach action potential firing frequencies higher than all other conditions. BRAFV600E neurons also have more depolarized resting membrane potentials, require less depolarizing current to fire first action potentials, and display a more hyperpolarized voltage threshold. Additionally, BRAFV600E neurons have increased hyperpolarizing current induced voltage SAG, and elevated rebound excitation. These phenomena are a result of increased hyperpolarization activated depolarizing conductance, which was evident in whole-cell voltage clamp in BRAFV600E neurons.

More importantly BRAFV600E neurons have decreased sustained tetraethylammonium sensitive potassium currents. The hyperexcitability, exhibited as increased AP firing frequency was decreased in BRAFV600E neurons when retigabin, a Kv7.2-7.5, potassium channel activator was applied. Preincubation of cortical slices in BRAFV600E inhibitor, Vemurafenib (PLX4032, PLX4720) decreased the AP firing frequency, however the effect was indistinguishable from the effect of similar amount of its solvent – DMSO. Action potential dependent excitatory spontaneous post-synaptic currents frequencies, recorded at -70 mV holding potential, were increased in BRAFV600E neurons, but they also were increased even more in their untransfected neighbors, showing that there may be non-cell autonomous network effects. Unsupervised hierarchical cluster analysis showed segregation of BRAFV600E expressing neurons from other conditions based on 20 electrophysiological parameters recorded in ex-vivo whole-cell patch-clamp experiments. Finally, I make a comparison to other
manipulations affecting neuroglial progenitors with MTOR regulatory component mutations, PIK3CA E545K and TSC1 loss of function using CRISPR-Cas9 system.

Chapter 4 presents caveats and future experiments and Chapter 5 presents concluding remarks.
CHAPTER 1 INTRODUCTION

Epilepsy is a spectrum disorder with more than 25 syndromes with varying types and severity of seizures. It is the fourth common neurological disorder affecting nearly 1 percent (~50 million people) of the population, decreasing the quality of life and increasing the risk of death. With approximately 2.8 million patients (5-8.4 of every 1,000 people) and 150,000 new cases (30-50 out of 100,000) diagnosed each year in US only, with about 15.5 billion-dollar estimated economic burden. The incidence of newly diagnosed epilepsy cases in childhood and early adulthood is increasing. About 30% of epilepsy patients have seizures that are refractory to anti-epileptic drugs (AEDs). (Fisher et al., 2017; Scheffer et al., 2017; CDC, August 3, 2017; World Health Statistics 2016: Monitoring health for the SDGs; Zack and Kobau, 2015; England et al., 2012; Russ et al., 2012; World Health Organization, 2006).

Epilepsy patients not responsive to at least two AEDs may be further referred for surgical resection of the affected brain area, excluding cases that involve eloquent or life sustaining brain centers (Blumcke et al., 2014; Alexandre et al., 2006). In 50-80% of the cases, depending on the extent of the affected area of resection, and the contribution of surrounding cortical tissue, surgical treatment is successful with no relapse over a two year follow up period (Wong, 2009; Alexandre et al., 2006). Amongst etiological causes the most frequent epilepsy resection surgery findings are malformations of cortical development (MCDs) and low-grade epilepsy associated neuroepithelial tumors (LNETs; previously – long-term epilepsy associate Tumors, LEATs) that involve varying degrees of cortical dyslamination and cellular dysmorphogenesis. LNETs are the second leading cause of epilepsy after FCD in children and adolescents 0-19 years of ages and after HS in adults (Blumcke et al., 2016; Barkovich et al., 2015; Blumcke et al., 2014; Wong, 2013; Blumcke and Wiestler, 2002).
1.1 LOW GRADE EPILEPSY ASSOCIATED NEUROEPITHELIAL TUMORS AND MCDs

LNETs were first described in patients with a long history (>2 years) of intractable epilepsy. Due to their slow growth and frequent presence in epilepsy surgery patients, the initially introduced term was long-term epilepsy associated tumors (Luyken et al., 2003). With the new, less stringent clinical definition of epilepsy - MRI lesion findings with shorter history of seizures, and drug refractory epilepsy has been considered a sufficient criteria for epilepsy diagnosis (ILAE, 2017; Blumcke et al., 2016), The International League Against Epilepsy (ILAE) task force suggested changing the definition to low-grade epilepsy associated neuroepithelial tumors (LNETs) (Blumcke et al., 2016). Cytoarchitectural changes in LNETs involve neuronal as well as glial components, with a predominant appearance in the temporal lobe (Thom et al., 2012). The presence of similar cytological component in LNETs and FCDs, including balloon cells and hypertrophic dysmorphic neurons, and cortical dyslamination suggested a common developmental origin. Due to multiple cases of LNETs with FCD in the surrounding tissue the new ILAE FCD classification, FCD type IIIb is defined by association with tumors (Blumcke et al., 2011).

Slowly growing LNETs are present in 25-80% of drug refractory epilepsy cases treated with resecting surgery (Pelliccia et al., 2017; Blumcke et al., 2016; Zanello et al., 2016; Barkovich et al., 2015; Guerrini et al., 2015; Nagarajan et al., 2015; Blumcke et al., 2014; Rossi, 2014; reviewed in Thom et al., 2012; Luyken et al., 2003; Blumcke and Wiestler, 2002; Schramm et al., 2001; Blumcke et al., 1999; Morris et al., 1998; Wyllie et al., 1998; Wolf and Wiestler, 1993; Silver et al., 1991; Rich et al., 1985; Spencer et al., 1984; Cavanagh, 1958). The major subtypes of LNETs - predominant in young patients ganglioglioma (GG) – about 5% of pediatric brain tumors (Blumcke et al., 2016; Barkovich et al., 2015), and dysembryoplastic neuroepithelial tumors
(DNETs) in about 20-36% of cases are associated with FCD (Blumcke et al., 2014). FCD is considered a common cause for drug refractory epilepsy and is found in 25-46% of cases in both children and adults (Blumcke et al., 2014; Rossi, 2014; Wong, 2013; Bast et al., 2006; Cepeda et al., 2006; Cataltepe et al., 2005; Blumcke and Wiestler, 2002; Morris et al., 1998; Wyllie et al., 1998). Although these statistics refer to the selected population of patients, which are surgically treated for epilepsy, FCDs and LNETs with varying severity may be present in epilepsy patients responsive to AEDs. In some cases, people diagnosed with FCD or LNETs may not develop epilepsy (Maynard et al., 2017; Leach et al., 2014). One of the main questions raised by these studies is – how and whether multiple common components in different types of malformations induce seizures and subsequently epilepsy in some patients, and not in others? Knowledge of the minimum extent of the cortical malformation that is required for ictogenesis and epileptogenesis, and if there is an increase in a specific malformation component, dysmorphic neurons, for instance, that makes neuronal networks more seizures prone may help address these questions. Also, are there intrinsic neuronal properties that change in dysmorphic neurons and are they present in regular formed neighboring neurons, and what is the contribution of the glial component to seizures? Finally, does the somatic genetic alteration that shifts neuronal network to ictogenesis and subsequently to epileptogenesis in dysplastic cortex only involves malformed cells in a cell-autonomous way, or does it involve non-malformed neurons and glia in a cell non-autonomous way?

LNET co-occurrence with FCD (FCD IIIb) present an additional conundrum. Despite an extensive research on LNETs and FCDs and their relationship there is no consensus on whether the co-occurrence is a dual pathogenicity or whether there is a primary malformation that causes the secondary to occur. The source of the epileptogenesis in the co-occurrence cases is also not
well understood (Palmini et al., 2013; Schwartzkroin and Wenzel, 2012; reviewed in Thom et al., 2012; Kohling et al., 2006). To better understand the relation of LNETs and associated FCDs to epilepsy and its’ recurrence after the surgery there is a need to adopt a common/standardized methodological description of the extent of malformation (MRI, EEG, ECoG during the surgery), the extent of its’ resection, differential malformations susceptibility of the affected brain areas, and what dysregulated molecular mechanisms are involved in developmental tumorigenesis and surrounding cortical malformation (Blumcke et al., 2016; Schwartzkroin and Wenzel, 2012; Wong, 2009).

Only recently has some consensus been reached on methods for classifying LNETs (Blumcke et al., 2016; Blumcke et al., 2014; Japp et al., 2013). Surgical resection of epileptogenic brain areas, the only available treatment for drug refractory epilepsy, is not suitable for a significant number of patients, either due to epileptogenic foci proximity to eloquent or life sustaining brain centers or simply unavailability of the procedure in many developing countries (World Health Organization, 2006). Importantly, following the surgical procedure seizures may relapse and tumors may recur (Blumcke et al., 2014; Thom et al., 2012; Wong, 2009). Hence there is a need to understand pathophysiological genetic and molecular mechanisms for elucidating the elements that increase probability of neuronal networks epileptogenesis. This may help to develop new treatments. This may be achieved through combined investigative methodologies and development of accurate animal models based on recent discoveries of recurrent somatic mutations in LNETs and FCDs (Schwartzkroin and Wenzel, 2012; Wong, 2009).
1.2 GENETIC ETIOLOGY

Shared histopathological features between MCDs and LNETs led some to include one of the LNETs (GG) into the MCDs broad group of pathologies (Barkovich et al., 2015). The earliest documented cases date back around 200 years (Sims, 1835). Since then reports on more than 100 genes associated with MCDs have been published. Alterations in genes that participate in cell-cycle regulation, apoptosis, cell-fate specification, cytoskeletal structure and function, cell migration and post-migrational organization and growth have been implicated in MCDs and LNETs (ILAE, 2017; Parrini et al., 2016; Guerrini and Parrini, 2015; Barkovich et al., 2015; 2012).

Genetic mutations in several molecular pathways involved in cortical development have been identified in known MCDs and LNETs (Barkovich et al., 2015; Lim and Crino, 2013; Zhang et al., 2013; Aronica et al., 2008a). Reduced cell proliferation found in microcephaly, or abnormal proliferation and possible neuro-glial differentiation of progenitors as in LNETs (Blumcke et al., 2014), tuberous sclerosis complex (TSC) disorders (Crino, 2013), type II FCDs (Blumcke et al., 2011); or abnormal cell migration, differentiation and growth as in megalencephaly (Roy et al., 2015; Lee et al., 2012; Riviere et al., 2012) are pathogenic mechanisms that might cause both – MCDs and LNETs. However, it is still unclear whether epilepsy is a direct consequence of abnormal development of cortical cytoarchitecture, or whether the specific intrinsic cellular excitability mechanisms are changed, independent of malformation creating cells that are easily excited by synaptic inputs and/or spontaneously active to induce seizure prone networks. Some evidence that epileptogenicity is independent of brain malformation comes from recent PIK3CA mutated mouse models finding that epileptic foci are found outside the malformed cortex, and the identified by MRI FCD cases without epilepsy (Maynard et al., 2017; Roy et al., 2015; Kohling et al., 2006). However, the type of malformation in question, its severity (Palmini et al., 2013; Cepeda
et al., 2006; Sisodiya, 2004; Frater et al., 2000), the population of progenitor cells involved within
the boundaries of affected developmental stages (Way et al., 2009; Cepeda et al., 2006), genetic
etiology, epigenetic modulation (Kobow and Blumcke, 2017; Becker et al., 2001) and
environmental effects may all be contributing factors to epileptogenicity induction. Additionally,
the “two-hit” hypothesis (Hino and Kobayashi, 2017; Stark and Otto, 2016; Knudson, 1996;
Knudson, 1971; Nicholls, 1969) of Epilepsy suggests that there are second factors, for instance,
pathological abnormalities of an autosomal dominant mutation (TSC) (Crino, 2013) (either
germline or somatic) that must combine with – “environmental factors” to induce second somatic
mutation (Feliciano et al., 2011; Wong, 2009; Takahashi et al., 2004; Wenzel et al., 2004). The
possibility of multiple hits in cancer causation has been considered as well (Tomlinson et al.,
2001). Understanding the molecular, genetic and epigenetic factors contributing to or directly
causing epilepsy may be possible with animal models.

Ideal animal models of MCDs should capture key aspects of the abnormal cortical development
– structural histopathology as well as electrophysiological cellular and network mechanisms,
which increase propensity for epileptogenesis. The significant diversity in structural abnormalities
found in different MCDs allowed development of multiple animal models capturing only partial
characteristic pathologic findings while epileptogenesis been the main clinical manifestation of
MCDs represented a relatively rare phenotype. In this case epileptogenic agents are used to
demonstrate lower Ictogenic threshold, or, in-vitro electrophysiological and histochemical
techniques used to characterize changes in cellular and network excitability (Schwartzkroin and
Wenzel, 2012; Feliciano et al., 2011; Wong, 2009; Cepeda et al., 2006). Recently developed
animal models demonstrate spontaneous ictogenesis and epileptogenesis, that might be
independent of histopathological features of malformations, with genetic etiologies, present a
promising platform for epilepsy treatment development (Baek et al., 2015; Nguyen et al., 2015; Roy et al., 2015; Lim et al., 2015; Ljungberg et al., 2009; Zeng et al., 2009). Consistent with the diverse histopathology in different MCDs and LNETs, the cellular and network electrophysiological mechanisms that cause seizures in each MCD or LNET type and affected age group may be different (Cepeda et al., 2006).

Some histopathological findings are shared in different conditions (Crino, 2013; Lee et al., 2012). Balloon cells observed in FCD IIb resemble “giant” cells found in TSC disorder and ganglion cells found in GG. Dysmorphic mislocalized neurons were found in FCD II, TSC, GG, and DNETs. Disruption of cortical cytoarchitecture is present in those disorders with different levels of severity (Crino, 2013; Thom et al., 2012; Blumcke et al., 2011; Thom et al., 2011a). This led researchers to hypothesize that activation of common, major molecular pathways that regulate cellular proliferation, migration, differentiation and growth may result in seizures. One of the key pathways activated by growth factors, in which high frequency of mostly de-novo somatic mosaic mutations has been found in MCDs is the mechanistic target of rapamycin pathway (mTORC1,mTORC2 complexes, PI3KCA-AKT3-mTOR) (Blumcke and Sarnat, 2016; Hevner, 2015; Crino, 2013; Lim and Crino, 2013; Wong, 2013). In respect to mutations found in MCDs (Crino, 2015), ligand binding to receptor tyrosine kinase leads to activation of phosphatidylinositol 3-kinase catalytic alpha subunit (PI3KCA) that phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) converting it to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Gross and Bassell, 2014). That phosphorylation cause binding and activation AKT3 (Protein kinase B) (Choe et al., 2003). Another enzyme, phosphatase and tensin (PTEN) homolog converts PIP3 back to PIP2 reducing activation of AKT3 (Kreis et al., 2014). AKT3 phosphoinhibit TSC2 (tuberin) thus disrupting its binding with TSC1(hamartin) and causes activation of mTOR1 complex (Inoki et
al., 2002; Manning et al., 2002). AKT3 has more than 100 substrates and can affect cell growth and survival, by inhibiting the forkhead box O pathway (FOXO), and cell proliferation through inhibition of glycogen synthase kinase 3 (GSK3) pathway. The second pathway, extensively studied in melanoma and papillary thyroid cancers (Chen et al., 2012; McCubrey et al., 2012) and is hyperactivated in LNETs is RAS-RAF-ERK (MAPK) pathway. This pathway may be activated in parallel to mTOR pathway downstream of receptor tyrosine kinase (RTK – FGFRs, IGF1R, TRKB) (Blumcke et al., 2016; Hevner, 2015; Kumar et al., 2005). Those two pathways may interact (Pernice et al., 2016) through ERK1/2 phosphorylation of TSC2 and subsequent activation of mTORC1 (Ma et al., 2005). Additionally, BRAF may regulate AKT3 through, possibly structural, kinase independent interaction with rictor-mTORC2 complex (rapamycin insensitive component of mTOR) and prevention of mTORC2 enzymatic activity, or through raptor of mTORC1 complex protein (regulatory associated protein of mTOR) (Chen et al., 2012). Also RAS protein may directly participate in activation of mTOR pathway (Kumar et al., 2005) (Figure 1 - Molecular Pathways in MCDs and LNETs).

Figure 1 - Molecular Pathways in MCDs and LNETs

![Figure 1](image-url)
PI3K-AKT3-MTOR PATHWAY IS UPREGULATED IN MCDs AND LNETs

Increasing number of pathological conditions are been shown to have mutations in mTOR signaling pathway. mTOR is a serine/threonine kinase that forms two protein complexes (mTORC1 and mTORC2) and is involved in regulation of most cellular growth, proliferation, migration and metabolic processes (Crino, 2016; 2015; Bockaert and Marin, 2015; Liko and Hall, 2015; Laplante and Sabatini, 2012; Zoncu et al., 2011; Yuan and Cantley, 2008). Activation of mTOR pathway is environmentally regulated and occurs in response to a wide variety of signals. Upon activation of mTOR pathway through RTKs or G-protein coupled receptors (GPCRs) by Insulin or insulin-like growth factors (IGFs) there is an increase in protein and lipid synthesis that may lead to increased cell size and dysmorphogenesis (McCubrey et al., 2012; Laplante and Sabatini, 2009; Kumar et al., 2005; Gao and Pan, 2001).

mTOR pathway is regulated by multiple extracellular as well as intracellular signals. Downregulation of mTOR pathway may result from intracellular changes in oxygen and cellular energy levels, DNA damage (Heberle et al., 2014; DeYoung et al., 2008), and synaptic transmission (Ling et al., 2017; Kenney et al., 2015; Weston et al., 2012; Fraser et al., 2008; Tavazoie et al., 2005). Changes in those processes may signal through adenosine-monophosphate-activated protein kinase (AMPK) (Budanov and Karin, 2008; Inoki et al., 2003) and/or regulation of DNA damage response 1 (REDD1) (Lipina and Hundal, 2016). Any activating upstream to mTOR signal requires presence of amino acids, particularly leucine and arginine, and this is independent of TSC1/2 complex regulation (Chantranupong et al., 2016; Wolfson et al., 2016; Smith et al., 2005).
Possible activation of mTOR pathway has been previously shown in LNETs. Using single-strand conformation polymorphism (SSCP) and sequence analysis of PCR products together with laser assisted microdissection of single cells Becker et al. (Table 2. 2001) described increased high frequency of genetic polymorphism with mostly silent or in the non-coding regions of TSC2 gene in 15 out of 20 dissected GG patients tissue samples (most affected are intron 4 and exon 41) and, additional, interesting, new somatic mutation in intron 32 of TSC2 (C.148C->T). The mutation in intron 32 of TSC2 was specific to glial cell population but not dysplastic neuronal cells. Additional findings included 7 silent polymorphisms or polymorphisms in the non-coding regions in TSC1 gene. The finding of new somatic mutation in intron 32 of TSC2 in glial population provides a possible support to the hypothesis of the neoplastic transformation of glial component in GG.

Latter study by Boer et al. (2010b) using immunocytochemistry demonstrated phosphoactivated protein members of mTOR pathway in 9 cases of GG. Amongst those proteins are pPDK1, a protein kinase upstream to AKT (in about 27% of all neurons in the field), and AMOG, an additional upstream activator of AKT, coincided with CD34 expression pattern. In the control tissue AMOG expression was restricted to perivascular astrocytes (GFAP positive). In DNETs, on the other hand, AMOG expression was evident in only few astrocytes. Ganglioglioma tissue also had relatively high expression of phosphorylated pAKT (in about 58% of all neurons in the microscope field). Ezrin, radixin and moesin (ERM) – actin binding proteins that are involved in cell adhesion and growth control and interact with TSC1 were found to be expressed in about 57% of all neuronal population of microscope field in GG. Phosphorylated mTOR was expressed in about 66% of all neurons in the microscope field. About 17% of neurons in GG were positive for p4EBP1, a downstream target of mTORC1. Phosphorylated eLF4G a downstream target of 4EBP1 was expressed in 28% of all neurons in the microscope field in GG.
Phosphorylated S6 in 33%, pS6K-T389 in 28%, p-S6K-T229 in 25%. In contrast to GG the highest expression of mTOR pathway components in 9 DNET cases was for pPDK1 – about 13%, 5% for pS6, 2% for pS6K-T229 and 2% for pAKT. Importantly, peritumoral dissected tissue (from three GG and two DNETs) showed higher expression of all mTOR pathway phosphorylated components compared to DNETs. The neoplastic glial component of GG was not consistently immunoreactive to phosphorylated mTOR pathway components. Interestingly that recent work by Kakkar et al. (2016) does show positive p-S6 and 4EBP1 staining in 89% of DNETs cases (57 patients) with 10 to more than 50% of cells stained. The difference may lay in pre-operative invasive evaluation procedures, for instance, epilepsy monitoring using depth electrodes implantation that has been shown to phoshoactivate pS6 (Sosunov et al., 2008). It’s possible that increased “stress” to brain parenchyma may increase phosphoactivation of pS6, which could be differentiated by IHC for phoshoactivated SAPK/JNK as shown by Sosunov et al. (2008). Avoiding such confusion initially by examining and quantifying the staining distribution in the tissue farther away from the implanted electrodes may represent a possible solution with care description of tumor affected area.

Samadani et al. (2007) has shown an increased activation of mTOR pathway in resected GG tissue. By Immunohistochemical staining against phosphorylated protein S6 of 40S ribosomal subunit and S6 kinase, a downstream target of mTOR, this group estimated that about 30% of all cells in each low magnification (X20) microscope field were positive for pS6K and pS6. Hutt-Gabevazas et al. (2013) using IHC staining against phoshoactivated components of mTOR pathway in formalin fixed/paraffin embedded tissue samples reported moderate to strong staining for pS6 in 107 cases out of 177 of pediatric low-grade glioma consisting mostly of pilocytic astrocytoma cases (133). Additionally, those authors showed various number of cases stained positive for
p4EBP1 (35 out of 115), pELF4G (66 out of 112), mTOR (53 out of 113), RAPTOR (64 out of 102), RICTOR (48 out of 101), and pAKT (63 out of 103) and complete loss of PTEN (7 out of 101). Immunohistochemical staining showed PTEN loss in another LNET - papillary GNET (PGNET, 2 out of 4 cases) (Myung et al., 2011). PCR amplification of DNET dissected and formalin fixed tissue showed 4 out of 73 studied cases with Loss of Heterozygosity (LOH) in 10q of PTEN locus (Thom et al., 2011a).

A case report using PCR analysis and literature review showed PIK3CA E545K mutation in one case with a mixed rosette forming glioneuronal tumor - RGNT/DNET finding (Eye et al., 2017). These authors using Pubmed and Embase search from 1946 to November 2015 also reported on another six cases of RGNTs published mutations in PIK3CA: 1) E542K, and 2 cases of H1047R (Ellezam et al., 2012), 2) H1047R (Thommen et al., 2013), 3) E545K (Cachia et al., 2014) 4), H1047R (Gessi et al., 2014). Fibroblast Growth Factor Receptor 1 (FGFR1) N546K mutation reported in RGNT by Gessi et al. (2014) using pyrosequencing and Sanger sequencing upon relapse also demonstrated aforementioned PIK3CA H1047R. There was one additional case with FGFR1 K656E mutation (initially diagnosed as pilocytic astrocytoma – PA). Overall these studies demonstrate significant activation of mTOR pathway either due to dysfunction of regulator-inhibitor (PTEN) component, increased downstream targets phosphorylation (pS6, pS6K, PELF4G), or presence of activating mutations (PIK3CA E545K, H1047R) in LNETs.

**RAS-RAF-ERK PATHWAY IS UPREGULATED IN LNETS**

Another pathway that is activated in LNETs and may have interactions with mTOR pathway is mitogen activated protein kinase (MAPK) pathway. MAPK pathway major components RAS-RAF-ERK are used on par for the pathway nomenclature. RAS-RAF-ERK is one of the major
molecular pathways involved in cell proliferation, survival/apoptosis, gene regulation, migration and growth. As mTOR pathway dysregulation increased recognition as a pathological cause of MCDs in the recent decade, the dysregulation of RAS-RAF-ERK pathway also has been linked to MCDs, particularly to LNETs (Hong et al., 2015; Kim and Choi, 2015; McCubrey et al., 2012; Lawrence et al., 2008; Roberts and Der, 2007; McCawley et al., 1999).

Binding of the ligands (growth factors, insulin, cytokines, interleukins) to RTK results in recruitment of Src homology 2 domain containing adaptor protein to C-terminus of activated RTK. Src homology domain 2 recruits Grb2 (growth factor receptor-bound protein 2) and SOS (son of sevenless – a guanine nucleotide exchange factor, GEF). Son of sevenless is a homolog protein that loads the membrane bound GDP:GTP exchange protein (GTPase) Ras with GTP. Ras then recruits the serine/threone kinase Raf to the membrane where it becomes activated. Raf phoshoactivate mitogen activated protein kinase kinase-1 (MEK1), that in turn phoshoactivate extracellular-signal regulated kinase 1/2 (ERK1/2). Phoshoactivated ERK1/2 has more than hundred substrates. Amongst its activation targets are transcription cellular machinery - P90 ribosomal six kinase-1 (P90RSK1), that activate cAMP response element binding protein (CREB)) in the nucleus. ERK1/2 also activates translational process through P90RSK1 activation of MAPK signal integrating kinases (Mnk1/2), that activates eukaryotic translation initiation factor 4E (ELF4B). Also ERK1/2 activates P70 ribosomal S6 kinase (P70S6K), which phoshoactivates Ribosomal Protein S6 (PS6)) in different cellular compartments (Pernice et al., 2016; McCubrey et al., 2012; Xing et al., 1996). Additionally, ERK1/2 exerts multiple inhibiting feedback loops with different components of RTK-RAS-RAF-ERK pathway to prevent its activity. Some of the examples are described here. ERK1/2 can loop back on another member of RAF family - CRAF either activating it or inactivating it depending on the site of phosphorylation (Sturm et al., 2010;
Balan et al., 2006; Dougherty et al., 2005). It can phosphoinhibit MEK1 (Catalanotti et al., 2009), and cause dissociation of SOS/Grb2 complex from tyrosine phosphorylated sequences that prevents Ras activation (Buday et al., 1995). ERK1/2 can also phosphoinhibit RTK (Li et al., 2008b), or induce dual specificity phosphatases (DUSPs) transcription that serves as a negative regulators of RAS-RAF-ERK pathway (Ekerot et al., 2008).

Activating mutations in RAS-RAF-ERK pathway are found in multiple components including the RTKs in LNETs (Stone et al., 2017; Rivera et al., 2016; Blumcke et al., 2014; Thom et al., 2012). Here are described few recent cases as an example of most frequent mutations. Chappe et al. (2013) using PCR-high resolution melting (PCR-HRM) with Sanger sequencing and IHC showed retrospectively BRAFV600E activating mutation in about 30% of DNETs, 38.7% of GG, 12.5% of PA and 60% of PXA. Using IHC Koelsche et al. (2013) showed that 58% of GGs have stained positive for BRAFV600E clone VE1 specific antibody. This was further confirmed by Sanger sequencing in DNA available samples with 32 out of 34 VE1 positive tumors. Dahiya et al. (2014) using VE1 IHC showed positive staining for BRAFV600E in 38% of GGs. BRAFV600E mutation represent the most frequent mutation in LNETs with up to 70% of cases (Prabowo et al., 2014; Schindler et al., 2011). Additionally, BRAF copy number increase was shown in 33% of DNETs (Kakkar et al., 2016). Fusion with KIAA-1549 gene arising from tandem duplication of about 2 Mbp fragment of chromosome 7q arm producing protein with N-terminus of KIAA-1549 and BRAF kinase domain, was shown in about 70% of PA cases (Collins et al., 2015; Jones et al., 2013). In a few cases, fusion with other genes that resulted in the loss of regulatory N-terminus and retention of BRAF kinase domain was reported as well: FAM131B, RNF130, CLCN6, MKRN1, GNAI1, QKI, FZR1, and MACF1 (Jones et al., 2013; Zhang et al., 2013; Cin et al., 2011). Interestingly, alterations (insertion of three amino-acids
p.Arg506_insValLeuArg) that stabilize dimeric form of BRAF, which is active independent of Ras stimulation, has been shown to increase pERK1/2 signal comparable to BRAFV600E mutation in NIH3T3 cells (Jones et al., 2013).

Other driver mutations (McCubrey et al., 2012) in RAS-RAF-ERK pathway in LNETs include RTK (Fibroblast Growth Factor Receptor 1: FGFR1) N544K, N546K, K656E, R659L (Qaddoumi et al., 2016; Jones et al., 2013). Alteration in autophosphorylation results in higher kinase activity increasing RAS-RAF-ERK activation, which probably works through its substrate FGF2. Alterations in RAS (KRAS), the same allele affecting E63K + R73M and L19F + Q22K mutations was described in two different cases of PAs (Jones et al., 2013). CRAF alterations include SRGAP3-CRAF fusion in PAs that leads to increased kinase activity of the product (Jones et al., 2009). Activating mutations in the platelet-derived growth factor receptor α (PDGFRα) K385I and K385L were reported in 2 LNETs, 1 DNET, and 1 oligoastrocytoma (Qaddoumi et al., 2016). New fusion of genes SLMAP-NTRK2 (sarcolemma associated protein-neurotrophic receptor tyrosine kinase 2) was found in GG (Qaddoumi et al., 2016). Passenger mutations and mutations in genes found in more aggressive tumors are not discussed here.

**RAF ONCOGENES AND PROTEINS FUNCTION IN DISEASE.**

Of the RAS-RAF-ERK pathway alterations, the RAF oncogene subfamily of serine/threonine kinases elicit the most interest. A single missense somatic mutation in BRAFV600E and multiple BRAF fusions has been found in different pathophysiological conditions, specifically in up to 70% of LNET cases (Ballester et al., 2017; Chasseur et al., 2017; Ko et al., 2017; Lee et al., 2017; Oishi et al., 2017; Vanden Borre et al., 2017; Domingo and Schwartz, 2016; Kakkar et al., 2016; Prabowo et al., 2014; Koelsche et al., 2013; Cin et al., 2011; Schindler et al., 2011).
RAF GENE FAMILY PROTEINS AND MOLECULAR INTERACTIONS.

The mammalian RAF (Rapidly Accelerated Fibrosarcoma) gene family consist of three genetically independent isoforms: ARAF, BRAF and CRAF (also RAF-1). ARAF is located on the sense strand of chromosome Xp11.2-p11.4, depending on the sequence source (GCID:GC0XP047562). BRAF is on the antisense strand of 7q34 (GCID:GC07M140719) and CRAF is on the antisense strand of 3p25.2-3p25 (GCID:GC03M012583). All three RAF proteins are activated by Ras-GTP binding and serve as activators of mitogen-activated protein kinase/ERK kinase (MEK1/2) (Papin et al., 1995b) with differential sensitivity to RAS and Src tyrosine kinase phosphorylation requirement, and differences in activation potency and interaction preferences (Lavoie and Therrien, 2015; Hekman et al., 2002; Marais et al., 1997; Wu et al., 1996). The BRAF is RAS-activated, Src-independent with the highest basal kinase activity, and the most potent activator of MEK. While CRAF and ARAF are both Src and Ras activated and ARAF is the least potent activator of specifically MEK1 and not MEK2 (Wu et al., 1996). There are 21 Ras isoforms and only HRAS, KRAS and NRAS has been shown to activate BRAF in HEK293T cells, while ARAF responded also to RRAS3 and CRAF to RRAS3, RIT and TC21. This Ras isoform interaction with ARAF and CRAF may be cell-type dependent (Rodriguez-Viciana et al., 2004; Vossler et al., 1997).

CRAF(RAFT-1) – cellular proto-oncogene was the first homolog of that family cloned in 1985 by Bonner et al. (Bonner et al., 1985), however the first description of RAF homologue, dates to 1983 as a retroviral oncogene, v-RAF, transduced by murine sarcoma virus (MSV) isolate 3611 recovered from mouse that developed histiocytic lymphoma and lung adenocarcinoma (Rapp et al., 1983). Before 2002 CRAF was the primary focus of research of those genes family. The discovery of BRAF mutation in 66% of malignant melanomas and at the lower frequency in
multiple human solid tumors (Davies et al., 2002) shifted attention to biological functions and biochemical interactions of that isoform. BRAF was first identified by Ikawa et al. (1988) and is the phylogenetic oldest isoform that is absent in yeast and appears in invertebrates (Aken et al., 2017; Ensembl, 2016).

Figure 2 - RAF protein and gene structure

2A. (numbered residues for PKA) adopted from Kornev et al. (2006) & reviewed by Roskoski (2010) 2B. is from Wan et al. (2004). A. Diagram of the inferred interactions between human BRAF kinase catalytic core residues, ATP, and MEK (the protein substrate). Catalytically important residues that are in contact with ATP and MEK occur within the light khaki background. Secondary structures and residues that are involved in regulation of catalytic activity occur within the gray background. Hydrophobic interactions between the HRD motif, the DFG motif, and the aC helix are shown by black arrows while polar contacts are shown by dashed lines. Phos, phosphate. B. Schematic of BRAF primary structure, showing functional domains and position of 32 observed cancer-associated mutants of BRAF. The amino acid substitutions are color coded according to their activity class. (Figure 4 in Wan et al., 2004)

All RAF isoforms have 3 functionally distinctive conserved domains (CR1-3, Figure 2, adopted from Wan et al. (2004)). CR1 contains two RAS-GTP binding sites (RBD and CRD – cysteine rich domain or a zing finger structure, a secondary RAS binding site), and is involved in membrane recruitment, and, through CRD interaction with kinase domain in autoinhibition (Tran.
et al., 2005). CR2 when phosphorylated at inhibitory phosphorylation sites (S214 for ARAF, S259 for CRAF and S365 for BRAF) negatively regulates RAS-GTP binding and Raf activation (Dhillon et al., 2002). CR3 is a kinase and phosphoactivation domain.

BRAFV600E mutation activates BRAF kinase by mimicking phosphorylation of the activation segment of CR3 that releases its inhibitory interactions with the ATP binding P-loop at the same kinase domain (Roskoski, 2010; Kornev et al., 2006; Wan et al., 2004). Conversely, Rheb protein, which activates mTORC1 at the lysosome, suppresses BRAF and CRAF kinase activity through phosphorylation reduction of N-region of CR3 domain and subsequent disruption of dimerization of RAF proteins (Karbowniczek et al., 2006; Im et al., 2002).

Although heterodimers of CRAF with BRAF are found at the lower concentrations in COS-1 cells (about 0.3% of CRAF and 0.1% BRAF total cell pool), it occurs as a part of physiological activation and accounts for most of the kinase activity compared to homodimers and monomers. Heterodimerization results in about 30-fold increase in kinase activity, functionally serving as additional regulatory mechanism. This heterodimerization requires intact kinase activity, is stabilized by binding of 14-3-3 proteins when CRAF is activated by Ras and destabilized by ERK feedback phosphorylation of T753 in BRAF C-terminus (Rushworth et al., 2006; Weber et al., 2001). During heterodimerization BRAF can transphosphorylate and activate CRAF given that it is phosphorylated at two residues (T491 and S494) in activation loop and is bound to 14-3-3 proteins (Garnett et al., 2005). While BRAF wild type protein requires RAS activation of CRAF for dimerization - BRAFV600E induce constitutive RAS independent dimerization with CRAF in COS-7 cells (Garnett et al., 2005).

Interestingly, more than 80% of mutations located either within the ATP binding P-loop or the activation segment, with V600 site mutations occurring in 98% of oncogenic BRAF and V600E
mutation representing over 97% of those mutations (Lavoie and Therrien, 2015; see supplementary Table1 Wan et al., 2004).

BRAF gene comprised of 18 exons and is expressed in all tissues with specifically high expression levels in neuronal tissue of CNS, testes and parathyroid gland as shown by RNA-seq and IHC, single cell RNA-seq, as well as RT-PCR (Uhlen et al., 2016; 2015; Linnarsson, 2015; Zeisel et al., 2015; Barnier et al., 1995). Alternative splicing of BRAF generates 10 different spliceosomes in murine and 5 in human tissues, contributing to tissue specific RAS-RAF-ERK pathway regulation (Aken et al., 2017; Ensembl, 2016; Barnier et al., 1995). Eight out of ten spliceosomes are expressed in murine brain and nine in spinal cord. Two of the BRAF spliceosomes (containing exon 8b; and neuron specific – 10) expressed in the brain tissue at much higher levels than in other organ tissues (Barnier et al., 1995; Papin et al., 1995a).

**FUNCTION OF RAF PROTEINS THROUGH MOUSE MODELS.**

Knock-out (KO) mouse models demonstrate a differential functionality of RAF isoforms (Matallanas et al., 2011). CRAF complete KO has been shown to have increased apoptosis of embryonic tissue. Specifically, high effects were observed in the liver with hypocellularity, due to hepatoblasts decrease. Those mice die by mid-gestation (E11.5-13.5, 16.5), which is dependent on mixed genetic background (Mikula et al., 2001). Conditional placenta excluded CRAF KO did not have rescue effect (Galabova-Kovacs et al., 2006). Knockin mutation of the endogenous 340/341 tyrosines to phenylalanine in CRAF, which disrupts kinase domain activity on MEK, produce adult mice with no apparent defects (Huser et al., 2001). This substitution results in only up to 70% reduction of induced activity, in which case the residual activity might be sufficient to sustain life and normal phenotype of affected mice (Barnard et al., 1998). BRAF complete KO results in death
in mid-gestation (E11.5) due to vascular malformation in the placenta and could be rescued by conditional placental excluded KO. This was consistent with the higher expression levels of BRAF in embryonic placenta of E11.5 mice, while CRAF showed higher expression levels in embryo proper and ARAF was equally expressed in embryo proper and the placenta (Galabova-Kovacs et al., 2006). The BRAF KO mice also have a substantial growth deficits and neuronal defects at E10.5 that are not attributed to developmental arrest (Galabova-Kovacs et al., 2006; Wojnowski et al., 1997). Interestingly, conditional knockin mouse model of BRAFV600E mutation also cause embryonic death by gestational day E7.5. At even low expression levels under the control of IFN-inducible promoter in the mouse are lethal with overall survival up to post-natal day P28 (Mercer et al., 2005). This indicates that deregulation of BRAF activity in growth and development is detrimental for survival. ARAF KO survive within the range of P7 to P21 exhibiting a wide neurological and intestinal pathologies. This is also genetic-background dependent, with C57Bl/6 background producing lethal young mice and when bred on 129/OLA background producing adult mice that has neurological defects (Pritchard et al., 1996).

The oncogenic potential of all three RAF isoforms is also different. CRAF and ARAF mutations are rare in human cancers (Rebocho and Marais, 2013; Zebisch et al., 2006; Emuss et al., 2005; Lee et al., 2005), while BRAF mutations has been established as a primary oncogene (Davies et al., 2002). Although overactivated CRAF in tissue-specific manner in transgenic mice under SP-C promoter can cause lung adenoma (Kramer et al., 2004; Kerkhoff et al., 2000) and its overexpression also found in a variety of human cancers, such as lung, liver, prostate, myeloid leukemia and head and neck squamous cell carcinoma (Mukherjee et al., 2005; Riva et al., 1995; Schmidt et al., 1994), current theory suggest a mediating RAS and RTK overactivation role for CRAF (Dumaz et al., 2006; McPhillips et al., 2006; Zebisch et al., 2006; Emuss et al., 2005; Porter
and Vaillancourt, 1998; Miwa et al., 1994). Considering scarce findings of CRAF mutations in naturally occurring cancers on one hand and CRAF overexpression found in some cancers on the other hand, more thorough examination is required to exclude the possibility of the allelic alterations and random mutations in cases with overactivated CRAF and to determine its role in oncogenesis. Rare ARAF mutations in cancer and their kinase inactivating nature suggest a passenger mutation or signaling specific context (Rebocho and Marais, 2013).

**OTHER PATHWAYS, GENES AND CHROMOSOMAL ALTERATIONS IN LNETs**

Previous studies have shown multiple alterations in PIK3CA-AKT3-mTOR and RAS-RAF-ERK pathway leading to their aberrant activation in LNETs, however in few cases other pathways which may interact with the aforementioned ones are pathologically altered. Additionally, multiple studies suggest that global gene expression is altered and possible chromosomal reorganization happens in LNETs (Qaddoumi et al., 2016; Prabowo et al., 2015; Aronica et al., 2008a; Fassunke et al., 2008; Hoischen et al., 2008; Becker et al., 2006).

One of the additional altered pathways in GG is the reelin pathway that is involved in neuronal development, migration, morphogenesis, neuronal plasticity and neurodegeneration (Bock and May, 2016; Ishii et al., 2016; Lee and D'Arcangelo, 2016; Sekine et al., 2014). Kam et al. (2004) showed one case of mutation/polymorphism in p35 gene (L302I) and overall lower expression levels of two major components of the reelin pathway p35 (21%) and dab1 (28%) (disabled-1) in GGs compared to controls of normal brain tissue with equivalent parts of white and grey matter. The protein encoded by p35 is an activator of cyclin-dependent kinase-5 (CDK5), another important component of the reelin pathway. Earlier Becker et al. (2002) showed lower expression
(mRNA levels normalized to GAPDH) of CDK5 (55%) and another major component of the reelin pathway - doublecortin (DCX, 30%) in GGS tumor tissue samples compared to epilepsy patients without MCDs. CDK5 functions in neuronal maturation and migration and many more other essential biochemical processes (Dhavan and Tsai, 2001). DCX is a phosphoprotein that is associated with microtubules and responsible for neuronal maturation, migration and cytoskeletal stabilization during neurite outgrowth (LoTurco, 2004).

Fusion of nuclear phosphoprotein transcription factor MYB involving mostly exon 15, but in some cases also exon 9 to QKI RNA-binding protein gene at exon 5 was found in 41% of diffuse astrocytomas (DA), and 87% (13/15) of angiocentric gliomas by whole-exome-sequencing (WES), whole-genome (WGS) and transcriptome sequencing (RNA-seq) (Qaddoumi et al., 2016). Using Sanger sequencing for hot-spot mutation analysis authors showed one DA case with h3 histone family member 3A (H3F3A) K27M mutation, which is involved in formation of nucleosome structure of chromosomal fiber. Authors also reported of IDH1/2 mutation that was found in 4 out of 20 diffuse oligodendroglial tumors (d-OT), new fusion of genes in GGS – EWSR1-PATZ1 (EWS RNA binding protein 1- POZ/BTB and at hook containing zink finger 1). In the same study additional frequent genetic alteration was found in d-OTs - chromosome 1p/19q co-deletion.

Gain of chromosomes 5 (16-18%), 7(21-23%), 6 (10%), 8 (13%), 12 (12%); and loss of 22q (16%), 9(10%), 10 (8%) mostly in glial components was found in GGs and DNETs. With gain of chromosome 5 been the most frequent finding in GGs (Prabowo et al., 2015; Hoischen et al., 2008). However, Prabowo et al. (2015) found no correlation of chromosomal 5,7 and 6 gains to DNETs and GG IHC features or clinical data. Hoischen et al. (2008) using unsupervised cluster analysis also showed separation of GGS into two subgroups, one with complete gain of chromosome 7 combined with 5, 8 and 12 and the other with loss of chromosome 9 and 22q, although the
alterations found in the second subgroup are not exclusive to it. Interestingly, methylation profiling with principle-component analysis of 91 samples of LNETs showed segregation of genetic mutation subtypes into four distinct groups - adult-type glioma, BRAF, FGFR1, MYB/MYBL1 pathologies (Qaddoumi et al., 2016). Supporting the finding of BRAF and FGFR1 groups segregation, another study using DNA methylation arrays combined with RNA sequencing of 111 samples from GG, DNET and non-specific glioneuronal tumors showed two distinct groups, one with astrocytic differentiation and is driven by BRAF mutations, while the second group displayed oligodendroglial differentiation driven by FGFR1 mutations (Stone et al., 2017)

Gene expression analysis using U133 plus 2.0 microarray with 38,500 human genes spanning 54,000 probe sets identified 2874 overexpressed and 3255 underexpressed probes at p<0.01 and false detection rate of 3.9% (each probe has a 3.9% chance of been false positive) in 4 GGs compared to 3 fresh autopsy acquired control samples (Aronica et al., 2008a). Aronica et al. (2008a) showed that immune and inflammatory genetic mechanisms were the most increased in GGs - numerous interleukins, TGF, Toll-like receptor pathway and T-cell receptor signaling pathway, multiple complement pathway genes, and coagulation cascade genes together with cell adhesion and extracellular matrix genes like laminin, collagens, thrombospondins, CD9, CD44. Angiogenesis genes were also increased. The authors also showed fivefold increased expression of GFAP and vimentin, indicative of reactive astrogliosis in GGs. Increased expression of cyclin D1, D2 and cyclin-dependent kinases 4 and 6 (CDK4 and 6) while decreased CDK5 and its regulatory subunit – P35 and deregulated Wnt signaling pathway that controls cell fate and cycle confirms abnormal cell development in GGs. Surprisingly genes associated with synaptic transmission and neuronal excitability represented the most prominent underexpressed group - decreased Voltage-Gated Potassium Channels was concurrent with decreased calcium channel
auxiliary subunits and some sodium channels auxiliary subunits underexpression. Interestingly though that SCN9A that encodes Nav1.7 and SCN7A were overexpressed in GGs. Multiple GABA receptor subunits together with gephyrin, a protein assisting in post-synaptic clustering were also underexpressed. Additionally, increase in expression for sodium-potassium co-transporter NKCC1 and decrease in potassium-chloride co-transporter KCC2 indicate possible change in GABARs modulation of neuronal excitability (Aronica et al., 2008a). To avoid inter-patients’ genetic variability Fassunke et al. (2008) using adjacent normal cortical samples from 6 GG patients and 7 DNETs and transcriptome-wide expression on U133A microarray analysis showed a differential expression of 94 genes with affected chromatin state and transcription factors, Intracellular and extracellular signal transduction, cell adhesion, cell cycle and proliferation, development and differentiation.

Gain on chromosome 3p26.2-p26.3 encompassing two activating ligands of notch - CNTN6 and CNTN4 suggest a possible dysregulation of this signaling pathway in GGs (Hoischen et al., 2008). Notch-signaling pathway play important role in embryogenesis and cortical development (Carrieri and Dale, 2017) The presented examples of chromosomal and genetic alterations in LNETs provide a significant amount of information about those changes and demonstrate importance of further organization, classification and elucidation of epileptogenic mechanisms of subgroups of LNETs for development of personalized treatment approach (Blumcke et al., 2016; 2014; Holthausen and Blumcke, 2016; Thom et al., 2012).
1.3 EXCITABILITY MECHANISMS CONTRIBUTING TO

EPILETOGENESIS IN MCDs AND LNETs.

Heterogeneity of LNETs may also point to multiple different molecular mechanisms responsible for epileptogenesis. Considering that LNETs are the second most frequent pathology finding in surgically treated adult patients after HS, and in children after FCD, it is necessary to find their primary causes. The genetic aberrations and the associated pathological hyperexcitability and hypersynchrony mechanisms have a direct link to epilepsy. Investigation of those mechanisms may further contribute to improvement of patients’ treatment with purpose of developing targeted personalized therapy with minimal effect on their quality of life. The next section discusses current theories of epileptogenic mechanisms in LNETs and MCDs.

PROLIFERATION, MIGRATION, DIFFERENTIATION AND CELL GROWTH

Cortical hexalaminar formation is a tightly regulated process. Initial proliferation of neuronal stem cells (NCS) in the ventricular zone (VZ), asymmetric and symmetric division, differentiation to radial glia in mid-gestational period (E33 in humans and E10.5 in rodents), and migration along the radial glia axis, or tangential migration from ganglionic eminence (GE) of interneurons to appropriate cortical laminae creates complex cortical structure. Glial and neuronal cells originating from radial glia progenitors pool, generating synaptic contacts with subcerebral and thalamic areas and function to create different sensory modalities and complex social behavior (Lim and Alvarez-Buylla, 2016; Breunig et al., 2011; Bystron et al., 2008; Molyneaux et al., 2007; Kriegstein and Parnavelas, 2003; Rakic, 2003). Interruption of this intricate process during proliferation leads to
changes in cell composition, size, dyslamination and synaptic connections (Roy et al., 2015; Alkuraya et al., 2011; Bakircioglu et al., 2011). Disrupted migration keeps cells from reaching designated laminae (Chew et al., 2013; Cushion et al., 2013; Poirier et al., 2010). Inappropriate differentiation and growth leads to alterations in a post-synaptic neurotransmitter receptors and changes in intrinsic excitability properties (Judkins et al., 2011; Andre et al., 2010; Andre et al., 2004; Cepeda et al., 2003; Crino et al., 2001). This may lead to neuronal networks with lower threshold for seizure generation.

Changes in neuronal and glial cell populations may stem from reduced proliferation or increased apoptosis, increased proliferation or decreased apoptosis, and/or abnormal proliferation. The molecular pathways that are involved in cell cycle progression and DNA repair, and subsequently affect neurogenesis and gliogenesis are altered in disorders with presumed defects in proliferation of progenitor cell population, like FCD II, TSC, HME, ME, GG, microcephaly. (Parrini et al., 2016; Blumcke et al., 2014; Barkovich et al., 2012). The mechanisms of epileptogenicity in abnormal proliferation MCDs is less clear. The suggested mechanisms are abnormal synaptic contacts with increased excitatory neurons and decreased inhibitory interneurons content, and support of the network with increased seizure susceptibility by astroglial dysplastic cells (Andre et al., 2004; Crino et al., 2001; O'Connor et al., 1998; Cornell-Bell et al., 1990). Additionally, current theory suggests that epileptogenicity source may arise outside the malformation foci, or be independent of cortical malformation (Maynard et al., 2017; Roy et al., 2015; Kohling et al., 2006; Patt et al., 2000).

Migrational defects may arise from injury to ventricular zone epithelium (migration start point) or pial limiting membrane that cause detachment of radial glia and disruption of migratory pathway. Included in these group are different types of heterotopia, lisencephalies, cobblestone
malformations (Parrini et al., 2016; Barkovich et al., 2012). Interesting that in periventricular nodular heterotopia expression of layer specific markers shows that the heterotopic population of migrating neurons is the early one. The neurons that designated to populate layers 6 (Rorβ) through 4 (Nurr1) are the ones that forms this malformation (Garbelli et al., 2009). The mechanisms of epileptogenicity is not clear in those disorders as well, with similar hypothesis of synaptic dysregulation and probably dyslamination independent seizures (Farhan et al., 2017; Diaz-Alonso et al., 2016; Hsieh et al., 2016).

Injury to the brain that happens after the cell migration period is ended may disrupt cortical cytoarchitecture and cause FCDI or in some instances FCDII, polymicrogyria and schizencephaly (Barkovich et al., 2012). In those disorders the epileptogenicity mechanisms are also not known.

1.4 HISTOPATHOLOGICAL FEATURES AND THEIR HYPEREXCITABILITY IN MCDs AND LNETs

Shared histopathological features in MCDs with LNETs may be the source of ictogenesis and subsequent epileptogenesis. Following sections presents common features and their possible contribution to neuronal and network hyperexcitability.

**DYSMORPHIC CYTOMEGALIC NEURONS**

Dysmorphic cytomegalic neurons in resected human brain tissue have a range of 16-43 μm diameter of the soma compared to 12-25 μm in normal-appearing pyramidal neurons in layer 3, tortuous processes and presumed to represent inappropriate differentiation and growth and almost always co-occur with cortical dyslamination. Those dysmorphic cytomegalic neurons were suggested to be the source of epileptogenicity (Cepeda et al., 2006; 2005; 2003). Those neurons are present in FCD Type II, hemimegalencephaly (HME), TSC, GG, complex DNETs, diffuse
DNETs and mixed DNETs, lack in FCD Type I (Crino, 2013; Thom et al., 2012; Blumcke et al., 2011; Thom et al., 2011b; Palmini et al., 2004) and simple DNETs (Thom et al., 2011b). In this case other morphologically and altered components and/or cortical structure disruption, for instance – immature, misoriented neurons and dyslamination may be the source of epileptogenesis. Increased excitability of dysmorphic cytomegalic cells may be due to increased L-type Ca\(^{2+}\) conductance (Cepeda et al., 2003). Relatively hyperpolarized resting membrane potential and reduced postsynaptic receptors densities in dysmorphic cytomegalic neurons contradict the hypothesis of those cells been epileptogenic source. Additionally, as it has been shown in FCD patients, ECoG ictal zone and interictal spiking zones were the least expected location for dysmorphic neurons to be found after resection. Cortical regions enriched in dysmorphic neurons displayed background high voltage slow rhythmic waves, while FDG-PET/MRI showed thickening of cortical area (pachygyria). Although those findings indicate that dysmorphic cytomegalic neurons are less likely to be the epileptogenic source, their presence correlated with CD severity and worst ECoG scores (Cepeda et al., 2005). Possible alterations in molecular mechanisms that may cause hyperexcitability in dysmorphic neurons is increased Pannexin 1 expression shown with Western Blot and immunohistochemistry in resected brain tissue from FCD IIa and IIb patients (Li et al., 2017). Dysmorphic cytomegalic neurons were shown to have decreased Mg\(^{2+}\) sensitivity of postsynaptic NMDA receptors due to altered, dysmature receptor subunit composition (Andre et al., 2004). Cytomegalic neurons from FCD II showed increased GABA induced peak currents, but decreased current densities. The GABA induced currents had longer kinetics and decreased sensitivity to zolpidem and zinc compared to non-dysplastic tissue and FCD I resected tissue pointing to different subunit composition of GABA\(_A\) receptors or delayed clearance of GABA from synaptic cleft (Andre et al., 2008).
In support of dysmature brain development hypothesis (Cepeda et al., 2007; 2006), where seizures may result from interactions of dysmature cells with normally developed and properly migrated pyramidal cells, cytomegalic interneurons may also be hyperexcitable and contribute to ictal discharges (Andre et al., 2010; Andre et al., 2008; Andre et al., 2007; Avoli et al., 2005). GABA\(_A\) mediated chloride driven synaptic currents may switch from inhibition (Cl\(^-\) influx) to excitation (Cl\(^-\) efflux) in GG, HME, FCD Type I and Type II, TSC and HS with medial temporal lobe epilepsy (MTLE) due to increased expression of Na\(^+\) K\(^+\) -2Cl\(^-\) cotransporter - NKCC1. This NKCC1 cotransporter increases the internal chloride concentration. Additionally, decreased/ altered expression of K\(^+\) - Cl\(^-\) cotransporter, KCC2 that extrudes Cl\(^-\) outside the cell based on established K\(^+\) gradient by Na\(^+\)/K\(^+\) ATPase pump was also reported in the aforementioned disorders (Puskarjov et al., 2014; Talos et al., 2012; Jansen et al., 2010; Aronica et al., 2007; Munakata et al., 2007; Cohen et al., 2002). Support to the dysmature brain development hypothesis was also shown in immunohistochemical study of resected brain tissue from FCD Type II patients with clustering of parvalbumin (PV) and GAD stained terminals around cytomegalic neurons (Andre et al., 2008; Spreafico et al., 2000). In those studies, however, the overall PV and GAD staining was reduced in the examined specimens. It is not known if cytomegalic interneurons release more than one type of neurotransmitters.

**IMMATURE MISORIENTED NEURONS**

Immature misoriented neurons are relatively scarce, and more frequently found in young patients with severe FCD. Those neurons have a diameter of less than 18 µm and soma area of less than 250 µm\(^2\), have underdeveloped dendritic arbors with relatively few spines, and frequently observed in vertically oriented microcolumns in FCD Type Ia (Blumcke et al., 2011; Hildebrandt
et al., 2005; Palmini et al., 2004; Cepeda et al., 2003). In FCD Ia immunohistochemistry showed that Pannexin 1 was distributed in microcolumn organized neurons, in FCD IIa and IIb it was shown in dysmorphic neurons, balloon cells and reactive astrocytes. Pannexin 2 levels were increased only in FCD IIb (Li et al., 2017). Quantitative RT-PCR and western blot analysis showed increased mRNA expression and protein levels of Pannexin 1 in the resected brain tissue of FCD Type Ia, FCD IIa, and FCD IIb patients compared to autopsy controls (Li et al., 2017). Pannexin 1 is a large pore ion channel, which is involved in release of multiple signaling molecules like Ca$^{2+}$, glutamate, arachidonic acid and ATP (Aquilino et al., 2017). Although Pannexin channels may both enhance and reduce seizure susceptibility the current data mostly in animal models with one study of resected brain tissue from Rasmussen syndrome patients suggest that blocking Pannexin channel is anticonvulsant (Aquilino et al., 2017; Cepeda et al., 2015). Immature misoriented neurons also receive strong rhythmic depolarizing GABAergic input (Cepeda et al., 2003).

**BALLOON CELLS**

Balloon cells are categorized by their large cytoplasm to nucleus ratio, pale eosinophilic appearing on H&E staining, lack of Nissl substance, with frequent finding of multinucleated forms. They are not specific to any cortical area or location and are often found in an underlying white matter. They can be found in small aggregates and within a “normal” brain tissue.(Blumcke et al., 2011). Intermediate neuronal filaments – vimentin and nestin that are more characteristic of progenitor cells and radial glia in neuroepithelium are frequently accumulate in balloon cells. Those cells are less frequently stained with GFAP (Urbach et al., 2002). The lack of extensive processes and electrophysiological neuronal features – no action potential generation, no synaptic inputs, suggest that they are not the “pacemaker” of epileptogenic activity, nor they able to sustain
However, their more glial morphology shows that they may be the source of extrasynaptic glutamate that contributes to epileptogenicity of the proximal cortical region (Cepeda et al., 2006). Additionally, their presence in cortical circuitry may disrupt “normal” synaptic contacts and create aberrant hyperexcitable neuronal networks.

1.5 CHANGES OF INTRINSIC NEURONAL EXCITABILITY MECHANISMS IN MCDs

Neuronal intrinsic excitability – ability to fire action potential, either spontaneously or in response to synaptic inputs is dependent on both “passive” as well as “active” cell electrophysiological properties. The mode of firing – bursting, anode break excitation (hyperpolarization induced rebound depolarization and AP firing), depolarization block and non-adapting high frequency firing are probably more dependent on “active” electrophysiological properties. Those active electrophysiological properties include channel densities and their distributions and channel gating properties - activation, inactivation, and voltage-dependence of the latter. Additionally, ion concentration inside and outside the cell and changing voltage “driving force” helps to establish resting membrane potential and contribute to the activation or inactivation of ion conductances (Bean, 2007). Passive electrophysiological properties of the cell include resting membrane potential (RMP), which is also established upon electrochemical equilibrium of all ions inside and outside the cell, membrane capacitance ($C_m$), input resistance ($R_{in}$) and membrane time constant ($\tau_m$). More depolarized RMP makes reaching AP voltage threshold, after which the cell fire AP, easier. Increased $R_{in}$ shows that to reach the same AP voltage threshold the cell will require smaller depolarizing input current ($I_m$). And shorter $\tau_m$ and smaller $C_m$ allows
faster $C_m$ charge and faster response of ion channels to changing voltage affecting the speed of AP propagation.

**PASSIVE PROPERTIES CHANGED IN MCDs**

Group of Cepeda et al. (2014; 2012; 2010; 2007; 2005; 2003; Andre et al., 2007) showed that different populations of cells in MCD resected tissue from a cohort of young patients (FCD I, II, III, FCD with HME, Heterotopia, TSC, and TSC alone ages 0.19-14 years) have distinctive electrophysiological properties. Average membrane capacitance was significantly larger in cytomegalic neurons (309-397 pF), cytomegalic interneurons (290 pF, N=8) and in balloon/giant cells (217-269 pF) compared to neighbor “normal” pyramidal neurons (132-194 pF). At the same time immature small neurons had smaller capacitance (64,77 pF). Input resistance was much higher in immature cells (607,479 MΩ) and “normal” interneurons (329,523 MΩ), and smaller in cytomegalic neurons (50-71 MΩ) and cytomegalic interneurons (45, N=8) (Andre et al., 2007), it was only higher in balloon cells in two publications, described initially as atypical cells (553 MΩ, N=24) in (Cepeda et al., 2003) and balloon cells (also 553 MΩ, N=22) in (Cepeda et al., 2005), which may be misclassification of different abnormal type of cells; latter work from the same lab showed comparative to normal input resistance in balloon cells (148-210 MΩ) (Cepeda et al., 2012; 2010). Membrane time constant, $\tau_m$, was significantly longer in dysmorphic neurons (4.5 ms, N=12), cytomegalic neurons (4.5-5 ms), cytomegalic interneurons (4.2 ms, N=8), and balloon cells (2.9-3.6 ms), shorter in immature neurons (0.9,1ms) and “normal” interneurons (0.9,1.1ms).

On the other hand, RMP was more hyperpolarized in cytomegalic neurons (-65 - -71 mV) compared to “normal” pyramidal neurons (-55 - -70 mV) (Cepeda et al., 2012; 2010; 2005; 2003; Andre et al., 2007). RMP in balloon cells (-57 mV, N=24) was depolarized and comparable with
the “normal” pyramidal neurons (Cepeda et al., 2005). Interestingly newer work from the same lab shows balloon cells with more hyperpolarized RMP (-74 mV, N=47) in resected tissue samples from TSC patients, with one example of balloon cell from FCD IIb patient displaying more depolarized RMP (Table. 2 & Figure 3D. Cepeda et al., 2012). Comparing FCD II with TSC resected tissue, Cepeda (2012) also showed that cytomegalic neurons were more frequently found in FCD, while balloon/giant cells were more frequent finding in TSC, also balloon cells in FCD II were closer to the white matter than in TSC. The differences in passive electrophysiological properties were not observed in previous publications, which characterized mostly “normal” pyramidal neurons (Cepeda et al., 1999; 1993; 1992; 1991; Tasker et al., 1996; Dudek et al., 1995; Wuarin et al., 1990)

**ACTIVE PROPERTIES CHANGED IN MCDs**

In the previous electrophysiological studies of resected CD-tissue no abnormal firing patterns were found in examined cells, however in most cases only “normal” pyramidal cells were examined (Tasker et al., 1996; Dudek et al., 1995; Wuarin et al., 1990). In studies that did examine abnormal cells, no differences were found in firing frequencies between cytomegalic and “normal” pyramidal neurons from FCD II. Additionally, cytomegalic neurons did not generate spontaneous AP firing in whole-cell current clamp.(Cepeda et al., 2005). The authors suggested that Inspite of these cells been capable of exciting neighboring neurons thus amplifying seizure activity they are not hyperexcitable enough to initiate seizures. On the other hand, cytomegalic inhibitory GABAergic interneurons displayed spontaneous AP firing and rhythmic bursting upon suprathreshold depolarization in current-clamp, which may synchronize neuronal activity thus initiating seizures (Andre et al., 2007). The balloon cells also did not display any hyperexcitability
and did not fire AP upon application of any depolarizing current steps from -500 pA to 500 pA. Immature small pyramidal neurons in resected CD-tissue were suggested to be a possible hyperexcitable candidates (Cepeda et al., 2007) with a single example of a current-clamp recorded cell responding to lower rheobase with higher firing frequency of smaller amplitude APs. Normal pyramidal neurons in TSC resected cortical slices did not show significant changes in the firing frequencies (Cepeda et al., 2010). In the TSC1 Cre-mediated post-natal knockout mouse model in acute slices in hippocampal CA1 pyramidal neurons the rheobase, AP voltage threshold and latency to the first spike were increased and the AP firing frequencies were decreased compared to control neurons. The authors suggested that this was the result of a decreased input resistance and increased membrane capacitance (Bateup et al., 2013). Earlier Wang et al. (2007) showed that there were no differences in firing frequencies and AP properties between the control tissue and TSC1 synapsin1-Cre driven knockout normal appearing pyramidal neurons. Although there was an increase in SAG and rebound excitation that was not quantified.

Cumulative study over five years (Cepeda et al., 2003) showed that peak mixed Ca\(^{2+}\) conductances were significantly increased in cytomegalic neurons (4,008 pA, N=22, compared to 1,666 pA, N=183 in “normal” pyramidal neurons), with about 60% of current flow through L-type Ca\(^{2+}\) channels, and other 40% through P/Q (25%) and N (15%) types. No active ion conductances were detected in balloon cells. Andre et al. (Figure 5B, 2007) noted a delayed inward rectification (SAG) in interneurons in current clamp mode upon hyperpolarizing current pulse, which is indicative of hyperpolarization activated depolarizing current (I\(_{\text{h}}\)).

Inspite of those findings intriguing question of how the epileptic seizures are generated remains unanswered. The possible epileptogenic source - the cytomegalic dysmorphic neurons are found
only in about 60% of child FCD cases (Cepeda et al., 2006), which cannot explain epileptogenesis in remaining 40% of cases.

1.6 CHANGES OF SYNAPTIC EXCITABILITY IN MCDs AND LNETs

Multiple Immunohistochemical and electrophysiological studies in FCDs, TSC, HME and LNETs showed altered synaptic receptors densities, subunit composition that suggest possible immature properties of malformed components. The following sections present representative studies on alterations in synaptic mechanisms in MCDs and LNETs.

ROLE OF GABA RECEPTORS IN MCDs

Alterations in inhibitory and excitatory circuits have long been suggested as one of the possible pathophysiological mechanisms in epilepsy (Spreafico et al., 1998a; Marco et al., 1996; During et al., 1995; DeFelipe et al., 1994; 1993; Wolf et al., 1994; Dichter and Ayala, 1987; Lloyd et al., 1986; Ribak et al., 1979; Van Gelder et al., 1972). First investigated by Ferrer et al. (1992) in tissue from FCD patient and latter (Ferrer et al., 1994) in a mixed group of patients that included patients with neoplasms (not specified) and FCDs showed a decrease in parvalbumin, calbindin D-28K and somatostatin positive interneurons in the necrotic area but not in the dysplastic area. There was also selective to the neoplasm affected cortical area decrease in parvalbumin and calbindin D-28K immunoreactivity. Latter study in FCD II showed patchy reduction in PV and calbindin immunostaining in dysplastic tissue with islets of positive cells throughout all cortical layers compared to localized to layers II/III positive staining in non-FCD control tissue (Calcagnotto et al., 2005). In nodular heterotopia, FCD I, and IIb the changes in expression of postsynaptic
receptors showed that there was a reduced number of parvalbumin (PV, chandelier and basket cells marker) and glutamic acid decarboxylase (GAD, enzyme that catalyzes conversion of glutamate to GABA) positive interneurons and interneuronal terminals. In FCD IIb the cytomegalic neurons were surrounded by clusters of PV and GAD positive neuronal terminals. The authors also reported reduced number of GABA-transporter 1 (GAT1, transports GABA from synaptic cleft into neurons) staining in FCD IIb. (Andre et al., 2010; 2008; Spreafico et al., 2000; 1998a; 1998b). In FCD I there was reduced staining of PV, GAD and patchy distribution of GAT1 and no clusters of PV and GAD (Spreafico et al., 2000), which was shown statistically insignificant in the latter studies (Andre et al., 2010; 2008). Interestingly in tissue from TSC patients there was an increased PV staining and positive for GAD staining in 30% of cases of TSC-like lesions as reported by Wolf et al. (1994). Increased decay kinetics of spontaneous inhibitory post-synaptic currents (sIPSCs) in (Calcagnotto et al., 2005) suggest that there is an alteration in GABA reuptake in FCDII. Decrease in overall PV positive terminals was seen in FCD II except giant neurons located in the white matter, which were surrounded extensively by PV positive terminals (Alonso-Nanclares et al., 2005). Similar results for the GAD staining were obtained in FCD II cortical tissue, with fewer GAD positive but enlarged cells in the presence of cytomegalic neurons in 50% of cases (4/8). The GAD immunopositive patches was not observed in FCD I, FCD II without cytomegalic neurons and non-FCD tissue (Andre et al., 2008). In addition to alterations in the number of neurons positive for PV or calbindin, and changes in number of synaptic contacts, alterations of GABA$_A$ subunit composition has been studied in FCD as a possible mechanism of increased seizure susceptibility. Andre et al. (2008) showed that when GABA is applied to dissociated cytomegalic cells from FCD II the kinetics of the cells’ responses that are mostly mediated by GABA$_A$ are different. Zinc, which blocks GABA$_A$ receptors that lack $\gamma_2$ subunits
were less effective in cytomegalic neurons. Additionally, authors demonstrated reduced sensitivity (no increase in GABA\textsubscript{A} currents) to zolpidem, a type I benzodiazepine that binds with higher specificity to α1 subunit containing GABA\textsubscript{A} receptors in all cells sampled from FCD affected foci. The same group also showed in 2010 (Andre et al., 2010) that bretazenil, a partial agonist that increases GABA current in neurons containing α3, α4 and α5 subunits caused a larger change in dissociated “normal” neurons from FCD II compared to non-FCD neurons. Additionally, frequencies and amplitudes of sIPSCs were only increased in FCD II neurons, with larger increase seen in cytomegalic neurons compared to non-FCD tissue and to FCD I. Worth of note that the increased sIPSCs frequencies were inconsistent with Calcagnotto et al. (2005), that reported on decreased frequencies of sIPSCs. The authors suggested that this was due to the older patients’ population in the latter work. Also in the previous work comparison of sIPSCs frequencies in dissociated cells from mixed population of patients with different MCDs from the same group (Cepeda et al., 2005) did not show any statistically significant difference suggesting that grouping those MCDs together might obscure true pathological alterations specific to each type of lesion. This was evident in the latter studies comparing FCD IIb with TSC (Cepeda et al., 2012; 2010). In this work authors found that all neurons from FCD IIb patients possess higher GABA\textsubscript{A} mediated synaptic innervations compared to neurons in TSC patients. Activation of GABA\textsubscript{B} receptors by its agonist baclofen in FCD cortical tissue to inhibit ictal discharges was shown to have higher IC50 compared to tissue from medial temporal lobe epilepsy patients (D’Antuono et al., 2004). White et al. (2001) showed that GAD65, VGAT, GABA\textsubscript{A}R α1 and α2 subunits mRNA was decreased in tubers compared to adjacent cortical tissue, which was further confirmed by single cell mRNA analysis of large dysplastic neurons and “giant” cells. GAD65 mainly was located to large dysplastic neurons, no immunoreactivity was detected in “giant” cells. Additionally, increased
mRNA levels of GABA\(\alpha_4\) and decreased levels of GABA\(\beta_1\) were detected only in large dysplastic neurons.

GABA\(_A\) synaptic activity is responsible for pacemaker interictal events in the cortex (Cepeda et al., 2014; 2007; Avoli et al., 2005; D'Antuono et al., 2004). If the neurons innervated by this action potential dependent spontaneous pacemaker activity have immature properties with increased internal Cl\(^-\) concentration, those events will become depolarizing. This may promote AP firing and synchronize neuronal network inducing seizures (Puskarjov et al., 2014; Talos et al., 2012; Aronica et al., 2007). Although, the consideration of the experimental preparation causing physiological changes that alter the GABA\(_A\) action from hyperpolarizing to depolarizing needs to be taken into the account (Dzhala et al., 2012) with possible utilization of perforated patch clamp recording technique (Talos et al., 2012). This pacemaker activity was more frequently found (in 34%) in patients with FCD compared to non-CD (Rasmussen Encephalitis – 12.5%, no significant histopathology – 16.7%) and to TSC patients (in 3%) (Cepeda et al., 2014). It was also more readily found in more sever FCD types (in 27% in FCD I, 34% FCD II\(a/\)II\(b\), 75% FCD III associated with GG or DNET).

**ROLE OF GLUTAMATE RECEPTORS IN MCDs AND LNETs**

Several studies showed alterations in NMDA receptors subunit composition in MCDs. Most of them suggested a decrease in NR2A/B subunits expression in the CD affected area. Contradicting IHC and RT-PCR based findings in different studies still leave some questions unanswered. Spreafico et al. (1998a) was the first one to show a decreased staining in superficial cortical layers and segregation of NR1 positive neurons in deep layers and white matter in 2 (ages 20 and 14) out of 3 FCD patients. In the same work authors showed increased GluR2/3, an AMPA
receptors subunits staining in “giant” dysmorphic pyramidal neurons, and increased NR1 positivity in “giant” dysmorphic pyramidal neurons and 50-60% of balloon cells. In previous work immunopositive for NR1 balloon cells were shown to be present in 30% of cases of TSC-like lesions (Wolf et al., 1994). Andre et al. (2004) showed that NMDA currents were increased in dissociated cytomegalic neurons from resected CD (not specified) tissue which is concordant with previous studies that showed increased positive co-staining and mRNA levels of NR1 and NR2A/B in cytomegalic, but also “normal” neurons from CD tissue (Finardi et al., 2013; Najm et al., 2000; Mikuni et al., 1999; Ying et al., 1999; Babb et al., 1998) or GG and DNETs (Aronica et al., 2001), and almost no mRNA and weak staining from distal parts of the same tissue. The current densities per unit area were decreased in this study (Andre et al., 2004), which shows that the number of receptors per unit area is decreased compared to non-CD cells. Subsequently they found a decreased Mg^{2+} and ifenprodil (a selective NR2B inhibitor) sensitivity of cytomegalic neurons and “normal” neurons from CD tissue, which with the use of RT-PCR and IHC showed a decreased mRNA and protein expression in a subset of cytomegalic and “normal” neurons from CD compared to non-CD cells. The decrease in NR2A/B expression in dendrites and soma of neurons was also reported in nodular heterotopia (Battaglia et al., 2002). In contrast to previous studies (Aronica et al., 2001; Najm et al., 2000; Ying et al., 1999) Andre et al. (2004) using RT-PCR showed that almost all neurons from non-CD tissue, which included epileptic tissue from either a cerebral infarct, temporal lobe epilepsy, trauma, or Rasmussen encephalitis, were expressing NR1 NR2A NR2B mRNA. The authors argued that from a functional perspective it is improbable that native homomeric NR1 receptors exist, additionally, NR2C/D subunits which may co-assemble with NR1 to create a functional heteromeric NMDA receptors are minimally expressed in cerebral cortex. In other study by Moddel et al. (2005) authors found that ifenprodil almost completely
suppressed epileptiform discharges in CD tissue, with no change observed in non-CD slices. This showed that additional processing of tissue into dissociated cells (Andre et al., 2004) may cause alterations in cellular membrane proteins. NR2B and NR2D mRNA was also reported to increase in cortical tubers from TSC patients compared to adjacent cortex. Additionally, authors reported lower IC50 of ifenprodil binding together with use-dependent I-MK801 effect, an inhibitor specific to NR1/NR2A and NR1/NR2B, but not to NR1/NR2C nor NR1/NR2D in tuber homogenates. At the same time GluR1 mRNA was decreased in tubers (White et al., 2001). Subsequent single cell mRNA analysis showed higher relative abundance of GluR3, GluR4, GluR6, NR2B, and NR2C in large dysplastic neurons than in “giant” cells or in control neurons. Whereas GluR1 and NR2A were lower in large dysplastic neurons. Only NR2D subunit mRNA relative abundance was higher in “giant” cells compared to control neurons and large dysplastic neurons. Aronica et al. (2001) also showed positive for NR1 balloon cells, astrocytes, oligodendrocytes and fibrillar matrix in GG and DNETs. Additionally, Aronica et al. (2001) showed GluR1/2 and GluR5-7 and mGluR1α, mGluR5 positive staining in neuronal component of GGs and DNETs. Although no astrocytes were stained with mGluR1α, positive staining for mGluR5 was shown in oligodendrocytes and some astrocytes. Those cell types were negative for mGluR5 in “normal” cortical tissue. In the same work mGluR2/3 were found in less than 1% of neurons in GGs and DNETs, but mainly were observed in astrocytes of GG and not in oligodendrocytes. The mGluR7α staining was only found in the granular areas within the neuropil. In the perilesional area authors showed an increased staining with GluR5-7, mGluR2/3 and mGluR5 in reactive, GFAP and Vimentin positive astrocytes compared to astrocytes from normal cortex and white matter (Aronica et al., 2001). Talos et al. (2008) using western blot showed an increase in GluR1 and GluR4 in tubers homogenates from TSC patients compared to control autopsy tissue. Using IHC authors showed
that GluR1 increase was observed in “normal” pyramidal and dysplastic neurons but low in most “giant” cells, dysplastic astroglia and reactive astrocytes. In contrast GluR4 was increased in “giant” cells, astroglia, and reactive astrocytes but low in “normal” pyramidal and dysplastic neurons. Western blot for GluR2 and GluR3 showed lower levels in cortical tubers than in control tissue and cell specific IHC showed that it was abundantly expressed in “normal” pyramidal neurons but low in most dysplastic neurons, “giant” cells, dysplastic astroglia and reactive astrocytes. NR1 subunit levels and expression was not different between tubers and controls. Western blot showed no significant difference in NR2A between tubers homogenates and control ones, but with IHC authors saw high expression in “normal” pyramidal neurons, whereas it was low or absent in most dysplastic neurons and giant cells. Dysplastic astroglia and reactive astrocytes both expressed high NR2A levels. Total expression of NR2B and NR3A was significantly greater in cortical tubers relative to controls, with IHC NR2B expression was high in most “normal” and dysplastic pyramidal neurons, while it was low in “giant” cells dysplastic astroglia and reactive astrocytes. The NR2C subunit expression was low in “normal”, dysplastic pyramidal neurons and “giant” cells, but was high in most of the dysplastic astroglia and reactive astrocytes. The NR2D subunit expression was high in “giant” cells but was low or undetectable in other cell types. The NR3A subunit expression was high in all tuber cell types.

In summary, the different malformations may have different post-synaptic receptors affected in different malformed component. Increased GABAergic innervation that was observed in sever FCD cases (FCD I < IIa, IIb, < III), and in GG and decreased in TSC. NMDA receptors increase in normal components in TSCs but decreased in malformed components and in tubers. AMPA receptors immunoreactivity displayed similar to NMDA immunoreactivity distribution, and metabotropic glutamate receptors expression was also altered. Additionally, subunit
composition of the tested GABAergic, NMDA, AMPA, and mGluR post-synaptic receptors are altered not only in neuronal components but also in astrocytes.

1.7 NON-NEURONAL NETWORK HYPEREXCITABILITY MECHANISMS IN MCDs AND LNETs

Multiple studies on FCD II, TSC, GG and DNETs have reported on astrogliosis with increased expression of GFAP, a marker for reactive astrocytes, and microgliosis with Human Leukocyte Antigen - antigen D Related (HLA-DR), marker for activated microglia in the grey matter and one of the major histocompatibility complex (MHC) class II receptors. Additionally, those studies have shown increased expression of classical complement pathway components – C1q, C3c, C3d in astrocytes, glia and neurons. (Sun et al., 2016; Zhang et al., 2016; 2015; Aronica and Crino, 2011; Aronica et al., 2008b; 2005; 2001; Boer et al., 2010a; 2008; 2006; Iyer et al., 2010; Sosunov et al., 2008). Differences in the levels of activated microglia and astrocytes between FCD I, FCD II without difference in seizure frequency and duration (Iyer et al., 2010) suggest that this is not a simple result of seizure activity. Activation of mTOR pathway in FCD II but not FCD I may be a possible contributing factor to inflammatory process.

ASTROCYTES – NORMAL PHYSIOLOGICAL FUNCTION

Embryonic astrocytes derive from neuroepithelial radial glia neuronal progenitors and intermediate progenitors of the subventricular zone (SVZ) later in corticogenesis – around E15-E18 in mice (Siddiqi et al., 2014; Levison and Goldman, 1993). Second wave of astrogenesis happens latter at the postnatal stages from local differentiated astrocytes of a gray matter at P0-P21 (Ge et al., 2012). Astrocytes are diverse population of cells with brain region variable density and differential functions (Chai et al., 2017; Khakh and Sofroniew, 2015; Tsai et al., 2012). There
are two generally recognized classes of astrocytes in cortex – fibrous, with straight and long processes that mostly populate white matter and express high levels of GFAP. The second class are protoplasmic astrocytes that possess highly branched bushy processes, populate gray matter and in the healthy cortex do not express high levels of GFAP (Tabata, 2015). However, this classification may underestimate the number of different astrocytes types (Zhang and Barres, 2010). There are characteristic differences in astrocytes classes and organization between humans and mice. For instance, some classes of astrocytes are found only in primates like interlaminar astrocytes that populate layer I and astrocytes in layers 5 and 6 with long varicose fibers (Oberheim et al., 2009).

Due to inability to generate action potentials in undamaged tissue (O'Connor et al., 1998; Bordey and Sontheimer, 1998; Verkhratsky and Steinhauser, 2000) astrocytes have long been considered as a passive participants in central nervous system homeostasis (Ben Haim and Rowitch, 2017; Sontheimer et al., 1994). Their few of many passive functions include K⁺ ions balance maintenance through the uptake by Na⁺/K⁺-ATPase, Na⁺/K⁺/2Cl⁻ contrasporter, and spatial buffering, mainly, through inwardly rectifying Kir4.1 channels. The Kir4.1 is, evidently, coupled to water balance homeostasis through perivascular and subpial membrane anchored AQP4 channels (Devinsky et al., 2013; Amiry-Moghaddam et al., 2003; Simard and Nedergaard, 2004), with the subsequent K⁺ ions redistribution to the syncytium through coupled gap junctions formed by connexin hemichannels Cx43, Cx30 and Cx26 (Nagy and Rash, 2003; Steinhauser and Seifert, 2012). Additional passive roles include glutamate clearance from extracellular environment mostly through EAAT1 (GLAST-1 in rodents) and EAAT2 (GLT-1 in rodents) transporters (Malarkey and Parpura, 2008; Heja, 2014). Oxygen, glucose and its metabolites redistribution and delivery through gap junctions for synaptic transmission maintenance (Rouach
et al., 2008; Kowianski et al., 2013). Stimulation of axonal growth and regeneration, synaptogenesis, regulation of local blood flow (Smith et al., 1986; Ullian et al., 2001; Kowianski et al., 2013). Last two decades were flushed with an extensive research of astrocytic active functions. Discovery of Ca$^{2+}$ dependent glutamate release by astrocytes in cultures (Parpura et al., 1994) that evidently affects extrasynaptic NMDA receptors (Araque et al., 1998), which latter was corroborated by Bezzi et al.(1998) in acute hippocampal slice preparation, led to the concept of “tripartite synapse” (Araque et al., 1999b; Araque et al., 1999a; Perea et al., 2009). In addition to glutamate astrocytes are capable of a vesicular release of D-serine, adenosine, ATP, GABA, TNF$\alpha$ (Panatier et al., 2006; Martineau et al., 2008; Henneberger et al., 2010; Pascual et al., 2005; Martin et al., 2007; Zhang et al., 2003; Butt, 2011; Kozlov et al., 2006; Stellwagen and Malenka, 2006; Beattie et al., 2002; Kowianski et al., 2013); and slower lysosomal release of ATP (Li et al., 2008a; Jaiswal et al., 2007; Zhang et al., 2007). Although the debate on vesicular release is still ongoing (Sloan and Barres, 2014; Devinsky et al., 2013).

Alternative release mechanisms - including reversal of glutamate transporters, unpaired connexin/pannexin hemichannels on the cell surface, pore-forming P2X7 receptors and swelling-induced activation of volume-regulated anion channels, have also been investigated (Malarkey and Parpura, 2008). These multiple functions of astrocytes in different processes might be altered in pathophysiological states like epilepsy (Binder and Steinhauser, 2006).

In response to injury and infection or tissue degeneration and cell death seen in degenerative disorders and epilepsy astrocytes can increase their proliferation, alter their morphology, molecular machinery, function and gene expression, with increased expression of GFAP and Vimentin serving as a marker of these changes. The changes that happens in reactive astrocytes are dependent upon the nature and the severity of the insult (Sofroniew, 2009).
**ASTROCYTES ACTIVATION IN MCDS AND LNETS**

Astrogliosis in TSC patients was described to have similar morphological and immunophenotypical characteristics as HS with MTLE patients. The immunoreactivity to anti-GFAP, anti-CD44 and anti-S100β Ab, proteins that are expressed in white matter fibrous type astrocytes was increased in both TSC and HS MTLE tissue compared to perituberal and non-sclerotic hippocampal tissue. The significant decrease in immunoreactivity to anti-EAAT1 and anti-EAAT2 – glutamate transporters, anti-Glutamine Synthetase that transforms glutamate to glutamine, and anti-Kir4.1, an inward rectifying potassium channel, proteins that are expressed in protoplasmic grey matter astrocytes was evident in both TSC and HS MTLE tissue. Lucifer Yellow filled astrocytes in TSC and HS MTLE tissue also demonstrated a similar morphology. In contrast to HS there was a second population of patches of Vimentin and Nestin positive astrocytes that were surrounding giant cells in tubers. Additionally, there was an increased staining in astrocytes in tubers for superoxide dismutase (Mn-SOD), a mitochondrial enzyme, compared to HS MTLE tissue (Sosunov et al., 2008). Boer et al. (2008) showed increased immunoreactivity to C1q, C3c and C3d in astrocytes of TSC resected cortical tubers compared to autopsy controls. Additional activation of inflammatory cascades may be through HMGB1, a chromatin component that is released by injured cells and controls transcription of different genes including proinflammatory. Upon secretion it can bind receptor for advanced glycation end products (RAGE), TLR2 and TLR4. Binding of HMGB1 to TLR4 plays a role in generation and sustenance of seizures through tyrosine phosphorylation of NMDA receptor NR2B subunit in MTLE (Maroso et al., 2010). Zurolo et al. (2011) using qRT-PCR, *in-situ* hybridization and IHC showed increased TLR4 receptor and transcription factor, and RAGE in FCD, TSC and GG cases in astrocytes (GFAP positive cells), dysplastic neurons, but only occasional staining in balloon and giant cells. Occasional TLR2 was
seen in astrocytes as well. The HMGB1 mRNA was not significantly increased as tested by qRT-PCR, however increased immunoreactivity was observed in all cells of dysplastic FCD, TSC tubers and GG tumors, with additional cytoplasmic staining in glial cells. Control autopsy and peritumoral tissue had similar expression profiles of HMGB1, which were lower than the dysplastic/tumor/tuber tissue. This pathway was investigated in an additional study of fetal TSC resected tissue by Prabowo et al. (2013) that showed increased TLR2, TLR4 and RAGE immunoreactivity in giant cells, that were also positive for GFAP, vimentin and nestin and mTORC1 activation markers (pS6, pS6K, p4EBP1).

Murine model of astrocyte specific conditional deletion of TSC1 (exons 17 and 18) driven by GFAP-Cre expression showed increased astrogliosis and, after the first month of age displayed electrographic seizures (Wong et al., 2003; Uhlmann et al., 2002). The same CKO mice species were used by Zhang et al. (2015) to show increased mRNA and protein levels of IL-1β, CCL2 and CXCL10 with qRT-PCR and IHC in neocortex and hippocampus and was reflected in astrocytic cultures, which was reversed by treatment with mTORC1 inhibitor rapamycin. Using IHC authors showed that IL-1β colocalize with GFAP positive astrocytes in brain slices of four-week-old TSC^{GFAP} CKO mice. Using Western blot for CXCL10 authors show that the protein levels were increased in four-week-old TSC^{GFAP} CKO mice compared to control mice, similar results were obtained in astrocytic cultures, and rapamycin and epicatechin-3-galate treatment decreased its levels either in-vivo or in astrocytic cultures in-vitro. Importantly the increased expression of cytokines was observed before the onset of seizures. Blocking IL-1β and CXCL10 with epicatechin-3-galate at least partially reversed the elevated levels of cytokines and chemokines, reduced seizure frequency and prolonged survival of TSC^{GFAP} KO mice. These early changes were presumed to be a result of innate immune system in the brain and not infiltration of peripheral
macrophages. Another murine model of epilepsy with the predominant in the brain β1-integrin transmembrane receptor subunit conditional knockout in radial glia, the protein that is required for astrocytes acquisition of non-reactive state (Robel et al., 2015; Robel et al., 2009), showed increased excitability as evident by spontaneous seizures during the first six postnatal week. This was complemented by whole-cell patch-clamp recording in acute slices from single pyramidal neurons in layers 2/3 that showed depolarized RMP, increased input resistance Rin, shorter latency to interictal and ictal events in Mg²⁺ free media, and increased AP firing in response to depolarizing current steps compared to control cells. This was correlated with increased protein levels of NKCC1 at six weeks of age and decreased protein levels of KCC2 at six months of age as shown with Western blot assay and redistribution of KCC2 to perisomatic region as evident with IHC. Importantly, there was no cytoarchitectural changes and neuronal specific deletion of β1-integrin did not induce hyperexcitability. (Robel et al., 2015). Astrocytes can be a source of local glutamate dependent interictal paroxysmal depolarization shifts that cause neuronal synchronized burst firing (Tian et al., 2005).

Intrinsic molecular mechanisms responsible for astrogliosis may be regulated by the local environment (Schipke et al., 2008). Interestingly, astrocytes proliferation is partially controlled by MAPK pathway (Tien et al., 2012), with BRAFV600E expression at physiological levels contributing to increased proliferation in spinal cord. Conditional deletion of BRAF exon 12 containing the kinase domain in radial glia caused a decrease in astrocytes proliferation, as evident by dividing cells DNA incorporation of 5-Ethynyl-2’-deoxyUridine (EdU) in astrocytes. The contribution of MAPK pathway activation to gliogenesis in spinal cord was confirmed in cortex through manipulation of MEK1/2 in NestinCre and hGFAPCre mouse lines, also shown with
introduction of mutated RAS BRAF (V600E) and MEK1/2 in Neurog2 and Ascl1 mouse lines (Li et al., 2014; Li et al., 2012).

**MICROGLIA – NORMAL PHYSIOLOGICAL FUNCTION**

Microglia are the primary immune and cytokine-producing cells of CNS. Microglia possess a wide variety of shapes from ramified with extending processes to amoeboid macrophage-like shape and it can change from one shape to another depending on the environmental signals, with current view of amoeboid shape as an activated form and ramified as a silent form, although both forms may perform different functions. (Lloyd et al., 2017; Salter and Stevens, 2017; Werneburg et al., 2017; Masuda and Prinz, 2016; Miyamoto et al., 2016; Wake et al., 2013; Kreutzberg, 1996).

Derived from yolk sack myeloid progenitors microglia start to populate CNS at E9 through E14.5 and with the second wave between E14-E16 in mice and at gestational week 4.5 and 12-13 in humans (Reemst et al., 2016; Matcovitch-Natan et al., 2016; Ginhoux et al., 2010; Verney et al., 2010; Monier et al., 2007). In CNS microglia have been shown to participate in such diverse array of processes as subpial dopaminergic neurons axonal tract formation, DAP12 (Kiialainen et al., 2005; Lanier and Bakker, 2000), a transmembrane adaptor protein, and Cx3cr1 fractalkine receptor (Liu et al., 2009; Harrison et al., 1998) dependent Lhx6 and PV positive fast-spiking interneuron migration (Squarzoni et al., 2014). Microglia was also shown to participate in DAP12 and Cx3cr1 dependent neurite-growth and corpus collosus formation (Pont-Lezica et al., 2014), and in Ca$^{2+}$ dependent and actin filament mediated synaptogenesis (Miyamoto et al., 2016). Paolicelli et al. (2011) showed that microglia also participate in synaptic pruning partially through Cx3Cr1 fractalkine receptor, although the pruning may be delayed in that case due to decreased microglia cell densities in CNS. Stevens et al (2007) showed that microglia participate in synaptic
pruning through a complement pathway component C1q and C3 upregulation. Paolicelli et al. (2011) also showed microglia involvement in synaptic maturation. Riazi et al. (2015) showed that microglia affect basal synaptic transmission, and functional synapse plasticity. Other studies (Bessis et al., 2007; Rakic and Zecevic, 2000) showed microglia participation in promotion of programmed cell death and designated neurons phagocytosis. Ziv et al. (2006) showed microglia participation in maintenance of neurogenesis. Nimmerjahn et al. (2005) showed that microglia, through their dynamic ramified processes participate in immune surveillance response to injury. Microglia also participate in immunological signaling (Salter and Stevens, 2017; Aloisi, 2001).

**MICROGLIA ACTIVATION IN MCDs AND LNETs**

Sun et al. (2016) showed that in FCD IIb and TSC resected brain tissue there is a decrease in mRNA and protein levels of CD47, its receptor SIRPα and CD200, but not its receptor CD200R that is mainly expressed by microglia. CD47/SIRPα (Gitik et al., 2011; Oldenborg et al., 2001) and CD200/CD200R (Hoek et al., 2000; Wright et al., 2000) exert inhibitory effects on microglia phagocytosis, hence authors hypothesized that in malformed components of FCD IIb and TSC (dysmorphic neurons, balloon cells and giant cells) these molecules levels would be decreased compared to autopsy controls hence dis-inhibiting microglia. This decrease was shown by IHC. Increased expression of IL-1β in microglia and neurons was found in FCD, GG and DNET (Ravizza et al., 2006). Aronica et al. (2005) showed increased HLA-DR immunoreactive microglia in GG and DNETs with some clustering around neurons compared to control autopsy and peritumoral area. The increase of HLA-DR was higher in peritumoral area compared to the autopsy control. The increase in HLA-DR positive microglia correlated with the duration of epilepsy and the preoperative seizure frequency. Iyer et al. (2010) showed increased HLA-DR immunoreactive
microglia in FCD II dysplastic area compared to autopsy cases with viral encephalitis and multiple sclerosis and FCD I. While HLA-DR immunoreactive microglia numbers were not significantly different between FCD I and epilepsy control cases. In TSC resected brain tubers Sosunov et al. (2008) did not observe a widespread activation of microglia using HLA-DR antibody staining. On the other hand, Boer et al. (2008) reported on increased HLA-DR positive microglia in TSC resected brain tubers compared to autopsy controls and perituberal tissue. Those HLA-DR positive microglia were clustered around dysplastic neurons, blood vessels, and balloon cells. The authors also showed that number of HLA-DR positive microglia was correlated with the preoperative frequency of seizures. Additionally, the C1q, C3c and C3d a part of a complement system was observed in microglia, astrocytes, neurons and giant cells of resected tubers. The IL-1β and its receptor IL-1RI showed similar pattern of immunoreactivity to the complement system. In the previous work on FCD II resected tissue Boer et al. (2006) demonstrated similar to TSC increase of HLA-DR positive microglia in dysplastic tissue compared to autopsy control and correlation with preoperative seizure frequency and duration of epilepsy. Zurolo et al. (2011) using qRT-PCR in-situ hybridization and IHC showed increased expression of TLR2 and RAGE in microglia (HLA-DR positive), balloon, and giant cells, with only occasional immunoreactivity to TLR4 in FCD, TSC and GG resected tissue compared to autopsy controls. HMGB1 was evident in cytoplasm of microglia compared to nuclear localization in neuronal component in FCD dysplastic tissue, TSC tubers, and GG tumors.

Using murine model of TSCGFAP conditional knockout Zhang et al. (2016) showed increased numbers of iba1 positive microglia. The increase in iba1 positive microglia correlated with the earliest age of seizure onset. These microglia cells were larger than their counterparts in control mice. They also showed that inhibition of microglia activation with minocycline (Yrjanheikki et
al., 1998), an anti-inflammatory broad spectrum tetracycline antibiotic, prevented the increase in iba1 positive microglia and the increase in cell size but did not affect astrogliosis (increased number of cells positive for GFAP), and cytokine/chemokine (IL-1β, CCL2, CXCL10) production as evident by qRT-PCR and Western blot, and did not have a significant effect on seizures progression and survival. However, there was a trend towards less frequent seizures. The effects of minocycline did diminish by 7 weeks of mice age, and the higher doses caused premature death probably associated with liver toxicity (Zhang et al., 2016).

Increased inflammatory reaction in CNS causes morphological and molecular changes in microglia, increases proliferation and size that can increase neuronal excitability through a direct effect on ionotropic glutamate receptors (Zhan et al., 2014) or ion channels (Zhou et al., 2011).
CHAPTER 2 RESEARCH OBJECTIVES AND APPROACH

Recent discovery of BRAFV600E activating somatic mutation in 30-50% of LNETs (Barkovich et al., 2015; Blumcke et al., 2014; Koelsche et al., 2013; Schindler et al., 2011), which has been extensively described in solid non-brain tumors (Mikhailenko et al., 2017; Taieb et al., 2016; Singh et al., 2015; Chaft et al., 2012; Janku et al., 2011) allows to develop and study a new, etiologically accurate animal models of drug refractory epilepsy associated with LNETs. Application of Immunohistochemical markers that has been highly associated with LNETs, examination of synaptic network activity and ion channels together with single-cell ex-vivo electrophysiology, electro-corticographic recordings (ECoG) and RNA-sequencing allowed elucidation of pathogenic mechanisms that caused neuronal hyperexcitability. Based on recent data we tested the overall hypothesis that constitutive activation of RAS-RAF-ERK and/or mTOR pathway alters neuronal and synaptic excitability as well as cytoarchitectural cortical organization thus shifting cortical networks to seizure prone states. We hypothesized that constitutive BRAF activation directly cause definable changes in neuronal conductance and synaptic mechanisms by pathway overactivation, and that these can be rescued by experimentally downregulation or upregulation of the affected mechanisms.

The goals of the current work were:

Goal 1. To determine whether BRAFV600E mutation and increased copy number variant of its wild type – BRAFwt alter neocortical glial and/or neuronal morphology and cortical cytoarchitecture and whether those alterations correlate with increased inflammatory reaction.

I used a newly developed LNETs model in our lab in which introduction of human BRAFV600E mutations or BRAFwt into glial and neuronal progenitors is accomplished by
piggyBac-mediated transposition and *in-utero* electroporation. We used two promoters-helpers/donors plasmids driven systems, Glast-PB and Nestin-PB, to test whether cortical cytoarchitecture alterations dependent on the population of progenitor cells affected by BRAFV600E expression. Immunohistochemistry was used for GFAP and iba1 markers that are increased in LNETs and in FCD (Aronica and Crino, 2011; Aronica et al., 2008b; Aronica et al., 2005; Boer et al., 2006) to assess changes in reactive gliosis and activation of microglia.

**Goal 2. To Identify the alteration in transcriptome profiles of BRAFV600E expressing animals**

RNA-sequencing was performed to examine alterations of tissue-wide gene expression in the transfected, EGFP positive, cortical patches from three experimental conditions, Glast+BRAFV600E, Glast+BRAFwt, Glast+ control-FP (EGFP only electroporated). Isolation of whole RNA from transfected, mostly containing somatosensory cortices were done using ambion RNA recovery kit. We used Illumina NextSeq-500 library preparation, together with HISAT2 to map the sequences to mouse genome (Pertea et al., 2016), Stringtie to quantify gene abundance (Pertea et al., 2015) and DESeq2 to explore differentially expressed genes (Love et al., 2014).

**Goal 3. To determine whether constitutively active BRAFV600E and/or BRAFwt alter electrophysiology of Neocortical Pyramidal Neurons.**

Whole-cell patch clamp recordings were used to assess the changes in neuronal excitability. I tested the hypotheses that constitutive activation of BRAF in dysplastic neurons can result in depolarization of resting membrane potential, hyperpolarized AP voltage threshold, lower rheobase (current threshold), increased number of APs fired to given current step (Input-Output), increased hyperpolarization activated current (Ih), increased calcium current (ICaT, ICaL); or decreased potassium currents (IKA, IKDr, IKs). Examination of changes in those mechanisms have
shown how the excitability in the affected neurons is increased. Additionally, I tested the hypothesis of decreasing the increased excitability by activating the voltage gated tetraethylammonium sensitive sustained potassium conductances.

To test alteration in synaptic properties of BRAFV600E expressing neurons and whether there were alterations in the presynaptic release machinery, and post-synaptic responses I performed whole-cell patch-clamp recording of action potential dependent spontaneous post-synaptic currents (sPSCs), and action potential independent miniature PSCs and examined the frequencies and amplitudes of those events.

To test whether those neuronal specific changes are sufficient to cause seizures I used depolarizing channelrhodopsin cation channel (pCAG-ChR2) (Lin, 2011) responsive to blue light that was electroporated together with pPB-BRAFV600E and pGlast-PB at E14.5-E15.5 embryonic stages in mice and after they reached adolescence performed ex-vivo whole-cell patch-clamp together with ChR2 stimulation to test for seizure generation threshold. Electrocardiographic (ECoG) recordings were performed to test whether BRAFV600E transfected mice do have spontaneous seizures in-vivo. Additionally, I tested whether acute inhibition of BRAFV600E with currently available inhibitor Vemurafenib/Zelboraf (PLX4032, PLX4720) prevents neuronal hyperexcitability in ex-vivo cortical slices.

The overall results from these experiments provided insights into intrinsic neuronal excitability mechanisms that may be involved in epileptic seizures generation in RAS-RAF-ERK and/or mTOR related LNETs. Additionally, RNA sequencing provided insights on tissue specific non-cell autonomous alterations related to expression of BRAFV600E, which may be compared to the previously published results from resected brain tissue of patients with GG, DNETs, FCD,
and TSC (Zhang et al., 2013; Boer et al., 2010a; Aronica et al., 2008a; Hoischen et al., 2008; Samadani et al., 2007)
CHAPTER 3 RESULTS

GOAL-1 CYTOARCHITECTURAL AND MORPHOLOGICAL ALTERATIONS IN MURINE CORTEX OF BRAFV600E TRANSFECTED ANIMALS.

Expression of BRAFV600E in Glast+ and Nestin+ neuroglial progenitors induced with In-Utero Electroporation in mice increased gliogenesis and decreased the percentage of mature differentiated neurons in Glast+ condition, and increased neurogenesis and decreased astrocytes percentage in Nestin+ condition (Figure 3C,3E,3F). In Glast+ BRAFV600E cortical slices increased number of neurons remaining in lower cortical layers was observed (Figure 3C). Balloon-like cells with round shape and increased cytoplasm to nucleus ratio were found in both Glast+ and Nestin+ BRAFV600E cortical slices, starting at P30 aggregates of those cells were also observed (Figure 3B,3D,3G). Some of the balloon-like cells were immunoreactive to both upper and lower cortical layer markers, Cux1 and Ctip2, some were positive for astroglial marker GFAP, but not for neuronal marker NeuN (Figure 3D). In Nestin+ BRAFV600E cortical slices soma size of the EGFP positive cells located in the vicinity of the ventricle was larger than neurons in layer 2/3 (Figure 4). The immunoreactivity to microglia and reactive astrogliosis markers - Iba1, GFAP was increased in BRAFV600E slices (Figure 5 - Increased activation of astrocytes in BRAFV600E transfected brains, Figure S1 Iba1 immunoreactivity.).
Figure 3 - Dysmorphogenesis, migrational delay, astrogenesis and neurogenesis in BRAFV600E brains.
**Figure 3.** BRAFV600E expression in neuroglial progenitors at embryonic ages E14-E15 causes neuron migrational delay and cellular dysmorphogenesis. A. In-Utero Electroporation (IUE) experimental design. B. Representative image of Glast+ BRAFV600E electroporated mouse brain slice at post-natal day 180 showing aggregates of balloon-like cells near the pia and aggregates of a crescent shaped cells in layers 4 and 5. C. Cux1, an upper cortical layer marker and Ctip2, a lower cortical layer marker shows that most of the Glast+ BRAFV600E transfected neurons reach appropriate cortical layers relative to embryonic age at IUE. Insets show zoomed in neurons positive for Cux1, and negative for Ctip2 in upper layer 2. Lower panel shows 3 neurons located in the lower cortical layers and positive for cux1. D. Balloon-like cells and aggregates (panels I and II, Roman numerals – R.n.) of balloon-like cells found in Glast+ BRAFV600E transfected brains. Panels II,IV,VI,VIII (even R.n.) show anti-HA stain confirmation of pPB-BRAFV600E presence, Panels III-IX with odd R.n. show balloon-like cell stained positive for both Cux1 and Ctip2. Panels XI-XVII, odd R.n. show a balloon-like cell stained positive for Glial Fibrillary Acidic Protein (GFAP) an astroglial markers. Panels XII-XVII, even R.n. show a balloon-like cell negative for NeuN, a neuronal marker. Panels XIX-XXI showing coronal section from Nestin+ BRAFV600E with balloon-like cells aggregates in a piriform cortex area (white arrowhead). E. Gross neuron counts (left panel) and scaled to max neuron counts (right panel) - distance to pia measurement shows that there is a decrease in EGFP positive neuronal content in Glast+ BRAFV600E transfected mouse cortical slices and that higher number of the BRAFV600E transfected EGFP positive neurons targeted for upper cortical layers under both Glast+ and Nestin+ do not reach their designated location compared to Glast+ control-FP transfected brain slices (p<0.001), and to Glast+ BRAFwt transfected brain slices (p<0.001), there was also higher number of Glast+ BRAFV600E transfected neurons that did not reach designated cortical layers compared to Glast+ control-FP (p<0.001); significant difference in neuronal distance to pia was also present between Glast+ BRAFV600E and Nestin+ BRAFV600E (p=0.001). Due to non-homogenous variance - Levene test (3, 28606) = 1674.918, p<0.0001, Welch test with Games-Howell post-hoc correction were used (3, 9803.913) = 970.974, p<0.001. The results were confirmed with cumulative distribution nonparametric Mann-Whitney U test – Glast+ BRAFV600E to Glast+ control-FP, U= 31122376, p<0.001; Glast+ BRAFV600E to Glast+ BRAFwt, U= 18003030, p<0.001; Glast+ BRAFwt to Glast+ control-FP, U= 26624785, p<0.001; Nestin+ BRAFV600E to Glast+ control-FP U= 11954144.5, p<0.001; Nestin+ BRAFV600E to Glast+ BRAFwt, U= 7186563, p<0.001; Nestin+ BRAFV600E to Glast+ BRAFV600E, U= 12109290.5, p<0.001. F. Neuron to astrocytes percent ratio showing increased astrogliosis (EGFP positive cells) in BRAFV600E electroporated murine cortical slices. Due to non-homogenous variance (Levene test (3, 243) = 4.574 and 4.575 for neurons and astrocytes respectively p=0.004), Welch test F(3, 106.49)=183.71, p<0.0001; for single neurons, 500 µm and 50 µm zoomed in images, Lower panel – 100 µm for the wide field view, 50 µm for single neurons, 10 µm for single zoomed in neuron; for D. I.X – 50 µm; II-IX, and XI-XVIII, R.n. – 20 µm; for F. 1 mm. * - p<0.05, ** - p<0.01, *** - p<0.001. Error bars are ±2SEM.
Figure 4 - Nestin+ BRAFV600E large cells remained closer to the lateral ventricle

**Figure 4.** BRAFV600E expression in Nestin+ neuroglial progenitors. A. Representative image of EGFP positive cells in subventricular area compared to cells in layer 2/3 of somatosensory cortex that are quantified in B. Area measured in Nestin+ BRAFV600E EGFP positive cells in A in the upper panel, and diameter of those cells in the lower panel showing increased size of subventricular located cells (n=17) compared to layer 2/3 neurons (n=11, paired sample T=7.883, p<0.001 for area and T=8.224, p<0.001 for diameter). Scale bars for A – 500 µm * - p<0.05, ** - p<0.01, *** - p<0.001. Error bars are ±2SEM.

Astrocytes immunoreactivity to characteristic astrocytic markers - ALDH1L1, S100β was not different from the murine control cortical slices transfected with EGFP only (Figure 5).
Figure 5 - Increased activation of astrocytes in BRAFV600E transfected brains

**Figure 5.** BRAFV600E transgene in Glast+ neuroglial progenitors increases gliogenesis and induce reactive astrogliosis. **A.** Whole-slice image showing increased GFAP immunoreactivity in somatosensory cortex transfected with Glast+ BRAFV600E compared to Glast+ control-FP and to Glast+ BRAFwt transfected brains (upper 3 panels). Whole-slice image showing increased GFAP immunoreactivity in more frontal part of somatosensory cortex transfected with Nestin+ BRAFV600E (lower panel). **B.** GFAP positive cells were also immunopositive to astrocytes marker ALDH1L1 (white arrowheads). Scale bars for A (upper panel) 1 mm; (lower panel) 500 µm. B. - 50 µm; * - p<0.05, ** - p<0.01, *** - p<0.0001. Error bars are ±2SEM.
GOAL-2 RNA SEQUENCING OF TRANSFECTED WITH GLAST+

BRAFV600E, BRAFWT, CONTROL-FP BRAINS TISSUE.

In order to determine whether the increased immunoreactivity to GFAP and Iba1 observed was due to reactive gliosis and potential inflammatory responses in the regions of mutation bearing cells, I performed an RNA-sequencing experiment to compare the gene expression profiles of patches of cortex containing BRAFV600E, BRAFwt, or EGFP only transgenes in Glast+ condition. Previous studies have shown that approximately 5-10% of cells are transfected with IUE (Figueres-Onate et al., 2015; Rice et al., 2010), and so the majority of any change in transcripts is likely driven by changes in gene expression profiles in untransfected reacting cells in those patches. Using an unsupervised hierarchical clustering analysis of all genes in 12 samples, 4 in each transgene condition, I found that BRAFV600E, control-FP, and BRAFwt conditions clustered separately (Figure 6A). Differential expression and gene ontology analysis indicated a significant increase in the expression of genes in the inflammatory immune response pathway (H2-Aa, CD74, H2-Ab1, CD48, CD109, Cxcl16, Ccr1), and classic complement pathway components (C3, Serpinf1, C4b, C1s1, C1ra, Serpina3i, Serpina3b, Serpina3n) (Figure 6D, 6G; Table 1). Iba1 and CD74 (HLA-DR), two markers of microglia activation were also increased in Glast+ BRAFV600E condition compared to control-FP (Figure 6G), it was also increased compared to BRAFwt at p=0.011 for Iba1 and p=0.007 for CD74. Similarly, markers of astrocyte activation, GFAP and Vimentin, were also significantly upregulated in the RNA-seq profiles of the four BRAFV600E samples relative to the other transgene conditions. Overall, the pattern of gene expression changes in cortical tissue containing a subpopulation of cells expressing BRAFV600E is consistent with these cells causing a glial activation and neuroinflammatory response (Figure 6B, F, G).
Interestingly, the decreased expression of potassium channels (Table 2) is consistent with previous study by Aronica et al. (2008a).
Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I

Figure 6 - RNA sequencing of Glast+ BRAFV600E shows inflammation and activation of complement cascade.

A

B

C

D

E

F

G

H

I
Figure 6. Unsupervised Hierarchical Clustering Analysis of Glast+ BRAFV600E, Glast+ control-FP and Glast+ BRAFwt tissue-wide expression profile. A. Four clusters of three conditions with four replicates each (Lamarre et al., 2018), Glast+ BRAFV600E, Glast+ control-FP, Glast+ BRAFwt, 17,037 genes with at least one of 12 replicates having TPM >=1 were used. B. Expression values in transcripts per million (TPM) of GFAP and vimentin in three conditions; GFAP is increased in BRAFV600E (20.78 fold increase, p=0.000197, FDR=0.014) and BRAFwt (7.99 fold increase, p=0.0166, FDR=0.32) compared to control-FP; BRAFV600E to BRAFwt (2.67 fold increase p=0.03066, FDR=0.185); vimentin was also increased in BRAFV600E compared to control-FP (11.07 fold increase p=0.0000804, FDR=0.014) and to BRAFwt (3.63 fold increase p=0.00421692, FDR=0.131); BRAFwt to control-FP (3.13 fold increase p=0.025, FDR=0.35); while GAPDH was insignificantly changed, BRAFV600E to control-FP (1.10 fold decrease p=0.025, FDR=0.175), to BRAFwt (1.03 fold decrease p=0.52, FDR=0.718); BRAFwt to control-FP (1.13 fold decrease p=0.31, FDR=0.7). C. Representantive astrocytes in BRAFwt immunoreactive to ALDH1L1 and GFAP. Lower panel zoomed in astrocytes from upper panel. Although in Figure 2 BRAFwt coronal slice is shown without a significant GFAP immunoreactivity, in some cortical slices of some animals there were patches of highly immunoreactive to ALDH1L1 and GFAP astrocytes. D. Scatter plot for BRAFV600E to control-FP showing fold change and p-values, data with p<0.01 is in red and was used for further functional enrichment analysis. Some of the genes with the highest fold change are shown. E. Scatter plot for BRAFV600E to BRAFwt showing fold change and p-values, data with p<0.01 is in red and was used for further functional enrichment analysis. Some of the genes with the highest fold change are shown. F. Venn diagram of all the upregulated genes with at least log2 fold change=1, and p<0.01 showing some genes from D. E. F. and G. according to their expression profile. G. Zoomed in y-axis scatter plot of BRAFV600E to control-FP gene expression fold change showing increased expression of classic complement pathway, microglial marker Iba1 (black due to the red background), GFAP and vimentin. H. Zoomed in y-axis scatter plot of BRAFV600E to BRAFwt gene expression fold change showing smaller increase in gene expression of complement pathway (Serping1, Serpind1), a chemokine (Cxcl16), and significant decrease of Dusp7 (dephosphorylation of ERK, JNK, and p38 MAPK), C3 and Serpina3n expression was not significant at p<0.01. I. Venn diagram of all the downregulated genes with at least log2 fold change=1, and p<0.01. Scale bar 50 µm in C.
TABLE 1 - GOTERM biological protein production pathways enrichment in Glast+ BRAFV600E compared to control-FP and to Glast+ BRAFwt from 402 upregulated genes at p<0.01

<table>
<thead>
<tr>
<th>Category GOTERM_BP_DIRECT</th>
<th>Count</th>
<th>%</th>
<th>Fold Enrichment</th>
<th>Benjamini p-value corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>collagen fibril organization</td>
<td>11</td>
<td>2.770781</td>
<td>14.16681</td>
<td>0.0000007152</td>
</tr>
<tr>
<td>response to hypoxia</td>
<td>19</td>
<td>4.785894</td>
<td>4.970457</td>
<td>0.000062725</td>
</tr>
<tr>
<td>immune system process</td>
<td>24</td>
<td>6.04534</td>
<td>3.147433</td>
<td>0.002011456</td>
</tr>
<tr>
<td>response to mechanical stimulus</td>
<td>10</td>
<td>2.518892</td>
<td>7.972663</td>
<td>0.00226683</td>
</tr>
<tr>
<td>positive regulation of angiogenesis</td>
<td>13</td>
<td>3.274559</td>
<td>5.396373</td>
<td>0.002342004</td>
</tr>
<tr>
<td>wound healing</td>
<td>10</td>
<td>2.518892</td>
<td>5.343381</td>
<td>0.038195949</td>
</tr>
<tr>
<td>inflammatory response</td>
<td>19</td>
<td>4.785894</td>
<td>2.774209</td>
<td>0.058961929</td>
</tr>
</tbody>
</table>

Table 2 - GOTERM Biological protein production pathways enrichment in Glast+ BRAFV600E compared to Glast+ control-FP and to Glast+ BRAFwt from 262 downregulated genes at p<0.01.

<table>
<thead>
<tr>
<th>Category GOTERM_BP_DIRECT</th>
<th>Count</th>
<th>%</th>
<th>Fold Enrichment</th>
<th>Benjamini p-value corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>neuron projection development</td>
<td>10</td>
<td>3.802281</td>
<td>5.807425</td>
<td>0.023973</td>
</tr>
<tr>
<td>cytoskeleton organization</td>
<td>9</td>
<td>3.422053</td>
<td>6.985663</td>
<td>0.027481</td>
</tr>
<tr>
<td>protein phosphorylation</td>
<td>20</td>
<td>7.604563</td>
<td>2.802889</td>
<td>0.031236</td>
</tr>
<tr>
<td>potassium ion transport</td>
<td>10</td>
<td>3.802281</td>
<td>6.356159</td>
<td>0.035131</td>
</tr>
<tr>
<td>ephrin receptor signaling pathway</td>
<td>6</td>
<td>2.281369</td>
<td>11.2637</td>
<td>0.044375</td>
</tr>
<tr>
<td>phosphorylation</td>
<td>20</td>
<td>7.604563</td>
<td>2.638014</td>
<td>0.044999</td>
</tr>
</tbody>
</table>
**Goal-3 BRAFV600E Transfected Neurons Are Hyperexcitable in Ex Vivo**

In current-clamp recordings BRAFV600E neurons displayed significantly higher action potential (AP) firing frequencies to 1 second depolarizing current pulses (Figure 7A upper panel, 7B, p<0.001 for 20-300 pA current steps). This significantly increased firing rate was observed in neurons from both the Nestin+ and Glast+ progenitor populations. Neither BRAFwt nor neighboring untransfected neurons in BRAFV600E conditions showed elevated firing frequencies above fluorescent protein transfected controls (control-FP). Instantaneous AP frequency (ISF) measured at +300 pA 1 second current step was significantly higher in BRAFV600E neurons (Figure 7C). In addition, in 4 out of 59 Glast+ BRAFV600E neurons and 1 out of 9 Nestin+ BRAFV600E neurons I observed an unusual bursting pattern and post-action potential depolarization waves that were not observed in any of the non-BRAFV600E conditions (Figure 7G left panel). The number of neurons with those events was increased when I recorded potassium currents with Co2+ (1 mM) substituting Ca2+ (1 mM) in aCSF solution (5 out of 24). In addition to the AP firing at a significantly higher frequency BRAFV600E neurons also had lower rheobase (n=49), minimal depolarizing current step required to elicit a first AP, and a lower voltage threshold to fire action potentials (Figure 8B, C, E). Passive membrane properties were also significantly different in BRAFV600E neurons. The resting membrane potential (RMP) was more depolarized in BRAFV600E neurons (n=61, -64.91 ± 0.76 mV) compared to untransfected neighbor neurons (n=23, -73.33 ± 1.55 mV, p<0.001), and input resistances measured to hyperpolarizing and depolarizing current pulses were significantly increased in BRAFV600E neurons (n=61) compared to all other non-BRAFV600E conditions (Figure 8F, G, H). The elevated resting membrane potential did not explain the increased firing rates in BRAFV600E.
neurons, as untransfected neighboring neurons (n=5) did not achieve similar AP firing frequencies to BRAFV600E neurons when depolarized to -60 mV. The depolarized RMP in BRAFV600E neurons also did not correlate with more hyperpolarized AP voltage threshold (Figure 7 - BRAFV600E neurons are hyperexcitableD, Figure S2 Action Potential voltage threshold correlation to RMP). Additionally, the few BRAFV600E neurons with more negative resting membrane potentials (n=14, average RMP=-70.66 ± 0.58 mV) generated high frequency trains of action potentials like more depolarized BRAFV600E neurons (data not shown as a separate figure). Also, subthreshold input resistances did not correlate significantly with action potential frequencies in either BRAFV600E or control neurons (data not shown). Taken together, BRAFV600E transgenes significantly alter the electrophysiological properties of pyramidal neurons making them more excitable.
Figure 7 - BRAFV600E neurons are hyperexcitable

A

GLAST+ control-FP
GLAST+ untransfected neighbor
GLAST+ BRAFwt
GLAST+ BRAFV600E

50 mV

10 mV

1 s

+300 pA step

Excitation

-40 pA step

SAG

B

NESTIN+ control-FP
NESTIN+ untransfected neighbor
NESTIN+ BRAFV600E

Output (avg AP N-Hz)

35

30

25

20

15

10

5

0

Input (pA)

0

50

100

150

200

250

300

11

12

13

16

20

***

***

***

***

C

AP ISF at +300 pA (Hz)

180

160

140

120

100

80

60

40

20

0

AP #

D

Output (avg AP N-Hz)

35

30

25

20

15

10

5

0

130

300 pA

130

300 pA

70**120 pA

30**40 pA

E

SAG=(1-AV_1/AV_m)_100 (%)

100

80

60

40

20

0

-20

-40

-60

-80

-100

F

Rebound Excitation (mV)

10

5

0

-5

-10

-15

-20

***

***

***

***

***

***

***

G

4/59 - 6.8%

12/59 - 20.33%

-60 mV

+140 pA

1 s

-40 pA

1 s

0.5 s
**Table 7.** **BRAF**V600E expressing neurons are hyperexcitable. A. Representative traces of four Glast+ neuronal experimental conditions (upper panel). Response to -40 pA 1 sec current step showing SAG ratio and rebound excitation measurement (lower panel). B. Input-Output curve shows more than 2 times higher AP frequency firing in Glast+ BRAFV600E transfected neurons (n=54 T= range of 4.37-6.73, p<0.001) and Nestin+ BRAFV600E transfected neurons (n=8 T= range of 5.53-9.64, p<0.001) compared to all other conditions, Glast+ untransfected neighbor (n=8), Glast+ control-FP (n=11), Glast+ BRAFwt (n=13), Nestin+ control-FP (n=3, 240-300 pA), and Nestin+ untransfected neighbor (n=5, 130-300 pA). For statistical comparison for 10 pA - 150 pA steps, due to significant difference in variances (Levene’s test (18, 149) = range of 8.816-1.68, p<0.001, p=0.013 for 140 pA and p=0.05 for 150 pA) Welch test with Games-Howell post hoc correction was used; for 160-300 pA one-way ANOVA F(18, 150) = 13.42-16.55 with Tukey post hoc correction was used. Only cells with 7 and more APs at 300 pA 1 sec current step are chosen for the comparison. C. Instantaneous frequency (IF) of APs in the train at 300 pA 1 second depolarizing current step was significantly higher in Glast+ BRAFV600E transfected neurons compared to all other conditions (p<0.001). AP IF at +300 pA 1 second step due to non-homogeneous variance for AP #1 and AP #2, Levene test (8, 132)=5.08 and 3.97 respectively, Welch test (8, 22.91 and 23.18)=27.79 and 15.82 respectively with Games-Howell post hoc correction was used, from AP #3 to AP #20 one-way ANOVA F(7-8, 82-132)=2.14-19.6, p<0.001, with Tukey post hoc correction was used; there was significant difference in ISF between control BRAFV600E neurons (n=54) to Nestin+ untransfected neighbor (n=5, p=0.001) and Nestin+ control-FP (p=0.001); to Nestin+ BRAFV600E (n=8) to their untransfected neighbor (p<0.001) and Nestin+ control-FP (p<0.001). D. Input-output curve for Glast+ BRAFV600E, Glast+ BRAF wt held at -60 mV (n=4), and Glast+ untransfected neighbor neurons (n=5) held at -60 mV shows still significant difference in AP firing frequency. Due to non-homogeneous variance for 30 and 40 pA steps Levene (2, 53)=5.5 and 4.41, p=0.007 and p=0.017 Welch test with Games-Howell post hoc correction was used; for steps 50-300 pA one-way ANOVA with Tukey post hoc correction was used. For 30-40 pA steps Welch F(2,10.85) = 10.43, p=0.003 and (2, 10.22)=9.78, p=0.004 respectively. For 70-300 pA steps ANOVA F(2, 54)=3.82 – 13.84, p=0.028 and p=0.017 for 70 and 80 pA steps, p=0.007-0.001 for 90-120 pA steps; p=0.001 for 130-300 pA steps. E. One way ANOVA with Tukey post-hoc correction of SAG ratio values in different conditions shows larger SAG in most of the recorded Glast+ BRAFV600E (n=58) transfected neurons, F(3,105) = 35.98 (Glast+ BRAFV600E to Glast+ control-FP (n=15) – p<0.001; untransfected neighbor neurons (n=18) – p<0.01 , Glast+ BRAF wt (n=17) – p<0.001). SAG ratio was significantly larger in Glast+ BRAFV600E (n=58) compared to Nestin+ control-FP (n=5 T=2.94, p=0.005); to Nestin+ untransfected neighbor (n=4 T=3, p=0.002); Nestin+ BRAFV600E (n=8) to Nestin+ control-FP (n=5 T=4.48, p<0.001); Nestin+ BRAFV600E to their untransfected neighbor (T=4.87, p<0.001); Nestin+ BRAFV600E to Glast+ BRAF wt (T=5.53 p<0.001); Nestin+ BRAFV600E to Glast+ untransfected neighbor (T=7.3 p<0.001); Nestin+ BRAFV600E to Glast+ control-FP (T=5.8 p<0.001). F. Due to non-homogeneous variances (Levene’s test (3, 91) = 7.61, p<0.001) Welch test with Games-Howell post hoc correction was used for statistical comparison of rebound excitation values and shows larger rebound excitation in Glast+ BRAF V600E (n=42) transfected neurons compared to all non-BRAFV600E Glast+ conditions Welch (3, 42.36) = 20.83, p<0.001; Glast+ control-FP (n=14, p<0.001), and Glast+ untransfected neighbor neurons (n=18, p<0.001), Glast+ BRAF wt (n=17, p<0.001). Rebound excitation was larger in Glast+ BRAFV600E neurons (n=42) compared to Nestin+ untransfected neighbor (n=4 T=3.3, p=0.010); to Nestin+ control-FP (n=5 T=2.94, p=0.013); it was increased in Nestin+ BRAFV600E (n=8) compared to their untransfected neighbor (n=4 T=5.514, p<0.001); to Nestin+ control-FP (n=5 T=6.035, p<0.001). BRAFV600E neurons with rebound APs were omitted from statistical comparison. G. Left upper panel - representative whole-cell current-clamp recording traces of Glast+ BRAFV600E transfected neuron showing depolarization waves with smaller spikes riding on top of them following each full size AP (4 out of 58 neurons, 6.9%), this bursting was not observed in control conditions or BRAF wt condition. Left lower panel – zoomed in depolarization waves. Right panel – representative trace of rebound AP observed in 12 out of 58 (20.7%) Glast+ BRAFV600E transfected neurons. * - p<0.05, ** - p<0.01, *** - p<0.001. Error bars are ±SEM for B, C, and D; ±2SEM for E-F.
Figure 8 - Singe AP and passive electrophysiological properties.
**Figure 8.** Properties of first action potential at rheobase are altered in Glast+ and Nestin+ BRAFV600E ectopically expressing neurons

A. Averaged first APs at the rheobase traces aligned at RMP from all conditions with SEM zoomed-in on the left and right panels showing larger APs in untransfected neighbor neurons, and increased AP peak in untransfected neighbor neurons. B. First order derivative over time (dV/dt) of averaged APs (phase-space plot) from all conditions showing hyperpolarised AP voltage threshold in BRAFV600E transfected neurons, and increased AP peak in untransfected neighbor neurons. C. One-way ANOVA F(6, 116) = 7.97, p < 0.001 with Tukey post-hoc correction showed that AP voltage threshold at 50 V/s was more hyperpolarized in Glast+ BRAFV600E transfected neurons (n=54) compared to Glast+ control-FP (n=18, p<0.001), and untransfected neighbor (n=16, p<0.001); Glast+ BRAFwt (n=17, p=0.007) neurons; Nestin+ control-FP (n=5, p<0.001); it was lower but not statistically significant compared to Nestin+ BRAFV600E untransfected neighbor (n=4 T=1.81, p=0.076). AP voltage threshold at 50 V/s was significantly more hyperpolarized in Nestin+ BRAFV600E neurons (n=9) compared to Nestin+ control-FP neurons (n=5 p<0.001); to Nestin+ BRAFV600E untransfected neighbor (n=4 T=2.63, p=0.023); to Glast+ untransfected neighbor (p=0.001); to Glast+ control-FP (p<0.001); to Glast+ BRAFwt (p=0.012). D. One-way ANOVA F(6, 122) = 6.29, p < 0.001 with Tukey post-hoc correction showed that AP peak measured from RMP was larger in Glast+ untransfected neighbor neurons (n=16) compared to Glast+ BRAFV600E (n=58, p=0.001) and to Glast+ BRAFwt transfected neurons (n=18, p=0.04); to Nestin+ BRAFV600E (n=9, p<0.001). Glast+ control-FP (n=18) to Nestin+ control-FP (n=5, p=0.039). Nestin+ BRAFV600E to Nestin+ untransfected neighbor (n=4, p=0.033); to Nestin+ control-FP (n=5, p=0.002). E. Due to non-homogenous variance – Levene test (6, 114) = 5.55, p<0.001 Welch test with Games-Howell post-hoc correction was used to compare rheobase between experimental conditions, which showed lower current required to fire AP in Glast+ BRAFV600E transfected neurons (n=49) Welch test (6, 20.94) = 36.71, p<0.001, compared to control-FP (n=19, p<0.001), and untransfected neighbor neurons (n=17, p<0.001), to Glast+ BRAFwt (n=18, p<0.001). Rheobase was significantly lower in Glast+ BRAFV600E (n=49) compared to Nestin+ control-FP (n=5 p<0.001); to Nestin+ untransfected neighbor (n=4 T=6.46, p<0.001); Nestin+ BRAFV600E neurons (n=9) to Nestin+ control-FP (n=5 T=11.52, p<0.001); to their untransfected neighbor (n=4 T=6.66, p<0.001); to Nestin+ untransfected neighbor (p<0.001); to Glast+ control-FP (p<0.001); to Glast+ BRAFwt (p=0.004). F. Glast+ BRAFV600E transfected neurons (n=61) had more depolarized resting membrane potential compared to Glast+ control-FP neurons (n=20, p<0.001), and to their untransfected neighbor neurons (n=23, p<0.001); to Nestin+ untransfected neighbor (n=5, p=0.001); to Nestin+ control-FP (n=5, p=0.031). Glast+ BRAFwt transfected neurons (n=18) had more depolarized RMP compared to untransfected neighbor neurons (p=0.044); to Glast+ control-FP (p=0.05); to Nestin+ untransfected neighbor (p=0.034); to Nestin+ control-FP (T=2.25 p=0.036). Nestin+ BRAFV600E to Glast+ control-FP (p=0.033), and to their untransfected neighbor (p=0.005). One-way ANOVA F(6, 133) = 9.59, p<0.001 with Tukey post-hoc correction test was used for statistical comparison. In case of small n student t-test was used. RMP measured in current clamp before beginning of steps protocol. G. Due to nonhomogeneous variance (Levene test (6, 112) =5.58, p<0.001) Welch test (6, 24.56) = 7.04, p<0.001 with Games-Howell post-hoc correction was used for input resistance comparison. Averaged input resistance (Rin) as a function of membrane potential response (Vm) to depolarizing current (I) steps was significantly larger in Glast+ BRAFV600E transfected neurons (n=39) compared to Glast+ control-FP (n=21, p<0.001), and their untransfected neighbor neurons (n=23, p=0.009); to Glast+ BRAFwt (n=18, p=0.001). Glast+ BRAFV600E to Nestin+ untransfected neighbor (n=5, p=0.018); to Nestin+ control-FP (n=5, p=0.003). Average Rin from depolarizing current steps was significantly larger in Nestin+ BRAFV600E (n=8) compared to Glast+ control-FP (n=21 T=2.496, p=0.040); to Nestin+ untransfected neighbor (n=5 T=2.418, p=0.043); to Nestin+ control-FP (n=5 T=2.512, p=0.038). H. Linear fit of averaged membrane potential responses to current step protocol from -40 to +50 pA 1 second pulse with 10 pA increment shows a different input resistance in between the recorded conditions. I. AP 10 V/s voltage threshold for all BRAFV600E neurons compared to control conditions at -60 mV. J. AP 50 V/s threshold for all BRAFV600E neurons compared to control conditions at -60 mV. K. Scatter plot for AP 50 V/s voltage threshold correlation to RMP for all BRAFV600E neurons and control conditions. * - p<0.05, ** - p<0.01, *** - p<0.001. Error bars are ±SEM for A. and H, ±2SEM for C-G.
BRAFV600E decreases delayed rectifier potassium currents

The exclusively increased excitability in BRAFV600E expressing neurons may be a result of altered ionic conductances. To test this hypothesis, I have performed whole-cell voltage clamp in Glast+ BRAFV600E neurons and their untransfected neighbors only. Recording of potassium currents showed decreased sustained currents measured at the last 100 ms of 500 ms depolarizing voltage pulses across examined range of voltage steps in Glast+ BRAFV600E neurons (n=18) compared to their untransfected neighbors (n=8, p<0.01; Table 3). The kinetic properties of activation were not significantly altered (Figure 9A, B, C, D, F). However, when the maximal sustained currents were normalized to cell capacitance, the current densities were similar between two conditions (Figure 9G; Table 3). Additionally, potassium currents sensitive to 25 mM TEA, a known potassium channel inhibitor, were also decreased in BRAFV600E neurons compared to their untransfected neighbors (p<0.05) without significant effect on current density (Figure 9A, B, C, F, G; Table 4). The currents were recorded in the presence of 3 mM 4AP, a known inhibitor of fast activating potassium currents. To test the hypothesis of whether early activation of potassium conductances, that contributes to sustained currents can restore AP firing to control conditions in BRAFV600E neurons I recorded 6 neurons in whole-cell current clamp and applied 10 μM retigabine. Retigabine is an anti-convulsant medication that shifts voltage-dependent activation of M-potassium currents (carried through Kv7.2-Kv7.3 channels) to more hyperpolarized potentials thus causing earlier opening of the channels (Stas et al., 2016). This manipulation decreased the AP firing frequency of BRAFV600E neurons (Figure 9E, H), however it was still higher than in control conditions.
Figure 9 - BRAFV600E decrease sustained potassium currents

A

GLAST+ BRAFV600E

GLAST+ BRAFV600E in 25 mM TEA

TEA traces subtracted

B

GLAST+ untransfected neighbor

GLAST+ untransfected neighbor in 25 mM TEA

C

Sustained $I_{\text{K}}$, avg (nA)

D

Sustained $I_{\text{K}}$, activation

E

GLAST+ BRAFV600E

before Retigabine

10 μM Retigabine 5-17 min

F

Sustained $I_{\text{K}}$, peak (nA)

G

Sustained $I_{\text{K}}$, density (pA/pF)

H

before Retigabine

10 μM Retigabine 15 min

I

Cm (pF)

J

Rin (MΩ)

-66 mV

-40 pA

+80 pA

-10 pA

+290 pA

0.5s

$V_{\text{m}}$ = -5.26 mV

$k = 16$ mV$^{-1}$

$V_{\text{m}}$ = -2.94 mV

$k = 16.5$ mV$^{-1}$

$V_{\text{m}}$ = -2.91 mV

$k = 16.9$ mV$^{-1}$

$V_{\text{m}}$ = -0.93 mV

$k = 16.8$ mV$^{-1}$
**Figure 9.** Sustained potassium currents are decreased in Glast+ BRAFV600E neurons compared to their untransfected neighbors. **A.** Representative traces of potassium currents in Glast+ BRAFV600E neuron recorded in the presence of 3 mM 4AP, 1µM TTX, 10µM NBQX, 50µM D-AP5, 10µM SR, 50µM ZD7288 (5 min in, holding voltage is -80 mV, holding current -32.41 pA) with whole-cell capacitance compensated, grey bar indicate the region where the measurement was made in all conditions (upper panel); middle panel is showing the traces of the same neuron 9 min after application of 25 mM TEA with previous inhibitors cocktail (holding current -40.25 pA); lower panel is showing subtracted traces before and after 25 mM TEA with voltage step protocol. **B.** Representative traces of potassium currents in Glast+ untransfected neighbor recorded in the presence of the same inhibitors cocktail as for A (6 min in, holding voltage is -80 mV, holding current is -48.04 pA, upper panel) with whole-cell capacitance compensated; middle panel is showing traces from the same neuron 9 min after application of 25 mM TEA with previous inhibitors cocktail (holding current -71.67 pA); lower panel is showing subtracted traced before and after 25 mM TEA. **C.** Average sustained current activation curve showing decreased peaks at all tested voltages in Glast+ BRAFV600E neurons compared to their untransfected neighbor before and after application of 25 mM TEA. **D.** Normalized to the maximum current and averaged sustained current activation curve have similar kinetics between two conditions before and after application of 25 mM TEA. **E.** Average input-output curve of 6 Glast+ BRAFV600E neurons before and 5-17 min after beginning of 10 µM retigabine perfusion. **F.** Maximal sustained current measured at +20 mV voltage step showing lower values in Glast+ BRAFV600E neurons (n=18) compared to their untransfected neighbors (n=8, T=2.92, p=0.008), as well as after application of 25 mM TEA (T=2.41, p=0.028). It was also decreased in the same neurons when comparing before and after 25 mM TEA – Glast+ BRAFV600E (paired sample T=2.47, p=0.035); and their untransfected neighbor (paired sample T=3.82, p=0.006); and comparing untransfected neighbor neurons to Glast+ BRAFV600E after application of 25 mM TEA (T=6.91, p<0.001). **G.** Current density was not different in any comparison. **H.** Representative current-clamp traces from Glast+ BRAFV600E neuron before and 15 min after beginning of 10 µM retigabine perfusion (upper panel). Current-clamp steps protocol (lower left panel). The traces from the same cell at +290 pA step overlaid with traces 15 min after retigabine perfusion. **I.** Capacitance was measured from -5 mV steps at the beginning of each trace recording using built-in procedure in Axograph acquisition software before application of whole-cell capacitance compensation. There was no statistically significant difference in capacitance measurements between the experimental conditions. **J.** Input resistance was significantly increased in Glast+ BRAFV600E (n=18) compared to their untransfected neighbors (n=8, T=3.29, p=0.003), it was increased in Glast+ BRAFV600E neurons (n=7) compared to their untransfected neighbors (n=4), but not statistically significant (T=2.22, p=0.053); it was significantly increased in Glast+ BRAFV600E neurons (n=7) after application of 25 mM TEA compared to their untransfected neighbors before application of TEA (n=8, T=3.66, p=0.009). * - p<0.05, ** - p<0.01, *** - p<0.001. Error bars are ±SEM for C. and D, ±2SEM for E-H.

**Table 3 - Sustained K⁺ current averages of maximal values and their current density**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Iₖ-Sustained maximal (pA) last 100 ms of 500 ms +20 mV pulse</th>
<th>Iₖ-Sustained maximal density (pA/pF)</th>
<th>Cₘ (pF)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glast+ untransfected neighbor</td>
<td>4062.66 ± 210.81</td>
<td>34.94 ± 11.76</td>
<td>178.04 ± 31.74</td>
<td>8</td>
</tr>
<tr>
<td>Glast+ BRAFV600E</td>
<td>3034.19 ± 282.49**</td>
<td>21.30 ± 2.53</td>
<td>157.76 ± 13.29</td>
<td>18</td>
</tr>
</tbody>
</table>

** - T(23.5)=2.92, P=0.008
Table 4 – TEA sustained K⁺ current averages of maximal values and their current density

<table>
<thead>
<tr>
<th>Condition</th>
<th>I_{K_{sustained}} peak (pA) last 100 ms of 500 ms +20 mV pulse TEA sensitive</th>
<th>n</th>
<th>I_{K_{sustained}} density TEA sensitive (pA/pF)</th>
<th>Cm (pF)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glast+ untransfected neighbor</td>
<td>2484.56 ± 258.29</td>
<td>8</td>
<td>12.23 ± 3.85</td>
<td>239.88 ± 59.23</td>
<td>4</td>
</tr>
<tr>
<td>Glast+ BRAFV600E</td>
<td>1561.06 ± 266.57*</td>
<td>11</td>
<td>7.28 ± 1.36</td>
<td>187.18 ± 18.45</td>
<td>7</td>
</tr>
</tbody>
</table>

* - T(17)=2.41, P=0.028

**BRAFV600E INCREASE I_H CURRENT**

In response to hyperpolarizing current pulses in whole-cell current clamp mode BRAFV600E neurons in either Glast+ or Nestin+ condition displayed an initial deflection, SAG ratio that was absent in untransfected neighbor neurons, control-FP neurons, and in BRAFwt neurons (Figure 10A lower panel, 10E). SAG ratio was calculated as $SAG = \left( 1 - \frac{V_{RMP} - V_{ss}}{V_{RMP} - V_{min}} \right) \times 100\%$, $V_{RMP}$ – Resting Membrane Potential, $V_{ss}$ – stable-state voltage in the last 100 ms of 1 second -40 pA pulse, $V_{min}$ – minimal initial voltage deflection in response to 1 second -40 pA pulse. Glast+ BRAFV600E neurons (n=57) had average SAG ratio of 23.41 ± 1.33% that was significantly larger than in their untransfected neighbor neurons of 4.46 ± 1.48% (n=20, p=0.001); and in Glast+ control-FP neurons 6.82 ± 1.86% (n=15, p<0.001); and in Glast+ BRAFwt neurons 6.59 ± 1.94% (n=17, p<0.001) (Figure 7E). In Nestin+ BRAFV600E average SAG was 24.48 ± 2.27 % (n=8) and it was significantly increased compared to all non-BRAFV600E conditions (p<0.001, Figure 7F). In Nestin+ control-FP the average SAG ratio was 10.03 ± 1.77% (n=5); and in Nestin+ untransfected neighbor the average SAG ratio was 6.59 ± 2.41% (n=4).

Average rebound excitation measured as an overshoot above RMP at the end of 1 second -40 pA current step was also larger in Glast+ BRAFV600E neurons (n=42) 2.28 ± 0.24 mV
compared to their untransfected neighbor neurons 0.69 ± 0.18 mV (n=22, p<0.01); to Glast+ control-FP neurons 0.37 ± 0.11 mV (n=14, p<0.01); to Glast+ BRAFwt neurons 0.34 ± 0.06 mV (n=17, p<0.01); to Nestin+ untransfected neighbor 0.18 ± 0.27 mV (n=4, p<0.05); to Nestin+ control-FP 0.47 ± 0.13 mV (n=5, p<0.05). In Nestin+ BRAFV600E rebound excitation was increased, (n=7) 1.82 ± 0.17 mV compared to non-BRAFV600E conditions (to Glast+ BRAFwt - p<0.001; to Glast+ untransfected neighbor – p=0.02; to Glast+ control-FP – p<0.001; to Nestin+ untransfected neighbor – p<0.001; to Nestin+ control-FP – p<0.001) (Figure 7H). In 20.34% of Glast+ BRAFV600E neurons (12/59) and in 11.11% of Nestin+ BRAFV600E (1/9) rebound excitation resulted in AP firing (Figure 7G upper right panel).

Increased SAG ratio and rebound excitation has been previously shown in layer 5 cortical, hippocampal and non-cortical neurons in mice, rats and cats to be associated with hyperpolarization activated conductances (Guan et al., 2015; Hawkins et al., 2015; Jung et al., 2010; Schridde et al., 2006; Timofeev et al., 2002). To test whether BRAFV600E ectopically expressing neurons have increased hyperpolarization activated conductance I recorded cells in whole-cell voltage clamp configuration. The BRAFV600E expressing neurons had increased Ih compared to all other conditions and have half activation voltage of \( V_{1/2} = -82.79 \) mV and the slope factor \( k = 11.58^{-1} \) mV using recording protocol that holds the cell at -50 mV and the first voltage step is at -120 mV with 5 mV increment for 1.5 seconds (Figure 10; Table 5). This current was blocked with application of 50 \( \mu \)M ZD7288, a known Ih inhibitor in perfusion system and recorded at least 5 minutes later. Ih peak was only significantly increased in Glast+ BRAFV600E neurons (n=17) compared to their untransfected neighbors (n=6, p<0.05), however when normalized to cell capacitance the Ih peak current density was significantly increased in Glast+ BRAFV600E neurons compared to their untransfected neighbors (p<0.001), and to Nestin+ control-FP (n=4, p<0.001).
In Nestin+ BRAFV600E neurons (n=4) the Ih peak current density was significantly increased compared to Glast+ untransfected neighbors (p<0.05), and to Nestin+ control-FP (p<0.05). Consistent with that application of ZD7288 decreased SAG and rebound excitation in BRAFV600E neurons (Figure 11).

Figure 10 - $I_H$ is increased in BRAFV600E neurons
Table 5 - Ih peak and current density.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$I_h$ peak -50 -120 mV (pA)</th>
<th>$I_h$ peak density -50 -120 mV (pA/pF)</th>
<th>$C_m$ (pF)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glast+ control-FP</td>
<td>-129.22</td>
<td>-1.02</td>
<td>126.94</td>
<td>1</td>
</tr>
<tr>
<td>Glast+ untransfected neighbor</td>
<td>-174.17 ± 58.24</td>
<td>-0.92 ± 0.30</td>
<td>193.03 ± 24.99</td>
<td>6</td>
</tr>
<tr>
<td>Glast+ BRAFwt</td>
<td>-66.22 ± 13.03*</td>
<td>-0.36 ± 0.08</td>
<td>253.49 ± 41.27</td>
<td>12</td>
</tr>
<tr>
<td>Glast+ BRAFV600E</td>
<td>-446.58 ± 58.24</td>
<td>-3.45 ± 0.37</td>
<td>135.95 ± 14.18</td>
<td>18</td>
</tr>
<tr>
<td>Nestin+ control-FP</td>
<td>-197.17 ± 93.19</td>
<td>-0.85 ± 0.28</td>
<td>214.29 ± 44.06</td>
<td>4</td>
</tr>
<tr>
<td>Nestin+ untransfected neighbor</td>
<td>-117.75</td>
<td>-1.13</td>
<td>104.00</td>
<td>1</td>
</tr>
<tr>
<td>Nestin+ BRAFV600E</td>
<td>-322.76 ± 54.45</td>
<td>-2.97 ± 0.63</td>
<td>84.34 ± 32.33</td>
<td>4</td>
</tr>
</tbody>
</table>

* - Ih was recorded with the first voltage step starting at -100 mV
Figure 11 - ZD7288 decrease SAG and rebound excitation

**Figure 11.** Effect of 50 μM ZD7288 on SAG and rebound excitation. A. Current-clamp traces of BRAFV600E neuron in response to 1s current pulses shown in the lower panel. B. The same neuron 3 min after application of 50 μM ZD7288 in perfusion showing more hyperpolarized membrane potential. C. overlay of A. and B. C. D. Effect of 50 μM ZD7288 on SAG in 3 BRAFV600E neurons. E. Effect of 50 μM ZD7288 on rebound excitation in 3 BRAFV600E neurons.
**BRAFV600E INCREASES FREQUENCY OF EXCITATORY POST-SYNAPTIC EVENTS**

Next, we tested how BRAFV600E alters synaptic activity in transfected cortex. I recorded spontaneous Post-Synaptic Currents (sPSCs) in whole-cell voltage clamp configuration in all conditions holding the cells at -70 mV and compared the instantaneous frequency and amplitude distributions with nonparametric Mann-Whitney U test (Figure 12C, D). I found that Glast+ BRAFV600E neurons had significantly higher sPSCs instantaneous frequency (ISF) (n=25, average of 20.36 ± 0.17 Hz) compared to Glast+ control-FP neurons (n=5 average of 15.93 ± 0.39 Hz, p<0.001), and to Glast+ BRAFwt neurons (n=9 average of 17.25 ± 0.28 Hz, p<0.001). Interestingly Glast+ BRAFV600E neurons had lower ISF compared to untransfected neighbor neurons (n=7 average of 24.29 ± 0.25 Hz, p=0.04). Untransfected neighbor neurons had higher ISF compared to Glast+ BRAFwt (p<0.001), and to Glast+ control-FP (p<0.001). Glast+ BRAFwt neurons had higher ISF compared to Glast+ control-FP (p=0.04). Nonparametric Mann-Whitney U test showed that amplitudes were only different in Glast+ control-FP compared to all Glast+ conditions (Figure 12E, F; p< 0.001). In all Nestin+ conditions the cumulative distribution of inter-events intervals (IEI) showed a significant left shift indicating a higher ISF compared to Glast+ conditions (p<0.001) except for Glast+ untransfected neighbor neurons that had higher ISF (p<0.001). I have also recorded miniature post-synaptic currents (mPSCs) in a few BRAFV600E expressing neurons that showed that the ISF of mPSCs was not significantly different from ISF of sPSCs (Figure S3 Miniature post-synaptic currents).
Figure 12 - sPSCs frequencies are increased in BRAFV600E neurons, and in their untransfected neighbors.
**Figure 12.** BRAFV600E expression under both Glast+ and Nestin+ alters spontaneous Post-Synaptic Currents frequencies. A. Representative traces. B. zoom in. C. Cumulative distribution of Inter-event intervals (IEI) compared with nonparametric Mann-Whitney U test showing that Glast+ BRAFV600E (red, n=25) had significantly lower ISF compared to their untransfected neighbor neurons (black left shifted curve, n=8 U=56162614, p<0.001); higher ISF compared to Glast+ control-FP (blue right shifted curve, n=6 U=14230311, p<0.001); higher ISF compared to Glast+ BRAFwt (magenta right shifted curve, n=10 U=3417926, p<0.001); lower ISF in Glast+ BRAFwt neurons compared to Glast+ untransfected neighbor (U=15082733, p<0.001); higher ISF in Glast+ BRAFwt compared to Glast+ control-FP (U=5015372.5, p=0.04); and higher ISF in Glast+ untransfected neighbor neurons to Glast+ control-FP (U=6221684.5, p<0.001). sPSCs frequencies are not significantly different in Nestin+ BRAFV600E (purple, n=8) compared to Nestin+ untransfected neighbor neurons (bright orange, n=5 U=3972278.5, p=0.25); compared to Nestin+ control-FP (brown, n=3 U= 3540666.5, p=0.96). sPSCs instantaneous frequencies were significantly increased in Nestin+ BRAFV600E neurons (n=8) compared to Glast+ BRAFV600E neurons (red, n=25 U= 35788328, p<0.001); to Glast+ untransfected neighbors (black, n=7 U= 19800832, p<0.001); to Glast+ control-FP (blue, n=5 U= 3943628.5, p<0.001); Nestin+ untransfected neighbor neurons (bright orange, n=5) to Glast+ BRAFV600E neurons (red n= 25 U= 11670220.5, p<0.001); to Glast+ BRAFwt (blue, n= 5 U= 1299202.5, p<0.001); Nestin+ control-FP (brown, n=3) to Glast+ BRAFV600E neurons (red, n=25 U= 10059716, p<0.001); to Glast+ untransfected neighbor neurons (black, n=8 U= 5534089, p=0.016); to Glast+ control-FP (blue, n=5 U= 1119629.5, p<0.001). D. Averaged sPSCs ISF per neurons. Comparison is as in C. E. Cumulative distribution of sPSCs amplitudes showing that amplitudes from Glast+ BRAFV600E neurons are not significantly different from their untransfected neighbor neurons (U=64347671, p=0.33); and Glast+ BRAFwt (U=38622212, p=0.82); but it was significantly increased compared to Glast+ control-FP (U=15598177.5, p<0.001); sPSCs amplitudes was not significantly different between Glast+ untransfected neighbor neurons and Glast+ BRAFwt (U=19867509, p=0.39); it was significantly increased in Glast+ untransfected neighbor neurons compared to Glast+ control-FP (U=8174115, p<0.001); and in Glast+ BRAFwt compared to Glast+ control-FP(U=4806537, p<0.001). Lower amplitudes in Nestin+ BRAFV600E (purple, n=8) and their untransfected neighbor neurons (bright orange, n=5) compared to Nestin+ control-FP neurons (brown, n=3 U= 3019414.5, p<0.001; and U= 935101, p<0.001 respectively). Significantly larger sPSCs amplitudes in Nestin+ BRAFV600E neurons (n=8) compared to Glast+ BRAFV600E neurons (red, n= 25 U= 36907544, p<0.001); to Glast+ untransfected neighbors (black, n=8 U= 18847821.5, p<0.001); to Glast+ control-FP (blue, n=5 U= 4639404, p<0.001); Nestin+ untransfected neighbor neurons (bright orange, n=5) to Glast+ BRAFV600E neurons (red n=25 U= 11751080.5, p<0.001); to Glast+ untransfected neighbor neurons (black, n=8 U= 6015340.5, p<0.001); to Glast+ control-FP (blue, n=5 U= 1481496.5, p<0.001); Nestin+ control-FP (brown, n=3) to Glast+ BRAFV600E neurons (red, n=25 U= 8523478, p<0.001); to Glast+ untransfected neighbor neurons (black, n=8 U= 4268880.5, p<0.001); to Glast+ control-FP (blue, n=5 U= 1113151, p<0.001). F. Averaged sPSCs amplitudes per neurons. Comparison as in E. * - p<0.05, ** - p<0.01, *** - p<0.001.
UNSUPERVISED HIERARCHICAL CLUSTER ANALYSIS INDICATES BRAFV600E TRANSFORMATION OF PYRAMIDAL NEURON CELL TYPE PHYSIOLOGY.

I used Hierarchical Cluster Analysis together with Principal Component Analysis to test whether the BRAFV600E ectopically expressing neurons segregate according to the electrophysiological parameters recorded in whole-cell patch-clamp experiments. After centering to the mean and dividing by standard deviation I used Gene Cluster 3.0 software with correlation (uncentered) setting HCA. Using Gene Pattern web software tool from broad institute of MIT, HCA showed that BRAFV600E transfected neurons segregate to the cluster according to their 20 electrophysiological properties recorded (Figure 13). Using Principal Component Analysis with XLSTAT Excel addon I found that the first two principal components explain 48.88% variability across electrophysiological parameters in 4 conditions. Major contributing parameters to the variability where AP Frequency at 300 pA 1s pulse, Rheobase, AHP, AP voltage threshold and maximal rising AP voltage slope. Some of them presented in 3D scatter plots in Figure 13. About 90% of the variability can be explained by the first 11 principal components as shown in Figure 13.
Figure 13 - Unsupervised hierarchical clustering of the electrophysiological properties showed segregation of BRAFV600E neurons from other conditions

A. Unsupervised Hierarchical Cluster Analysis was performed on 20 recorded electrophysiological parameters and showing that most of the BRAFV600E neurons segregate together by electrophysiological parameters recorded. The parameters are AP width at 50% height from RMP in ms, AP maximal decay slope in V/s, AP decay time from 100% to 50% height in ms, AP rise time from 10% to 90% height in ms, AP 50 V/s voltage threshold in mV, AP 10 V/s voltage threshold in mV, rheobase in pA, AHP measured at the end of +300 pA 1 second current step in mV, AP peak relative to RMP in mV, AP maximal rise slope in V/s, RMP in mV, Rin from hyperpolarizing pulses in MΩ, Rin from depolarizing pulses in MΩ, average sPSCs amplitude in pA, average sPSCs instantaneous frequency in Hz, mAHP measured relative to 10 V/s AP voltage threshold for rheobase APs in mV, SAG ratio in %, AP frequency at +300 pA 1 second current step in Hz, number of APs at rheobase, rebound excitation measured as an overshoot above RMP (mV).

B. Most contributing electrophysiological parameters to the variability in PCA shown in 3D plots – upper left panel SAG ratio on the Z axis, AP number at +300 pA 1 second pulse is on the X axis and rebound excitation is on the Y axis. C. SAG ratio on the Z axis, AHP at the end of +300 pA 1 second pulse on the X axis and rebound excitation on the Y axis. D. Rheobase on the Z axis, AP maximal rise slope is on the X axis, and AP 50V/s voltage threshold on the Y axis. E. AP number at +300 pA 1 second pulse on the Z axis, resting membrane potential (RMP) is on the X axis, Input resistance from depolarizing current pulses (Rin) is on the Y axis.

Figure 13. Glast+ and Nestin+ BRAF V600E ectopically expressing neurons segregate to separate clusters in HCA analysis of electrophysiological properties. A. Unsupervised Hierarchical Cluster Analysis was performed on 20 recorded electrophysiological parameters and showing that most of the BRAFV600E neurons segregate together by electrophysiological parameters recorded. The parameters are AP width at 50% height from RMP in ms, AP maximal decay slope in V/s, AP decay time from 100% to 50% height in ms, AP rise time from 10% to 90% height in ms, AP 50 V/s voltage threshold in mV, AP 10 V/s voltage threshold in mV, rheobase in pA, AHP measured at the end of +300 pA 1 second current step in mV, AP peak relative to RMP in mV, AP maximal rise slope in V/s, RMP in mV, Rin from hyperpolarizing pulses in MΩ, Rin from depolarizing pulses in MΩ, average sPSCs amplitude in pA, average sPSCs instantaneous frequency in Hz, mAHP measured relative to 10 V/s AP voltage threshold for rheobase APs in mV, SAG ratio in %, AP frequency at +300 pA 1 second current step in Hz, number of APs at rheobase, rebound excitation measured as an overshoot above RMP (mV). B. Most contributing electrophysiological parameters to the variability in PCA shown in 3D plots – upper left panel SAG ratio on the Z axis, AP number at +300 pA 1 second pulse is on the X axis and rebound excitation is on the Y axis. C. SAG ratio on the Z axis, AHP at the end of +300 pA 1 second pulse on the X axis and rebound excitation on the Y axis. D. Rheobase on the Z axis, AP maximal rise slope is on the X axis, and AP 50V/s voltage threshold on the Y axis. E. AP number at +300 pA 1 second pulse on the Z axis, resting membrane potential (RMP) is on the X axis, Input resistance from depolarizing current pulses (Rin) is on the Y axis.
OTHER SOMATIC MUTATIONS ASSOCIATED WITH FOCAL CORTICAL DYSPLASIAS CAUSE CHANGES IN ELECTROPHYSIOLOGICAL PROPERTIES DISTINCTLY DIFFERENT FROM BRAFV600E

To compare BRAFV600E electrophysiological properties to previously published MTOR related mouse models of brain overgrowth - a mutation in Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA E545K) (Roy et al., 2015), and CRISPR-Cas9 induced insertion-deletion in Tuberous Sclerosis 1/2 (TSC1/TSC2) genes (Lim et al., 2017) I directed PIK3CAE545K transgene into Glast+ neuronal progenitors, and used CRISPR guide-RNA T4 for TSC1 and T7 for TSC2 from Lim et al., 2017 to transflect neuronal progenitors at E14-E15. The AP firing frequency, AP ISF, rheobase, RMP and Rin was closer to control conditions in previous experiments (Figure 1A upper panel, 1B, 1C, 1D, 1F, 1G). However, AP voltage threshold was not different from Glast+ BRAFV600E neurons. The T7 TSC2 experiments did not show significant differences compared to their untransfected neighbors and to CRISPR-Cas9 without any guide-RNA controls, probably reflecting a minor difference in TSC2 sequences between mouse lines used in our experiments and in Lim et al., (2017). Comparison of action potential dependent mixed glutamatergic and GABAergic synaptic events showed that frequencies of those events were significantly higher in Glast+ PIK3CAE545K and TSC1 CRISPR-Cas-9 KD compared to Glast+ BRAFV600E but not to BRAFV600E untransfected neighbor neurons (Figure 15). This suggest that alterations in neuronal electrophysiological properties affected differently by pathological mutations in MTOR pathway key protein components.
Figure 14 - Different somatic mutations in MTOR and MAPK pathways have different effect on neuronal excitability

A

GLAST+ PIK3CA E545K  TSC1 CRISPR KD  GLAST+ BRAF V600E

B

C

D

E

F

G

AP N-Hz

AP ISF (Hz)

Rheobase (pA)

AP 50 Vss threshold (mV)

RMP (mV)

Rin from depol steps (MΩ)
**Figure 14.** Differential effect of three experimental manipulations on whole-cell current-clamp properties. **A.** Representative traces from Glast+ neurons ectopically expressing PIK3CA E545K (blue) and BRAFV600E mutations (red), and CRISPR knockdown of TSC1 gene showing AP firing at +300 pA (upper panel), and membrane potential response to hyperpolarizing current step of -40 pA. **B.** Average AP firing frequency in all three conditions, BRAFV600E (n=54), TSC1 KD (n=11), PIK3CA E545K (n=6). Cells with maximal values are shown for PIK3CA E545K (half-filled blue circles), and for TSC1 KD (brown spheres). **C.** AP instantaneous frequency at +300 pA current step except TSC1 KD, which is shown for +450 pA current step with maximal value cell shown for +300 pA current step (brown spheres). **D.** Rheobase for all three conditions was compared with Welch test (2, 14.38) =72.98 due to nonhomogeneous variance (Levene test (2, 68) =15.36, p<0.001), together with Games-Howell posthoc correction BRAFV600E to TSC1 KD (p<0.001), BRAFV600E to PIK3CA E545K (p=0.011, due to small sample for PIK3CA E545 student T=3.68, p=0.004 was used). **E.** AP 50 V/s voltage threshold is not different compared with Welch (2, 19.59) =2.48, p=0.11) together with Games-Howell BRAFV600E to TSC1 KD (p=0.57), BRAFV600E to PIK3CA E545K (p=0.12); Levene test (2, 73) =4.93, p=0.01. **F.** Resting Membrane Potential (RMP recorded before application of current steps) was compared with One-way ANOVA F(2,86) =28.72, together with Tukey posthoc corrections BRAFV600E to TSC1 KD (p<0.001), and BRAFV600E to PIK3CA E545K (p<0.001). **G.** Input resistance (Rin) from depolarizing current steps (due to Ih activation in BRAFV600E) was compared with Welch (2, 25.70) =74.48, due to nonhomogeneous variance (Levene test (2, 61) =3.40, p=0.04), together with Games-Howell posthoc correction BRAFV600E to TSC1 KD (p<0.001), and BRAFV600E to PIK3CA E545K (p<0.001). * - p<0.05, ** - p<0.01, *** - p<0.001.
Figure 15 - SPSCs frequencies are increased in Nestin+ BRAFV600E neurons compared to Glast+ BRAFV600E neurons

**Figure 15.** Spontaneous Post-Synaptic Currents (sPSCs) frequencies in different experimental conditions. **A.** sPSCs recorded in GLAST+ BRAF V600E neurons (n=25), in their untransfected neighbors (n=8), in GLAST+ BRAFwt (n=10), GLAST+ control-FP (n=6), Glast+ PIK3CAE545K (n=8), their untransfected neighbors (n=3), CRISPR-Cas9 TSC1 KD (n=13), their untransfected neighbors (n=5), CRISPR-Cas9 TSC2 KD (n=11), their untransfected neighbors (n=5). **B.** Averages of sPSCs frequencies per cell. **C.** Averages of sPSCs amplitudes per cell. The dashed grey line is aligned to the average of GLAST+ control-FP.
CHAPTER 4 CAVEATS AND FUTURE EXPERIMENTS

In current work I have tested the effect of acute BRAFV600E inhibition with specific blocker Vemurafenib (PLX4032, PLX4720) on excitability in BRAFV600E neurons. This FDA approved cancer medication was developed for unresectable or metastatic melanoma treatment (Bollag et al., 2010; Lee et al., 2010). Preincubation of BRAFV600E transfected cortical slices in 10-50 µM of Vemurafenib for 1-5h did decrease action potential firing frequency, but this effect was indistinguishable in slices preincubated in comparable amount of solvent (DMSO). This was also consistent with previous work examining the effect of DMSO on neuronal excitability in layer 2 of perirhinal cortex (Tamagnini et al., 2014). Similar results were obtained for Rapamycin experiments, which was also dissolved in DMSO. Further experiments to test different RAS-RAF-ERK pan-inhibitors that are soluble in other solvents may show the effect of acute inhibition of BRAFV600E on neuronal excitability. The decreased sustained potassium currents in BRAFV600E neurons, probably resulted from deactivation of potassium channels, since early activation of some of the potassium channels contributing to the sustained currents with acute (5-17 minutes) retigabine application decreased AP firing frequency significantly more than Vemurafenib or DMSO. This suggest that the hyperexcitability in BRAFV600E driven LNETs may be ameliorated with retigabine, or retigabine-like agents with less side-effects and toxicity (Rubi et al., 2017; Brickel et al., 2012).

Histopathological examination of immunopositivity to CD34, a hematopoietic stem cell marker previously shown to label extensively LNETs (reviewed in Thom et al., 2012), showed only few immunopositive neuronal cells.
The Nestin+ conditions presented here have small sample size, but comparison of hyperexcitability, dysmorphogenesis, activation of astrocytes with other conditions shows that these few examples did partially capture the effects observed in Glast+ BRAFV600E cells with distinct differences. Additionally, five brains out of six from different litters had larger cells located closer to the lateral ventricle, suggesting that the effect of BRAFV600E may be progenitors’ populations dependent. However, more experiments are needed to make a better estimation of such an effect.

Stimulation of BRAFV600E transgenic neurons with ChR2 in Nestin+ condition with high power blue laser under high extracellular potassium concentration may differentiate BRAFV600E condition from control-FP based on the amount of stimulation required to initiate ictal activity (threshold) (Chung et al., 2018). Stimulation with the blue laser of ChR2 and BRAFV600E transfected cortical slices perfused with 6 mM extracellular potassium did not show ictal-like discharges, suggesting that reduced connectivity in slice preparation may present a challenge for testing epileptiform activity in slices in those experiments.

**CHAPTER 5 CONCLUSION**

BRAFV600E somatic mutation when expressed in neuronal progenitors that produce both neurons and astrocytes increase excitability of layer 2/3 neurons in mouse somatosensory cortex as evident by increased AP firing frequency, depolarized RMP, increased Rin and hyperpolarized AP voltage threshold. It also increases hyperpolarization activated depolarizing conductances (Ih). (Figure 7, Figure 10, Figure 11) The increased AP firing frequency is not solely due to depolarized RMP, and depolarizing holding potential to around -60 mV for the control conditions and BRAFwt neurons did not increase the AP firing frequency to the same levels as in BRAFV600E expressing
neurons (Figure 7). Increased sPSCs frequency in BRAFV600E expressing neurons, which accounts for 5-10% of all cortical neurons (Rice et al., 2010) occurred together with increased sPSCs frequency in their untransfected neighbor neurons suggesting non-cell-autonomous effect of BRAFV600E expression (Figure 12). Unsupervised hierarchical Cluster Analysis showed that using 20 electrophysiological properties of the recorded 154 neurons BRAFV600E mostly segregate closer together with interesting few untransfected neighbor neurons interspersed in between (Figure 13). Those untransfected neighbor neurons either had non-cell autonomous effect, or they actually were transfected with BRAFV600E but not EGFP considering that the rate of co-transfection of multiple plasmids in IUE is around 90-95% (Figueres-Onate et al., 2015). Expression of BRAFV600E, which increases activation of MAPK pathway also increases percentage of astrocytes compared to neurons (Figure 3), these results corroborate previous work on MAPK pathway activation by Li et al. (2012). Li et al. (2012) showed that constitutively active MEK1, a downstream effector protein to BRAF when electroporated at E15.5 under ubiquitously active pCAG promoter increases gliogenesis of transfected neuroglial progenitor population. Additional support to that comes from another study by Li et al. (2014) that used ectopic expression of BRAFV600E in Neurog2+ and Ascl1 mouse lines. Those astrocytes are activated as evident through increased immunopositivity for GFAP in transfected area of cortical slices (Figure 5). Additionally, some of the BRAFV600E expressing neurons destined for upper cortical layers 2/3 mislocalized to the lower cortical layers 4-6 (Figure 3, Figure 4). Cellular morphological alterations observed in BRAFV600E transfected mouse somatosensory cortices included balloon-like cells that were observed at all examined post-natal ages. The aggregates of those cells were observed starting at P30 post-natal age. Those balloon-like cells may be immunopositive for both cortical layer markers – Cux1 and Ctip2, and some may not express neuronal nuclear marker
NeuN. Inflammatory immune system is overactivated in BRAFV600E mouse cortices together with classic complement pathway. The increased gene expression of innate inflammatory immune system, classic complement pathway and microglia markers (Iba1, CD74 – HLA-DR) are in line with findings of other studies in human resected GG tumors (Aronica et al., 2008b). More interestingly, they also in line with findings from resected human tubers from TSC patients (Boer et al., 2010a). Video and electrocorticographic recording showed epileptiform activity with behavioral manifestation suggesting that this mouse model did capture the symptomatic pathophysiology of LNETs. Those include cellular dysmorphogenesis, neuronal mislocalization, increased astrogliosis and activation of microglia, increased innate inflammatory response with complement pathway activation and seizure-like ictal electrographic discharges with motor manifestation (supplement video1).
Figure S1. **Immunoreactivity to Iba1.** Upper 3 panels show Immunoreactivity to Iba1 in Glast+ conditions. Lower panels show balloon-like cells surrounded by Iba1 positive cells. Scale bars 500 µm upper panels. 50 µm lower panels.
Figure S2 Action Potential voltage threshold correlation to RMP

A. Scatter plot of AP voltage threshold vs. RMP showing low correlation coefficient B. Same as A, excluding the single outlier and including other conditions. C. AP 10 V/s voltage threshold (closer to the kink on dV/dt plot). Two tailed T(60)=5.043, p<0.001 for BRAF V600E to controls combined; T(14)=2.757, p=0.015 for BRAFwt to controls combined. D. AP 50 V/s voltage threshold. Two tailed T(61)=3.466, p=0.001. * - p<0.05, ** - p<0.01, *** - p<0.001
Figure S3 Miniature post-synaptic currents

A. Cumulative distribution of the inter-events interval and average instantaneous frequencies of spontaneous post-synaptic currents (sPSCs) and miniature post-synaptic currents (mPSCs). Miniature post-synaptic currents were recorded in the same neurons after at least 3 min in 3 µM TTX in the perfusion system.

B. sPSCs and mPSCs amplitudes cumulative distribution and averages per cell.

Figure S3. Miniature post-synaptic currents in Glast+ BRAFV600E neurons. A. Cumulative distribution of the inter-events interval and average instantaneous frequencies of spontaneous post-synaptic currents (sPSCs) and miniature post-synaptic currents (mPSCs). Miniature post-synaptic currents were recorded in the same neurons after at least 3 min in 3 µM TTX in the perfusion system. B. sPSCs and mPSCs amplitudes cumulative distribution and averages per cell.
REFERENCES


expression in epileptogenic cortex from human periventricular nodular heterotopia.

Epilepsia 43 Suppl 5:209-216.


Blumcke I, Sarnat HB (2016) Somatic mutations rather than viral infection classify focal cortical dysplasia type II as mTORopathy. Curr Opin Neurol 29:0.


CDC, (August 3, 2017) August 12, 2017:


Cepeda C, Andre VM, Yamazaki I, Hauptman JS, Chen JY, Vinters HV, Mathern GW, Levine MS (2010) Comparative study of cellular and synaptic abnormalities in brain tissue samples
from pediatric tuberous sclerosis complex and cortical dysplasia type II. Epilepsia 51 Suppl 3:160-165.


modulation by dopamine of responses evoked by excitatory amino acids in human cortex. 
Synapse 11:330-341.


CS, Ladanyi M, Kris MG (2012) Coexistence of PIK3CA and other oncogene mutations in 
lung adenocarcinoma-rationale for comprehensive mutation profiling. Mol Cancer Ther 
11:485-491.

Chai H, Diaz-Castro B, Shigetomi E, Monte E, Octeau JC, Yu X, Cohn W, Rajendran PS, 
Astrocytes: Transcriptomic, Proteomic, Morphological, and Functional Evidence. Neuron 
95:531-549.e9.

Gygi SP, Sabatini DM (2016) The CASTOR Proteins Are Arginine Sensors for the 

Chappe C, Padovani L, Scavarda D, Forest F, Nanni-Metellus I, Loundou A, Mercurio S, Fina F, 
with pleomorphic xanthoastrocytomas and gangliogliomas BRAF(V600E) mutation and 


BRAF (v-raf murine sarcoma viral oncogene homolog B1). 2004-9:


Ensembl (2016) BRAF phylogenetic tree ensemble

GRCh38.p7 (Genome Reference Consortium Human Build 38), INSDC Assembly GCA_000001405.22, Dec 2013 Version 87.38.


Neuropathological spectrum of cortical dysplasia in children with severe focal epilepsies.


ILAE (2017) Epilepsy Diagnosis.


Linnarsson S (2015) BRAF Cortex and hippocmapal expression from single cell RNA-seq.


World Health Organization (2006)

World Health Statistics 2016: Monitoring health for the SDGs


