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PHARMACOLOGICAL CHARACTERIZATION OF AN *IN VITRO* MODEL OF *STATUS EPILEPTICUS*

Dissertação de Mestrado em Bioquímica, orientada pelo Professor Carlos B. Duarte e pela Doutora Miranda Mele, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Dissertação apresentada à Universidade de Coimbra para cumprimentos dos requisitos necessários à obtenção do grau de Mestre em Bioquímica realizada sobre a orientação científica do Doutor Professor Carlos B. Duarte (Departamento de Ciências da Vida, Faculdade de Ciência e Tecnologia, Universidade de Coimbra) e da Doutora Miranda Mele (Centro de Neurociências e Biologia Celular, IIIUC).

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“No sense in calling it a dream, every reason to call it a path”

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ABBREVIATIONS

4-AP	4-Aminopyridine
Abi-1	Abelson-interacting protein 1
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Action potential
AP-2	Activating protein 2
APV	D-2-amino-5-phosphonovalerate
Arc	Activity-regulated cytoskeleton-associated protein
ATP	Adenosine triphosphate
BAR	Bin-Amphiphysin-Rvs
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium ion
CamK	Calmodulin-dependent protein kinase
CK2	Casein kinase 2
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CNS	Central Nervous System
COP	Coat protein
CPG2	Carboxypeptidase G2 precursor
CTD	Carboxyterminal Domain
DDBM	2-(1,1-Dimethyl-1,3-dihydro-benzo[e]indol-2-ylidene)-malonaldehyde
DG	Dentate gyrus
DIV	Days *in vitro*
EAAT	Excitatory amino acid transporter
ECS	Electroconvulsive shock
EEG	Electroencephalogram
EIS	Electroshock-induced seizures
E_{Na}	Sodium equilibrium potential
EPSC	Excitatory Post-Synaptic Current
EPSP	Excitatory Post Synaptic Potential

ER	Endoplasmic reticulum
ERK	Extracellular signal–regulated kinase
G-proteins	Guanine nucleotide-binding proteins
GRASP-1	GRIP associated protein 1
GRIP1	Glutamate receptor interacting protein
HBSS	Hank's balanced salt solution
hnRNP K	Heterogenous Nuclear Ribonucleoprotein K
IEG	Immediate early gene
iGluRs	Ionotropic glutamate receptors
K+	Potassium ion
KA	Kainic Acid
KAR	kainate receptors
KH	K Homology domains
KI	K Interactive domain
KNS	Nuclear shuttling domain
LTD	Long-term depression
LTP	Long-term potentiation
MAGUK	Membrane-associated guanylate kinase
MEK	Mitogen-activated protein kinase kinase
Mg^{2+}	Magnesium ion
mGluRs	Metabotropic glutamate receptors
MK-801	Dizocilpine
mRNP	Messenger ribonucleoprotein
mTOR	Mechanistic target of rapamycin
Na^{+}	Sodium ion
NBM	Neuronal Basal Medium
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NEEP21	Neuronenriched endosomal protein 21
NLS	Localization signal
NMDAR	N-methyl-D-aspartate receptor

NSF	N-ethylmaleimide sensitive factor
NSF	N-ethylmaleimide-sensitive factor
NTD	Aminoterminal Domain
PDZ	PSD-95/DlgA/ZO-1
PI3K	Phosphoinositide 3-kinase
PICK1	Protein Interacting with C Kinase - 1
PKA	Protein kinase A
PKC	Protein Kinase C
PLCγ	Phosphoinositide phospholipase Cγ
PP1/2A/2B	Protein phosphatase
PPS	Perforant Path Stimulation
PSD-95	Post-synapyic density 95
pTrkB	Phosphorylated TrkB
Rab4	RAS-related GTP-binding protein
RER	Rough endoplasmic reticulum
SAP	Synapse-associated protein
SE	Status Epilepticus
shRNA	Short hairpin RNA
SNAP	Soluble NSF Attachment Protein
SNARE	Soluble NSF attachment protein receptors
SRS	Spontaneous recurrent seizures
Stx	Sintaxin
TARP	Transmembrane AMPAR regulatory protein
TLE	Temporal Lobe Epilepsy
TrkB	Tropomyosin receptor kinase B
TTX	Tetrodotoxin
UPR	Unfolded protein response
VGCC	Voltage-gated calcium channel
vGlut	Vesicular Glutamate Transporter

KEWORDS

Status Epilepticus

TrkB Receptors

BDNF

Epilepsy

Spontaneous Recurrent Seizures

PALAVRAS-CHAVE

Estado de mal epilético

Recetores TrkB

BDNF

Epilepsia

Crises Epiléticas Espontâneas

ABSTRACT

Epilepsy is a chronic condition characterized by recurrent unprovoked seizures. The disease affects approximately 50 million people worldwide. Hyperexcitability is considered as the permissive factor for the genesis/propagation of epileptic seizures. In many epileptic patients, seizures are controlled with conventional antiepileptic drugs that act by blocking sodium channels or by enhancing the function of GABA as a neurotransmitter. However, 30% of epileptic cases become resistant to the treatment (pharmacoresistance). Several *in vitro* models of epilepsy were developed in the past, using brain slices or cultured neurons, to investigate the mechanisms underlying seizures. Different factors were found to be involved in the pathogenesis of epilepsy. In particular, numerous studies have shown that activation of ionotropic and metabrotopic glutamate receptors, as well as the TrkB receptors for BDNF, contribute to epileptogenesis.

Non-published results from our laboratory showed synchronous $[Ca^{2+}]_i$ transients in cultured hippocampal neurons incubated transiently in a magnesium-free medium, to mimic *Status Epilepticus in vitro*. These alterations in intracellular calcium levels are possibly concomitant with bursts in electrical activity. In this study, we aimed to further characterize this model through whole-cell path clamp, investigating the nature and the mechanisms behind seizures. Moreover, we investigated the alterations in BDNF signaling induced by *Status Epilepticus*. We observed that APV and CNQX, which inhibit NMDA and non-NMDA glutamate receptors, respectively, reduce the firing rate during *status epilepticus*. Likewise, we found that the rhythmic burst activity following *status epilepticus* was prevented by APV and CNQX. Additionally, we observed alterations in the rise and decay tau of the action potentials during and after *status epilepticus*, which however were not statistically significant. NMDA and non-NMDA glutamate receptors may also influence the hyperpolarization phase of the action potential under conditions mimicking *Status Epilepticus*, being the contribution of the latter receptor population more robust.

Given the role of BDNF in epileptogenesis, we investigated the putative alterations in the phosphorylation of the TrkB receptors for the neurotrophin in cultured hippocampal

neurons subjected to *in vitro Status Epilepticus*. An increase in TrkB phosphorylation was observed under these conditions, but the effect was not statistically significant. Interestingly, this effect was not abrogated by TrkB-Fc which chelates free BDNF and prevents the activation of TrkB receptors by the extracellular neurotrophin. These results suggest that there might be other mechanisms involved in phosphorylating TrkB receptors in the absence of BDNF during *status epilepticus*.

Future follow up studies using the model implemented in this work may contribute to the understanding of the mechanisms underlying seizure activity. This work may also lead to the development of new therapies to better act on *Status Epilepticus*.

RESUMO

A epilepsia é uma patologia crónica caracterizada por crises epiléticas espontâneas. Esta patologia afeta aproximadamente 50 milhões de pessoas mundialmente. A hiperexcitabilidade é considerada um fator facilitador da génese/ propagação do ataque epilético. Em muitos dos pacientes epiléticos, é possível controlar as convulsões com medicamentos antiepiléticos convencionais que atuam como bloqueadores dos canais de sódio ou por aumento da função de GABA como neurotransmissor. No entanto, 30% dos doentes epiléticos tornam-se resistentes ao tratamento (farmacoresistência). Têm sido desenvolvidos vários modelos de epilepsia *in vitro* recorrendo a fatias do cérebro ou culturas neuronais, as quais permitem investigar os mecanismos responsáveis pelas crises epiléticas. Mais especificamente, vários estudos têm demonstrado que a ativação de recetores ionotrópicos e metabotrópicos do glutamato, bem como os receptores TrkB que ligam ao BDNF, contribuem para a epileptogénese.

Resultados não publicados do nosso laboratório mostraram oscilações sincronizadas de $[Ca^{2+}]_i$ em neurónios do hipocampo em cultura incubados num meio sem magnésio, que mimetiza a fase de *status epilepticus in vitro*. Estas alterações nos níveis de cálcio intracelular, são possivelmente concomitantes com *bursts* na atividade elétrica celular. Este estudo teve como objetivo caracterizar mais exaustivamente este modelo, utilizando *whole-cell patch clamp*, de forma a investigar a natureza e os mecanismos responsáveis por crises epiléticas. Além disto, investigámos as alterações na sinalização pelo BDNF induzidas por *status epilepticus*. Observámos que APV e CNQX, que inibem os recetores de glutamato do tipo NMDA e não-NMDA, respetivamente, reduzem a frequência de disparos de potenciais de ação durante a fase de *status epilepticus*. Em consonância, verificou-se que os *bursts* rítmicos que procedem o *status epilepticus* foram suprimidos na presença de APV e CNQX. Observámos também alterações no tau ascendente e descendente dos potenciais de ação durante e após o *status epilepticus*, apesar dos resultados obtidos não serem estatisticamente significativos. Os resultados obtidos sugerem também que os recetores de glutamato podem influenciar a fase de

hiperpolarização do potencial de ação sob condições que mimetizam *status epilepticus*, sendo que a contribuição dos recetores não-NMDA foi mais proeminente.

Considerando o papel do BDNF no processo de epileptogénese, investigamos as alterações nos níveis de fosforilação dos recetores para a neurotrofina em neurónios do hipocampo incubados em condições que mimetizam *in vitro* as condições de *status epilepticus*. Observou-se um pequeno aumento da fosforilação do recetor TrkB, ainda que não significativo, durante a fase de *status epilepticus*. Curiosamente, este efeito não foi abolido por TrkB-Fc, uma proteína de fusão que liga BDNF livre impedindo a ativação do recetor TrkB pela neurotrofina. Estes resultados sugerem que pode haver outros mecanismos envolvidos na fosforilação dos recetores TrkB na ausência de BDNF durante a fase de *status epilepticus*.

Futuramente, o modelo implementado neste trabalho pode contribuir para a compreensão dos mecanismos responsáveis pela atividade epilética. Este estudo pode também contribuir para o desenvolvimento de novas terapias para uma melhor atuação na fase de *status epilepticus*.

I

INTRODUCTION

1.1. GLUTAMATERGIC SYNAPSE

Excitatory neurotransmission in the Central Nervous System (CNS) is largely mediated by glutamate. The neurotransmitter is synthetized by the presynaptic neuron and accumulated in the nerve terminals into small synaptic vesicles through the activity of specific vesicular glutamate transporters (vGluts). These vesicles fuse with the plasma membrane at active zones by exocytosis following activation of the presynaptic neuron, thereby releasing glutamate into the synaptic cleft (Fig. 1.1) (Popoli, Yan, McEwen, & Sanacora, 2011). The latter process is induced by Ca^{2+}-entry through voltage-gated calcium channels (VGCCs) and is regulated by several proteins. Intracellular calcium sensors are responsible for the fast release of glutamate whenever there is a calcium influx (Volk, Chiu, Sharma, & Huganir, 2015), and the SNARE (soluble NSF attachment protein receptors) complex is thought to be the machinery regulating glutamate vesicles fusion with the presynaptic membrane. The latter complex is formed by the interaction of two synaptic membrane proteins and a vesicular protein: syntaxin 1 or syntaxin 2 and SNAP25, synaptobrevin 1 or synaptobrevin 2, respectively (Popoli et al., 2011).

After exocytosis, the glutamate uptake machinery associated with the plasma membrane of neurons and astrocytes regulates the glutamate concentration at the synaptic cleft and consequently the period of activation of glutamate receptors (Volk et al., 2015). Glial cells have excitatory amino acid transporters (EAATs) which are responsible for glutamate uptake from the extracellular compartment; these transporters also exist in neurons but in less quantity. When glutamate reaches glial cells it is converted into glutamine by glutamine synthase; this amino acid by turn is transported to the glutamatergic neuron where it is hydrolysed into glutamate by glutaminase (Popoli et al., 2011) .

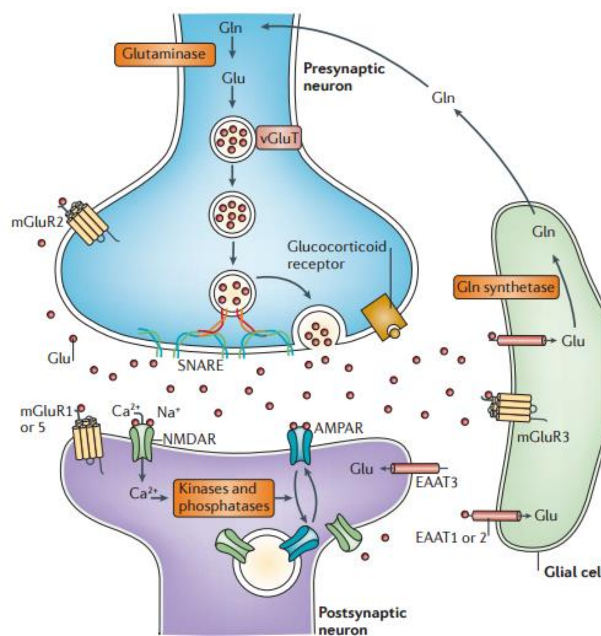

Figure 1.1 – The glutamatergic synapse: synthesis and reuptake of glutamate. Neuronal glutamate is synthesized de novo from glucose (not shown) and from glutamine supplied by glial cells. Then, it is packed into synaptic vesicles by vesicular glutamate transporters (vGluts); these vesicles fuse with the presynaptic membrane in a process mediated by SNARE complex proteins. The effects of glutamate are mediated by activation of AMPARs (AMPA Receptors), NMDARs (NMDA Receptors) and metabotropic glutamate receptors (mGluR1 to mGluR8), which may be associated with the plasma membrane of both postsynaptic and presynaptic neurons and/or glial cells. Upon binding of glutamate, the receptors initiate various responses, including membrane depolarization, activation of intracellular messenger cascades, modulation of local protein synthesis and, eventually, regulation of gene expression (not shown). Surface expression and function of NMDARs and AMPARs is dynamically regulated by protein synthesis and degradation, as well as by receptor trafficking between the postsynaptic membrane and endosomes. The insertion and removal of receptors from the postsynaptic membrane provides a mechanism for long-term modulation of synaptic strength. Glutamate is cleared from the synapse through excitatory amino acid transporters (EAATs) on glial cells and, to a lesser extent, on neurons. Within the glial cell, glutamate is converted to glutamine by glutamine synthetase and glutamine is then released by System N transporters and taken up by neurons through System A sodium-coupled amino acid transporters to complete the glutamate–glutamine cycle (Popoli et al., 2011).

Glutamate activates two major classes of postsynaptic receptors: the ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels, and the metabotropic glutamate receptors (mGluRs), whose effects are mediated through regulation of guanine nucleotide-binding proteins (G-proteins) (Purves, 2008). Ionotropic glutamate receptors are classified in three distinct groups, based on their molecular, electrophysiological and pharmacological properties: NMDA (N-methyl-D-aspartate) receptors (NMDAR), AMPA (α-amino-3-hydroxy-5-methyl-4- 4 isoxazolepropionic acid) receptors (AMPAR), and kainate receptors (KAR) (see figure 1.2) (see next section).

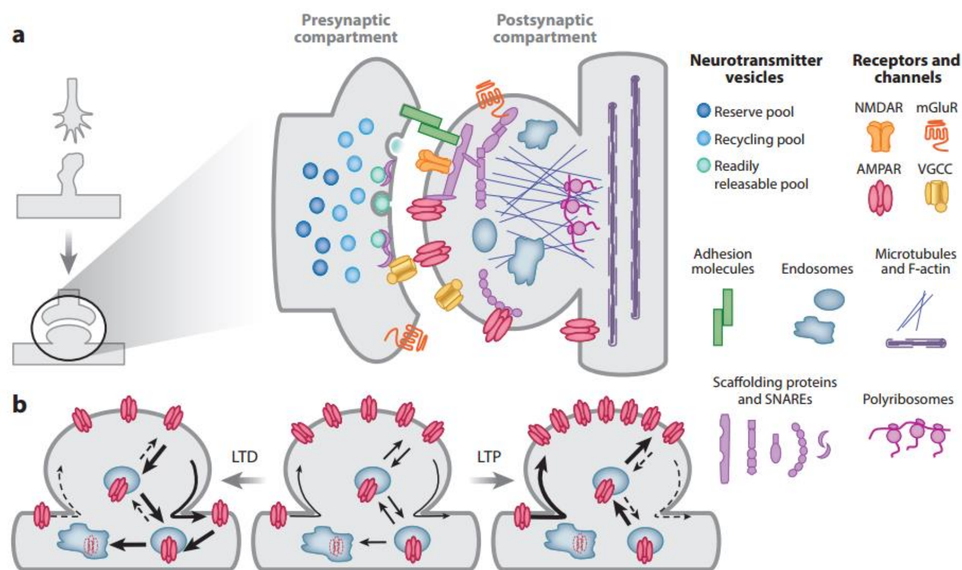

Figure 1.2 – Development of glutamatergic synapses and synaptic plasticity. (a) Synapse formation starting with the interaction and stabilization of an axonal growth cone and dendritic filopodia. Synapses maturate in pre- and postsynaptic compartments. The presynaptic compartment is composed of several membrane-associated proteins with different functions (e.g., voltage-gated ion channels, metabotropic autoreceptors and cell adhesion molecules), neurotransmitter-filled synaptic vesicles, and SNARE protein complexes for vesicle fusion and glutamate release. The main groups of plasma membrane proteins at the postsynaptic spine are: ionotropic receptors (AMPAR and NMDAR), mGluRs, voltage-gated ion channels and cell adhesion molecules. Moreover, an extensive network of scaffolding proteins, endosomes, and local protein translation machinery are present in this compartment. (b) Postsynaptic alterations in synaptic plasticity. Activity-dependent, long-term changes in synaptic strength at postsynaptic sites are determined predominately by alterations in the number of AMPARs at the synapse. An increase in lateral diffusion and/or direct insertion of AMPAR into the postsynaptic membrane yields LTP, whereas an increase in lateral diffusion out of the spine and/or internalization and degradation of synaptic AMPARs is associated with LTD. Thick arrows represent an increase (relative to the basal state) and dashed arrows represent a decrease in the indicated receptor trafficking step (Volk et al., 2015).

In summary, synaptic transmission in most excitatory synapses in the CNS consists in the release of glutamate from the presynaptic compartment and consequent activation of AMPAR and NMDAR. Both receptor types are ionic channels that, when opened, produce a depolarizing current. The resulting activity consists in an Excitatory Post Synaptic Potential (EPSP). Moreover, different spine sizes correspond to a different number of receptors and distinct AMPAR/NMDAR ratios (Di Maio, Ventriglia, & Santillo, 2015).

1.1.1. AMPA receptors

AMPAR are ionotropic glutamate receptors responsible for the mediation of the majority of fast excitatory synaptic transmission in the CNS, contributing to long-lasting changes in synaptic strength (Earnshaw & Bressloff, 2008). These receptors show a widespread

distribution at the postsynaptic membrane of excitatory glutamatergic synapses, and an upregulation in the surface synaptic expression of AMPA receptors contributes to long-term synaptic strengthening.

The AMPA-type glutamate receptors are permeable to cations and expressed throughout the brain. They have a tetrameric structure, i.e. they are formed by the assembly of four subunits, GluA 1-4 (Anggono & Huganir, 2012), encoded by the genes GRIA1-GRIA4 (Hollmann & Heinemann, 1994; Traynelis et al., 2010). The AMPAR subunits share several conserved domains, thus are highly homologous (Collingridge, Isaac, & Wang, 2004). Each subunit is constituted by an aminoterminal (N-terminal) extracellular domain, three transmembrane domains (M1, M3 and M4), a M2 transmembrane domain forming the channel pore loop, a carboxyterminal (C-terminal) intracellular domain and a ligand-binding domain which is formed by a sequence located at the N-terminal loop together with part of the extracellular loop between the M3 and M4 transmembrane domains. In the ligand binding domain there is a pocket-like structure where glutamate binds (see figure 1.3).

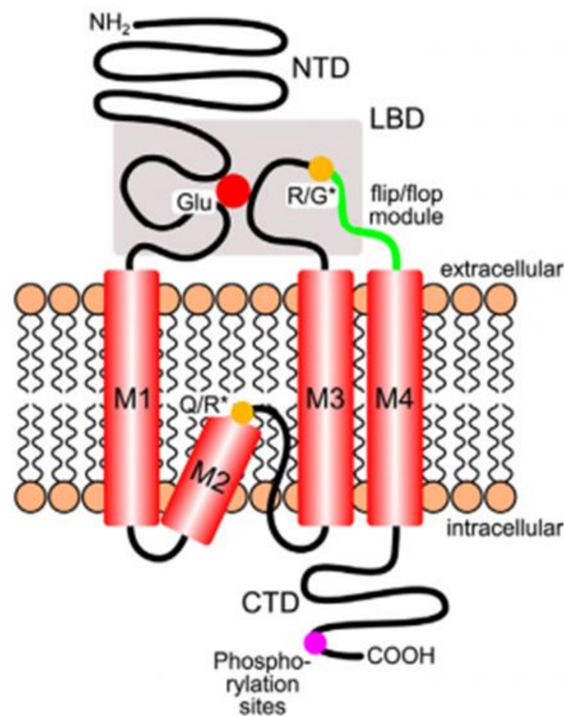

Figure 1.3 – Structure of the AMPA receptor subunits. Each AMPAR subunit contains an extracellular N-terminal domain (NTD) and an intracellular C-terminal domain (CTD). The ligand binding domain (LBD) of AMPAR is composed by an amino acid sequence located at the NTD and an extracellular loop located between M3 and M4, as indicated by the grey box. The figure shows a glutamate molecule (Glu; red circle)

bound to the receptor. M1, M3 and M4 are transmembrane domains and M2 is a plasma membrane-associated domain (Freudenberg, Celikel, & Reif, 2015).

The major difference in the amino acid sequence of AMPAR subunits is found in their C-terminal intracellular tails, due to alternative splicing, resulting in variants with different receptor desensitization properties (Lambolez, Ropert, Perrais, Rossier, & Hestrin, 1996). Layers of the extracellular domain of the receptor subunits can reorganize in response to channel activation and desensitization (Krieger, Bahar, & Greger, 2015). For instance, the GluA2 subunit prevents calcium influx due to a positively charged arginine residue located at the pore region (Greger, Khatri, Kong, & Ziff, 2003). AMPA receptors containing GluA2 subunits have a higher conductance as well as a faster rise and decay kinetics when opened (Chater & Goda, 2014). GluA1 and GluA2 are the most abundant subunits in adult brain, hence most AMPAR are impermeable to calcium, even though this depends on the brain region and cell type (Cull-Candy, Kelly, & Farrant, 2006).

In summary, AMPAR properties differ according to their subunit composition, which determines the dynamics and efficiency of AMPAR-mediated signaling (Collingridge et al., 2004).

1.1.1.1. AMPAR trafficking and recycling

AMPAR are very dynamic in the plasma membrane, moving in and out the synapse by lateral diffusion. The quantity of receptors in the plasma membrane and at the synapses is highly regulated, and their trafficking is a key mechanism in the plasticity of glutamatergic synapses (Volk et al., 2015).

The synthesis and assembly of AMPAR occurs at the endoplasmic reticulum (ER) (Volk et al., 2015), and from this compartment the receptors then transit to the Golgi apparatus where they suffer post-translational modifications giving rise to their mature form. In the ER, the NTD of the receptor subunits is cleaved and this is followed by glycosylation of the extracellular domain. GluA1/2 exit the ER (Endoplasmic Reticulum) faster than GluA2/3. Also, ER chaperones, highly associated with GluA2, may control ER retention. In addition, there are also several proteins interacting with AMPAR with a possible role in ER retention. GluA2 has a PDZ (PSD-95/DlgA/ZO-1)-binding motif within the CTD which can interact with proteins containing a PDZ domain, for instance PICK1 (Protein

Interacting with C Kinase 1). Also, the GluA1 CTD has a PDZ motif that can interact with SAP97 (synapse-associated protein 97). Another possible player is stargazing: stargazer (a mouse with a mutation in stargazin's gene granule cells) exhibits upregulated ER UPR (unfolded protein response), suggesting that stargazin has a role in AMPAR's folding and assembly.

After exiting the ER and Golgi apparatus, vesicles containing AMPAR travel through microtubules and get coupled with recycled endosomes, forming a pool of AMPAR that are exocytosed at a later point by a regulated mechanism. This process is mediated by different proteins, like GRIP1 (glutamate receptor interacting protein), an AMPAR interacting protein, and is also regulated by phosphorylation of the receptor subunits. Additional proteins that play a role in the trafficking of AMPAR are NEEP21 (neuron enriched endosomal protein 21), Rab4, Syntaxin 13, PICK1 and GRASP-1 which will be described later on. PICK1 and GRIP1 are both AMPAR-interacting proteins which interact with Syntaxin 13 and NEEP21. The first is an endocytic/recycling protein while the latter protein has a general role in recycling and interacts directly with Syntaxin 13.

AMPAR contained within recycling endosomes can be transported back to the plasma membrane through actin cytoskeleton, in a process mediated by Rab4, while receptor exocytosis is mediated by the SNARE complex. For example, GluA2 CTD binds to NSF (N-ethylmaleimide sensitive factor), an ATPase typically involved in membrane fusion which dissociates PICK1 from the receptor and allows rapid exocytosis. From here receptors suffer lateral diffusion in a stimulus-dependent manner. Once the receptors arrive at the post-synaptic density (PSD) they get trapped there (Groc & Choquet, 2006).

After playing their role at the synapse AMPAR are internalized. Endocytosis of the receptors requires the intracellular C-terminal domain of AMPA receptor subunits. The process is clathrin-mediated, and dynamin and endophilin take part in endosome formation, along with AP-2, a clathrin-adaptor molecule (Groc & Choquet, 2006).

AMPAR internalization is mediated by Arc, an IEG induced by neuronal activity. Another IEG involved in the process in CPG2, which mediates internalization specifically at excitatory synapses. Afterwards, endosomes are sorted, and they can be recycled or degraded. Under the latter conditions the receptors travel to late endosomes before arriving at the lysosomal compartment where they are degraded. If recycled, the receptors are moved to early endosomes, and can be directly inserted back into the plasma membrane, a process named fast recycling, or alternatively they may be incorporated into recycling endosomes following the process described above (see figure 1.4) (Shepherd & Huganir, 2007).

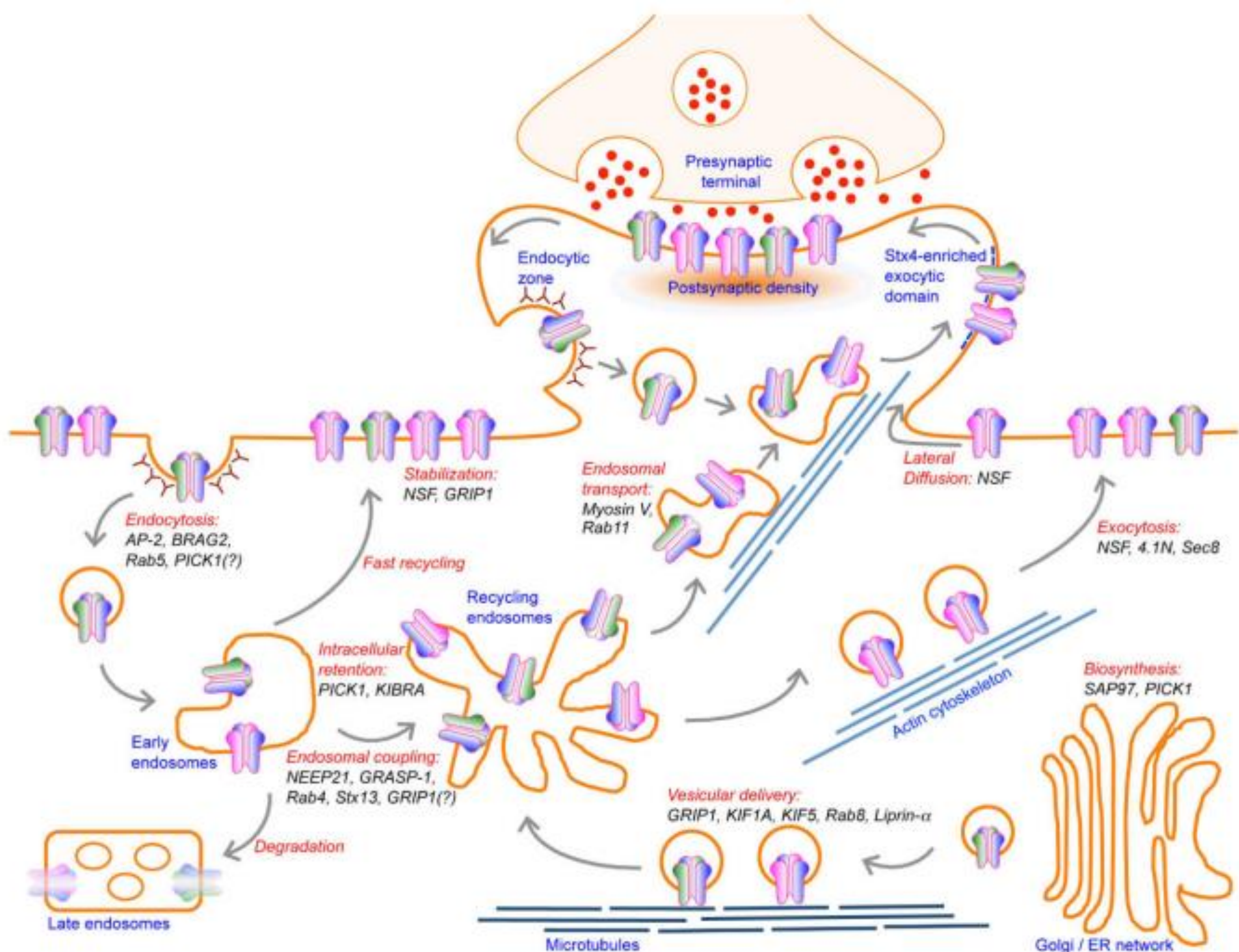

Figure 1.4 – Schematic overview of AMPAR trafficking. AMPAR are assembled in the ER and maturated in the Golgi apparatus. At a later point the receptors are inserted into the plasma membrane where they can diffuse laterally in an activity-dependent manner. From the plasma membrane the receptors can be internalized and recycled back to the membrane or be degraded (Anggono & Huganir, 2012).

The mechanisms that control the trafficking of AMPAR depend on their subunit composition: short-tailed heterodimers (GluA2/3) keep a continuous cycle, maintaining

the intracellular pool of AMPAR (Passafaro, Piech, & Sheng, 2001), while receptors containing the long-tailed subunits (GluA1/2 and GluA2/4) are inserted in the plasma membrane in an activity-dependent manner (Shi, Hayashi, Esteban, & Malinow, 2001). Also, GluA1/GluA2 heteromers exit the ER faster than GluA2/GluA3 heteromers. A subpopulation of GluA2-containing AMPAR remains in the ER due to presence of a charged arginine residue at the pore region (Greger, Khatri, & Ziff, 2002). From the ER, AMPAR travel to the Golgi apparatus before they are delivered to dendrites (or to axons) (Shepherd & Huganir, 2007) by a mechanism that depends on the activity of several kinesin motor proteins (Perestenko, Ashby, & Henley, 2003) and protein linkers between the receptors and microtubule motor proteins (Groc & Choquet, 2006).

Once at the plasma membrane, AMPAR can diffuse laterally to arrive at the synapse. Plasma membrane associated AMPAR may be endocytosed and degraded or, alternatively, they are recycled back into the membrane from an intracellular pool (Chater & Goda, 2014) (Shi et al., 2001).

After insertion into the plasma membrane AMPAR can diffuse laterally to the synapses (Adesnik, Nicoll, & England, 2005). Trafficking of AMPAR is regulated by TARPs (Transmembrane AMPA Receptor Regulatory Proteins) (Adesnik et al., 2005)and PSD-95 (Colledge et al., 2003), two proteins important in AMPAR anchoring at the synapse. Accordingly, modifications on TARPs or PSD-95 lead to alterations in the pattern of expression of AMPAR in the membrane (Adesnik et al., 2005; Hegde, 2010). The traffic of AMPAR also depends on their subunit composition. Short-tailed heterodimers (GluA2/3) keep a continuous cycle maintaining the intracellular pool (Passafaro et al., 2001), while long-tailed (GluA1/2 and GluA2/4) are inserted in an activity-dependent manner (Shi et al., 2001). Also, GluA1/GluA2 heteromers exit the ER faster than GluA2/GluA3 heteromers. Although not all GluA2 subunits leave the ER, some stay forming a pool of receptors in this compartment; this is due to the presence of a charged arginine residue at the pore region (Greger et al., 2002).

AMPAR trafficking is also regulated by post-translational modification of the receptor subunits. For AMPAR to exit ER it is required that the PDZ motif of the C-terminal domain of GluA2 subunit interacts with the PDZ domain of another protein, for instance PICK1 (C-kinase 1). The same happens between GluA1 and SAP97 (synapse associated protein-

97), a member of the MAGUK family which interacts with the protein kinase A (PKA) anchoring molecule AKAP79 as a way to enhance the phosphorylation of GluA1 required for LTP (Groc & Choquet, 2006).

The increase or decrease in the number of AMPAR in the membrane is related with synaptic strength, long lasting or not (Luscher et al., 1999), and concomitantly with spine size and morphology (Matsuzaki et al., 2001). The best characterized consequences of these changes are long-term synaptic potentiation (LTP) and long-term synaptic depression (LTD), two forms of synaptic plasticity that are thought to underlie learning and memory formation (Chater & Goda, 2014).

1.1.2. NMDA receptors

NMDAR are ionotropic receptors permeable to sodium, potassium and, more importantly, calcium ions (Malenka & Nicoll, 1993; Sanz-Clemente, Nicoll, & Roche, 2013). Therefore, these receptors for the neurotransmitter glutamate are essential in excitatory neurotransmission in the central nervous system (Sanz-Clemente et al., 2013)and for activity-dependent synaptic plasticity (Ye et al., 2000). They also play a role in synapse stability (Alvarez, Ridenour, & Sabatini, 2007).

These receptors have a tetrameric structure and are comprised by heteromeric channels formed by the assembly of different subunits: GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B (see figure 1.5) (Cull-Candy, Brickley, & Farrant, 2001; Paoletti, Bellone, & Zhou, 2013). These subunits are differentially expressed in the brain and affect the receptor kinetics, magnesium sensitivity, ion conductance and molecular interaction partners (Paoletti et al., 2013). For instance, The GluN1 and GluN3 subunits bind glycine (and D-serine), whereas glutamate (and NMDA) interacts with GluN2 subunits (Clarke & Johnson, 2006).

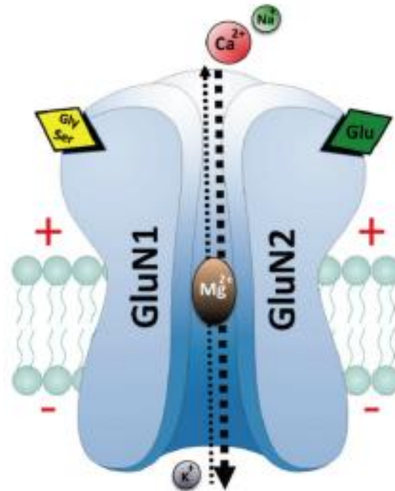

Figure 1.5 – Structure of the NMDA receptors. NMDA receptors are ionotropic glutamate receptors composed of two GluN1 subunits and two GluN2 or GluN3 subunits. NMDARs are permeable to Ca^{2+}, Na^{+} and K^{+}. In order to be activated, NMDAR needs to bind to glutamate (via GluN2 subunits) and glycine (via GluN1), and to release the Mg^{2+} ions from the NMDAR channel. The release of Mg^{2+} from the NMDAR channel depends on the depolarization of the membrane (Sanz-Clemente et al., 2013).

Each subunit of the NMDAR is composed by an extracellular N-terminal and an intracellular C-terminal domain, as well as three transmembrane domains and one membrane domain. The ligand binding domain is formed by part of the two extracellular regions, S1 and S2, which are located in the N-terminal domain and the loop between M3 and M4 respectively (see figure 1.6). The binding of glutamate to this region of the GluN2 subunit activates the NMDAR, but this process also requires the presence of glycine (or D-serine) which binds as co-agonist to an allosteric site on the receptor. Activation of the receptor also requires the removal of magnesium from the receptor channel. Moreover, the M2 loop, which is part of the channel pore, contains an asparagine residue responsible for determination of calcium permeability and mediates the magnesium blockade (Sanz-Clemente et al., 2013).

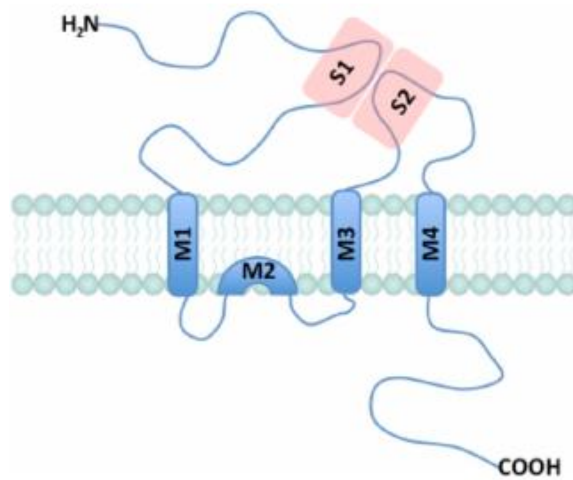

Figure 1.6 – Structure of NMDAR subunits. Each NMDAR subunit is composed by two extracellular domains, three transmembrane domains (M1, 3 and 4), one re-entrant loop (M2) and one cytoplasmic region. Glutamate binds the pocket created by two extracellular regions (S1-2). S1 is part of the N-terminal tail and S2 is located in the loop between M3 and M4. The C-terminus is cytoplasmic and varies in length between subunits (Sanz-Clemente et al., 2013).

The NMDAR NTD functions as a periplasmic binding protein allosteric module, being stabilized in a closed-cleft conformation upon binding of zinc to the cleft, which reduces the probability of channel opening. In tightly packed N-terminal domains of NMDAR subunits there are extensive interactions between the LBD and the NTD, which allow allosteric coupling and hence changes in the channel open probability in response to the interaction with NTD ligands (Sanz-Clemente et al., 2013).

1.1.2.1. NMDAR trafficking

NMDAR subunits are translated and assembled in the rough endoplasmic reticulum (RER), before they are transported to the dendrites within vesicles. Fusion of these vesicles with the plasma membrane allows the insertion of the receptors in the spines. These receptors can be directly inserted in the synaptic membrane, or at non-synaptic locations, depending on the stimulus. The mechanism by which this process occurs depends on the NMDAR subunit composition. The exchange between NMDAR is dynamic and related to neuronal activity. In total, three different NMDAR pools can be identified in neurons: (i) the synaptic pool, (ii) the extrasynaptic pool (NMDAR associated with the plasma membrane in the vicinity of the synaptic plasma membrane), and (iii) the non-synaptic pool (NMDAR present in cell body and dendritic shaft) (Cercato et al., 2014).

The GluN1-1 splice variant impairs NMDAR exiting from the ER, and the same applies to the C2 domain, but not the C2′ variant. The latter variant binds to the COPII coat complex increasing trafficking and surface delivery of NMDAR, by a mechanism that is stimulus-dependent (Groc & Choquet, 2006). Export from endoplasmic reticulum and synaptic delivery of NMDAR depends on GluN1 C-terminal splicing, GluN1 as several isoforms according to splice variants, each has specific gating, pharmacological and trafficking properties (Paoletti et al., 2013). Intracellular and surface trafficking is also controlled by different motifs of the CTD of GluN2A. In order for GluN2B-containing NMDAR to get trapped at the synapse, the CTD has to bind with MAGUKs (or PDZ domain containing scaffold proteins). This is not the case of GluN2A, which can bind to postsynaptic sites independently of PDZ (see figure 1.7). Surface mobility also differs between receptor subtypes: GluN2B can move more readily than GluN2A, possibly due to phosphorylation of the PDZ-binding motif (Paoletti et al., 2013).

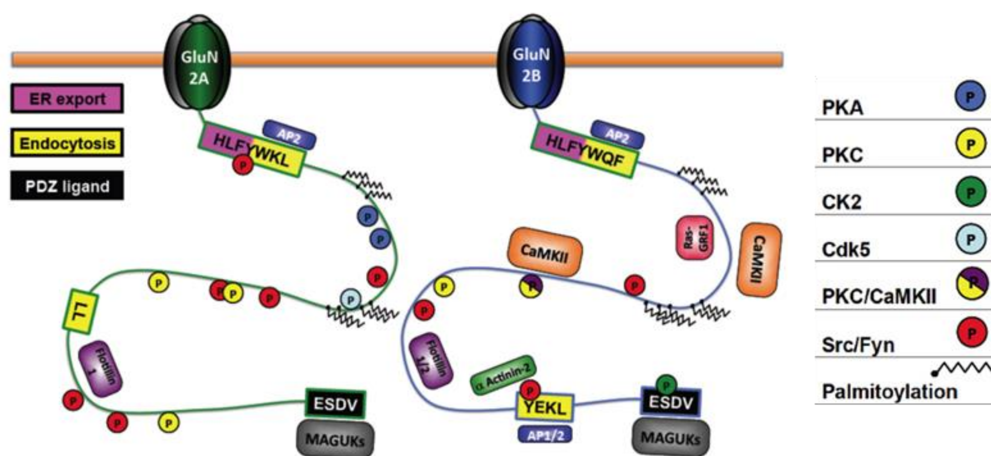

Figure 1.7 - The GluN2A and GluN2B C-termini. It contains distinct regulatory motifs, phosphorylation sites, and protein-protein interaction domains. GluN2A contains PKA, PKC and Src/Fyn phosphorylation sites; GluN2B has sites phosphorylated by CK2 and PKC/CaMKII, which are not found in GluN2A. Palmytoilation sites are different among NMDAR subunits and are more abundant in GluN2B C-termini (Sanz-Clemente et al., 2013).

NMDAR trafficking is also regulated by different posttranslational modifications of GluN2A and GluN2B subunits, including palmitoylation, nitrosylation and phosphorylation (Koles et al., 2016). CaMKII is thought to interact more strongly with GluN2B than with the GluN2A CTD. As mentioned above, CaMKII has an important role in LTP and therefore it is thought that the former interaction has major implications in

synaptic plasticity, as well as in synapse maturation and in the regulation of AMPAR content during brain development (Paoletti et al., 2013).

Summing up, GluN2A and GluN2B subunit composition determines the trafficking of NMDAR. GluN2B has higher surface mobility, endocytosis ratio and diffusion coefficient possibly because its PDZ binding domain is phosphorylated by CK2 (casein kinase 2) which disrupts the association with scaffolding proteins. Furthermore, GluN2B is sorted to recycling endosomes while GluN2A is directed to late endosomes, indicating that the former subunit gets recycled while the latter is preferentially degraded (Paoletti et al., 2013).

1.2. SYNAPTIC PLASTICITY

Synaptic plasticity is a concept that translates the brain ability to modify synapses favoring some neuronal pathways and weakening others. These alterations lead to changes in behavior (Hebb, 1949), memory and learning (Purves, 2008). Synaptic plasticity can be regulated at the pre- and/or postsynaptic side, changing the efficacy of neurotransmitter release or the properties of neurotransmitter receptors, respectively (Kessels & Malinow, 2009). In particular, in the mature hippocampus and cerebral cortex, synaptic plasticity is related to learning, adaptive behavior and memory creation (Lohmann & Kessels, 2014).

There are several factors related with the ability of synapses to change:

(i) the synaptic structure - the presence of dendritic spines allows synapse compartmentalization, with the consequent formation of semi-autonomous domains, which provide a microenviroment adequate for segregating postsynaptic chemical responses;

(ii) the malleability of synaptic transmission determined by the local molecular machinery, which is different among spines;

(iii) equally important are the neurotransmitter receptors in terms of their abundance at the membrane and function. They can determine if a pathway is potentiated or depressed and being ion channels ionotropic glutamate receptors are responsible for the alterations in membrane potential;

(iv) intracellular signaling cascades are extremely important due to the fact that they are the last effective response related with the receptor channel activity; these effects are mediated by kinases like PKA, protein kinase C (PKC) and CaMKII (Ca^{2+} -calmodulin dependent kinase), and by phosphatases such as PP1 (protein phosphatase 1), PP2A and PP2B (protein phosphatases 2A and 2B, respectively). Phosphorylation of NMDAR and AMPAR by protein kinases alters their interaction with binding partners (Lohmann & Kessels, 2014). In particular, phosphorylation of GluA4 and GluA1 by PKC promotes their surface expression (Gomes, Correia, Esteban, Duarte, & Carvalho, 2007; Lin et al., 2009) and LTP (Boehm et al., 2006), together with activation of CaMKII by calcium influx (Lisman, Yasuda, & Raghavachari, 2012). In contrast, the phosphorylation of GluA2 induces its internalization (Henley & Wilkinson, 2013). Furthermore, phosphorylation of GluA1-containing AMPARs by PKA increases their surface expression levels and lowers the LTP threshold (Lohmann & Kessels, 2014). Thus, there are several important factors involved in changing the strength of glutamatergic synapses (Lohmann & Kessels, 2014).

Various forms of synaptic plasticity have been described: short-term plasticity, which is considered a form of rapid, activity-dependent modulation of synaptic efficacy, and long-term plasticity, a slow, activity-dependent modulation of synaptic strength (Citri & Malenka, 2008). The two forms of long-term plasticity, named long-term potentiation and depression (LTP and LTD), are related to an increase or decrease of the synaptic response, respectively, and can be induced by distinct patterns of neuronal activity. LTP is characterized by an increase in the number of AMPAR in the postsynaptic membrane with consequent enlargement of the postsynaptic spine. On the other hand, LTD forms resulting from a small but prolonged influx of calcium is associated with a decrease in the number of AMPAR in the postsynaptic membrane followed by shrinkage of spines (Holtmaat et al., 2005). In contrast, short-term plasticity can be triggered by small bursts of activity resulting from stimuli such as sensory inputs, which are coupled to the accumulation of calcium in the presynaptic region and to the induction of brief adaptations (Citri & Malenka, 2008).

1.3. PROPAGATION OF THE GLUTAMATE SIGNAL THROUGH THE ACTION POTENTIAL

The resting membrane resting potential is around -60 mV to -85 mV (Bean, 2007); under these conditions the neuronal plasma membrane shows a low permeability to Na^+. A stimulus that allows the influx of Na^+ ions depolarizes the plasma membrane, and an action potential can be generated if the threshold is reached. This stimulus may come from a synaptic input or may be part of sensory transduction mechanisms; alternatively, a spontaneous action potential may be generated by some cells through their endogenous pacemaker activity. Independently of the mechanism that initiates the action potential, the action potential will travel down the axon (Purves, 2008). For instance, the release of glutamate into the synaptic cleft activates AMPAR channels allowing influx of Na^+ which would cause a local depolarization of the membrane.

During the rising and overshoot phase of the action potential (see figure 1.8) the plasma membrane shows an increased Na^+ permeability due to the opening of inward Na^+ selective channels, which further changes the membrane potential to more positive values. At the same time, K^+ outward channels open, although at a slower rate, which allows a greater influx of Na^+ when compared to the efflux of K^+ (Purves, 2008). When the maximum depolarization is reached, voltage-gated Na^+ channels become inactivated with a consequent decrease in the influx of the cation. This event initiates the repolarizing phase of the action potential (see figure 1.8), a period when the outward K^+ currents are still observed. The K^+ channels that mediate those currents remain open after the membrane reaches its resting potential originating the hyperpolarization phase of the action potential (see figure 1.8). During this period, the membrane potential is slightly more negative when compared with the resting conditions, and forces the Na^+ and K^+ channels to close. Subsequently the plasma membrane recovers its resting potential (Purves, 2008).

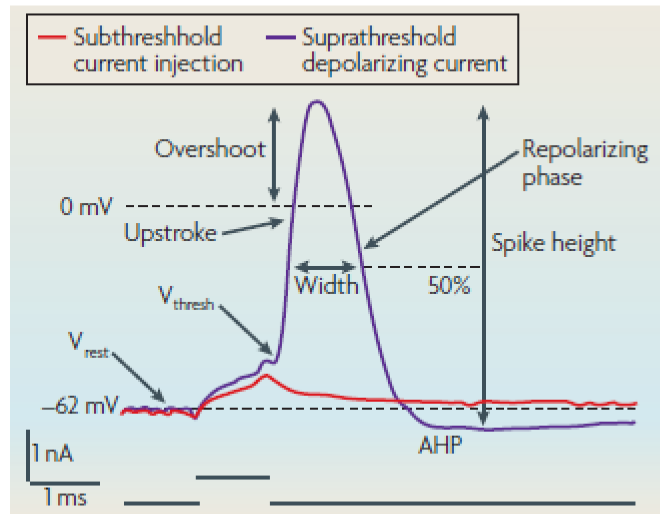

Figure 1.8 – Alterations in the membrane potential during an action potential. Under resting conditions, the membrane potential is around -60 mV to -85 mV. Depolarization of the membrane may result from different types of stimulation, and an action potential is initiated once a certain threshold voltage is reached. The overshoot phase is triggered when the membrane potential is depolarized down to 0 mV: the alteration of the membrane potential is fast and thus the depolarization spike is also tight and fast. Subsequently, the repolarization phase is initiated, often reaching a more negative voltage when compared with the resting conditions (hyperpolarization) (AHP) (Bean, 2007).

Another feature supporting the self-propagation of the action potential is the voltage dependence of the ionic conductance, i.e. the inverse of the resistance to ion movement across the membrane, which also explains the "all or none" behavior. The increase in Na^+ influx is irrevocably related to an increase in its conductance. The maximum depolarization is reached when the Na^+ equilibrium potential (E_{Na}) is also achieved, contributing to the initiation of the repolarization phase due to a decrease in the Na^+ driving force. Likewise, the K^+ conductance is voltage dependent and contributes to the self-propagation of the action potential, which is an intrinsic property of the neuron (Purves, 2008).

Voltage-dependent Ca^{2+} channels are also activated near the action potential peak and Ca^{2+} inward currents reach their maximum during the repolarization phase, when channels are opened thereby enhancing the Ca^{2+} driving force. Moreover, voltage-dependent calcium channels play a role in determining the shape and firing pattern of the action potential which differs between distinct types of neurons (Bean, 2007).

1.4 EPILEPSY AND *STATUS EPILEPTICUS*

Epilepsy is a prevalent neurological disorder, affecting more than 50 million people worldwide according to the estimations of the WHO (World Health Organization). Although this is the 4th most common neurological disease according with the Epilepsy

Foundation about 30% of the patients do not respond to the available therapies, making urgent the identification of new therapeutic targets.

Epilepsy is characterized by spontaneous recurrent epileptiform discharges, commonly known as seizures. The process by which a normal brain is functionally altered and becomes prone to generate abnormal electrical activity culminating in chronic seizures is referred as epileptogenesis (Goldberg & Coulter, 2013), and may result from brain injury or genetic abnormalities, among other causes, which may be coupled to changes in one or more large-scale molecular signaling pathways. In recent years, master regulators of epileptogenesis have emerged including the brain-derived neurotrophic factor (BDNF)-tropomyosin-related kinase B (TRKB; also known as NTRK2) signalling (Binder, 2007; McNamara, Huang, & Leonard, 2006), the mTOR pathways (Crino, 2011; Laplante & Sabatini, 2012; Wong, 2010), the REST pathway (Brooks-Kayal, Raol, & Russek, 2009) in addition to other transcriptional regulators (Laplante & Sabatini, 2012).

The process of epileptogenesis is complex and multifactorial. It is difficult to determine whether the changes in gene regulation characteristic of the epileptic condition are consequential to or causative of the disease aetiology. However, it is well accepted that an initial precipitating event such as *status epilepticus* (SE) can increase the risk of later development of epilepsy. *Status epilepticus* was defined in the late 90s, in adults and older children (>5 years old), as 5 min of continuous seizures or two or more seizures between which there is incomplete recovery of consciousness (Lowenstein, Bleck, & Macdonald, 1999). More recently, in 2015, it was defined as a seizure lasting longer than 5 min, resulting from the failure of mechanisms to terminate the attack or from the initiation of mechanisms leading to prolonged seizures. After 30 min of ongoing seizure activity there can be long-term consequences for the individual as neuronal death, neuronal injury and alteration of neuronal networks (Trinka et al., 2015).

It is a condition in which there is failure of the endogenous mechanisms that serve to terminate a typical generalized tonic-clonic seizure (Lowenstein et al., 1999). Under normal conditions, a single seizure terminates by inhibitory feedback and synaptic plasticity events which change the brain and make it unsubmissive to more seizures. However, once the SE is established it is very hard to stop (A. M. Mazarati & Wasterlain, 1999). Even though the SE concept is usually related to epilepsy it also applies to seizures with other causes (Trinka et al., 2015) and, often, SE leads to epilepsy later in life (French et al., 1993).

The mechanisms underlying SE are still under investigation, but it is thought to be caused by failure of inhibitory mechanisms and/or enhancement of excitatory activity (A. M. Mazarati & Wasterlain, 1999; Scharfman, 2007; Trinka et al., 2015). During SE there is increased firing of action potentials, starting with high-frequency bursts of action potentials (APs) in several neurons (Bromfield, Cavazos, & Sirven, 2006). Until recently it was thought that this could result in an hypersynchronous network. However, recent studies brought to discussion the possibility of desynchronous neuronal activity (Cash, 2013). In any case, if the high number of APs raises the membrane potential to a given threshold there is a sustained depolarization which leads to a burst of APs (Bromfield et al., 2006) [series of rapid action potentials with very short interspike intervals (less than ~5ms) (Bean, 2007)] that cause a seizure. This is considered epileptiform activity and is reflected as a spike discharge in an EEG (Bromfield et al., 2006). Throughout the burst there is activation of voltage-gated Na^+ channels, which contributes to the generation of more APs. The long-lasting depolarization of the membrane also causes a prolonged influx of Ca^{2+} which is coupled to the regulation of signaling pathways that lead to the insertion of more AMPA and NMDA receptors in the plasma membrane. At the presynaptic level, the burst activity also leads to the accumulation of Ca^{2+} in the nerve terminal with a consequent enhancement of glutamate release and activation of glutamate receptors. The continuous efflux of K^+ also makes more difficult the hyperpolarization of the plasma membrane (Bromfield et al., 2006).
Epileptogenesis is also associated with an impairment of GABAergic neurotransmission. While under normal physiological conditions the activation of GABAergic synapses causes an hyperpolarization of neurons mediated by $GABA_A$ receptors, due to the influx of Cl^-, such mechanism does not operate in SE, further contributing to the propagation of seizures (Bromfield et al., 2006). This is due to a disruption in Cl^- homeostasis, at least in part resulting from an impairment of the activity of the KCC2 (potassium-chloride transporter) chloride transporters. Under normal conditions the KCC2 transporters mediate Cl^- extrusion from neurons but the consecutive depolarization of the plasma membrane can overwhelm these transporters and disrupt this mechanism of Cl^- regulation (Raimondo, Burman, Katz, & Akerman, 2015). In addition, kindling induction was found to downregulate the expression of KCC2 transporters in the hippocampus (Rivera et al., 2002; Woodin, Ganguly, & Poo, 2003), and similar evidence was obtained upon increased neuronal activity. Overactivation of BDNF-TrkB receptor signaling through the PLCγ pathway was found to account for the observed downregulation of the KCC2 transporter under the latter conditions (Rivera et al., 2004) (see section 1.5.1). The

impairment in Cl^- transport mediated by KCC2 disrupts the transmembrane Cl^- gradient, increasing its intracellular concentration. In this scenario, activation of $GABA_AR$ channels leads to the efflux of Cl^-, rather than influx, further contributing to membrane depolarization (Raimondo et al., 2015).

1.4.1. Models of *Status Epilepticus*

Several different pathways have been implicated in SE, but the results obtained in laboratory are often dependent of the model used: method of induction, brain region analysed and time-point of analysis. Valid experimental models for the study of human temporal lobe epilepsy (TLE) are considered animals in which a *status epilepticus* (SE) is induced via injection of drugs (pilocarpine or kainate) or electrical brain stimulation (Ben-Ari, 1985; Lothman, Bertram, Kapur, & Stringer, 1990)(Cavalheiro et al., 1991). These models display characteristic neuropathological aspects: hippocampal sclerosis (neuron loss and gliosis), synaptic reorganization, such as mossy fibre sprouting, and the recurrence of seizures. Moreover, these models are characterized by the development and recurrence of spontaneous seizures (Bertram & Cornett, 1994; Bouilleret et al., 1999; Cavalheiro et al., 1991; Hellier, Patrylo, Buckmaster, & Dudek, 1998; Nissinen, Halonen, Koivisto, & Pitkanen, 2000; Stafstrom, Thompson, & Holmes, 1992).

Animal models of epilepsy have been very useful to investigate the underlying mechanisms of epileptogenesis and to test novel therapeutic strategies. *In vivo* models, in which animals exhibit electroencephalographic seizures, mimic the clinical symptoms of human epilepsy in a more reliable manner. However, simpler biological systems, such as brain slices and neuronal cultures may be advantageous to study the molecular mechanisms of epilepsy. However, none of the models is perfect to study the disease and ultimately each model system must be carefully evaluated for its specific advantages and limitations when studying the different aspects of epilepsy (Wong, 2011).

1.4.1.1. *In vitro* models

Primary cultures of dissociated neurons and/or organotypic hippocampal slice cultures are also good *in vitro* models to study the response to epileptogenic stimulation. This type of model systems together with electrophysiology techniques allow to investigate the mechanism of action of anti-epileptic drugs or molecules at the receptor and channel levels (Reddy & Kuruba, 2013). The three most common stimuli used to induce *in vitro* SE are: incubation of cultured neurons or slices in Mg^{2+}-free solution, high K^+ solution and the K^+ current blocker 4-AP (4-Aminopyridine) (Reddy & Kuruba, 2013).

The incubation of dissociated neuronal cultures/slices in a Mg^{2+}-free salt solution allows the activation of NMDA receptors due to the removal of the Mg^{2+} blockade of the receptor channels (Fueta et al., 2005; Reddy and Kuruba, 2013) (see figure 1.9). In this model, after prolonged periods of stimulation the epileptiform activity leads to the development of resistance to benzodiazepine (Reddy & Kuruba, 2013).

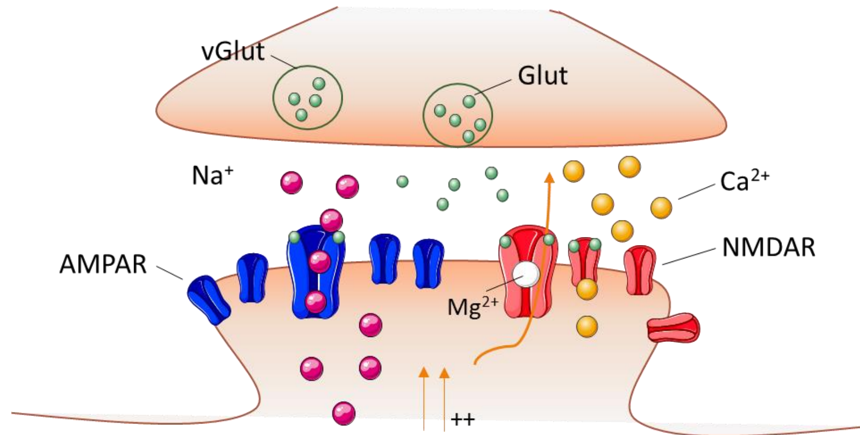

Figure 1.9 - Schematic representation of the synaptic alterations resulting from the incubation of neurons in a Mg^{2+}-free solution, a model to study epileptogenesis. Glutamate is released from the pre-synaptic neuron and binds to AMPA and NMDA receptors. AMPAR become activated allowing the influx of Na^+ which depolarizes the membrane. If the depolarization is high enough the Mg^{2+} blockade of NMDAR is relieved which allows entry of Ca^{2+}. The lack of Mg^{2+} in the medium increases the activity of NMDAR and enhances the rate of firing of action potentials (Reddy & Kuruba, 2013).

In the high potassium *in vitro* model of epilepsy, the increased extracellular K^+ concentration shifts the equilibrium potential for the cation to a more positive value. Therefore, under these conditions the neuronal resting membrane potential increases towards the threshold for firing of action potentials. As a result, neurons become hyperexcitable and spontaneous epileptiform bursts can be induced (Reddy & Kuruba, 2013). Similarly, the K^+ channel blocker 4-aminopyiridine (4AP) induces spontaneous discharges in neurons due to the transient increase in the extracellular $[K^+]$ (Reddy & Kuruba, 2013; Rutecki, Lebeda, & Johnston, 1987).

1.4.1.2. *In vivo* models

Chemoconvulsants: Kainic Acid and Pilocarpine

Kainic Acid (KA) and Pilocarpine induce spontaneous seizures in animals, approximately 2 weeks after the initial *status epilepticus* (Turski, Ikonomidou, Turski, Bortolotto, &

Cavalheiro, 1989). For this reason, both treatments are considered valid TLE models. In fact, they reproduce the typical histopathological alterations and spontaneous chronic seizures seen in epileptic patients (Levesque & Avoli, 2013).

Kainic acid is an analog of L-glutamate and an agonist of the ionotropic kainic acid receptors. Stimulation of ionotropic glutamate receptors with kainate induces recurrent depolarizations of the neurons causing seizures that target preferentially the hippocampus (Kandratavicius et al., 2014). Injection of kainate in mice induces massive neuronal loss in ipsilateral CA1, CA3 and dentate hilus, in addition to the death of dentate granule cells (Twele, Bankstahl, Klein, Romermann, & Loscher, 2015).

Pilocarpine is a muscarinic acetylcholine receptor agonist. Although the drug induces cholinergic hyperactivation, the observed continuous seizure activity is thought to carry on through glutamatergic mechanisms (Reddy & Kuruba, 2013). Unlike kainate this drug can cause lesions, as sclerosis, in neocortical areas as well, as observed in TLE in humans. Overall, pilocarpine-induced TLE mimics better the alterations at the network and neurochemical levels in humans, such as upregulation of neurotrophins, cognitive and memory deficits and generation of interictal activity (Kandratavicius et al., 2014).

Electrical Stimulation: Electroshock-induced seizures and Kindling

Electrical stimulation reproduces epileptogenic features with low mortality and high reproducibility, and allows studying the postictal alterations in the brain, e.g. intracellular cascades. However, this method does not allow cell-type specificity (Kandratavicius et al., 2014).

EIS (electroshock-induced seizures) is one of the most studied models along with Kindling. This method can affect the whole-brain and is usually applied through corneal electrodes or auricular stimulation, not requiring surgery. Depending on the applied intensity it affects different structures at distinct intensities (Kandratavicius et al., 2014).

Kindling induces epilepsy by repeated after discharges (focal electrical stimulation) induced through stimulation of implanted electrodes, building up seizure susceptibility which culminates in spontaneous seizures and established epilepsy (Kandratavicius et al., 2014). This model is widely used to study molecular mechanisms involved in epileptogenesis since it slowly builds up the epileptic state unlike the models using

chemoconvulsants which start with SE, a refractory prolonged seizure with great neuronal loss (Kandratavicius et al., 2014).

1.4.5. Mechanisms underlying *status epilepticus*

As discussed previously, the pathophysiological cascade behind seizures is thought to involve the activity of neuronal voltage- and ligand-gated ion channels (McCormick & Contreras, 2001). Antiepileptic drugs (AEDs) suppress the occurrence of seizures by acting on these ion channels to reduce hypersynchronization, inhibiting the spreading of abnormal neuronal firing (Rogawski & Loscher, 2004a). The three major mechanisms of AED action are: (i) modulation of voltage-gated ion channels (including sodium, calcium and potassium channels), (ii) enhancement of synaptic inhibitory neurotransmission, and (iii) attenuation of brain excitation (Kwan, Sills, & Brodie, 2001; Mulley, Scheffer, Petrou, & Berkovic, 2003; Rogawski & Loscher, 2004a). In this work, we focused on the alteration of excitatory neurotransmission in epileptic condition.

An enhanced excitatory receptor sensitivity, involving NMDA receptors, has been identified in kindled rodents and in focal epilepsy in humans. In patients with refractory convulsive *status epilepticus* it was found, through tissue collection, an increase in GluN2B (2543% $\pm$ 1363) receptors and in the ratio GluN2A/ GluN2B (3520% $\pm$ 751), while no substantial effects were detected on GluA1 or GluA2 (Loddenkemper et al., 2014). Complementary studies were also performed in samples from patients with refractory electrical SE during sleep and refractory epilepsy. In the first group there was only an increase in GluA1/GluA2 ratio while in the second there was an upregulation of GluA1 and GluN2A/GluN2B ratio (Loddenkemper et al., 2014). Experiments performed in pilocarpine model of epilepsy using rats showed a slower decay of NMDA EPSCs (Excitatory Post-Synaptic Currents) in response to stimulation of lateral and medial perforant pathways (Scimemi, Schorge, Kullmann, & Walker, 2006). These results point to an upregulation of NMDAR subunits and an increased probability of NMDAR opening due to a change in the phosphorylation state of the NMDA receptor (Kohr, De Koninck, & Mody, 1993; Lieberman & Mody, 1999). The upregulation of NMDAR may lead to a greater influx of calcium which can account for the *status epilepticus*. In contrast, a different study reported a decrease of GluN2A and GluN2B expression in animal models of seizures (flurothyl model) and SE [TNTX (tetanus toxin) model] (Swann, Le, & Lee, 2007), which is consistent with the 18%-30% decrease in CaMKII activity during SE, with

no alteration of the protein levels, both in the cerebral cortex and hippocampus (Singleton, Holbert, Lee, Bracey, & Churn, 2005).

NMDAR antagonists like MK-801 (0.5mg/kg) decrease significantly the time spent seizing, and the occurrence of the last seizures, in rats with SE induced by 60 min of Perforant Path Stimulation (PPS) (A. M. Mazarati & Wasterlain, 1999). Treatment with ketamine (10 mg/kg), a different antagonist of NMDAR, also reduced by 88% the seizures in studies where SE was induced by kainate injection into the CA1 area of the hippocampus (Twele et al., 2015). However, adverse side effects were reported in this case. DDBM [2-(1,1-Dimethyl-1,3-dihydro-benzo[e]indol-2-ylidene)-malonaldehyde], a new NMDAR antagonist, was also tested in studies where it was administered to rats 6 hours before the induction of *status epilepticus* through electroconvulsive shocks (ECS). Treated rats presented a decreased duration of seizures, reduced neurodegeneration and almost the same number of neurons as control rats, showing a neuroprotective effect of the NMDAR antagonist in CA1, CA3 and DG (dentate gyrus) (Rothan et al., 2017).

Seizure activity also involves the participation of AMPA receptors, as shown in studies with specific inhibitors of this class of glutamate receptors. Thus, the AMPAR antagonist NBQX (30 mg/kg) suppressed the spontaneous recurrent seizures after SE in an adult mouse model of mesial temporal lobe epilepsy induce by kainic acid (Fornai, Busceti, Kondratyev, & Gale, 2005; Twele et al., 2015), although it was without effect on the epileptogenic activity (Twele et al., 2015). Furthermore, inhibition of AMPAR with CNQX (10 nmol in 0.5 µl), administered 10 min after 30 min PPS, strongly decreased seizing but it lost effectiveness at 4 h - 5 h after administration (A. M. Mazarati & Wasterlain, 1999). Globally, NBQX can exert a partial anti-seizure effect but mice maintain convulsive seizures indicating that inhibition of AMPAR does not have an antiepileptogenic effect (Twele et al., 2015). Perampanel (2 mg/kg), a non-competitive inhibitor of AMPAR, reduced by 96 % the seizures induced by kainate injection into the right CA1 area of the dorsal hippocampus. However, the compound exerted adverse behavioral effects in both control and epileptic mice (Twele et al., 2015). Although several stage III clinical studies suggest otherwise, perampanel was used as adjunctive treatment to approved antiepileptic drugs with accepted levels of efficacy, safety and tolerability (Loscher & Schmidt, 2012; Zaccara, Giovannelli, Cincotta, & Iudice, 2013).

Overall, inhibition of glutamate receptors is an effective, although transient, strategy to inhibit seizure activity. However, glutamate receptor antagonists do not prevent epileptogenesis and several side effects were observed, possibly due to the widespread

distribution of these receptors throughout the brain and their important physiological roles. This makes difficult the design of effective drugs targeting glutamate receptors that would succeed in pre-clinical trials for antiepileptic agents (Rogawski & Loscher, 2004b). Some of the variability in the results available in the literature may depend of the diversity of models used to study epilepsy (Rogawski, 2011; Zaccara et al., 2013).

1.4.6. Spontaneous seizures after *Status Epilepticus*

As previously mentioned, SE is a long seizure that may arise from different possible causes and after a latent period (pre-epileptic state in which non-convulsive seizures can occur) leads to spontaneous recurrent seizures (SRS) characteristic of chronic epilepsy, (Williams, Hellier, White, Staley, & Dudek, 2007). SE can be induced in animal models, usually by kainate or pilocarpine injection which trigger the development of SRS (Williams et al., 2007); in these models, a latent period of over a week is observed for most rats (Hellier et al., 1999).

Studies performed in rats showed that only 71% of the animals showed SRS upon induction of SE with pilocarpine. These studies showed no correlation between the induction of seizures and the density of mossy fiber sprouting (Furtado, Castro, Del Vecchio, de Oliveira, & Garcia-Cairasco, 2011), in contrast with the hypothesis proposing that mossy fiber sprouting is a consequence of epilepsy (A. Mazarati et al., 2002). Also, the frequency of seizures increases with time, after the latent period (Williams et al., 2009), and seizure clusters emerge (Goffin, Nissinen, Van Laere, & Pitkanen, 2007; Williams et al., 2009). Spontaneous seizures cause continuous increase in neuronal damage and death in the models of SE induced by electrical stimulation or kainic acid (Drexel, Preidt, & Sperk, 2012; Pitkanen et al., 2002), as well as in *in vitro* models of SE (Deshpande et al., 2007). On the other hand, mild neurogenesis was also observed after SRS (Cha, Akman, Silveira, Liu, & Holmes, 2004).

The electrophysiological properties of dentate gyrus granule cells are also changed before SRS induced by pilocarpine in rats. These cells receive excitatory input from mossy fibers, and they were shown to exhibit four patterns of activity: 34% increase firing before seizure onset, 32% decreased firing before seizure onset, 16% increase firing only after seizure onset and 19% show no alterations. The decrease in firing activity is probably due to stimulation of inhibitory currents (Bower & Buckmaster, 2008). Accordingly, a different study performed in humans recorded alterations (increase and decrease) of the firing rate before seizures. Furthermore, the distribution of spikes and seizures was similar i.e. when there were seizures there was also an increased firing rate (Karoly et al., 2016).

Moreover, interneurons displayed synchronous behavior just after seizure onset including increased firing rate and synchronicity with field potential variations, in contrast with the results obtained in pyramidal cells (Grasse, Karunakaran, & Moxon, 2013).

Studies performed in hippocampal neuronal cultures showed a reduction in the activity of CaMKII after induction of SE, which may affect the $[Ca^{2+}]i$ homeostasis thereby contributing to SRS (Blair, Churn, Sombati, Lou, & DeLorenzo, 1999; Carter et al., 2006). Unpublished results from our laboratory showed that in cultured hippocampal neurons SE induces synchronous calcium transients (see figure 1.10). The oscillations in the $[Ca^{2+}]_i$ observed with Fluo-4 is possibly concomitant with burst activity.

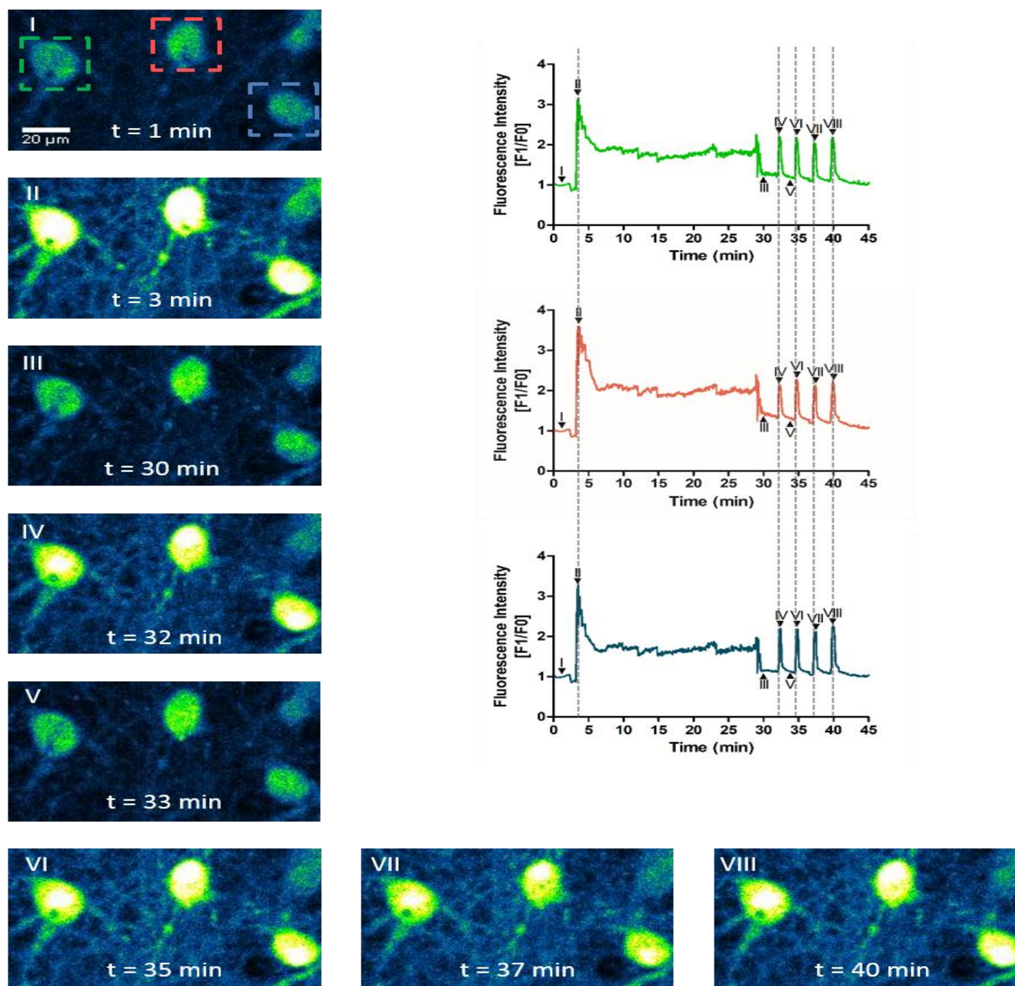

Figure 1.10 - SE induces the development of synchronous and spontaneous calcium spiking. Single Cell Calcium Imaging using the fluorescent Ca^{2+} indicator Fluo-4, coupled to Spinning Disk microscopy, was used to analyse the alterations in the $[Ca^{2+}]_i$ in cultured hippocampal neurons. I - The cells were incubated with Sham medium (Na^+-salt solution containing Mg^{2+}) for 3 min to determine the baseline levels of calcium. II - After 3 min, the Sham medium was replaced with $[Mg^{2+}]_0$ medium (SE) for 30 min. III/VIII - the SE medium was replaced by Sham medium for 15 min. Fluo-4 fluorescence was recorded for the duration of the incubation. The results are represented as the normalized intensity of Fluo-4 fluorescence (Fluorescence for

a given time point / the baseline fluorescence). Calcium dynamics is represented in a time course visual representation of three different neurons. These neurons are indicated by a coloured square and the corresponding alterations in Fluo-4 fluorescence are shown in the traces (right). The dashed lines portray the synchronous neuronal calcium fluctuation pattern (Vieira, 2015).

1.5. BDNF AND EPILEPSY

1.5.1. BDNF and TrkB Receptors

Brain-derived neurotrophic factor (BDNF), is a member of neurotrophin family which participates in many aspects of neuronal physiology, including in the survival, differentiation and growth of several types of neurons (Leal, Afonso, Salazar, & Duarte, 2015). Two different classes of receptors are responsible for mediating BDNF signaling: $p75^{NTR}$ and TrkB (Shimada, Otterness, & Stitt, 1994). The immature form of BDNF (ProBDNF) binds to the $p75^{NTR}$ receptor while the mature form of BDNF has a high affinity to TrkB (Bollen et al., 2013). BDNF binding to TrkB plays important roles in neuronal survival, neuronal plasticity, and neurogenesis (Leal et al., 2015; Shimada et al., 1994). In contrast, the $p75^{NTR}$ receptor is more associated with apoptosis (Wurzelmann, Romeika, & Sun, 2017). The mature form of BDNF can also bind to $p75^{NTR}$ receptor when there are high concentrations of BDNF (Boyd & Gordon, 2001). Both BDNF receptors can be found in the same cell, coordinating and modulating neuronal responses.

Upon binding to the TrkB receptor, BDNF induces dimerization and transphosphorylation of receptors and initiates several parallel intracellular signaling cascades (Levine, Dreyfus, Black, & Plummer, 1996). These signaling cascades include the Ras/ERK (extracellular signal–regulated kinases), PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) and PLCγ (phospholipase Cγ). The PI3K pathway activates protein kinase B (Akt), promoting cell survival by inhibiting Bad and by inducing the expression of anti-apoptotic proteins, such as BcL2 (Yoshii & Constantine-Paton, 2010). Phosphorylation of Akt also results in the suppression of pro-apoptotic proteins, pro-caspase-9 and Forkhead (Kaplan & Miller, 2007). The PLCγ pathway leads to the release of calcium from intracellular stores by activating the inositol 1,4,5-trisphosphate (IP3) receptor, and contribute to increase the calmodulin kinase (CaMK) activity. This pathway also plays an important role in synaptic plasticity through regulation of the transcription factor CREB (cyclicAMP-response element binding protein) (Leal, Comprido, & Duarte, 2014). The effects of BDNF on synaptic potentiation in the hippocampus are mediated by activation of pre- and post-synaptic receptors (Gartner et al., 2006). Postsynaptically, BDNF has been shown to change gene expression (e.g. IEGs [immediate early genes]) and mRNA translation,

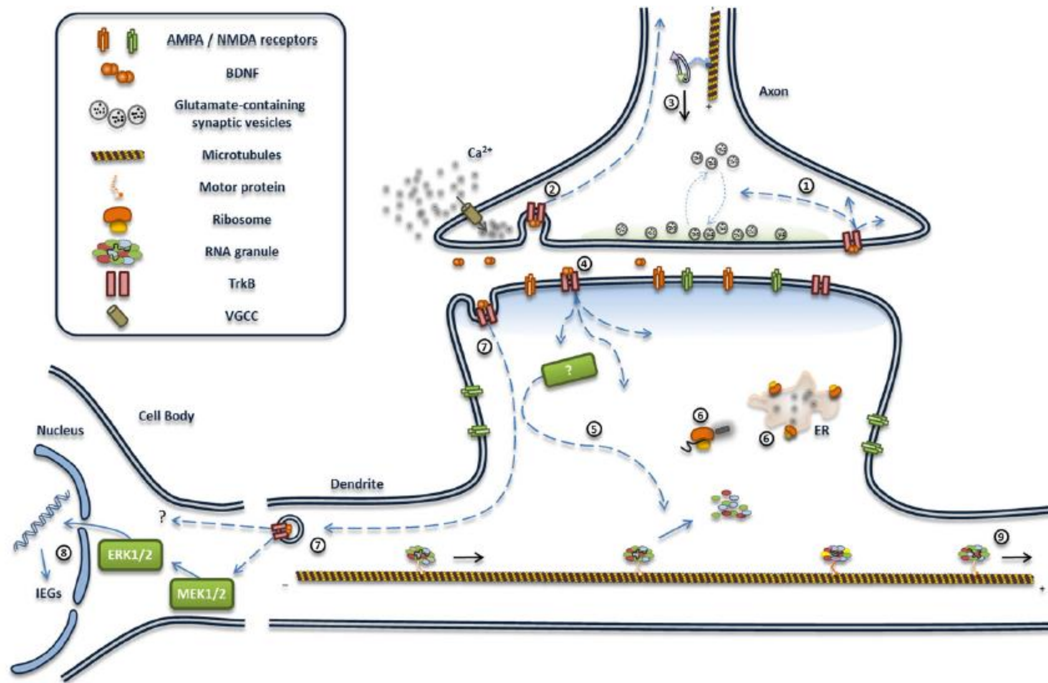

Figure 1.11 - BDNF increases the presynaptic accumulation of synaptic vesicles containing glutamate, thereby potentiating synaptic transmission. In the postsynaptic neuron BNDF can induce three pathways activating translation (process explained in figure 1.12). The complex BDNF-TrkB receptors can be internalized and travel towards the soma where it activates ERK1/2 and MEK1/2, inducing the transcription of IEGs. mRNAs can be transported along dendrites in a repressed state within the RNA granules. These granules may be unrepressed by BDNF-induce pathways at the synapse, making the transcripts available for local translation (Leal et al., 2014).

including local translation at the synapse (Figure 1.11) (Leal et al., 2014). Furthermore, the neurotrophin may enhance synaptic transmission through activation of signaling pathways coupled to the release of calcium from internal stores and by inducing the delivery of NDMAR and AMPAR to the membrane (see figure 1.12) (Leal et al., 2014).

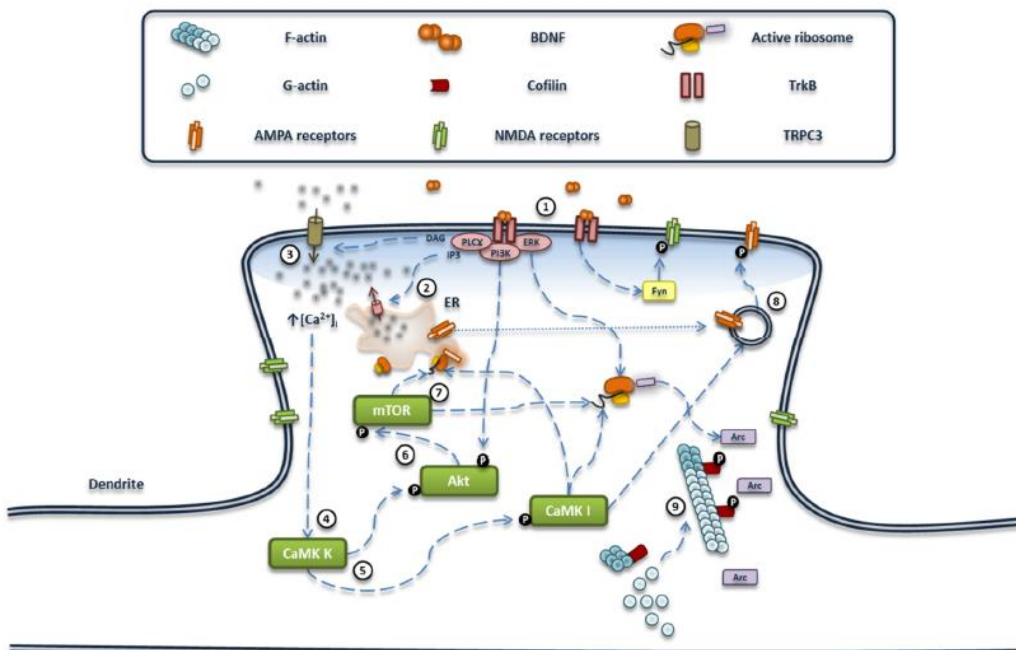

Figure 1.12 - Stimulation of TrkB receptors by BDNF activates the PLCγ pathway with the consequent formation of DAG or IP_3. DAG activates protein kinase C and induces the opening of plasma membrane-associated Ca^{2+} channels (TRPC channels), while IP_3 induces the release of calcium from the endoplasmic reticulum. The resulting increase of the intracellular calcium levels leads to the activation of Ca^{2+}- and calmodulin-dependent protein kinase kinase (CaMKK) which activates Akt and Ca^{2+}- and calmodulin-dependent protein kinase I (CaMK I). The latter enzyme is responsible for the delivery of AMPAR to the plasma membrane. BDNF also activates the PI3K pathway with a consequent stimulation of Akt. This kinase activates mTOR, thereby enhancing translation. An additional mediator of the effects of BDNF is the ERK pathway which is also coupled to the activation of translation. Moreover, the BDNF-TrkB complex evokes the delivery of NMDAR to the plasma membrane through Fyn (Leal et al., 2014).

Studies performed with the pilocarpine model of temporal lobe epilepsy showed an upregulation in BDNF protein levels in the dendritic compartment of CA1 pyramidal neurons (Tongiorgi et al., 2004) and activation of TrkB receptors by BDNF was also shown to play an important role in epileptogenesis (Gu et al., 2015).

1.5.2. BDNF, TrkB receptors and *Status Epilepticus*

Several studies suggest that BDNF plays an important role in the control of neuronal excitability in the adult brain (Leal et al., 2015; Thoenen, 1995). The neurotrophin may therefore play an important role in the control of neuronal excitability in the epileptic brain since the mRNA and protein levels of BDNF were found to be upregulated in several animal models of epilepsy, including kindling (Elmer et al., 1998; Ernfors, Bengzon, Kokaia, Persson, & Lindvall, 1991), kainic acid (Rudge et al., 1998) and pilocarpine (Mudo, Jiang, Timmusk, Bindoni, & Belluardo, 1996; Poulsen, Lauterborn, Zimmer, & Gall, 2004; Roberts, Hu, Lund, Brooks-Kayal, & Russek, 2006). A decrease in the methylation of the BDNF promoter accounts, at least in part, for the observed increase in the mRNA and protein levels of BDNF (Ryley Parrish et al., 2013). The upregulation in BDNF protein levels is associated with an increase in TrkB signaling activity as shown by the increase in receptor phosphorylation, particularly in excitatory synapses in the dentate gyrus as well as in synapses made by CA1 pyramidal neurons, following injection of kainic acid into the amygdala, an *in vivo* model of TLE (temporal lobe epilepsy; the same as limbic epilepsy) (Helgager, Liu, & McNamara, 2013).

The evidence showing that BDNF up-regulation is associated with seizure activity suggests that BDNF may play a role in epileptogenesis. In fact, $BDNF^{+/-}$ mice display a marked reduction in the rate of kindling development. Moreover, intraventricular

infusion of TrkB-Fc inhibits the development of kindling, whereas infusion of TrkA-Fc or TrkC-Fc does not (Binder, Routbort, Ryan, Yancopoulos, & McNamara, 1999), suggesting that signaling through the TrkB receptor specifically promotes epileptogenesis (Gu et al., 2015; Liu et al., 2013).

The upregulation of BDNF protein levels during SE induction was also found to enhance neuronal damage (Unsain, Montroull, & Masco, 2009). Similarly, induction of SE in transgenic mice overexpressing TrkB (Lahteinen, Pitkanen, Koponen, Saarelainen, & Castren, 2003) or overexpressing the BDNF mRNA (Croll et al., 1999) leads to a more severe SE and neuronal loss. However, in the former study no difference was found in what concerns epileptogenesis, severity of epilepsy, network reorganization or number of immature neurons after 4.5 months of kainate-induced epilepsy. Together, these results indicate that TrkB overexpression does not have a long-term effect after induction of SE (Lahteinen et al., 2003).

In vivo studies using the pilocarpine model of TLE showed that SE downregulates TrkB receptors before CA1 neuronal injury (Unsain, Nunez, Anastasia, & Masco, 2008). A decrease in TrkB protein levels was also observed in the motor cortices (but not the auditory cortex) at 1 week after SE induced by the kainic acid model of TLE. In this study, regions with decreased levels of TrkB were concomitant with dendritic atrophy, which might lead to cognitive impairment but, on the other hand, may decrease seizure propagation (Ampuero et al., 2007).

In accordance with the role played by BDNF-TrkB signaling in the control of neuronal excitability, inhibition of TrkB activation with a drug administered after SE, for two weeks, prevented recurrent seizures evoked by the kainic acid model of TLE (Liu et al., 2013). Furthermore, infusion of function-blocking antibodies against BDNF in the hippocampus prevented TrkB downregulation and neuronal degeneration in the *in vivo* pilocarpine model of TLE. Inhibition of BDNF mRNA translation also prevented neuronal damage (Unsain et al., 2009), while infusion of BDNF after SE increased neuronal degeneration (Unsain et al., 2009). More recently, the many effectors of TrkB activation were also studied as potential antiseizure targets: PLCγ1 was found to be the most relevant in the context of epilepsy: uncoupling this effector from TrkB receptors prevented TLE while preserving the neuroprotective effects of TrkB signaling (Gu et al., 2015).

In contrast with the hypothesis proposing a role for BDNF-TrkB signaling in the control of neuronal excitability after SE, the increased levels of BDNF detected in P17 mice under

these conditions were not correlated with an upregulation of pTrkB, raising the question of whether there are other mechanisms involved in the activation of the neurotrophin receptors (Danzer, He, & McNamara, 2004). Additionally, continuous administration of BDNF in the hippocampus with osmotic minipumps (Scharfman, Goodman, Sollas, & Croll, 2002) or microinjections (Xu, Michalski, Racine, & Fahnestock, 2004) inhibited epileptogenesis, suggesting a decrease in the affinity between BDNF and TrkB receptors upon sustained activation of the receptors. Accordingly, the BDNF-induced inhibition of epileptogenesis was not observed when the neurotrophin was used at lower concentrations and applied intermittently (Xu et al., 2004). Increasing TrkB activation through a change in BDNF levels has yielded conflicting results in what concerns the role of TrkB and BDNF in epileptogenesis. Clearly, this is an area that needs additional investigation given the complexity of the results obtained with different animal models and experimental protocols.

1.6. hnRNP K, BDNF AND EPILEPSY

As discussed in section 1.5.1, activation of BDNF-TrkB signaling at the synapse may release transcripts that are transported along dendrites within RNA granules (mRNPs) (Figure 1.11). These structures contain dendritic mRNAs possessing a cis-acting element, which are recognized by specific RNA-binding proteins making a functional mRNP. Once mRNPs arrive at their final destination, the translational block is relieved in response to synaptic activity, and mRNAs are translated (Leal, 2013). The BDNF-TrkB complexes may also be internalized and travel towards the soma where they activate signaling pathways that ultimately regulate transcription activity (Leal, 2013).

The RNA-binding protein hnRNPK (Heterogenous Nuclear Ribonucleoprotein K) is present in mRNPs and transports dendritic transcripts in a repressed state (Leal et al., 2014). The presence of hnRNP K was detected in synaptoneurosomal fractions (Liao et al., 2007) and at the postsynaptic densities of hippocampal neurons (Proepper et al., 2011). Furthermore, studies performed in cell lines showed that hnRNP K localization and activity are modulated by phosphorylation of the protein (Habelhah et al., 2001). The RNA-binding protein acts as docking site for 114 binding partners (Mikula et al., 2006) hence its functions are varied: mRNA transcription, splicing, regulation of mRNA stability and translation. hnRNP K is capable of interacting both with RNA and ssDNA due to its three K Homology domains and K Interactive domain (see figure 1.13); these regions are

capable of recruiting kinases and factors that regulate the previous mentioned processes (Bomsztyk, Denisenko, & Ostrowski, 2004).

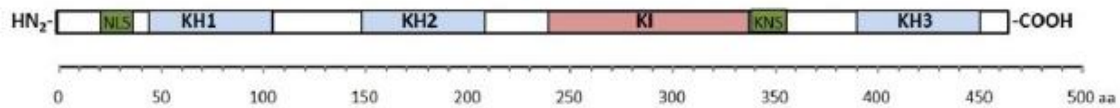

Figure 1.13 - hnRNP K structure. hnRNP K contains one localization signal (NLS), three K Homology domains (KH), one K Interactive domain (KI) and a nuclear shuttling domain (KNS) (Mikula et al., 2010) (Image from Leal, 2013).

hnRNP K binds thousands of transcripts, including mRNAs encoding GluA1, GluN1, CamKIIβ and BDNF (Comprido, 2011). It is involved in dendritic arborization through Abi-1 (Abelson-interacting protein 1) (Proepper et al., 2011), regulates density and molecular composition of dendritic spines, and is required for MEK1/2 and ERK 1/2 cascade activation (Folci et al., 2014). Moreover, knockdown of hnRNP K increases filopodia formation, decreases the number of mature synapses and impairs synaptic plasticity. The effect of synaptic plasticity was attributed to a decrease in GluA1 delivery to the plasma membrane following the inactivation of MEK1/2-ERK1/2 cascades at the post-synaptic membrane (Folci et al., 2014). Activity of the ERK pathway is required for PKA-mediated phosphorylation of GluA1 and AMPAR insertion at synapses (Song et al., 2013). The abundance of PSD-95 and GluA2 is also decreased upon knockdown of hnRNP K (Folci et al., 2014).

BDNF was shown to upregulate dendritic levels of hnRNP K (Leal, 2013) and to decrease the interaction of 51% of hnRNP K-bound transcripts (Comprido, 2011) in hippocampal neurons. Furthermore, the effect of BDNF on the interaction of hnRNP K with several transcripts was also observed in hippocampal synaptoneurosomes, a subcellular fraction containing the pre- and post-synaptic regions (Comprido, 2011). More recently, we showed that hnRNP K is a key mediator of the effects of BDNF on the surface synaptic NMDAR receptors (Afonso, 2016; De Luca, 2016). Given the important role of BDNF as a mediator of the synaptic alterations induced by SE, it is tempting to suggest that hnRNP K contributes to some of the effects of the neurotrophin.

II
OBJECTIVES

Excessive and/or hypersynchronous activity of neuronal networks in the epileptic brain are responsible for starting seizure activity and for their propagation. One of the players involved in regulation of neuronal activity is the neurotrophic factor BDNF, a neurotrophin that has been shown to play an important role in the development of TLE. Accordingly, BDNF and TrkB receptor phosphorylation are upregulated in epileptic conditions (Liu et al., 2013), and BDNF potentiates glutamatergic synaptic transmission (Leal et al., 2015) as well as neural activity in limbic circuits (Helgager et al., 2013). Evidence from our laboratory shows that BDNF upregulates NMDA receptor-mediated mEPSC in cultured hippocampal neurons by a mechanism dependent on the RNA binding protein hnRNP K (Heterogenous Nuclear Ribonucleoprotein K) (Leal, 2013).

In this study, we investigated the nature and the mechanisms underlying seizures using an *in vitro* model of epilepsy resulting from the transient incubation of cultured hippocampal neurons in a Na^+-salt solution lacking Mg^{2+}. Under these conditions hippocampal neurons develop synchronous and spontaneous calcium spiking (Vieira, 2015). Here we hypothesized that BDNF may contribute to the neuronal hyperactivity characteristic of epilepsy through the upregulation of synaptic NMDAR by a mechanism mediated by the RNA binding protein hnRNP K. To further investigate the nature and the mechanisms behind seizures *in vitro* we set the following objectives:

(i) To validate the used model studying the SE-induced rhythmic activity in cultures of hippocampal neurons incubated in a Na^+-salt solution lacking Mg^{2+} by whole-cell current clamp. Specific chemical inhibitors were used to characterize the role of NMDA and AMPA receptors in the development of SE-induced rhythmic activity;

(ii) To investigate the alterations in BDNF signaling induced by *Status Epilepticus*. For this purpose, the levels of TrkB receptor activation were assessed in *in vitro Status Epilepticus*, by western blot analysis using an antibody specific for the phosphorylated form of the receptor. Moreover, TrkB-Fc (BDNF activity inhibitor) was used to antagonize the putative effect of BDNF on SE-induced activity.

(iii) To evaluate the role of RNA binding protein hnRNP K in the effect of BDNF on neuronal activity, cultured hippocampal neurons were transduced with an shRNA targeting hnRNP K, and the SE-induced alteration in intracellular calcium were evaluated by single-cell Ca^{2+} imaging.

Overall this work will contribute to characterize the events underlying abnormal neuronal activity responsible for seizures and their propagation.

III

EXPERIMENTAL PROCEDURES

3.1. Cultures of hippocampal neurons

Cultures of hippocampal neurons with a density of 80.0 x 10^3 cells/cm^2 were prepared from Wistar rat embryos (E18-E19). Hippocampi were dissected from diencephalic structures and washed with Mg^{2+}- free Hank's balanced salt solution (HBSS, 5.56 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM HEPES and 0.001% phenol red). The tissue was then digested with trypsin [0.06% (GIBCO – Life Technologies) in HBSS] for 15min at 37ºC with gentle shaking, followed by a washing step with HBSS supplemented with 10% fetal bovine serum (GIBCO Invitrogen) to stop trypsin activity. Hippocampi were then transferred to Neuronal Basal Medium (NBM, supplemented with SM1 [1:50 dilution; Stem Cell Technologies], 25 µM glutamate, 0.5 mM glutamine and 50 µg/ml gentamycine) and dissociated with a 5 ml pipette. The suspension was filtered (70 µm filter) and the cells were counted and plated on 6 well plates, previously coated with poly-D-lysine (0.1 mg/mL), or on poly-D-lysine coated glass coverslips. Cultures were maintained for 15-16 days at 37ºC and 5% CO_2/ 95% air. After 3 days in culture the medium was supplemented with FDU (5-fluoro-2'-deoxyrudine, 10 µM; Sigma Aldrich) and at day 7 in culture fresh culture medium lacking glutamate (one third of the total volume) was added to the wells.

3.2. Induction of *in vitro Status Epilepticus*

Cultured hippocampal neurons (15 DIV) were incubated with a $[Mg^{2+}]_0$ medium (SE medium: 148 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 10 mM Glucose, 10 mM HEPES, pH = 7.4) in an incubator with 5% CO_2 at 37ºC, for 30 minutes. For experimental conditions with a post-incubation period, the $[Mg^{2+}]_0$ medium was replaced by Sham medium (same composition of the $[Mg^{2+}]_0$ medium and supplemented with 2 mM $MgCl_2$,) and analysis were performed during the indicated period. The same medium was used for Sham control conditions. Sham and SE medium have an osmolarity of ± 300 mOsm/l.

3.3. Viral Production and hippocampal neuronal cell infection with shRNA for hnRNP K

shRNA target sequences (see table 3.1) were inserted into pFUChW nontargeting shRNA plasmid from David Chan (Addgene plasmid #78522) (Rojansky, Cha, & Chan, 2016). Briefly, shRNA target sequences were designed and cloned into the BamHI and EcoRI sites of the pFUChW nontargeting shRNA vector. Upon restriction analysis, the selected positive clones were sequenced to confirm the insertion of the shRNA target sequence

into the vector.

Table 3.1 - shRNA sequences targeting the mRNA of hnRNPK (shRNA 6) or scrambled sequence (shRNA1) inserted into pFUChW.

	Sense Oligo	**Antisense Oligo**
shRNA 1 **Control shRNA**	5′GATCCgactagaaggcacagagggATTC AAGAGATccctctgtgccttctagtcTTTTT3′	3'GctgatcttccgtgtctcccTAAGTTCTCTA gggagacacggaagatcagAAAAActta5'
shRNA 6 **hnRNPK shRNA**	5′GATCCgtaactattcccaaagattTTCAAG AGAaatctttgggaatagttacTTTTTG 3′	3'GcattgataagggtttctaaAAGTTCTCT ttagaaacccttatcaatg AAAAACTTAA 5′

For the generation of lentivirus capable to transduce shRNA targeting hnRNPK, HEK293T cells were transfected with the lentiviral expression vector (pFUChW-shRNA-hnRNPK or pFUChW-shRNA-non targeting sequence) after reaching 80% confluence, in 10 cm petri dishes, using the calcium phosphate transfection protocol. Three lentiviral packaging vectors pLP1, pLP2 and pLP-VSVG were used, for the expression of gag/pol genes, rev gene and the vesicular stomatitis virus G (VSVG) envelope glycoprotein gene, respectively.

The medium of the cells containing virus particles was collected at 24 h, 48 h and 60 h after transfection and centrifuged at 86.000 g, using an SW40Ti rotor (Beckman Coulter ultracentrifuge), for 2 h at 22ºC. The viral pellet was then re-suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) with 1% BSA and stored at -80ºC.

In order to determine the volume of virus to transduce cultured neurons, viral titers and multiplicity of infection (MOI) was calculated according with the following formulas:

$$\text{Titer (TU/ml)} = \frac{\text{Target cell number x \% of GFP} - \text{positive cells}}{\text{Volume of viral supernatant (ml)}/100}$$

$$\text{MOI} = \frac{\text{Volume of viral supernatant (ml)x titer (TU/ml)}}{\text{Target cell number}}$$

Lentivirus were added to the neuronal culture medium 4 days before imaging, with a MOI of 5, which results in 80% of infection, as previously tested (Morais, 2014).

3.4. Whole-cell current clamp electrophysiology

Cultured hippocampal neurons after 15 DIV were whole-cell current clamped to -60mV/-70mV at room temperature for 25 min in three sets of experiments. (i) In the first set cells were incubated in SE solution for 15 min and recorded in the same solution with or without the following inhibitors: TTX (tetrodotoxin, 500 nM; Tocris), APV (D-2-amino-5-phosphonovalerate, 50 µM; Enzo Life Sciences) and CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione, 20 µM; Tocris). (ii) In the second set, cells were incubated in SE solution for 30min, the cells were post-incubated in Sham solution and the recording performed in presence or in absence of the inhibitors listed above. (iii) Finally, in the third set of experiments hippocampal neurons were incubated in SE solution for 30 min in presence of TrkB-Fc (1:100) and recorded in Sham solution.

The conductive electrode solution had the following composition: 100 mM K-gluconate, 20 mM KCl, 20 mM HEPES, 13.6 mM NaCl, 3mM MgATP, 1mM EGTA, 0.2 mM $CaCl_2$ (300mOsm). Recording electrodes were made of borosilicate glass capillaries and pulled on a horizontal stage Sutter Instrument P-97 puller (resistances: 3-4 MΩ). Seals (1-10 GΩ) were formed by applying gentle suction to pipettes. Recordings were performed using an Axon CNS, Multiclamp 700B amplifier, Axon Digidata 1550 A acquisition board and pClamp software (version 10.6; Molecular Devices). Quantification was done through Clampfit (10.7; Molecular Devices).

3.5. Single Cell Calcium Imaging

Hippocampal neurons cultured on coverslips and infected with viruses containing pFUChW-shRNA-hnRNPK or pFUChW-shRNA-non targeting sequence were pre-incubated with fluo-4/AM (5 µM) (fluo-4 acetoxymethyl ester) prepared in Sham solution supplemented with 0.2% Pluronic F-127. Coverslips were then washed with Sham medium to remove residual probe and assembled on a stage filled with Sham medium. The stage was placed in a Cell Observer Spinning Disk Microscope for recording. The baseline fluo-4 fluorescence was recorded for 3 min and after this period the sham medium was replaced by $[Mg^{2+}]_0$ medium and the cells were further incubated for 30min. Finally, the $[Mg^{2+}]_0$ medium was replaced by Sham medium for the rest of the experiment. In control experiments the cells were always maintained in Sham solution.

Fluo-4 fluorescence was recorded in a Zeiss Cell Observer Spinning Disk microscope with a Plan-Apochromat 20x objective coupled to the highly sensitive electron multiplying

camera, EM-CCD Evolve Delta. The microscope stage was maintained at 37ºC while Fluo-4 fluorescence was monitored and the preparation was excited at 488 nm using a solid state laser 100mW. Fluo-4 fluorescence was recorded in intervals of 10 sec during the first 3 min and 3 sec for the rest of the experiment. Analysis was performed with ImageJ analysis software.

Fluo-4/AM is a membrane-permeable fluorescent calcium indicator. The probe crosses the membrane and the acetoxymethyl groups are cleaved in the cytosolic compartment by esterases, giving rise to Fluo-4. Ca^{2+} binding to the probe increases the emission of fluorescence. Fluo-4 has a low Ca^{2+}-binding affinity and thus it is appropriate to assess fast changes in the intracellular calcium concentration, such as in calcium spikes.

3.6. Western blot

Total cell extracts were prepared after washing the cells twice with ice-cold PBS. Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS, at a final pH of 7.5) supplemented with a cocktail of protease inhibitors (0.1 mM PMSF, 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain and 1 μg/mL pepstatin) and phosphatase inhibitors (50 mM NaF, 1.5 mM sodium orthovanadate). The suspension was collected and frozen at -80ºC and then stored at -20ºC.

To determine protein concentration of the samples, the lysates were centrifuged for 10 min at 16,000 x g. The supernatants were collected and the protein concentration in the samples was quantified using the BCA (bicinchoninic acid) method. Protein concentration was equalized to the least concentrated sample and the samples were diluted with a denaturing buffer (200 mM Tris-HCl, 8% glycerol, 1.6% SDS, 0.001% bromophenol blue and 5% β-mercaptoethanol). Samples were heated at 95ºC for 5 min and the proteins were separated by SDS-PAGE using a 7.5% polyacrylamide gel. Proteins were transferred to PVDF (Polyvinylidene fluoride) membranes overnight at 40 V, followed by a 1 h pulse at 100V. Membranes were blocked in 3% BSA prepared in TBS-T (20 mM Tris, 13.7 mM NaCl, 0.1% Tween, pH = 7.6) for 1 h at room temperature. After blocking, membranes were incubated overnight with the primary antibody, pTrkB (1:500), in a TBS-T supplemented with 1% BSA. Membranes were then washed and incubated with an anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:20000) in TBS-T supplemented with 1% BSA solution, for 1h at room temperature. Alkaline phosphatase

activity was visualized using the ECF system on the Storm 860 gel and blot imaging system. Tubulin was used as loading control (1:10000).

IV

RESULTS

4.1. Characterization of the Mg^{2+}-free *in vitro* model of *Status Epilepticus*

Incubation of brain slices or cultured neurons in a Mg^{2+}-free (or a low Mg^{2+} concentration) medium has been used as an *in vitro* model of *Status Epilepticus* (Tancredi, Hwa, Zona, Brancati, & Avoli, 1990). These conditions are expected to increase the activity of NMDAR by removing the Mg^{2+} blockade of the receptor channels, thereby enhancing the frequency of action potentials, which mimics the SE state, and overactivating the downstream signaling cascades.

To validate the Mg^{2+}-free *in vitro* model of *Status Epilepticus* we analyzed the frequency of firing of action potentials in high density primary cultures of rat hippocampal neurons, using whole-cell current clamp electrophysiology. Neuronal activity was measured in hippocampal neurons incubated in a Na^{+}-salt solution lacking Mg^{2+}, as well as in neurons that had been maintained under those conditions before an additional incubation in a solution containing physiological levels of Mg^{2+}. In control experiments, neuronal activity was monitored in cells maintained in a Na^{+}-salt solution containing Mg^{2+} (Sham solution).

Neurons maintained in SE condition presented a higher frequency of action potentials compared to control: 3.8-fold increase (Fig. 4.1) indicating that the removal of Mg^{2+} from the extracellular medium mimics the SE state.

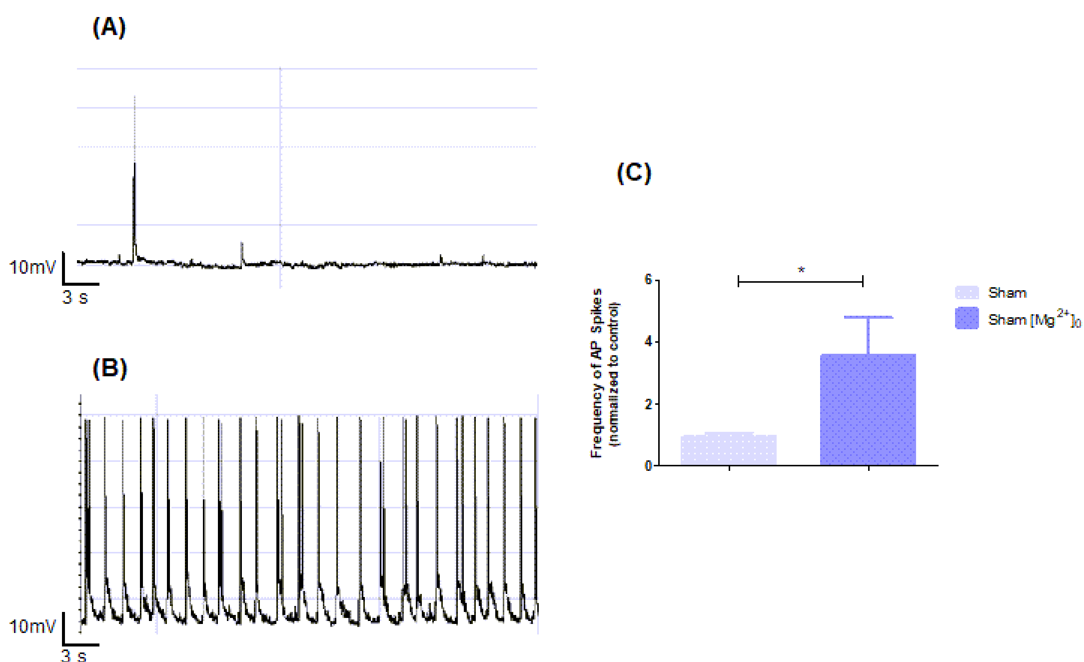

Figure 4.1 - Incubation of cultured hippocampal neurons in a Mg^{2+}-free salt solution during 15min increases the rate of firing of action potentials, mimicking *Status Epilepticus*. (A) Representative recording of a neuron in Sham condition. (B) Representative recording of a neuron in Sham $[Mg^{2+}]_0$ condition. (C) Quantification of the mean frequency of AP spikes obtained in the Sham (n = 20) and SE conditions (n = 16). Results were normalized with the Sham control of each batch. The bars represent the fold change mean ± SEM of 9 independent experiments performed in different preparations. Statistical analysis was performed using the Student's *t*-test (*$p < 0.05$).

Inhibitors of AMPAR and NMDAR have been used to prevent the increase in firing rate during SE (Rogawski, 2011). Here, we aimed to characterize the contribution of these channels to the pattern of neuronal activity observed under Mg^{2+}-free conditions to mimic *Status epilepticus*. With this objective, hippocampal neurons were incubated in Mg^{2+}-free medium for 15 min and afterwards whole-cell current clamp recordings were performed in the same medium and in the presence or absence of the following inhibitors: APV (50 µM; NMDAR antagonist) and/or CNQX (20 µM; AMPAR antagonist), and TTX (500 nM; blocker of voltage-gated Na^+ channels). The increase in the frequency of firing of action potentials in the SE condition when compared with the Sham control (Fig. 4.2A, B, G) was abolished upon incubation with CNQX alone or together with APV (Fig. 4.2D, E, G). Although APV also decreased the frequency of action potentials recorded under SE conditions, the effect was not statistically significant (Fig. 4.2C, G). As expected, TTX completely blocked the firing activity induced by *Status Epilepticus* (Fig. 4.2C, G).

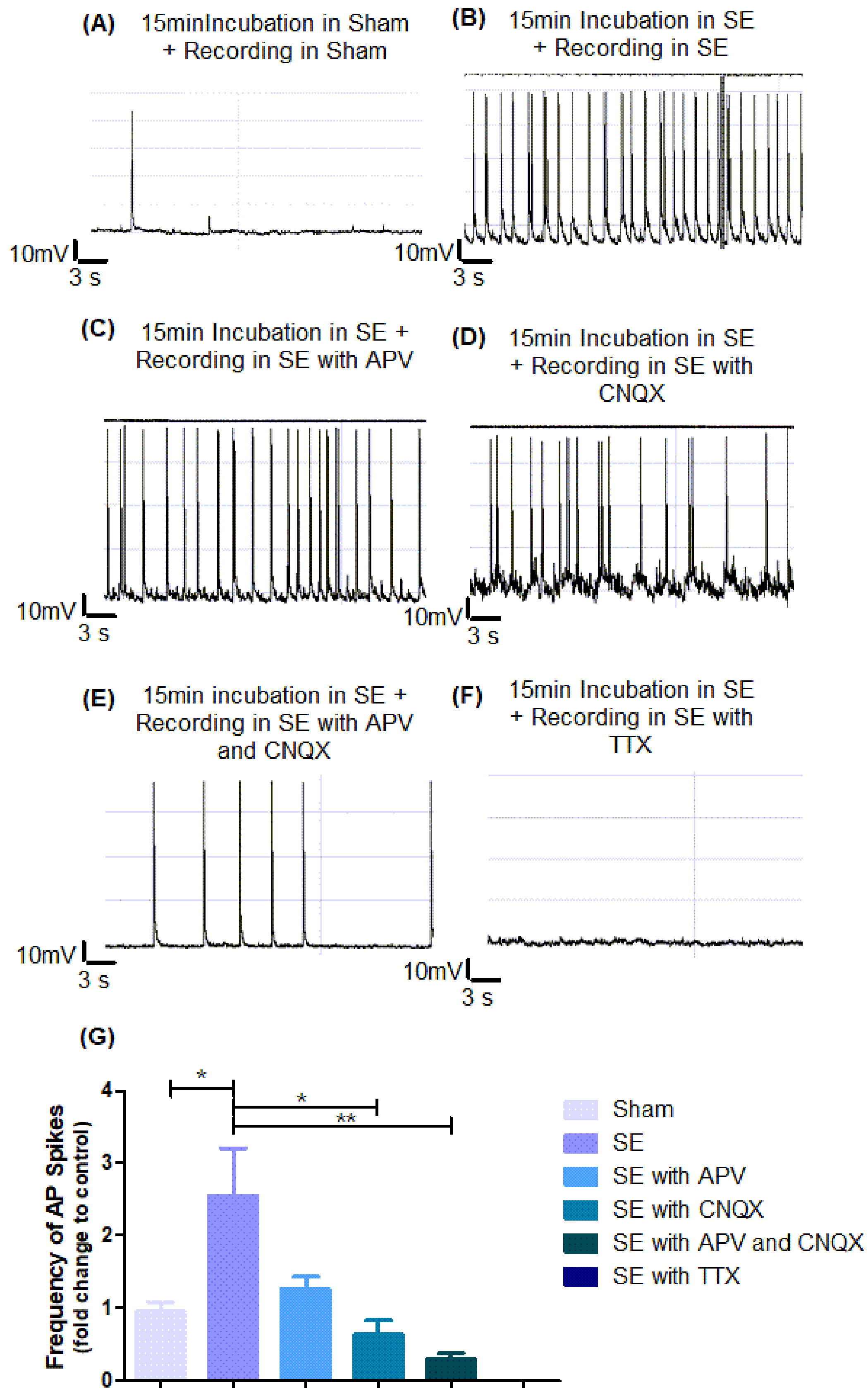

Figure 4.2 - Incubation of hippocampal neurons in Mg^{2+}-free salt solution increased the frequency of action potentials by a mechanism dependent of the activity of ionotropic glutamate receptors. Representative profile of a 25 min recording after 15 min of incubation of hippocampal neurons in Mg^{2+}-

free Na^+ salt solution. (A) Representative recording in Sham condition (n = 20); (B) Representative recording in SE condition (n = 16); (C) Representative recording in SE condition in the presence of APV (50 μM; NMDAR inhibitor) (n = 10); (D) Representative SE recording in the presence of CNQX (20 μM; AMPAR inhibitor) (n = 9); (E) Representative recording SE condition in the presence of CNQX (20 μM) and APV (50 μM) (n = 8); (F) Representative recording in SE condition in the presence of TTX (500 nM) (n = 3). (G) Analysis of AP frequency of spikes in cultured hippocampal neurons maintained in Mg^{2+}-free medium and in the presence and absence of APV and CNQX. Traces are representative of at least 4 independent experiments performed in different preparations. Results were normalized with the Sham control of each batch. The bars represent the fold change mean ± SEM of at least 4 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA followed by Bonferroni test (* $p< 0.05$, ** $p< 0,01$).

4.2. Characterization of *Status Epilepticus*-induced rhythmic electrical activity in hippocampal neurons transiently incubated in the absence of Mg^{2+}

Unpublished results from our laboratory showed synchronized $[Ca^{2+}]_i$ spiking in cultured hippocampal neurons incubated for 30 min in Mg^{2+}-free medium (*Status Epilepticus*) and later returned to a Na^+ salt solution containing Mg^{2+}, as assessed by single cell calcium imaging (Fig 1.10). In this work, we aimed to further investigate the mechanisms leading to this synchronized activity using whole-cell patch clamp. We also assessed the role of NMDAR and AMPAR activation in the induction of rhythmic burst activity after induction of *status epilepticus in vitro*.

Hippocampal neurons (15 DIV) were incubated for 30 min in Mg^{2+}-free medium and post-incubated in Sham medium while recorded by whole-cell current clamp. As depicted in the Fig. 4.3, transient incubation in a Mg^{2+}-free salt solution (*Status Epilepticus*) followed by incubation in Sham medium induced a pattern of rhythmic bursting activity. In a 25 min recording, there was an average of 3 bursts occurring. During the bursting period the frequency of action potentials was 11.6 Hz (Fig. 4.4H) while in the whole recording a frequency of 0.6 Hz was calculated (Fig. 4.5). This result is in accordance with previous data showing synchronized calcium spikes after the same stimulus (see Fig. 1.10) and supports the hypothesis that transient incubation of cultured hippocampal neurons in Mg^{2+}-free salt solution is a good *in vitro* model of *status epilepticus*.

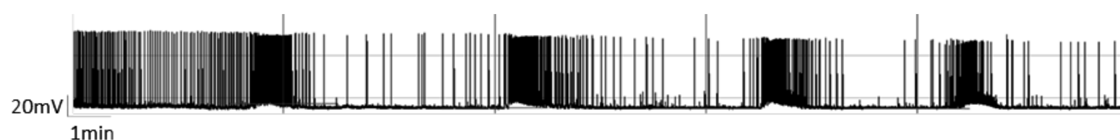

Figure 4.3 - SE induces rhythmic burst activity. Representative record of SE-induced rhythmic activity. Cultured hippocampal neurons were incubated in SE solution for 30 min and post-incubated in Sham solution for 25 min while performing whole-cell patch clamp.

Control experiments showed that the pattern of activity recorded in hippocampal neurons maintained in Mg^{2+}-containing Na^{+} salt solution (Sham) was stable throughout the recording period. Furthermore, neurons maintained in Mg^{2+}-free medium for the same time period maintained the high frequency of spiking activity. However, in some cells there was a decrease in activity (Fig. 4.4B) while others showed an increase in spike frequency. Burst analysis using the Poisson Surprise method (Devices, 2005) indicated that during the 25 min of incubation in Sham medium after SE there was an increase of rhythmic bursts when compared with the Sham condition (Fig. 4.4A, C and F).

4.2.1. AMPAR and NMDAR inhibitors prevent rhythmic activity and reduce the number of bursts induced by *Status Epilepticus*

In additional experiments we performed a pharmacological characterization of the bursts of action potentials in hippocampal neurons subjected to a transient incubation in the absence of Mg^{2+}, followed by incubation in a solution containing Mg^{2+}. The inhibitors of ionotropic glutamate receptors, APV and CNQX, decreased bursting activity, but the effect was statistically significant only in the experiments performed in the presence of the non-NMDA receptor antagonist. Both the average number of bursts (Fig. 4.4F) and the intraburst (within a burst) frequency of events (Fig. 4.4G) were significantly reduced by CNQX, while a significant increase in the intraburst interval was observed in the presence of the non-NMDA receptor antagonist (Fig. 4.4H). Additionally, recordings in the presence of TTX showed no neuronal activity (not shown).

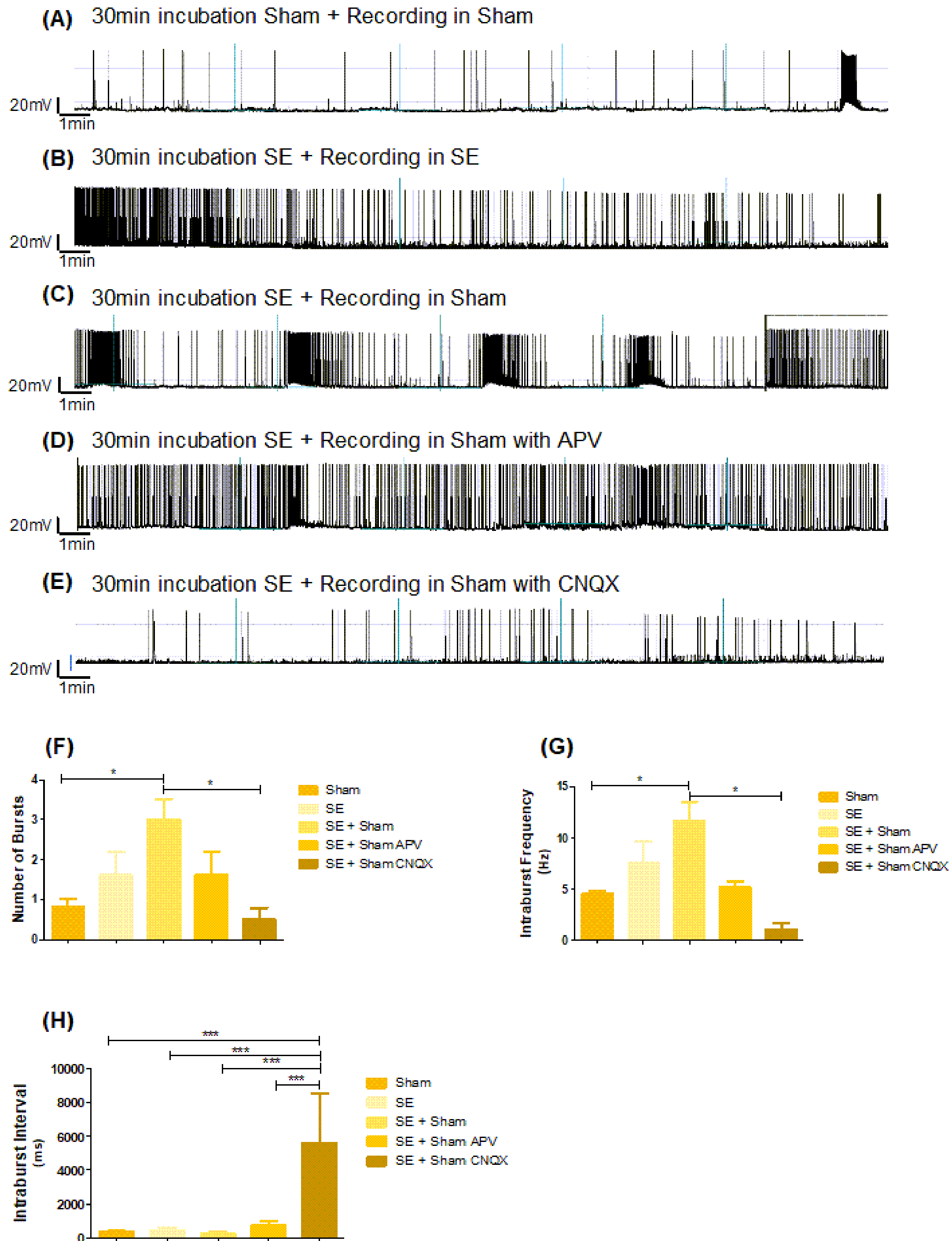

Figure 4.4 - Role of ionotropic glutamate receptors in the rhythmic bursting activity induced by *status epilepticus*. Representative 25 min recordings after 30 min stimulus mimicking SE. (A) Incubation and post-incubation in Sham solution (n = 7); (B) Incubation and post-incubation in SE solution (n = 5); (C) Incubation in SE and post-incubation in Sham; (D) Incubation in SE and post-incubation in Sham solution in the presence of APV (n = 5); (E) Incubation in SE and post-incubation in Sham solution in the presence of CNQX (n= 4); (F) Average number of bursts during the 25 min recording; (G) Mean frequency of intraburst events; (H) Time interval between AP spikes within a burst; Burst analysis was performed using the Poisson Surprise method. The bars represent the means ± SEM of at least 3 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni test (* $p < 0.05$, *** $p < 0.001$).

To further characterize the SE model in cultured hippocampal neurons, we analysed the frequency of action potentials spikes recorded in a Mg^{2+}-containing salt solution after a transient incubation in the absence of Mg^{2+}, and compared the results with those obtained in Sham conditions, both in the presence and absence of NMDA and non-NMDA inhibitors (Fig. 4.5), however the results show no significant differences among conditions.

Additional experiments are required to clarify the relative role of glutamate receptors in the bursting activity after incubation of hippocampal neurons in the Mg^{2+}-free salt solution.

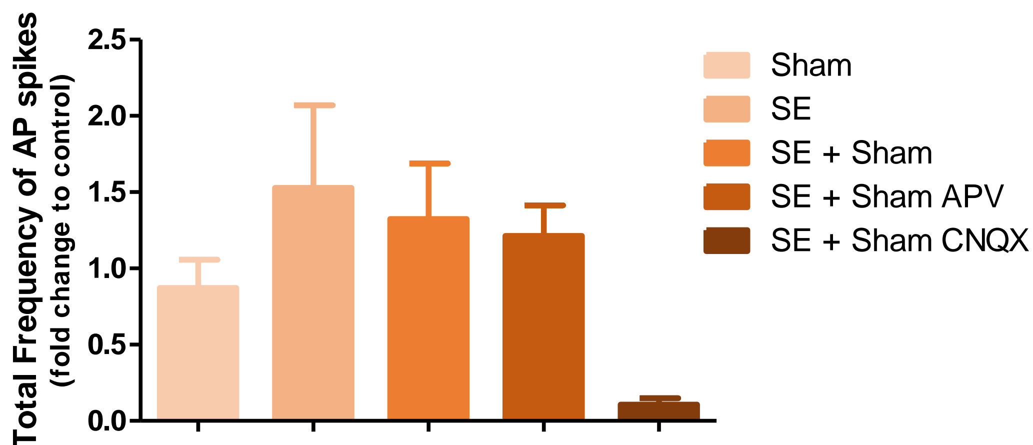

Figure 4.5 – Analysis of AP frequency of spikes in cultured hippocampal neurons transiently incubated in Mg^{2+}-free medium in the presence and absence of APV and CNQX Traces are representative of at least 4 independent experiments performed in different preparations. Results were normalized with the Sham control of each batch. The bars represent the fold change mean ± SEM of at least 3 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni test.

4.3. pTrkB in SE and SE- induced rhythmic activity model

Since BDNF is synthesized and released from hippocampal neurons in an activity-dependent manner (Gottmann, Mittmann, & Lessmann, 2009), and considering the role played by BDNF-TrkB signaling in the development of TLE, we investigated whether TrkB receptors are activated under the experimental conditions used in this work to mimic SE *in vitro*. Previous studies showed an increased expression of pTrkB both during (Gu et al., 2015) and after SE (Liu et al., 2013) in *in vivo* models of TLE. TrkB receptor activation by

BDNF sets in motion several parallel intracellular signaling cascades which lead to insertion of NMDA and AMPA receptors in the membrane (Leal et al., 2014).

4.3.1. *Status Epilepticus* induces a transient phosphorylation of TrkB receptor

We investigated the alterations of TrkB phosphorylation levels in hippocampal neurons incubated in Mg^{2+}-free Na^{+}-salt solution (SE) for 30 min, as well as during the recovery period in a Sham solution when rhythmic burst activity was detected. TrkB phosphorylation, a measure of its activity, was assessed by western blot with a phosphospecific antibody against pTrkB (Tyr816). The results were also compared with TrkB phosphorylation in hippocampal neurons maintained in culture medium (control). The respective controls were maintained in Sham medium for the whole period of the experiment. Western blot analysis showed an increase in TrkB phoshorylation in SE condition when compared to the respective control (Fig. 4.6), although the effect was not statistically significant. However, when hippocampal neurons were further incubated in a Na^{+}-salt solution for 15 min after the *in vitro* SE pTrkB protein levels were similar to the control, suggesting that the activation of TrkB receptors is a transient event (Sham 45 min). Although these results suggest that TrkB receptors are activated in *in vitro* SE, additional experiments are required to validate this hypothesis.

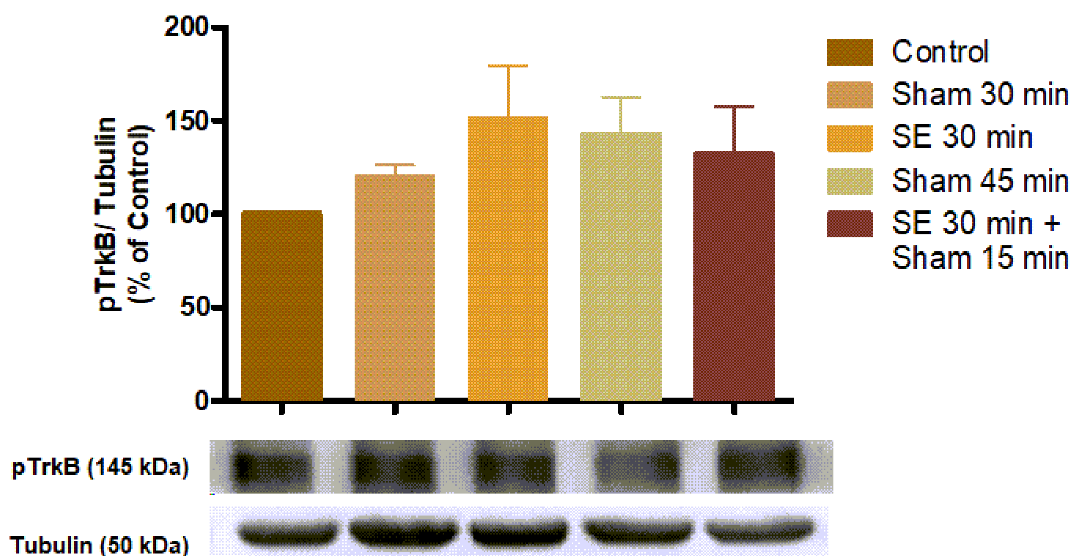

Figure 4.6 – Alterations in pTrkB phosphorylation in *in vitro Status epilepticus.* TrkB phosphorylation

was evaluated immediately after SE or 15 min later, by western blot using an antibody against the phosphorylated form of TrkB. Hippocampal neurons (15 DIV) were subjected to SE (incubation with $[Mg^{2+}]_0$ medium; 30 min) or maintained under control conditions (Sham medium). When a post-incubation was performed, the cells were maintained in culture-conditioned medium for 15 min. The results were normalized with the loading control β-tubulin and are expressed as percentage of the control. The bars represent the means ± SEM of at least 4 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni test.

Considering the results suggesting an increased phosphorylation of TrkB receptors during SE, we tested whether the inhibition of BDNF signaling using the TrkB-Fc peptide would abrogate this effect. TrkB-Fc chelates free BDNF thereby preventing the activation of TrkB receptors by the neurotrophin. Similarly to the previous set of experiments, the alterations in TrkB phosphorylation levels were tested during SE by western blot using a phosphospecific antibody. The results show no effect of TrkB-Fc on the phosphorylation of TrkB in hippocampal neurons subjected to *in vitro* SE (Fig. 4.7).

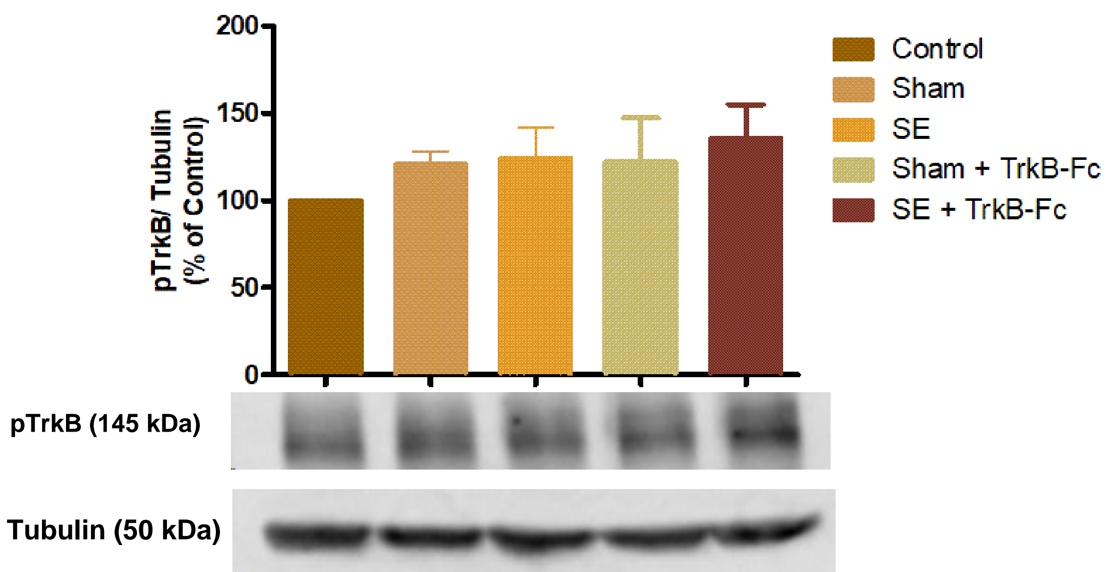

Figure 4.7 - TrkB-Fc inhibitor of BDNF signaling does not affect TrkB phosphorylation during *status epilepticus*. TrkB phosphorylation was evaluated immediately after SE by western blot using an antibody against the phosphorylated form of the TrkB receptor. Hippocampal neurons (15 DIV) were subjected to SE (incubation with $[Mg^{2+}]_0$ medium; 30 min) or maintained under control conditions (Sham medium). Where indicated the cells were incubated with TrkB-Fc (10µg/mL). Results were normalized with the loading control β-tubulin and were expressed as percentage of the control. The bars represent the means ± SEM of at least 3 independent experiments performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni test.

4.4. Infection of hippocampal neuron cultures with shRNA for hnRNP K

hnRNP K is a ribonucleoprotein that is found in mRNPs and it was shown to interact directly or indirectly with a large number of transcripts, including those coding for GluA1 (AMPA receptor subunit), GluN1 (NMDA receptor subunit), CamKIIβ and BDNF (Comprido, 2011). This interaction may be important to maintain the transcripts in a repressed state while they are transported along dendrites. Furthermore, recent studies from our laboratory showed that BDNF increases hnRNP K levels in dendrites and reduces its interaction with the transcripts (Leal, 2013). For example, hnRNP K was found to mediate the effects of BDNF on the synaptic expression of NMDA receptors, which are dependent of protein synthesis (Afonso, 2016; De Luca, 2016). Considering these evidence, and given the role of BDNF in epileptogenesis, we hypothesized that BDNF and hnRNP K may play a role in the induction of the spiking activity in hippocampal neurons incubated transiently in a medium lacking Mg^{2+}, which mimics SE.

Heretofore, we successfully infected hippocampal neuron cultures (Fig. 4.9) with pFUChW-shRNA-hnRNPK (86% of the cells were infected) or pFUChW-shRNA-non targeting sequence (81% of the cells were infected) to express an shRNA against hnRNP K (Afonso, 2016) together with mCherry (emission in red). These tools will allow performing single-cell calcium imaging experiments to investigate whether downregulation of hnRNP K affects the synchronized $[Ca^{2+}]_i$ oscillations observed in cultured hippocampal neurons exposed transiently to a Na^+ salt solution lacking Mg^{2+} (Fig. 1.10).

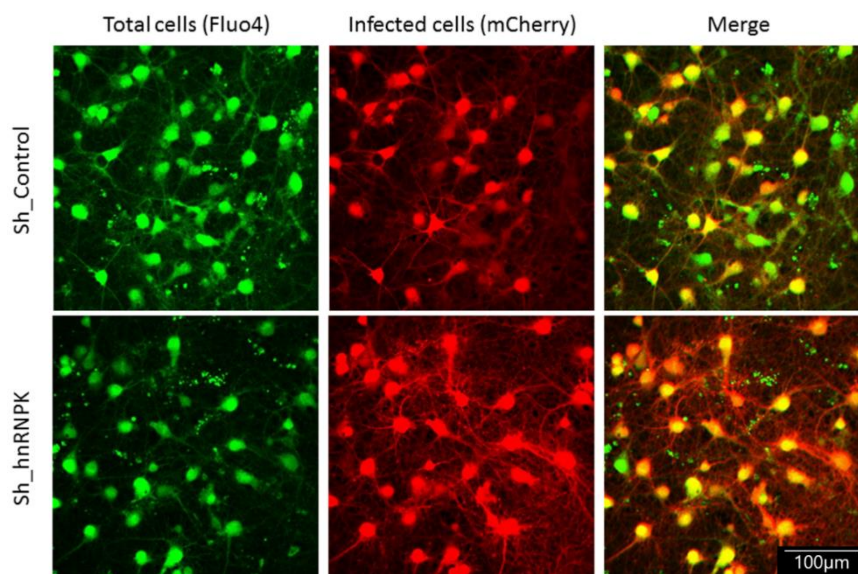

Figure 4.8 – Cultured hippocampal neurons transduced with an shRNA targeting hnRNP K. Neurons were infected with virus containing pFUChW-shRNA-hnRNPK or pFUChW-shRNA-non targeting sequence; both vector expressed mCherry as Tag protein. Images were taken in a Spinning Disk fluorescence microscope with an objective of 20x magnification (the red and green images represente the fluorescence of mCherry and Fluo-4, respectively).

V
DISCUSSION

Epilepsy is a neurological chronic disorder, the hallmark of which is recurrent unprovoked seizures translating burst discharges of the neuronal network. Different causes have been associated with epileptogenesis, which commonly culminate in an unbalance between excitatory and inhibitory neurotransmission. Pharmacoresistance remains a major challenge in epilepsy management since almost 30% of epileptic cases are resistant to the currently available pharmacological treatment. The mechanism of seizure generation has been only partially elucidated, and therefore additional studies are required to understand the basic mechanism underlying seizure activity. Current efforts also aim at the development of new antiepileptics to overcome pharmacoresistance.

In this work, we validated the Mg^{2+}-free *in vitro* model of *Status Epilepticus* in high density primary cultures of rat hippocampal neurons, analyzing the firing of action potentials with whole-cell current clamp electrophysiology. We have shown that transient *Status Epilepticus* induces a pattern of rhythmic bursting activity that is partly related with the activation of ionotropic glutamate receptors. Moreover, we addressed the putative role of BDNF signaling in the *Status Epilepticus*-induced firing activity. Although evidence was obtained suggesting that BDNF-TrkB receptor signaling may be activated in *in vitro* SE additional experiments are required to elucidate the role of the neurotrophin in seizure activity induced by *Status Epilepticus.*

5.1. Recovery from Status Epilepticus induces rhythmic burst activity

The most common form of epileptic activity that can be recorded in cortical or hippocampal neurons is a synchronized burst of action potentials known as interictal spike. Experimentally, epileptiform burst discharges can be evoked in neuronal cultures by removing extracellular magnesium (Mody, Lambert, & Heinemann, 1987), increase in extracellular potassium concentration (Somjen & Muller, 2000), inhibition of the sodium pump (Balestrino, Young, & Aitken, 1999) or antagonizing $GABA_A$ receptors (Hablitz & Heinemann, 1989). In this work, we validated the *in vitro* model of SE induced by incubation of cultured hippocampal neuron in Mg^{2+}-free medium for 30 min. This protocol prevents the blockage of NMDAR by extracellular Mg^{2+}, making the receptors more prone to be activated. Ultimately, these experimental conditions allow the influx of Na^+ into the intracellular compartment, facilitating the development of action potentials,

as well as the entry of Ca^{2+} with the consequent activation of Ca^{2+}-dependent signaling pathways. This method has been used for years to study the molecular mechanisms leading to the occurrence of spontaneous, recurrent epileptiform discharges (DeLorenzo, Pal, & Sombati, 1998). We showed that in the latter conditions, the frequency of action potentials recorded in cultured hippocampal neurons was significantly higher when compared with control condition, in accordance with the results obtained in previous studies (Blair, Sombati, Churn, & Delorenzo, 2008; Cao, Jiang, Liu, & Wu, 2003; DeLorenzo et al., 1998; Mangan & Kapur, 2004).

The main goal of the project was to further characterize the SE-induced rhythmic burst activity *in vitro*. Non-published results from our laboratory showed synchronized and spontaneous $[Ca^{2+}]_i$ spiking in 50% of the cultured hippocampal neurons after 15 to 30 min of *in vitro status epilepticus* (Vieira, 2015). These results are in accordance with the pattern of activity obtained in the present work, by whole-cell patch clamp, characterized by rhythmic bursts of action potentials with the same frequency. In a previous report, a similar spontaneous bursting activity was described in cultured hippocampal neurons incubated for 3 h in a $[Mg^{2+}]_0$ solution (Blair et al., 1999; Cao et al., 2003) or in a solution containing a low Mg^{2+} concentration (DeLorenzo et al., 1998). However, the results obtained in the present work showed 3 - 4 bursts in 25 min after 30 min of incubation in a Mg^{2+}-free solution, while the most similar study observed the same number of bursts within 15 min (Blair et al., 1999). This difference might be due to the protocol used for incubation in the $[Mg^{2+}]_0$ solution: in the present work, this incubation was performed for 30 min, while in the work by Blair et al. the incubation period mimicking SE lasted for 3 h. Together, these results suggest that the time period of high NMDAR activity, and the concomitant activation of downstream signaling pathways, is an important factor to determine the frequency of AP bursts.

Downregulation of CaMKII, a kinase that is regulated by the $[Ca^{2+}]_i$, induced spontaneous activity in cultured hippocampal neurons incubated in Na^+-salt solution, with 2 bursts recorded in 30 min (Carter et al., 2006). A distinct study showed a decrease in CaMKII activity immediately after incubation in a medium containing a low Mg^{2+} concentration (Blair et al., 2008). The accumulation of intracellular Ca^{2+} due to CaMKII underactivity was found to affect intracellular calcium homeostasis mechanisms independently of neuronal

activity (Carter et al., 2006). Together, these evidence suggest that the spontaneous burst activity described as consequents of *Status Epilepticus* is associated with a decrease in CaMKII activity and with increased intracellular calcium levels. Thus, after incubation of neurons in Mg^{2+}-free solution there is a decrease in CaMKII activity which deregulates the intracellular calcium homeostasis mechanisms with a consequent accumulation of intracellular calcium. The burst activity detected both in patch-clamp recordings and single-cell calcium imaging can be due to the release of calcium from both internal stores and/or calcium influx. However, additional studies are required to understand the molecular mechanism underlying the synchronized activity after incubation of neurons under conditions mimicking SE.

5.2. AMPAR and NMDAR inhibitors prevent SE induced alteration in neuronal activity

It is generally accepted that seizures result from the excessive excitatory activity in a given population of neurons in the brain or from deficient neuronal inhibition (A. M. Mazarati & Wasterlain, 1999; Scharfman, 2007; Trinka et al., 2015). This mechanism is thought to comprise hyperactivity of glutamatergic transmission and insufficient $GABA_A$ receptor-mediated neurotransmission, respectively (Bromfield et al., 2006). AMPAR and NMDAR inhibitors have been reported to prevent the increased neuronal activity during *Status Epilepticus* (DeLorenzo et al., 1998; Mangan & Kapur, 2004), similar to the results obtained in this work (Fig. 4.2). Hence, we tested the effect of glutamate receptor inhibitors in the recovery from *status epilepticus*.

Inhibitors of ionotropic glutamate receptors prevented the bursting activity, especially CNQX which blocks non-NMDA glutamate receptors (Fig. 4.4D, E). The effect of CNQX was observed on the number of bursts (Fig. 4.4F), the intraburst activity, the frequency of events within a burst (Fig. 4.4 G), as well as on the intraburst interval (Fig. 4.4H) (time interval between events within a burst). Furthermore, when the burst frequency was analyzed in the whole recording (Fig. 4.5) there was a decrease in the presence of the non-NMDA receptor antagonist, although the effect was not statistically significant. It is important to note that although the transient exposure of hippocampal neurons to conditions mimicking SE increased the spiking activity, when compared with the Sham

conditions, the frequency of AP recorded after returning to the solution containing Mg^{2+} was lower than that detected during the incubation in $[Mg^{2+}]_0$. This difference is probably due to the fact that after incubation in $[Mg^{2+}]_0$ the bursting activity alternate with the interburst periods characterized by a lower frequency of AP firing (Fig. 4.5). The results presented suggest that in the presence of inhibitors there are less bursts of AP (Fig. 4.4F). . These results are distinct from those previously published using a distinct protocol. In the latter study, cultured hippocampal neurons were incubated for 3 h in a medium lacking Mg^{2+} and the bursting activity was analyzed after 2 days of incubation in Neurobasal medium. In this model, the spiking activity was reduced to approximately control levels in the presence of 25 μM APV, with no burst activity. Furthermore, burst activity was observed in the presence of 10 μM CNQX, although with shorter duration (DeLorenzo et al., 1998). This contrasts with the results obtained in the present work showing (i) no burst activity in the presence of CNQX or APV, and (ii) a greater decrease in frequency of APs in the presence of CNQX.

Overall, when there were effects of the glutamate receptor antagonists of the AP, CNQX induced more robust alterations when compared with APV, suggesting an important role for AMPA receptors.These results show that the spontaneous bursting activity is dependent, although not necessarily exclusively, of NMDA and non-NMDA (possibly AMPA) glutamate receptors since the pattern of activity was sensitive to APV and CNQX. Nevertheless, AMPA receptors seem the most relevant in the process since the activity was severely impaired in the presence of CNQX (Fig. 4.4) (see also Fig. 4.5), although additional effects were observed in the presence of APV (Fig. 4.5). This is probably because in the presence of Mg^{2+}, NMDA receptors are blocked and their activity requires the depolarization produced by opening of the AMPAR channels (Fig. 1.9). Therefore, inhibition of AMPAR makes more difficult the opening of NMDAR.

5.3. pTrkB is transiently increased in SE condition

Seizures are produced by synchronized hyperactivity of neuronal populations due to the disruption of the balance between excitatory and inhibitory synaptic transmission (A. M. Mazarati & Wasterlain, 1999; Scharfman, 2007; Trinka et al., 2015). In epileptogenesis-

related brain areas, including the hippocampus, BDNF is up-regulated in the course of the development of epilepsy and induces the collapse of the balance between excitation and inhibition (Elmer et al., 1998; Ernfors et al., 1991; Helgager et al., 2013; Mudo et al., 1996; Poulsen et al., 2004; Roberts et al., 2006; Rudge et al., 1998). Moreover, intracerebroventricular infusion of TrkB receptor body which chelates BDNF was found to attenuate the development of epilepsy (Binder, Routbort, Ryan, et al., 1999).

In this work, we assessed the alterations of the BDNF pathway during *Status Epilepticus in vitro,* by analysing the alterations in the phosphorylation state of its receptor TrkB in cultured hippocampal neurons. Western-Blot analysis showed a transient alteration in TrkB receptor phosphorylation during SE, although the effects did not reach statistical significance. This small change in pTrkB may result from the activation of the synaptic population of receptors, which is likely to represent a small fraction of the total number of receptors expressed in cultured hippocampal neurons. The hypothesis suggesting the specific activation of synaptic TrkB receptors under SE conditions is supported with the evidence indicating the activity-dependent synaptic release of BDNF (Balkowiec & Katz, 2002). Additional experiments are required to clarify this issue.

The observed upregulation of pTrkB under conditions of *in vitro* SE is in accordance with previous studies performed in animal models reporting elevated pTrkB levels immediately after SE induced by kindling, kainic acid and pilocarpine (Binder, Routbort, & McNamara, 1999; He, Minichiello, Klein, & McNamara, 2002; Liu et al., 2013; Unsain et al., 2009). The increase in neuronal activity was shown to stimulate the production and to induce the release of BDNF (Kuczewski, Porcher, Lessmann, Medina, & Gaiarsa, 2009). BDNF binds to TrkB receptors and activates downstream cascades, thereby potentiating synaptic transmission (Gartner et al., 2006). BDNF was shown to promote the insertion of AMPA and NMDA receptors in the membrane (Leal et al., 2014), to release calcium from internal stores which may further induce signaling activity (Wang, Ward, Boswell, & Katz, 2006), and to enhanced translation activity (Leal et al., 2014). Together, this suggests that the enhanced synaptic transmission following activation of TrkB receptors is an important player in the enhanced synaptic transmission and neuronal synchronization observed in SE.

The lack of pTrkB alterations during the post-incubation period (Fig. 4.6), when the SE-induced rhythmic activity is observed, suggests that the role of the neurotrophin in epileptogenesis results from the transient activation of the receptors during the SE period characterized by enhanced neuronal activity.

5.4. TrkB-Fc does not influence pTrkB levels in this model of SE

Inhibition of the TrkB kinase and downstream signaling has been shown to prevent epilepsy in different studies performed in animal models (Binder, Routbort, & McNamara, 1999; Gu et al., 2015; Liu et al., 2013). Therefore, we hypothesized that TrkB-Fc, which prevents BDNF from binding to TrkB receptors, would influence the activation of TrkB receptors in hippocampal neurons incubated in a solution lacking Mg^{2+}. However, western-blot analysis showed no changes in TrkB phosphorylation under the latter conditions indicating that BDNF is not involved in the phosphorylation of the receptor under the experimental conditions used. This is in accordance with the results of studies in P17 mice, using the kainic acid model of SE, which showed an increase in BDNF levels which were not accompanied by an upregulation of pTrkB (Danzer et al., 2004). Additionally, hippocampal knock out of BDNF (-/-) did not prevent epileptogenesis or decreased pTrkB levels. This suggests that alternative mechanisms may be involved in the activation of TrkB receptors under the conditions used to mimic SE (He et al., 2004). This hypothesis is supported by experimental evidence indicating that G protein coupled receptors (e.g. PACAP receptors [PAC1] an adenosine [A_{2A}] receptors) are coupled to the activation of TrkB receptors by a BDNF-independent mechanism (Rajagopal, Chen, Lee, & Chao, 2004).

Additional studies are required to further characterize the rhythmic burst activity model and investigate the role of BDNF during and after *status epilepticus*. The number of electrophysiological recordings to characterize the *in vitro* model of SE should be increased, including the analysis of the effects of the glutamate receptor antagonists. The number of Western blot experiments should also be increased to characterize the alterations in TrkB phosphorylation under the same conditions. Finally, electrophysiology and single cell calcium imaging studies should allow determining whether the RNA

binding protein hnRNP K plays a role in synchronized bursting activity observed in hippocampal neurons transiently exposed to a Mg^{2+}-free salt solution to mimic SE *in vitro* (Fig. 4.8).

VI
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