UNIVERSITA' DEGLI STUDI DI MILANO
Department of Pharmacological and Biomolecular Science (DiSFeB)

PhD Course in Experimental and Clinical Pharmacological Sciences

XXX Cycle

CHARACTERIZATION OF A NOVEL ANTIEPILEPTIC THERAPY BY TARGETING THE eEF2K/eEF2 PATHWAY

BIO/14

Tutor: Prof. Monica Maria Grazia Di Luca

Co-Tutor: Dr. Carlo Sala

Coordinator: Prof. Alberico L. Catapano

Doctoral Thesis of

Laura Gritti

R10991

Academic Year 2016/2017

TABLE OF CONTENTS

1. ABSTRACT

eEF2K (eukaryotic Elongation Factor 2 Kinase) is an ubiquitous Ca^{++}/Calmoduline-dependent kinase that regulates protein translation by catalyzing the phosphorylation of eEF2 (eukaryotic Elongation Factor 2) at Thr56 (Nairn and Palfrey, 1987). In neurons, eEF2K is activated by Ca^{++} influx mediated by glutamate stimulation (Scheetz et al., 2000; Park et al., 2008), leading to an increased expression of certain protein involved in synapse formation and plasticity (Davidkova and Carroll; 2007; Park et al., 2008; Verpelli et al., 2010), whereas the general protein translation is decreased (Browne and Proud, 2002).

We recently demonstrated that eEF2K activity functionally regulates excitation/inhibition ratio in brain. In particular, eEF2K-knockout (*eEF2K*$^{-/-}$) mice display enhanced GABAergic transmission and tonic inhibition by the upregulation of protein involved in inhibitory synapses functioning and are less susceptible to epileptic seizures (Heise et al., 2017).

Accordingly to these data, we propose eEF2K/eEF2 pathway as possible target for antiepileptic therapies. In a previous work, indeed, we demonstrated that the inhibition of eEF2K was able to rescue the epileptic phenotype in a mouse model of genetic human epilepsy (*Synapsin1*$^{-/-}$ mice) (Heise et al., 2017). The aim of this project was to validate the effect of the eEF2K inhibition in models of Dravet syndrome, one of the most drug-resistant forms of epilepsy.

Dravet syndrome is characterized by febrile/hyperthermia seizures at onset and later development of afebrile seizures, cognitive impairment, elevated mortality, and ataxia (Dravet et al., 2005). About 80% of patients with Dravet Syndrome carry heterozygous missense or truncating mutations in the SCN1A gene encoding the $Na_V1.1$ sodium channel subunit (Claes et al., 2001). This subunit is mostly expressed parvalbumin-positive interneurons and loss of function mutations of NaV1.1 reduces interneurons activity (Ogiwara et al., 2007), leading to the development of a hyperexcitable neuronal network (Liautard et al., 2013).

We studied the effect of eEF2K deletion in *Scn1a*$^{+/-}$ mice, through a genetic and a pharmacological approach. For the genetic approach, we generated a mouse model by

crossing *Scn1a*$^{+/-}$ mice with *eEF2K*$^{-/-}$ mice. Since the enhancement of the GABAergic transmission has been demonstrated ameliorative for the most common symptoms of Dravet syndrome (epilepsy, ataxia and social behavior defects) in the mouse model of the pathology (Ogiwara et al., 2007; Han et al., 2012), we evaluated the effect of eEF2K depletion on the pathological phenotype of *Scn1a*$^{+/-}$ mice. First, we found that eEF2K deletion protected *Scn1a*$^{+/-}$ mice from the onset of epileptic seizures either under basal condition or under thermal stress, a condition known to trigger seizures in Dravet syndrome patients as well as in *Scn1a*$^{+/-}$ mice (Oakley et al., 2009). Also motor coordination defect, memory impairments and stereotyped behavior were reverted by eEF2K depletion. The analysis of spontaneous inhibitory postsynaptic currents (sIPSCs) suggested that the rescue of the pathological phenotype was driven by the potentiation of the GABAergic synapses. In addition, the analysis of eEF2 phosphorylation in samples from cerebral cortex and hippocampus of *Scn1a*$^{+/-}$ mice revealed that eEF2K/eEF2 pathway might play a role in the progression of the pathology.

For the pharmacological approach, we tested two selective inhibitors of eEF2K: JAN-384 and A-484954. We discovered that JAN-384 could not pass the brain blood barrier and, thus, it cannot be used in our experiments, but A-484954 inhibited efficaciously the activity of eEF2K in mouse brain. This compound will be tested on *Scn1a*$^{+/-}$ mice in order to validate a possible pharmacological therapy in Dravet syndrome.

1. Riassunto

eEF2K (eukaryotic Elongation Factor 2 Kinase) è una chinasi ubiquitaria che regola la traduzione proteica attraverso la fosforilazione di eEF2 (eukaryotic Elongation Factor 2) sulla Thr56. L'attivazione di eEF2K dipende dalla Ca^{++}/Calmodulina (Nairn and Palfrey, 1987). Nelle cellule neuronali, eEF2K è attivata dall'aumento di concentrazione di calcio intracellulare in seguito all'attivazione dei recettori per il glutammato (Scheetz et al., 2000; Park et al., 2008) che induce un'aumentata espressione di alcune proteine coinvolte nella formazione di sinapsi e nella loro plasticità (Davidkova and Carroll; 2007; Park et al., 2008; Verpelli et al., 2010), mentre la traduzione proteica, in generale, è rallentata (Browne and Proud, 2002).

Recentemente, abbiamo dimostrato che l'attivazione di eEF2K regola il rapporto tra trasmissione eccitatoria ed inibitoria nel cervello. In particolare, nel modello murino *eEF2K*$^{-/-}$, la trasmissione GABAergica e l'inibizione tonica sono incrementate dall'aumentata espressione di proteine coinvolte nel funzionamento delle sinapsi inibitorie; inoltre, i topi *eEF2K*$^{-/-}$ sono meno suscettibili all'insorgenza di crisi epilettiche (Heise et al., 2017).

In linea con queste premesse, proniamo il pathway di eEF2K/eEF2 come possibile bersaglio terapeutico per lo sviluppo di un farmaco antiepilettico; infatti, in un lavoro precedente, abbiamo dimostrato che l'inibizione di eEF2K è in grado di mitigare il fenotipo epilettico in un modello murino di epilessia gentica umana (topo *Synapsin1*$^{-/-}$) (Heise et al., 2017). Lo scopo di questo progetto è, pertanto, di validare l'effetto dell'inibizione di eEF2K su un modello murino di sindrome di Dravet, una delle maggiori forme di epilessia farmacoresistenti.

La sindrome di Dravet è caratterizzata da attacchi epilettici, il cui esordio è associato a febbre o innalzamento della temperatura, ma che successivamente si sviluppano spontaneamente in assenza di febbre, in concomitanza a difficoltà cognitive, alta mortalità e atassia (Dravet et al., 2005). Circa l'80% dei pazienti con la sindrome di Dravet sono portatori di mutazioni missenso o non senso in eterozigosi nel gene SCN1A che codifica per $Na_V1.1$, una subunità del canale per il sodio (Claes et al., 2001). Questa subunità è prevalentemente espressa dagli interneuroni positivi per la

parvalbumina e mutazioni loss of function di $Na_V1.1$ riducono l'attività di questi interneuroni (Ogiwara et al., 2007), causando lo sviluppo di una rete neuronale ipereccitabile (Liautard et al., 2013).

Abbiamo studiato l'effetto della delezione di eEF2K in topi *Scn1a*$^{+/-}$ tramite un approccio genetico e farmacologico. Per quanto riguarda l'approccio genetico, abbiamo incrociato topi *Scn1a*$^{+/-}$ con topi knock out per eEF2K. Poichè il potenziamento della trasmissione GABAergica è stato dimostrato efficace nel trattare i sintomi più comuni della sindrome di Dravet, come l'epilessia, l'atassia ed i disturbi comportamentali, nel modello murino (Ogiwara et al., 2007; Han et al., 2012), abbiamo indagato l'effetto della deplezione di eEF2K sul fenotipo patologico dei topi *Scn1a*$^{+/-}$. Innanzitutto, abbiamo confermato che la delezione di eEF2K protegge i topi *Scn1a*$^{+/-}$ dall'insorgenza di crisi epilettiche sia in condizioni basali sia in condizioni di stress termico, condizione nota per innescare attacchi epilettici sia nei pazienti affetti da sindrome di Dravet che nei topi *Scn1a*$^{+/-}$ (Oakley et al., 2009). La deplezione di eEF2K è anche in grado di alleviare anche difetti di coordinazione motoria, i problemi di memoria e i comportamenti stereotipati. Dall'analisi delle correnti inibitorie postsinaptiche spontanee (sIPSC), è possibile associare i miglioramenti del fenotipo patologico al potenziamento delle sinapsi GABAergiche. Inoltre, analisi sulla fosforilazione di eEF2 su campioni corticali ed ippocampali di topi *Scn1a*$^{+/-}$, indicano che il pathway di eEF2K/eEF2 potrebbe avere un ruolo sul decorso della malattia.

Per quanto riguarda l'approccio farmacologico, abbiamo testato due inibitori selettivi di eEF2K: JAN-384 and A-484954. Abbiamo scoperto che JAN-384 non può essere usato per gli esperimenti *in vivo* in quanto non passa la barriera ematoencefalica, al contrario di A-484954 che è in grado di inibire l'attività di eEF2K in maniera efficace nel cervello dei topi trattati. A-484954 sarà utilizzato su topi *Scn1a*$^{+/-}$ in esperimenti volti a validare l'inibizione di eEF2K come possibile terapia farmacologica della sindrome di Dravet.

2. INTRODUCTION

2.1. eEF2K

Eukaryotic elongation factor 2 kinase (eEF2K) is an ubiquitous, conserved protein involved in the control of protein translation by phosphorylating eukaryotic elongation factor 2 (eEF2), its only known substrate. eEF2K activity is calcium dependent, in fact its alternative and less used name is Ca^{2+}/Calmodulin-dependent kinase III (CaMKIII) (Nairn et al., 1985 ; Ryazanov et al., 1988).

2.1.1. Structure of eEF2K

eEF2K belongs to α-kinases family, a heterogeneous group of kinases associated by the high level of similarity in their catalytic region, which three-dimensional structure and sequence of their functionally relevant motifs are very similar to conventional protein kinases, indicating that they may probably have a mutual evolutionary origin and have similar functions in the cell (Pavur et al., 2000; Drennan and Ryazanov, 2004).

Human eEF2K is 725 aminoacids long for a molecular weight of 105 kDa (Ryazanov et al., 1997). It consists of an N-terminal catalytic domain, which includes the adenosine triphosphate (ATP)-binding site, and 2 main binding site, one N-terminal, the Calmodulin (CaM) binding site, and one C-terminal domain, necessary for eEF2 binding. Between the amino- and carbossi-terminal domain, there is an unorganized linker region that is an important site for the regulation of eEF2K via its phosphorylation by several upstream kinases such as AMP-activated protein kinase (AMPK), P70S6 kinase (p70S6K), and p90 ribosomal S6 kinase (p90RSK1) (Ryazanov et al., 1997; Diggle et al., 1999; Pavur et al., 2000, Pigott et al., 2012).

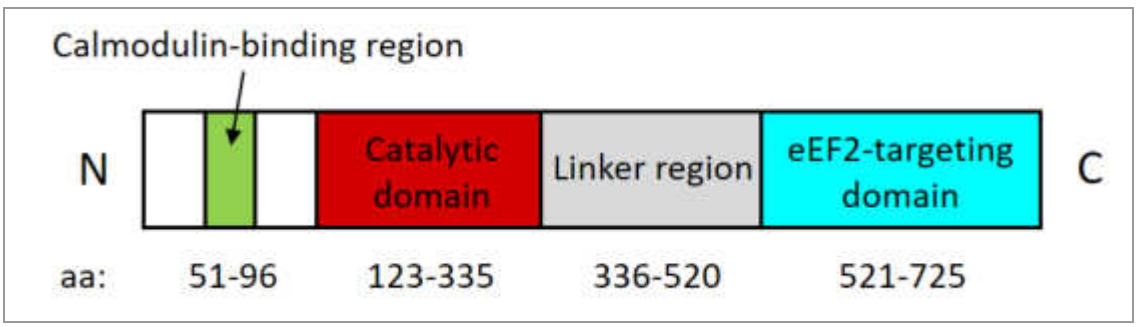

Figure 1. Schematic representation of eEF2K domains.

2.1.2. Regulation of eEF2K in mammalian cells

eEF2K activity is strongly regulated by a number of kinases regulated by defined cellular events (see Fig. 2) (Browne and Proud, 2002). Activated eEF2K phosphorylates eEF2 and, thus, slows the protein translation. Indeed in mammal cells, the decreased translation can be a strategy in response to cellular stress, such as low pH and low energy (i.e. a low availability of aminoacids): these events activate AMPK, which in turn activates eEF2K by phosphorylating it on Ser398 (Dorovkov et al., 2002; Browne et al., 2004) (Fig. 2).

eEF2K activity is also triggered by increased levels of the second messenger Ca^{2+} and the Ca^{2+}-dependent protein CaM (Nairn et al., 1985; Mitsui et al., 1993; Dorovkov et al., 2002) which activation is a downstream event of several signalling pathways (Clapham, 2007; Chuderland and Seger, 2008). In neuronal cells, for example, eEF2K is mainly activated by Ca^{2+} influx in response to N-methyl-D-aspartate receptor (NMDAR) which regulates neuronal protein translation (Scheetz et al., 1997, 2000). Ca^{2+} /CaM is required for eEF2K autophosphorylation in 5 main sites: Thr-348, Thr-353, Ser-445, Ser-474 and Ser-500. Interestingly, phosphorylation in Thr-348 is necessary for eEF2 binding and phosphorylation in Ser-500 appears to render eEF2K activity independent of Ca^{2+} /CaM (Tavares et al., 2012). Ser-500 is also a target for the phosphorylation mediated by PKA (cAMP-dependent protein kinase) in response to an elevated cytoplasmatic concentration of cAMP. This pathway reduces eEF2K activity dependency on Ca^{2+} and calmodulin (Diggle et al. 1998, 2001).

On the contrary, eEF2K activity is reduced by insulin and availability of aminoacids or energy in the cell, by increased activity of mammalian target of rapamycin (mTOR) signalling which triggers p70S6K-dependent phosphorylation of eEF2K on Ser366, a phosphorylation site that is associated with eEF2K inhibition (Wang et al., 2001; Proud et al., 2001). Ser366 is also a target for the phosphorylation through $p90^{RSK1}$, a mediator of MEK/ERK (mitogen-activated protein/extracellular signal-regulated kinase) signalling (Wang et al., 2001; Proud et al., 2001). Thus mTOR and $p90^{RSK1}$ activity balance the AMPK one, and act when protein translation is necessary during for example cell growth and other ATP-dependent processes in the cell that depend on protein synthesis.

Other sites and pathways are involved in eEF2K activity inhibition, for example, phosphorylation of Ser359 is triggered by TNF-α and anisomycin, a protein synthesis inhibitor, through p38δ in a mTOR-indipendent manner (Knebel et al., 2002).

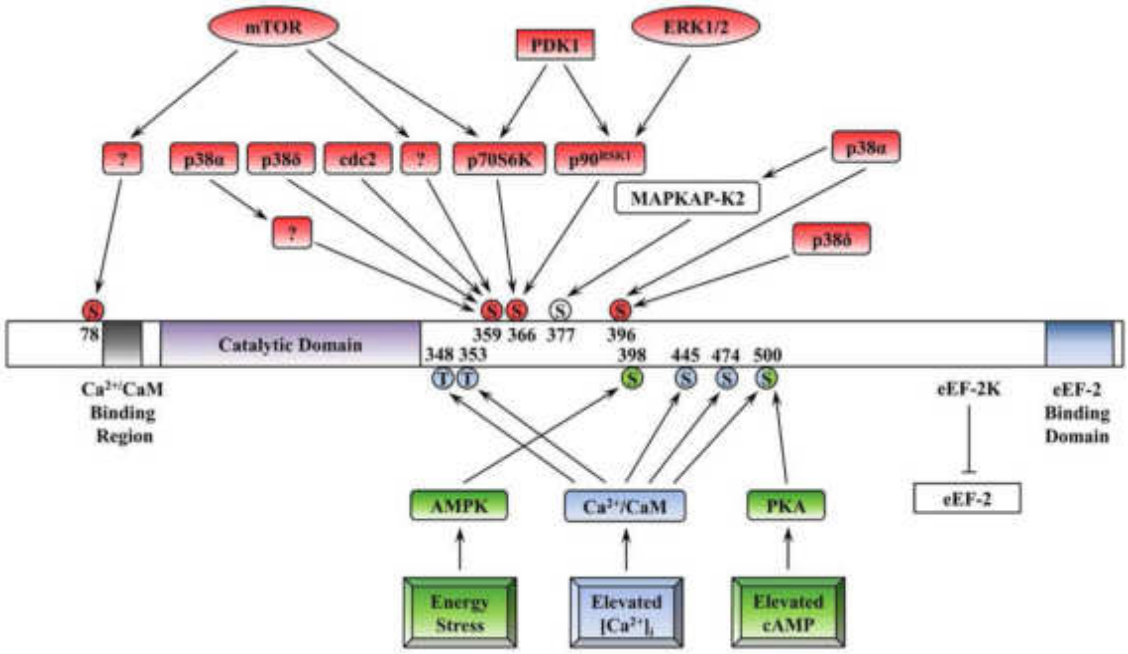

Figure 2. **Regulation of eEF2K activity by multisite phosphorylation in response to cellular stimuli.** The scheme summarizes the phosphorylated residues on eEF2K. The colour code suggests the effect of the molecule on eEF2K: the red ones are involved in negative regulation of eEF2K activity by an inhibitory phosphorylation, the green ones positively regulates eEF2K activity through activating phosphorylations and the blue ones are involved in eEF2K autophosphorylation (Scheme from Tavares et al., 2012)

2.1.3. eEF2K/eEF2 pathway and its regulation

eEF2K downregulates protein synthesis by phosphorylating eEF2 on Thr56 (from now to called P-eEF2 or phosphorylated eEF2) the only substrate of the kinase. More specifically, the phosphorylation prevents eEF2 binding to the ribosomes and it cannot catalyze the peptidyl-Transfer RNA (tRNA) translocation from the acceptor region to the peptidyl region, rendering eEF2 catalytically inactive and halting peptide chain elongation/protein translation at the elongation level (Diggle et al., 1999; Pigott et al., 2012).

eEF2K activity is antagonized by several phosphatases that reconvert P-eEF2 into eEF2, thus restoring peptide chain elongation. One of these phosphatases is protein phosphatase 2A (PP2A). The regulation of eEF2 activity and therefore protein chain elongation, is well controlled by an array of phosphatases and a single kinase, eEF2K (Browne and Proud, 2002).

2.1.4. eEF2K/eEF2 pathway in chemical synapses

Although eEF2K and eEF2 are ubiquitously expressed and, as described before, eEF2 phosphorylation is generally associated with a halt protein translation, in some particular cells, such as neurons, eEF2K/eEF2 pathway regulates the translation of some proteins in a particular manner.

Indeed, in neurons, it has been demonstrated that, even if eEF2 phosphorylation is also associated to a decrease in general protein translation, the expression of certain proteins located at the synaptic compartment is instead increased after eEF2K activation and eEF2 phosphorylation (Scheetz et al., 1997, 2000).

Because the Ca^{2+}-dependent activation of eEF2K in neurons occurs after glutamate receptors activation and other synaptic processes involve Ca^{2+} release (Breedlove et al., 2007; Sutton et al., 2007), it has been proposed a link between synaptic activation downstream processes and control of protein translation via eEF2K activity.

In several publication, for example, it has been demonstrated an inter-regulation between GluRs and eEF2K activity in different synaptic functions such as synaptic signal transduction and synaptic plasticity (Taha et al., 2013). eEF2K is involved in AMPARs endocytosis through two different pathways: one by upregulating MAP1b (Microtubule Associated Protein 1 b) (Davidkova and Carroll; 2007) and the other by the upregulation of Arc (activity-regulated cytoskeletal-associated protein) (Park et al., 2008). eEF2K pathway is also involved in dendritic spines maturation, mediating the upregulation of BDNF (Brain-derived neurotrophic factor) (Verpelli et al., 2010) and in synaptic plasticity, in mGluR-dependent-Long Term Depression (mGluR-LTD) (Park et al., 2008).

In synapses, the NMDA activation leads to Ca^{2+} dependent eEF2K activation, eEF2 phosphorylation and downregulation of protein synthesis, except for some protein

such as the α subunit of CaMKII which expression is instead increased (Scheetz et al., 1997, 2000). Thus synaptic spontaneous activity control protein synthesis at synapses by tonic NMDA dependent activation of eEF2K (Sutton et al., 2007). On the contrary, action potentials seem to reduce eEF2K activity, leading to an increase of protein translation (Taha et al., 2013). These data suggest that spontaneous and evoked neurotransmission regulate protein synthesis through eEF2K/eEF2 pathway.

eEF2K activity has a role in excitatory synapses, but also in inhibitory ones. In our laboratory, it has been demonstrated that eEF2K can modulate the GABAergic transmission. This issue will be examined in the next paragraph.

2.1.5. Characterization of *eEF2K*$^{-/-}$ mice

eEF2K/eEF2 pathway exists in every mammalian cell and is highly conserved (Kaul et al., 2011). As described before, it has been related to cellular processes, cytokine production and synaptic functioning in neurons (Kaul et al., 2011; Gonzalez-Teran et al., 2013; Leprivier et al., 2013; Taha et al., 2013, Dorovkov et al., 2002; Ryazanov, 2002).

Thus, it is quite surprising that eEF2K-knockout (*eEF2K*$^{-/-}$) mice do not show any obvious abnormal phenotypes in development and reproduction and that they are impaired only in a mild behavioural memory test (Ryazanov, 2002; Heise et al., 2017). Indeed Heise et al. demonstrated that *eEF2K*$^{-/-}$ are impaired in the contextual fear conditioning, but normal in many other memory and learning tests.

On the other hand, the *eEF2K*$^{-/-}$ mice have peculiar alterations in the synaptic properties that modify the excitation/inhibition balance in brain.

First, *eEF2K*$^{-/-}$ mice exhibit potentiated mIPSCs (miniature Inhibitory Post Synaptic Currents), both pre- and postsynaptically, but normal mEPSCs (miniature Excitatory Post Synaptic Currents), meaning that only inhibitory, but not excitatory transmission is changed. The enchanced GABAergic transmission correlates with the upregulation of Synapsin 2b and a specific increase of synaptic vesicle number in the GABAergic terminal. Second, in these mice there is also an upregulation of GABA-A Receptor-α5-subunit that causes a higher tonic inhibition (Heise et al., 2017). Thus, in wild type mice the eEF2K/eEF2 pathway modulates inhibitory synapses by downregulating

presynaptically the expression of synaptic vesicle-associated proteins like Synapsin 2b and postsynaptically by reducing the efficacy of GABAARs.

The potentiation of GABAergic synapses, obtained by eEF2K depletion, can be a potential therapy for epilepsy treatment, indeed many antiepileptic drugs enhance tonic inhibition which is lower in epileptic patient (Meldrum, 1989). Indeed Heise et al. demonstrated that genetic or pharmacological inhibition of eEF2K activity in a genetic mouse model of epilepsy, the Synapsin1 knockout mice (*Syn1*$^{-/-}$ mice) (Cambiaghi et al. 2013), can rescue the onset of epileptic seizures, even when triggered with chemoconvulsant drugs such as pentylenetetrazol (PTZ) or pilocarpine. Synapsin 1, as the other members of the family, is a vesicle-associated phosphoprotein involved in synaptic formation, function and plasticity (Valtorta et al., 2011). In patients, SYN1 gene deletion is associated with epilepsy, autism and aggressive behaviours (Cambiaghi et al. 2013). The onset of epilepsy in *Syn1*$^{-/-}$ mice is associated with a reduced amplitude of evoked inhibitory postsynaptic currents (PSCs) and a concomitantly increased amplitude of evoked excitatory PSCs (Chiappalone et al., 2009). Interestingly, *Syn1*$^{-/-}$ mice display a higher level of eEF2 phosphorylation in the hippocampus, suggesting a detrimental role of eEF2K/eEF2 pathway in the pathogenesis of the epileptic phenotype in these mice (Heise et al., 2017).

2.2 Epilepsy

Epilepsy is chronic disorder of the brain that affects 50 millions of people worldwide (WHO, updated February 2017). It is characterized by recurrent seizures (at least 2 unprovoked seizures or more), which are brief episodes of involuntary movement that may involve a part of the body (partial) or the entire body (generalized), and are sometimes accompanied by loss of consciousness and control of bowel or bladder function (ILEA, 2014). Seizure episodes are caused by an excessive electrical discharge in a group of brain cells that can originate from different parts of the brain. In epileptic patients, seizures can appear in various way, from the briefest lapses of attention or muscle twitch to severe and prolonged convulsions, and with different frequency, from less than 1 per year to several per day. Epileptic seizures can be associated to other symptoms depending on the site

where seizures arise such as loss of awareness or consciousness, and disturbances of movement, sensation (including vision, hearing and taste), mood, or other cognitive functions (WHO, updated February 2017).

According to a recent classification of International League Against Epilesy (ILEA), the etiology of epilepsy can be:

- Structural: when epilepsy is caused by a structural abnormality detected through neuroimaging techniques. At this group belong epilepsies occurred as a consequence of a stroke, a trauma or an infection that modifies brain structure;
- Genetic: when epilepsy is a direct consequence of a genetic mutation. An increasing number of *de novo* mutations are identified in both mild and severe epilepsies;
- Infectious: when epilepsy onset is linked to infective disease, such as meningitis, encephalitis, tuberculosis, HIV, cerebral malaria, subacute sclerosing panencephalitis, cerebral toxoplasmosis and congenital infections (i.e. Zika virus and cytomegalovirus);
- Metabolic, when epilepsy is a core symptom of a metabolic disorder that involves biochemical changes throughout the body such as porphyria, uremia, aminoacidopathies, or pyridoxine-dependent seizures;
- Immune, when epilepsy is associate with an autoimmune-mediated central nervous system inflammation. Some examples of known target for auto-antibodies are NMDAR and LGI1;
- Unknown, where are classified epilepsies with still unknown causes.

Moreover, epilepsy is in many case associated with comorbidities such as learning, psychological, and behavioral problems, ranging from learning difficulties to intellectual disability, to psychiatric disorders such as autism spectrum disorders and depression. Recognizing the etiology of the epilepsy and side symptoms are necessary for a successful therapy (ILEA, 2017).

About 70% of epileptic patients can control the onset of epileptic seizure through antiepileptic drugs (AED), but, even though the majority of them can conduct a normal life or even suspend the pharmacological treatment after some years, a part of patient can develop resistance to antiepileptic drugs. In addition, it has been estimated that 30-40% of epileptic patients do not respond to pharmacological therapy (Laxer et al., 2014). Since untreated recurrent epileptic seizures can damage brain leading to several psychiatric and neurological disorders, it is necessary to turn to others antiepileptic therapies (Hermann et al., 2002). In some well-defined cases, epileptic patients can be cured with surgery, such as resective surgery, corpus callosotomies or hemispherectomies; in other case patients can take advantage from neurostimulation (Laxer et al., 2014). Even the variety of available treatment, epileptic seizures in some patients are still untreatable, rendering the discovery of new target for antiepileptic treatment a necessity.

2.2.1. Dravet syndrome

Among genetic epilepsy syndromes, we focused our study on Dravet syndrome. Dravet syndrome, known also as severe myoclonic epilepsy of infancy (SMEI), arises at about 6 months of age with a prolonged generalised or hemiclonic febrile seizure and progresses to prolonged, clustered, or continuous seizures. Between the second and fifth years of life, patients develop other symptoms, which are psychomotor delay, ataxia, sleep disorder, autistic-like behaviours, and cognitive impairment (Dravet et al., 2005; Jansen et al., 2006). The long-term outcome of pathology is exacerbate by the difficulty in find an efficacious therapy for the patients, indeed Dravet syndrome is considered one of the most pharmacoresistant epilepsy syndrome (Chiron and Dulac, 2011). Dravet syndrome is also associated with a high rate of mortality related to status epilepticus or SUDEP (Sudden Unexpected Death in Epilepsy) that is very frequent in patients (Skluzacek et al., 2011).

Dravet syndrome is diagnosed in the 80% of patient with mutations (as represented in figure 3) in *Scn1a* gene that encodes for the α1 subunit of the voltage-gated sodium channel ($Na_V1.1$) (Claes et al., 2001; Mantegazza et al., 2005). Notably, mutations in *Scn1a* gene is also associated with milder phenotype defined as Genetic Epilepsy with

Febrile Seizures Plus (GEFS+) characterized by febrile seizures and mild to severe generalised epilepsies (Scheffer et al., 1997; Wallace et al., 2001). Other less common mutations associated with Dravet and Dravet-like syndromes involves GABRG2.8 and PCDH19 genes (Audenaert et al., 2006; Depienne et al., 2009).

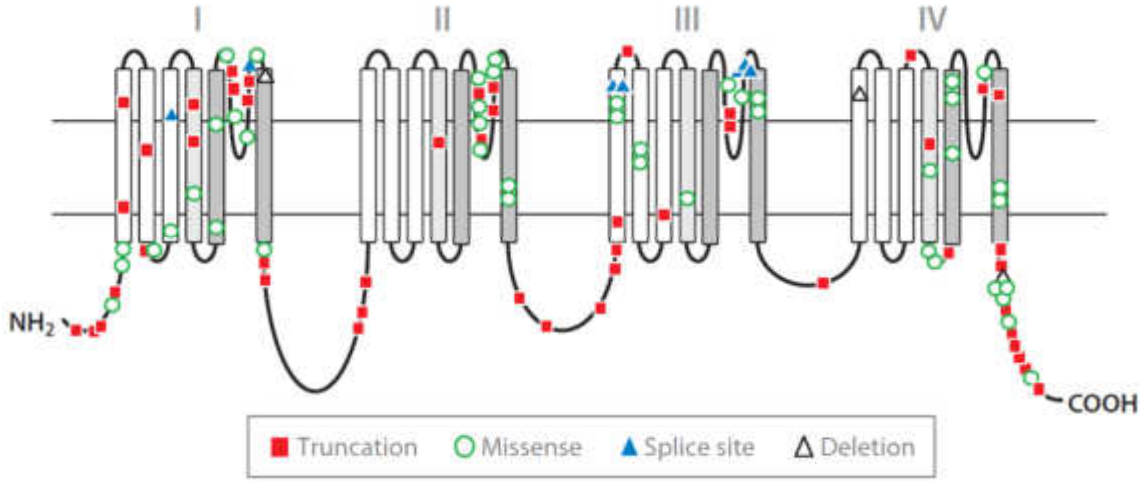

Figure 3. A schematic representation of mutations in $Na_V1.1$ in Dravet syndrome patients (adapted from Catterall, 2014)**.** A graphic summary of all known mutations discovered in Dravet syndrome patients and their relative localization in $Na_V1.1$ structure.

2.2.1.1. $Na_V1.1$ and Dravet syndrome

$Na_V1.1$ is one of the identified α subunit of sodium channels. Sodium channels are, indeed, formed by large α subunits of 260 kDa noncovalently associated to smaller β subunits of 30–40 kDa (Hartshorn and Catterall, 1984). Sodium channel α subunits are encoded by 10 genes, which are expressed in different excitable tissues (Goldin, 2001). In particular, $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.6$ are the more expressed genes in central nervous system.

As mentioned before, $Na_V1.1$ loss of function due to stop codons, deletions, or inactivating single residue mutations in the *Scn1a* gene is the well-recognized etiology of Dravet syndrome. $Na_V1.1$ is mainly expressed by GABAergic interneurons, in particular the parvalbumin-positive in developing neocortex and hippocampus. Loss of $Na_V1.1$ reduces sodium current in these interneurons and, thus, causes a loss of high-frequency firing of action potentials in hippocampal and cortical interneurons

(Ogiwara et al., 2007). Therefore, it is not surprising that $Na_V1.1$ haploinsufficiency, the common situation in patients, leads to a hyperexcitable neuronal network (Yu et al., 2006; Cheah et al., 2012; Liautard et al., 2013).

To better understand Dravet syndrome onset and progression, it has been generated mouse model, *Scn1a*$^{+/-}$ mouse, by deleting the last coding exon of S*cn1a* gene, which encodes domain IV downstream of the S3 segment and the cytoplasmic tail. While homozygous *Scn1a*$^{-/-}$ mice exhibit a severe form of ataxia that causes death within the postnatal day 15 (P15), the heterozygous mice mutation is sufficient to recapitulate in the murine model the main pathological features displayed by Dravet syndrome patients (Yu et al., 2006). First, *Scn1a*$^{+/-}$ mice are affected by spontaneous seizures from P21 that in some cases leads to death. Moreover, as well as Dravet syndrome patient, *Scn1a*$^{+/-}$ mice develop first epileptic seizures in the context of elevated body temperature (Oakley et al., 2009).

Scn1a$^{+/-}$ mice exhibit motor coordination impairment, indeed, Kalume et al. associated motor deficit with the reduction of about 58% of the amplitude of sodium currents in Purkinje neurons in *Scn1a*$^{+/-}$ mice (Kalume et al., 2007).

Finally *Scn1a*$^{+/-}$ mice, as well as patients, display also cognitive impairment, for example in spatial learning and memory, and autistic like traits, such as repetitive behaviours (Han et al., 2012).

2.2.1.2. Dravet syndrome therapy

Loss-of-function mutations in $Na_V1.1$ channels lead to an imbalance of excitation over inhibition in the brain. Thus, for a successful therapy, it is necessary to correct this unbalance by potentiating the GABAergic neurotransmission.

Commonly, in epilepsy treatment, one strategy consist in modulating sodium channels activity. Unfortunately, non-subtype-selective sodium channel–blocking antiepileptic drugs such as phenytoin, carbamazepine, and lamotrigine that are widely used to control epileptic seizures, exacerbate symptoms in Dravet patients (Guerrini et al., 1998; Chiron and Dulac, 2011). The activation of $Na_V1.1$ through $Na_V1.1$-selective sodium channel activators might be counter-productive because $Na_V1.1$ is also

expressed at low levels in excitatory neurons, thus, $Na_V1.1$ sodium channel activators could have proepileptic side effects.

GABAergic enhancement can be carried out also by the use of drugs that increase the concentration of GABA in the synaptic cleft by inhibiting its reuptake into nerve terminals and glia, such as tiagabine, or substances that increase the response of postsynaptic GABAA receptors to GABA, such as clonazepam (Chiron and Dulac, 2011). It has been shown that treating *Scn1a*$^{+/-}$ mice with a combination of tiagabine and clonazepam prevent the onset of epileptic seizure, even if thermally induced, and reduces the side effect of each compound (Oakley et al., 2013). Moreover, a low-dose of benzodiazepine is able to rescue also autistic-like behaviours in the mouse model of Dravet syndrome (Han et al., 2012).

Despite the standard treatment of Dravet syndrome patients consists in the combination of several antiepileptic drugs that enhances the GABAergic transmission, most patients are not seizure free, arising the necessity of new antiepileptic compound with novel molecular targets.

3. AIM OF THE STUDY

According to World Health Organization estimate, around 50 million people worldwide are afflicted by epilepsy. 70% of patients can efficiently control seizures through pharmacological or surgical treatments, but about 30% of patients do not still have an efficacious therapy (WHO, updated February 2017). The number of patients that are not responsive to therapies is dramatically increased in certain genetic epilepsy syndrome such as Dravet syndrome (Dravet et al., 2005; Chiron and Dulac, 2011). Untreated epileptic seizures and co-morbidities worsen the quality of life in patients, creating the exigency of new targets for the development of new antiepileptic drugs.

In a previous work of my laboratory, it has been demonstrated that eEF2K genetic deletion in mice leads to an enhancement in GABAergic transmission and, thus, mice were less susceptible to epilepsy seizures when triggered by proconvulsant drugs, such as pentylenetetrazol or pilocarpine. Moreover, it was also proven that eEF2K deficiency reduced the number of epileptic discharges in mouse model of epilepsy, *Syn1*$^{-/-}$ mice (Heise et al, 2017). These observations support that the eEF2K/eEF2 pathway may represent a possible therapeutic target for treating epilepsy. The aim of this study was to test this working hypothesis by studying whether eEF2K inhibition can rescue epileptic symptoms. In order to prove the potentiality of eEF2K inhibition in antiepileptic therapies, we tested the effect of eEF2K deletion in *Scn1a*$^{+/-}$ mice, a model of human Dravet syndrome.

We crossed *Scn1a*$^{+/-}$ mice with eEF2K$^{-/-}$ mice and we evaluated the outcome on epileptic seizures by electroencephalographic recordings and synaptic changes by electrophysiological analysis. Moreover, since it has been previously demonstrated that enhancing GABAergic transmission in *Scn1a*$^{+/-}$ mice rescued motor and cognitive deficits (Oakley et al., 2013; Han et al., 2012), we tested whether eEF2K deletion in the *Scn1a*$^{+/-}$ mice can rescue behavioural and motor symptoms typical of Dravet syndrome.

In order to develop possible pharmacological therapies we then tested two eEF2K inhibitors, JAN-384 and A-484954, for their efficacy in safely reducing eEF2 phosphorylation in brain in vivo.

These approaches will give us the possibility to test if eEF2K/eEF2 pathway is a potential new pharmacological target for the treatment of Dravet syndrome.

4. MATERIAL AND METHODS

4.1 Animals

Mice were housed under constant temperature (22 ± 1°C) and humidity (50%) conditions with a 12 h light/dark cycle, and were provided with food and water *ad libitum*. All experiments involving animals followed protocols in accordance with the guidelines established by the European Communities Council and the Italian Ministry of Health (Rome, Italy). Experimental procedures of EEG and behavioural analysis followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government decree No. 17/2013 and 980/2017. All efforts were made to minimize the number of subjects used and their suffering.

eEF2K$^{-/-}$ mice were provided by dr. Ryazanov as described in Ryazanov, 2002*; Scn1a*$^{+/-}$ mice were provided by dr. Mantegazza as described in Yu et al., 2006.

4.1.1. Mice genotyping

DNA was extracted from tails and analysed by PCR with the REDExtract-N-AmpTM Tissue PCR Kit (Sigma-Aldrich).

4.2. Electroencephalographic (EEG) analysis

4.2.1. Preparation of mice for electroencephalography analysis

10- to 12-week-old mice were anesthetized with chloral hydrate (Sigma-Aldrich, St. Louis, MO; 450 mg/kg) through intraperitoneal injection (i.p.). Four screw electrodes (Bilaney Consultants GMBH, Dusseldorf, Germany) plus one placed in the nasal bone as attachement were inserted In the skull (anteroposterior, +2.0–3.0 mm; left–right 2.0 mm from bregma) , as previously described (Manfredi et al., 2009) and in agreement to brain atlas coordinates (Franklin and Paxinos, 2008). The electrodes were connected through a support (Bilaney, Dusseldorf, Germany) and fixed with acrylic cement (Palavit, New Galetti and Rossi, Milan, Italy). Each mouse was allowed to recover for approximately 1 week under antimicrobial cover (ceftriaxone, Sigma-Aldrich; 50 mg/kg i.p.). EEG were recorded in awake freely moving mice placed in a Faraday chamber. The acquisition system used was Power-Lab digital acquisition system (AD Instruments, Bella Vista, Australia; sampling rate 100 Hz, resolution 0.2 Hz).

4.2.2. Electroencephalography analysis under baseline conditions

Basal cerebral activity recordings lasted 24 hours and eventual convulsions were recorded by a video camera put in the Faraday chamber. Epileptic discharges were recognized from EEG traces as an irregular event characterized by a high and rapid increase of the amplitude, at least 4,5 fold higher than the mean of the amplitude of the previously 200 ms. Segments with electrical noise or movements artifacts were excluded from statistical analysis (Manfredi et al., 2009).

4.2.3. Electroencephalography analysis under thermal stress

After EEG electrode implantation, mice body temperature was monitored and managed by a rectal temperature probe connected to a controller. Average mouse body temperature is 36.9°C and it was controlled by a heat lamp above the chamber. Mice were accustomed to the Plexiglas cage for at least 10 minutes at 37.5°C. Then, the body temperature was increased by 0.5°C every 2 minutes until a seizure occurred or 42.5°C was reached (Oakley et al., 2009).

4.3. Brain slice electrophysiology

4.3.1. Brain slice preparation

Hippocampal slices were prepared from P25–P35 mice using standard procedures. Briefly, mice were deeply anesthetized with isoflurane and decapitated. The brain was quickly removed and horizontal hippocampal slices (300 µm) were cut with a Vibratome in chilled (0–4°C) slicing solution containing 75 mM sucrose, 87 mM NaCl, 25 mM NaHCO3, 25 mM D-glucose, 2.5 mM KCl, 1.25 mM NaH2PO4, 0.5 mM CaCl2, 7.0 mM MgCl2, and pH 7.4. The slices were transferred to a storage chamber with the same solution and they were then incubated at room temperature for at least another 45 min before recording. All solutions were saturated with 95% O2 and 5% CO2. The slices were transferred to a recording chamber and perfuse at 28/30 °C with ACSF containing 129 mM NaCl, 3 mM KCl, 1.8 mM MgSO4, 1.6 mM CaCl2, 1.25 mM NaH2PO4, 21 mM NaHCO3, and 10 mM D-glucose, pH 7.4.

4.3.2. Brain slice recording

Whole-cell voltage-clamp recordings were performed on CA1 pyramidal neurons within hippocampal slices visualized under differential interference contrast (DIC)

optics, and near infrared illumination was used to identify individual neurons in a recording chamber located on an upright microscope. Patch electrodes were pulled from 1.5 mm outer diameter thin-walled glass capillaries in six stages on a Flaming-Brown micropipette puller. In the recordings of inhibitory postsynaptic currents (IPSCs), the patch electrodes were filled with intracellular solution containing CsCl (135 mM), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; 10 mM), ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; 0.2 mM), Mg-ATP (2 mM), ATP (4 mM), Phosphocreatine (10 mM), pH 7.2. Kynurenic acid (KA, 3 mM) was included in the recording solutions to block the excitatory synaptic transmission in IPSC recordings. The holding potential was -70 mV. When filled with intracellular solution, patch electrode resistance ranged from 3 to 5 MΩ. Recordings were obtained through a Multiclamp 700B amplifier (Molecular Devices) by the data-acquisition software (pCLAMP 8.0; Molecular Devices). Access resistance was continuously monitored for each cell. Only the cells with access resistance less than 20 MΩ were recorded, and recordings were terminated/discarded when a significant (>10%) increase occurred. Data from electrophysiology experiments were analyzed using Clampfit 10.5 (Molecular Devices) and Mini Analysis (Synaptosoft).

4.4. Behavioral tests

The behavioural test were performed on 3 months old male and female mice.

4.4.1. Spontaneous motor activity

The spontaneous motor activity was evaluated in an automated activity cage (43×43×32 cm) (Ugo Basile, Varese, Italy), located in a sound-attenuating room as previously described (Braida and Sala, 2000). Horizontal and vertical activity were detected by infrared photobeam break sensors put from 2.5 cm and 4 cm from the floor of the cage, respectively. Cumulative horizontal and vertical movements were counted for 3 hours.

4.4.2. Wire hanging test

The limb force was tested by positioning a mouse on the top of a wire cage lid (19 x 29 cm) that was turned upside down at approximately 25 cm above the surface of the bedding material. The grip of the mouse was ensured by gently waving three times

before rotating the lid as described in Olivan et al. (2015). The latency to fall onto the bedding was registered (cut-off= 300 s).

4.4.3. Pole test

Pole test evaluated motor coordination in mice. Mice were trained for 2 days (in the morning and in the afternoon) to descend a pole (90 cm length, 1 cm diameter). Every training consisted of three trials. Mice were accustomed to the room 20 minutes before trials and test. The test was performed the third day: the experimenter measured the time necessary to the mice in climbing down the pole until 60 seconds in five trials (Rial et al., 2014, with minor modifications).

4.4.4. Novel Object Recognition test

The novel-object recognition test was performed in an open plastic arena (60 × 50 × 30 cm), as previously described (Pan and Xia, 2008). The test had 3 phases: the habituation on the first day, when mice were accustomed to the test arena for 10 min, the familiarization and novel-object recognition the day after. In the familiarization phase, two identical objects were placed in the middle of the arena equidistant from the walls and from each other. Mice were placed between the two objects until it had completed 30 s of cumulative object exploration (20 minutes cut-off). Experimenter measured the time mice was within approximately 1 cm of an object with its nose toward the object. Climbing the object or pointing the nose toward ceiling near the object were not considered exploring behaviours. After familiarization, mice returned to the home cage until they were tested for novel recognition after 5 min, 120 minutes or 24 h. In the novel recognition phase, a novel object (never seen before) took the place of the one more explored in the familiarization phase. Scoring of object recognition was performed as during the familiarization phase. For each mouse, the role (familiar or new object) as well as the relative position of the two objects were randomly permuted. The objects used for the test was white plastic cylinders and coloured plastic Lego stacks of different shapes. The arena was cleaned with 70% ethanol after each trial. Performance was analyzed by calculating a discrimination index (N-F/N+F), where N = the time spent exploring the novel object, and F = the time spent exploring the familiar object.

4.4.5. Spatial object recognition test

Spatial object recognition test was performed in an arena according to Kenney et al. (2011), with minor modifications that consisted in an opaque white Plexiglass cage (58×50×43 cm) that was dimly lit from above (27 lux) and two visual cues were placed above two adjacent walls. In the center of the northern wall there was a black and white striped pattern (21×19.5 cm) and in the center of the western wall there was a black and gray checkered pattern (26.5×20 cm) and the object were placed across the visual cues. Mice were habituated to the arena for 10 minutes the day before the test.

At time 0, mice were allowed to familiarize with two different objects. The experimenter measured the time spent in sniffing both objects until the mouse completed 30 seconds in exploring objects (cut-off 20 min). Exploring behavior was defined as mouse having its nose directed toward the object and within approximately 1 cm of the object (Bevins and Besheer, 2006); climbing or sitting were not considered exploration behaviours. After 5 minutes, 120 minutes or 24 hours, mice were allowed to re-explore the cage where the object more explored the previous session was moved. The experimenter measured the time spent in sniffing both objects until the mouse completed 30 seconds in exploring objects (cut-off 20 min). Between two sessions, mice returned to their home-cage. Cage and object were carefully cleaned with acetic acid (0.1%) before and after all behavioral procedures. Performance was analyzed by calculating a discrimination index (N-F/N+F), where N = the time spent exploring the moved object during the test, and F = the time spent exploring the unmoved object during the test.

4.4.6. Repetitive self grooming

The spontaneous self grooming behaviour was assayed as described in McFarlane et al., 2008. Single mouse was placed into a standard cylinder (46 x 23.5 x 20 cm). Cylinders were empty to eliminate digging in the bedding, which is a potentially competing behaviour. The room was illuminated at about 40 lux. A front-mounted CC TV camera (Security Cameras Direct) was placed at circa 1 m from the cages to record the sessions. Sessions were video-taped for 20 min. The first 10 min of habituation was

not scored. Cumulative time spent grooming all the body regions during the second 10 min of the test session and the total number of grooming episodes was measured.

4.4.7. Sociability and preference for social novelty tests

The sociability tests were performed in a rectangular apparatus in transparent polycarbonate with three-chamber (width = 42.5 cm, height = 22.2 cm, central chamber length = 17.8 cm, and side chamber lengths = 19.1 cm) as previously described in Sala et al. (2011). In the 10-minutes habituation phase, each mouse was placed in the middle compartment and free to explore all chambers. Then, one side of the compartment was occupied from a DBA/2J unfamiliar male mouse and the other contained an empty wire cage. Immediately after sociability test, without cleaning the apparatus, it was performed the social novelty test putting an unfamiliar mouse in the empty wire cage. Every test lasted 10 minutes in which were measured the time spent exploring each chamber. The data were expressed in sociability index (SI) and social novelty preference index (SNI) as follows: SI = (time exploring novel mouse 1 – time exploring empty cage) / (time exploring novel mouse 1 + time exploring empty cage) and SNI = (time exploring novel mouse 2 – time exploring familiar mouse) / (time exploring novel mouse 2 + time exploring familiar mouse).

4.4.8. Tube test

The tube test assayed the aggressive behaviours of mice as described in Zhou et al. (2016). During the three days preceding the test, mice were accustomed to enter a transparent, Plexiglass tube (20 cm length, 3 cm diameter). 20 minutes before the test mice were habituated to the room. Two mice that did not have previous contact were placed at the extremes of the tube and reached the centre autonomously. The experimenter measured the time occurred to one mouse to move back to tube limit and exit with all four limbs (6 minutes cut-off), that was considered the loser mouse. Every mouse fought several matches with 60 minutes among matches.

4.4.9. Elevated plus maze

The Elevated Plus Maze paradigm was used to study anxiety related behavior. The apparatus had a central platform (10 cm x 10 cm) from which originated two opposite open arms (30 cm x 10 cm) and two enclosed arms (30 cm x 10 cm x 14 cm) according

to Lister (Lister, 1987). The apparatus was made of white wood, placed to a height of 60 cm above floor level in the center of a small quiet room under dim light (about 30 lux). The test was conducted in the morning, during the early light phase of the light cycle. After 20 min of familiarization to the novel environment, mice were placed individually onto the center of the apparatus, facing an open arm. Experimenter recorded for 5 minutes the number of open- and closed-arm entries and the time spent in open and closed arms.

4.4.10. Tail suspension test

Tail suspension test assayed depressive-like behaviours of mice as described in Steru et al., (1985). The test was preceded by a familiarization phase where mice were left in the room test at least 1 hour before. Tail suspension test was performed on an apparatus with a support at 35 cm from the basis where was fixed a hook where mouse tail was fasten at about 1 cm from the origin. The test lasted 6 min during which the experimenter measured time of mouse immobility.

4.5. Biochemistry protein analysis

4.5.1 Sample preparation

Brain areas were lysated in precooled "sucrose buffer" (0.32 M sucrose and 4 mM Hepes (Sigma Aldrich), pH 7.4, protease and phosphatase inhibitors (Roche)) and quantified the protein concentration through Bradford protein assay (Quick Start™ Bradford Protein Assay from Bio-Rad). A portion of total homogenate was collected and solubilized in loading dye 4X (250 mM Tris, 40 % glycerol, 0.008 % bromophenol blue; all Sigma-Aldrich), the remaining underwent to a fractionation process in order to obtain P2 fraction (enriched in excitatory pre- and post-synaptic components) as described in Huttner et al., 1983) that was quantified and solubilized as total homogenate. The P2 fraction resulted from a centrifugation of the total homogenate at 800 x *g* for 10 minutes at 4°C, followed by a centrifugation at 10000 x *g* for 15 minutes at 4°C of the supernatant obtained in the previous step. All samples were boiled at 95°C for 10 minutes. 7 µg of samples were loaded in the pockets of 7,5% polyacrylamide gels (home made with reagents from Bio-Rad) and proteins were

electrophoretically separated by sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE).

4.5.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotting

Proteins were electrophoretically seperated by SDS-PAGE under denaturing conditions (Laemmli, 1970) and electroblotted onto a nitrocellulose membrane using the Trans-Blot Turbo System (Bio-Rad). Then, the efficiency in protein transfer were ensured by staining the membrane with Ponceau S Stain (Sigma-Aldrich) and, after, membranes were washed in Tris-buffered saline-Tween (TBS-T) (20 mM Tris pH 7.4, 150 mM NaCl (both Sigma-Aldrich), and 0.1% Tween 20 (Bio-Rad)).

4.5.3. Western Blot analysis

After blocking membranes for at least 1 hour at room temperature with 5% Bovine Serum Albumin (BSA) in TBS-T, they were incubated with the primary antibodies in TBS-T containing 3% BSA for 2-3 hours at room temperature or overnight at 4°C. The membranes were washed three times with TBS-T and then incubated HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) in TBS-T and 3% BSA for 1 hour at room temperature. After three washes (10 min each), chemiluminescence was induced using an ECL kit (GE Healthcare) and visualized on X-ray films (GE Healthcare). Then, the films were scanned and the band intensity was analysed by ImageJ (US National Institutes of Health).

eEF2 phosphorylation was measured by calculating the ratio between P-EF2 (rabbit, Cell signalling, 1:2000) and total eEF2 (rabbit, Cell signalling, 1:500).

4.6. Preparation and neuronal differentiation of iPS cells

Peripheral blood mononuclear cells (PBMC) were isolated from human blood samples using Ficoll and growth in StemPro®-34 SFM Medium (Thermo Fisher Scientific), supplemented with L-Glutamine (2 mM), PenStrep (1%), SCF (100 ng/mL), FLT-3 (100 ng/mL), IL-3 (20 ng/mL), IL-6 (20 ng/mL), all from Thermo Fisher Scientific. To generate iPSC, PBMCs were reprogrammed with 2.0 Sendai virus, containing four Yamanaka factors using the integration-free CytoTune-iPS Sendai Reprogramming Kit (Thermo Fisher Scientific). After seven days, transduced

cells were plated on matrigel-coated cultures dishes and grown with Essential 8 medium (Thermo Fisher Scientific). Three to four weeks after the infection, iPSC colonies were picked and transferred onto matrigel-coated culture dishes (Corning) for further expansion or analysis. Then iPSCs were differentiated to neural stem cells (hNSC) using the Neural Induction kit (Thermo Fisher Scientific); proliferating hNSCs were differentiated into neurons by plating Neurobasal medium supplemented with B27 w/o vitA (2%, Thermo Fisher Scientific), PenStrep (1%, Thermo Fisher Scientific), Glutamax (2 mM, Thermo Fisher Scientific), NT-3 (10 ng/mL, Miltenyi Biotec), BDNF (10ng/mL, Miltenyi Biotec), GDNF (10 ng/ml, Miltenyi Biotec), Retinoic Acid (1 uM, Sigma-Aldrich). Cells were growth for 50 days changing the medium every 2-3 days. Neuron maturation was assayed by MAP2 expression (mouse Abcam, 1:500) through immunofluorescence labelling.

4.7. eEF2K inhibitors preparation and administration

For *in vitro* experiments, JAN-384 was dissolved in DMSO at a concentration of 5 µM.

For *in vivo* experiments, JAN-384 or A-484954 were dissolved in DMSO by sonicating for 30 minutes at 37°C and after Polyethylene glycol (PEG) was added to reach the ratio 10% DMSO + 90% PEG. Drugs were administered once a day with intraperitoneal injection to wild type mice.

4.8. Data analysis and display

Data is represented as means ± SEM or percentage. Statistical analysis and graphic design were carried out by Prism 5 software (GraphPad, San Diego, CA). The accepted level of significance was $p \leq 0.05$.

Figures were generated with Microsoft Office PowerPoint for Windows (Microsoft) in combination with Adobe Photoshop 6.0 for Windows (Adobe).

4.9. Acknowledgement of experimenters and corresponding institutions

Cross breeding to get *Scn1a*$^{+/-}$ *eEF2K*$^{-/-}$mice was carried out by Dr. Caterina Montani (CNR Institute of Neuroscience and BIOMETRA, University of Milan, Milan, Italy). Behavioural tests, EEG recordings and their analysis was carried out by Dr. Luisa Ponzoni (CNR Institute of Neuroscience and BIOMETRA, University of Milan, Milan,

Italy). Electrophysiological experiments was carried out by Dr. Paolo Scalmani (Department of Neurophysiology and Diagnostic Epileptology, IRCCS Foundation C. Besta Neurological Institute, Milan, Italy,). Dr. Carlo Sala and Dr. Chiara Verpelli (CNR Institute of Neuroscience and BIOMETRA, University of Milan, Milan, Italy) provided expertise for the biochemical experiments of the study and corrected the text. Dr. Mariaelvina Sala (CNR Institute of Neuroscience and BIOMETRA, University of Milan, Milan, Italy) provided expertise for behavioural experiments and the EEG analysis of the study. Dr. Paolo Scalmani and Dr. Massimo Mantegazza (Institute of Molecular and Cellular Pharmacology, Centre National de la Recherche Scientifique and Université Côte d'Azur, Valbonne, France) provided the expertise for the electrophysiological analysis and gave us *Scn1a*$^{+/-}$ mice.

5. RESULTS

5.1. Generation of double *Scn1a*$^{+/-}$ /*eEF2K*$^{-/-}$ mice

The first aim of this study was to test if the deletion of the eEF2K gene in the murine genetic model of Dravet syndrome could rescue epileptic phenotype. We used a mouse that carried a dominant mutation in *Scn1a* gene as described in Yu et al. (2006) and mimicked the Dravet syndrome described in human patients. To obtain the deletion of eEF2K in this model, we crossed *Scn1a*$^{+/-}$ mice with *eEF2K*$^{-/-}$ mice (generated by Ryazanov). As expected by the Mendelian law, in the first generation we obtained 50% of the mice with the genotype *Scn1a*$^{+/+}$ *eEF2K*$^{+/-}$ and 50% with the genotype *Scn1a*$^{+/-}$ *eEF2K*$^{+/-}$. We then crossed the *Scn1a*$^{+/-}$ *eEF2K*$^{+/-}$ mice and, among the genotype described in the figure 4, we obtained the three genotypes used for our studies: *Scn1a*$^{+/+}$ *eEF2K*$^{+/+}$ (from now called WT mice), *Scn1a*$^{+/-}$ *eEF2K*$^{+/+}$ mice (from now called *Scn1a*$^{+/-}$ mice*)* and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice.

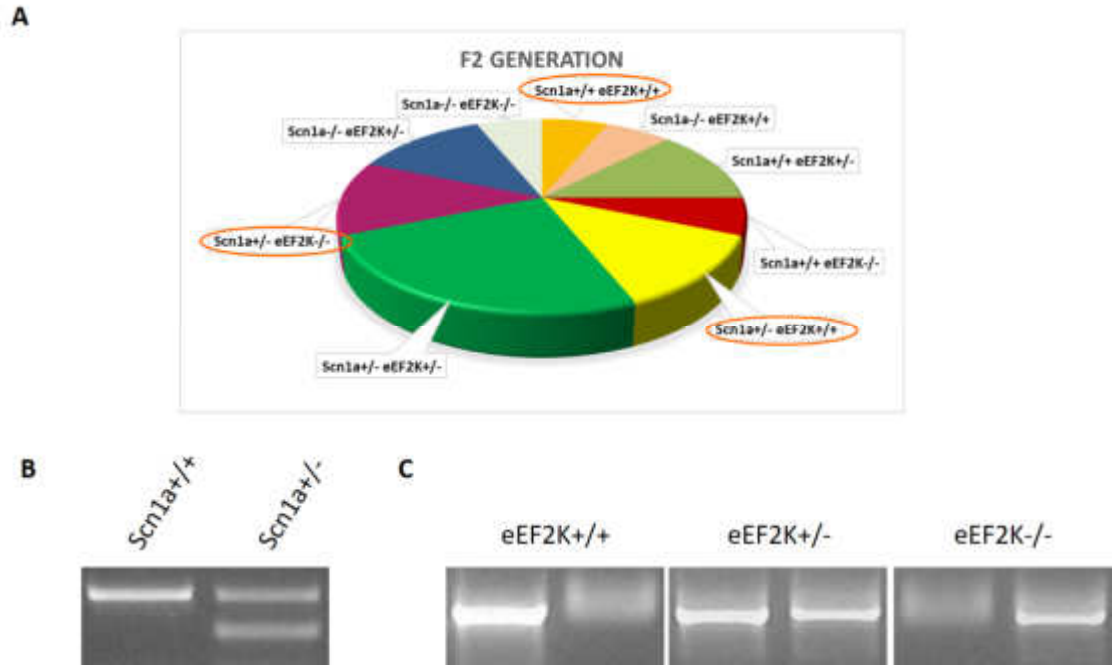

Figure 4. The generation of WT, *Scn1a*$^{+/-}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$mice. (A) The pie chart represents the various genotypes resulting by the crossing of *Scn1a*$^{+/-}$ *eEF2K*$^{+/-}$ mice. The ones used in our study are circled in orange. (B) A representative image of PCR for *Scn1a* gene. WT displayed a single band of 300 bp, heterozygous mice the one at 300 bp and another band at 150 bp. (C) A representative image of PCR for *eEF2K* gene. Since the length of the bands for WT and KO is 1,2 kb for both, we

perform two different PCRs for WT and KO genes. WT displayed a single band of 300 bp, heterozygous mice the one at 300 bp and another band at 150 bp.

5.1.1. Mice survival

We first monitored the survival of newly generated genotype. In a 5-months period of observation, there was no difference in the survival of the 3 groups of mice used in this study (WT, *Scn1a*$^{+/-}$ and *Scn1a+/-/eEF2K-/-*mice) after the weaning at Post natal day 21 (P21) (Fig. 5).

We monitored also *Scn1a*$^{-/-}$ mice survival in order to define if eEF2K depletion is able to increase the survival rate of *Scn1a*$^{-/-}$ mice. As described in literature, *Scn1a*$^{-/-}$ mice died within P15 because of a severe form of ataxia that prevent feeding (Yu et al., 2006). Also double *Scn1a*$^{-/-}$ *eEF2K*$^{-/-}$mice died within P15, meaning that eEF2K deficiency was not able to rescue the ataxia presented by the *Scn1a*$^{-/-}$ mice (data no shown).

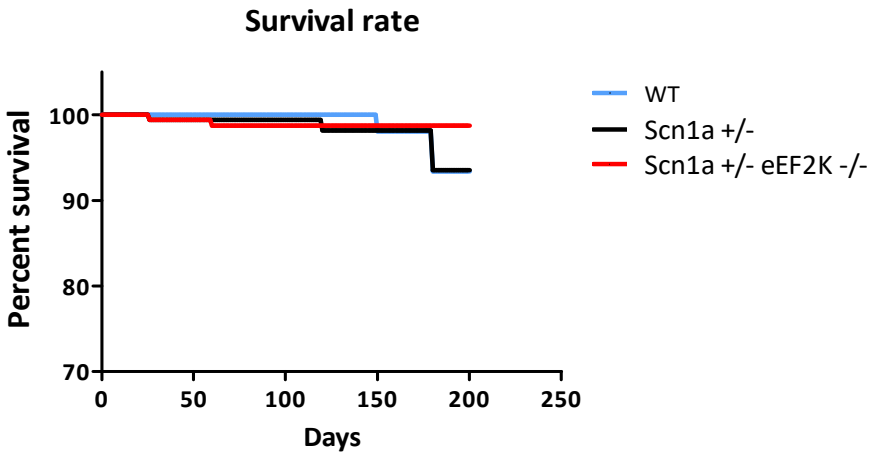

Figure 5. The survival rate of WT, *Scn1a*$^{+/-}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ *mice*. There were no difference in the survival among the three groups object of the study.

5.2. eEF2K deletion rescues the epileptic phenotype in *Scn1a*$^{+/-}$ mice by the potentiation of GABAergic transmission

5.2.1. eEF2K deficiency protects Dravet mice from epileptic seizures onset

The recurrent epileptic seizures are one of the major symptoms of Dravet syndrome, thus, we investigated the outcome of eEF2K deficiency on epileptic symptom. We recorded electroencephalographic traces from male mice for 24 hours and we counted the number of epileptic discharges that *Scn1a*$^{+/-}$ mice and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$

mice displayed compared to WT mice. *Scn1a*$^{+/-}$ mice displayed an increased number of epileptic discharges in basal condition compared to WT mice and double *Scn1a*$^{+/-}$ */eEF2K*$^{-/-}$ mice, suggesting that eEF2K depletion was able to reduce the number of epileptic episodes (Fig. 6A, left side). In Dravet syndrome, the onset of the epileptic seizures is triggered by an episode of fever and, usually, the following seizures arise without thermal stimuli (Jansen et al, 2006). This feature is maintained in *Scn1a*$^{+/-}$ mice (Oakley et al., 2009), therefore we triggered the convulsions by increasing mice body temperature and we counted the number of epileptic discharges 7 days after. The thermal stress induced epileptic episodes in *Scn1a*$^{+/-}$ mice (3.8 ± 1.7, mean ± SEM) whereas double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice behave as WT mice (0.5±0.5, 0.1±0, respectively), meaning that eEF2K deficiency reduced the susceptibility to epilepsy in Dravet mice (Fig. 6A, right side). Furthermore, eEF2K depletion significantly increased the minimum temperature necessary to trigger epileptic seizures (Fig. 6B).

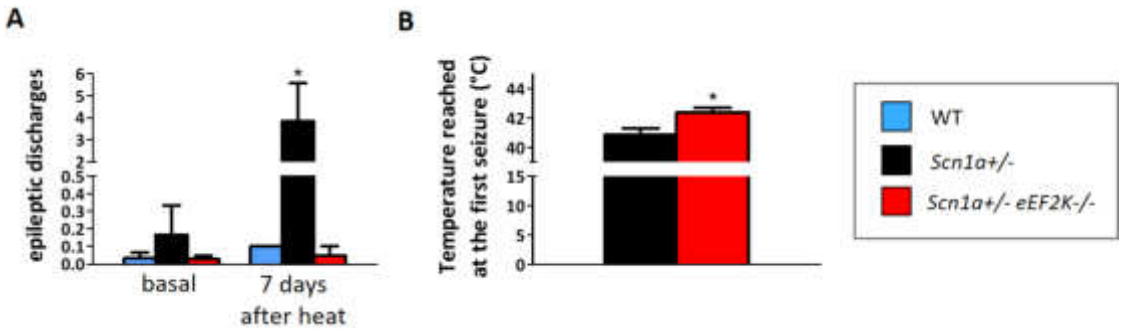

Figure 6: EEG recordings. (A) Number of epileptic discharges both in basal condition and 7 days after thermal stress in 24 hours. Data are presented as mean ± SEM. **p*< 0.05 versus corresponding WT; Two way ANOVA, Bonferroni test. (B) Temperature reached at the first seizure. Data are presented as mean ± SEM. **p*< 0.05 versus corresponding *Scn1a*$^{+/-}$; Student's t-test.

5.2.2. eEF2K deficiency enhances GABAergic transmission in *Scn1a*$^{+/-}$ mice

A hyperexcitable neuronal network is one of the major cause of the epileptic seizures in Dravet syndrome (Liautard et al., 2013). Given that *eEF2K*$^{-/-}$ mice GABAergic transmission is potentiated (Heise et al., 2017), we hypothesized that double *Scn1a*$^{+/-}$ */eEF2K*$^{-/-}$ mice could be less susceptible to develop epileptic seizure because of the potentiation of their GABAergic synaptic transmission measured against *Scn1a*$^{+/-}$ mice.

To verify this hypothesis, we recorded spontaneous inhibitory postsynaptic currents (sIPSCs) in the CA1 region of the hippocampus on brain slices from WT, *Scn1a*$^{+/-}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$mice. In the previous work, in experiments on *eEF2K*$^{-/-}$mice, we recorded mIPSCs, that are currents observed in the absence of presynaptic action potentials that are blocked with TTX (tetradotoxin); mIPSCs correspond to the response that is provoked by a single vesicle of transmitter (Pinheiro and Mulle, 2008). In this project, we decided to record related but different currents, sIPSCs, that are currents generated by action-potential-dependent and –independent release of neurotransmitter in the absence of experimental stimulation (Pinheiro and Mulle, 2008). We chose to record in the hippocampal region because it has been demonstrated that the onset of epileptic seizures in *Scn1a*$^{+/-}$ mice strongly involves the hippocampus and its hyperexcitable network and, thus, this area has been suggested as a targets for screenings of antiepileptic approaches (Liautard et al., 2013).

As expected, *Scn1a*$^{+/-}$ mice displayed reduced amplitude and instantaneous frequency in the recorded sIPSCs compared to *Scn1a*$^{+/+}$ mice, but eEF2K depletion enhances both amplitude and instantaneous frequency of sIPSC of *Scn1a*$^{+/-}$ mice. The rescue driven by eEF2K depletion was partial, indeed double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice showed increased iPSC amplitude and instantaneous frequency compared to *Scn1a*$^{+/-}$ mice, but these values were still significantly smaller compared to the value measured in WT mice (Fig. 7).

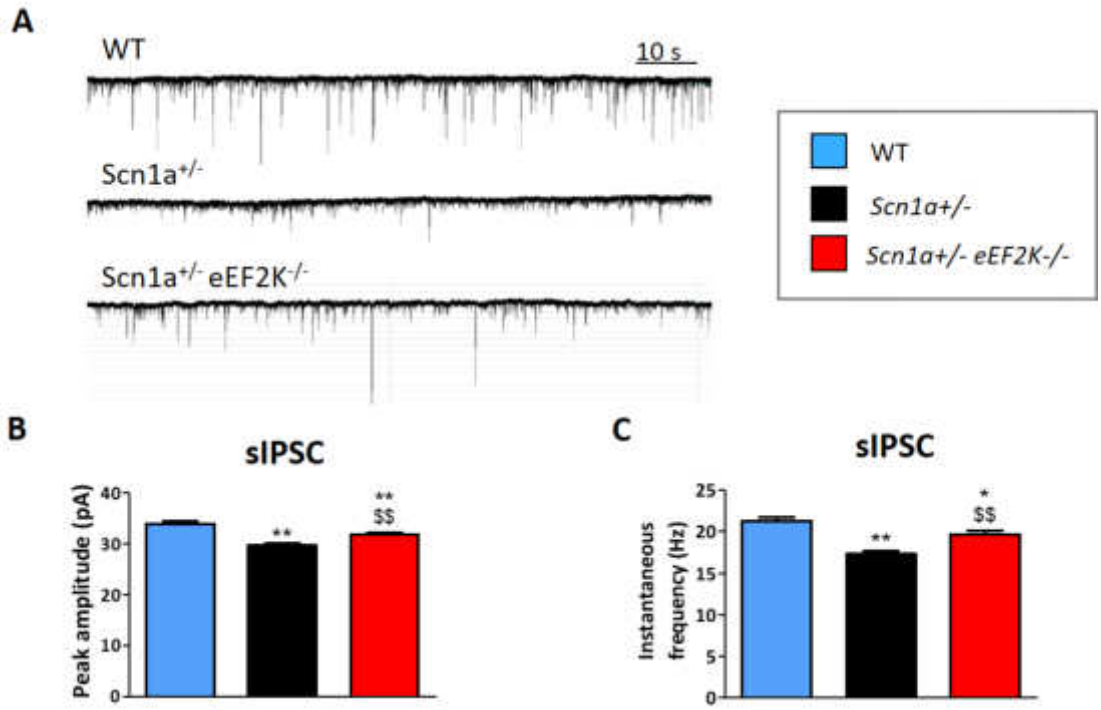

Figure 7. sIPSC in the CA1 region of the hippocampus of WT, *Scn1a*$^{+/+}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice. (A) A representative trace of sIPSC for each considered genotype. (B,C) Quantification of the peak amplitude (B) and the istantaneous frequency (C). All data are presented as mean ± SEM. **p*< 0.05, ***p*< 0.01 versus corresponding WT; $$ *p*< 0.01 versus *Scn1a*$^{+/-}$; One way-ANOVA, Tukey's test.

5.3. eEF2K deletion recovers motor and cognitive impairments in *Scn1a*$^{+/-}$ mice

5.3.1. eEF2K deprivation ameliorates motor coordination in *Scn1a*$^{+/-}$ mice

Motor deficits are common in Dravet syndrome: most of the patient are affected by ataxia, dysarthria, intention tremor and eye movement disorder (Genton et al., 2011). This feature is replicated in *Scn1a*$^{-/-}$ mice: the animals are so compromised that cannot feed themselves. NaV1.1 haploinsufficiency in mice leads to a milder motor phenotype that allows mice survival, but compromise *Scn1a*$^{+/-}$ mice performance (Yu et al., 2006; Han et al., 2012).

We tested WT, *Scn1a*$^{+/-}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice for motor function with the spontaneous motor activity test, the limb force with the wire hanging test and the motor coordination with the pole test, balance beam test and rotarod test.

First, we recorded horizontal and vertical movements of freely moving animals for 3 hours (Fig. 8A.). WT and *Scn1a*$^{+/-}$ mice movements in horizontal and vertical directions were similar, meaning that *Scn1a*$^{+/-}$ mice had no difficult in walking or climbing. Also double *Scn1a*$^{+/-}$ *eEF2K*$^{-/-}$ mice did not display difficulty in movements, even if the horizontal movements, unexpectedly, were significantly higher compared to the other groups of animals (Fig. 8A).

We also observed the latency to fall in wire hanging test as measure of limb muscle strength. The three groups of animals had no difference, meaning that muscle strength was not compromised in these animals (Fig. 8B). Finally, we assayed the motor coordination of these animals with the pole test as a representative test (the other ones confirmed the results obtained from the pole test). Measuring the latency to turn downwards and descend the pole in this test we observed that *Scn1a*$^{+/-}$ mice took longer time than WT mice (43,3±6,3; 13,2±5,1 respectively). Interestingly double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice behaved as WT mice, meaning that deletion of eEF2K was able to recover the motor coordination defect in the *Scn1a*$^{+/-}$ (Fig. 8C).

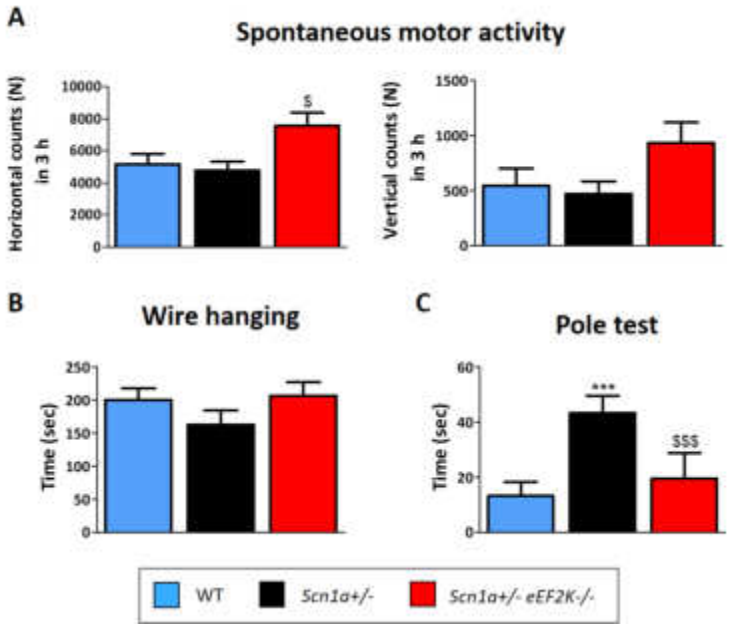

Figure 8. An array of motor test performed on WT, *Scn1a*$^{+/-}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice. (A) Counting of horizontal (left graph) and vertical (right graph) movements occured in 3 hours in the spontaneous motor activity test. (B) Latency to fall in wire hanging test measured over 300 seconds. (C) Time taken to complete the

task in pole test. (A – C) All data are presented as mean ± SEM. ***p< 0.001 versus corresponding WT; $ p< 0.05; $$$ p< 0.001 versus *Scn1a*$^{+/-}$; One way-ANOVA, Tukey's test.

5.3.2. eEF2K deficiency ameliorates episodic and spatial memory

Another typical trait of Dravet syndrome is cognitive impairments (Dravet et al., 2005; Jansen et al., 2006).

We tested mice for episodic and spatial memory using novel object recognition test and spatial object recognition test, respectively. Mice had to recognize the unfamiliar or displaced object after 5 minutes, 120 minutes or 24 hours from familiarization phase.

In the novel object recognition test *Scn1a*$^{+/-}$ mice showed a reduced discrimination index when tested 5 min, 120 min and 24 hour after familiarization compared to WT, meaning that *Scn1a*$^{+/-}$ mice were unable to recognize the new object as unfamiliar. On the contrary, double *Scn1a*$^{+/-}$ */eEF2K*$^{-/-}$mice, as well as WT mice, displayed a strong preference for the unfamiliar object, implicating that eEF2K deletion had a beneficial effect on *Scn1a*$^{+/-}$ mice episodic memory (fig. 9A). In the spatial object recognition test *Scn1a*$^{+/-}$ mice displayed preference for the stationary object, showing a negative discrimination index. This cognitive impairment was rescued by the deletion of eEF2K because the double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ performed in the test similarly to the WT mice, spending more time exploring the displaced object (fig 9B).

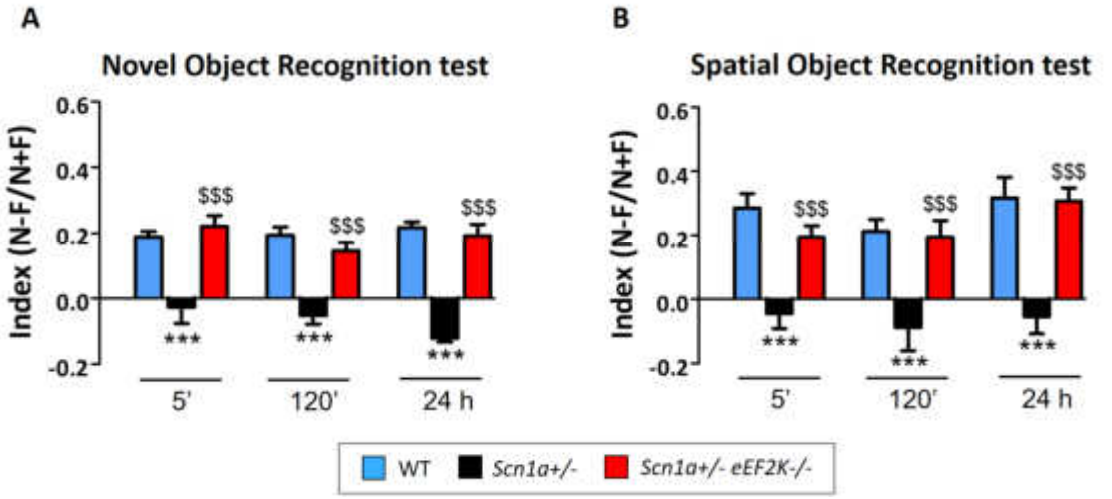

Figure 9: Memory tests: novel object recognition test and spatial object recognition test. (A, B) Discrimination index evaluated in the novel object recognition test (A) and in the spatial object recognition test (B). All data are presented as mean ± SEM. ***p< 0.001 versus corresponding WT; $$$ p< 0.001 versus *Scn1a*$^{+/-}$; Two way ANOVA, Bonferroni test.

5.3.3. eEF2K deletion can rescue the stereotyped behaviour, social novelty preference, but not the aggressive/dominant comportment

Dravet syndrome patient are affected also by important and diverse patient-related co-mobility symptoms such as hyperactivity, anxiety and autism spectrum disorders. Behavioural tests demonstrated that some of these stereotypic and social alterations are present in the *Scn1a*$^{+/-}$.

We measured stereotyped behaviours by self-grooming test, counting the number of episodes and the time spent in this activity. We observed that *Scn1a*$^{+/-}$ mice spent more time in self-grooming than WT mice, but eEF2K deletion in the *Scn1a*$^{+/-}$ mice reduced both the number of episodes and the time occupied in self-grooming to value similar to the WT mice (Fig. 10A).

We then analysed the social behaviour of WT, *Scn1a*$^{+/-}$ and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice using the sociability and social novelty preference test. *Scn1a*$^{+/-}$ mice exhibited more interest for the unfamiliar mouse than for the empty cage, as well as WT and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice, demonstrating a normal attitude in social interaction. However, in the social novelty test, *Scn1a*$^{+/-}$ mice, differentially to WT mice, spent the same time in exploring familiar and unfamiliar mice, indicating an impairment in the

social recognition. Interestingly the deletion of eEF2K in the *Scn1a*$^{+/-}$ was able, but not significantly, to rescue this behavioural alteration because double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice remained closer to the unfamiliar stranger, similarly to WT mice (Fig. 10B).

We tested the three groups of animals for aggressive/dominant behaviour with the tube test. *Scn1a*$^{+/-}$ mice won 60% of matches against WT mice. Also double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice won 60% matches against WT mice and the number of won matches between *Scn1a*$^{+/-}$ mice and double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice were similar, as well as the latency to win the match, indicating that *Scn1a*$^{+/-}$ mice, but also double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice, were more aggressive/dominant compared to WT mice. Thus, eEF2K deletion was unable to lead to an improvement or rescue of this behavioural phenotype (Fig. 10 C-E).

Finally we tested *Scn1a*$^{+/-}$ mice also for anxiety-like and depression like behaviour through the elevated plus maze test (Fig. 10F) and the tail suspension test (Fig 10G), respectively, no differences were found.

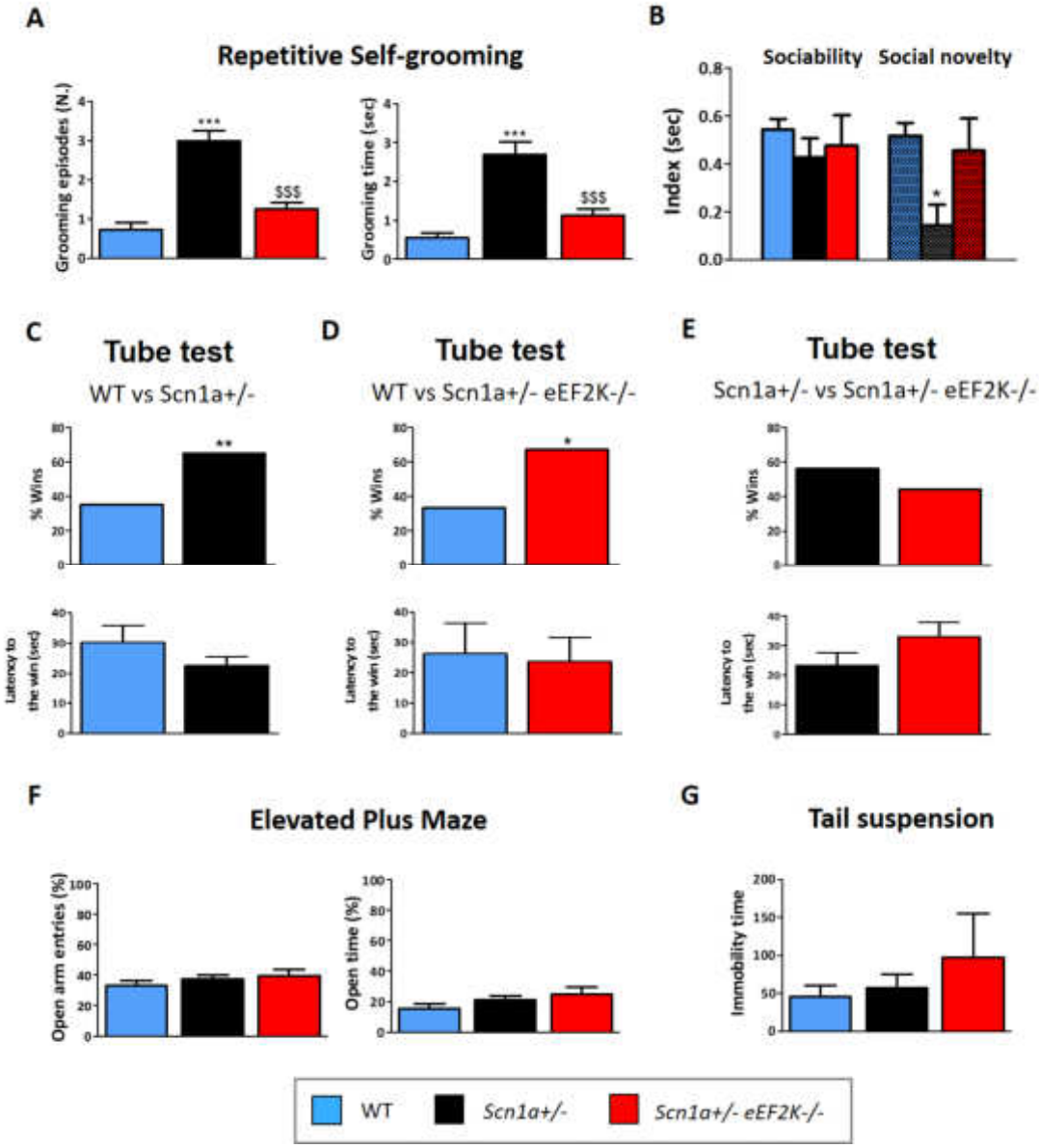

Figure 10. Tests to evaluate repetitive behaviour, sociability and social novelty and aggressiveness/dominant behaviour. (A) Total number of grooming episodes (left) and time spent grooming (right). ***p< 0.001 versus corresponding WT mice; $$$ p< 0.001 versus *Scn1a*$^{+/-}$; One way ANOVA, Tukey's test. (B) Sociability index and social novelty preference index evaluated in sociability and social novelty test. *p< 0.05 versus corresponding WT mice, One way ANOVA, Tukey's test (C-E) Percentage of won matches (upper row) and mean latency to the first attack (bottom row) in the tube test. *p< 0.05, **p< 0.01 versus corresponding WT; Fisher's exact test. (F). Open arm entries (left) and time in open arms (right), evaluated in the elevated plus maze test One way ANOVA, Tukey's test. (G) Time spent in immobility in tail suspension test. One way ANOVA, Tukey's test. (A-B, F-G) Data are presented as mean ± SEM.

5.4. eEF2 phosphorylation in *Scn1a*$^{+/-}$ mice

It has been demonstrated that in other genetic murine models of epilepsy, the *Synapsin1*$^{-/-}$ mice, eEF2 phosphorylation, assayed in brain samples, is strongly increased, suggesting that in these mice the activation of eEF2K is altered and this might contribute to the excitation/inhibition unbalance and epileptic phenotype in these mice (Heise et al., 2017). Thus, we wondered if the activity of eEF2K is altered also in the *Scn1a*$^{+/-}$ mice by looking to the level of phospo-eEF2.

We analysed the level of eEF2 phosphorylation in total homogenate and in P2 fraction (a fraction enriched in excitatory synaptosome) of two main brain areas: hippocampus and cerebral cortex. We collected brain samples at different time-points (3, 5 and 9 months) in order to detect eventual changes of P-eEF2 in ageing.

Western blots on brain samples showed that the level of P-eEF2 raised during time, in fact, even if at 3 months-old mice displayed an amount of P-eEF2 was similar between WT and *Scn1a*$^{+/-}$ mice, 6 months later the level of eEF2 phosphrylation was strongly increased in *Scn1a*$^{+/-}$ mice, suggesting that alterations in eEF2K/eEF2 pathway can further contribute to the imbalance of excitation over inhibition occurred in these mice (Fig. 11).

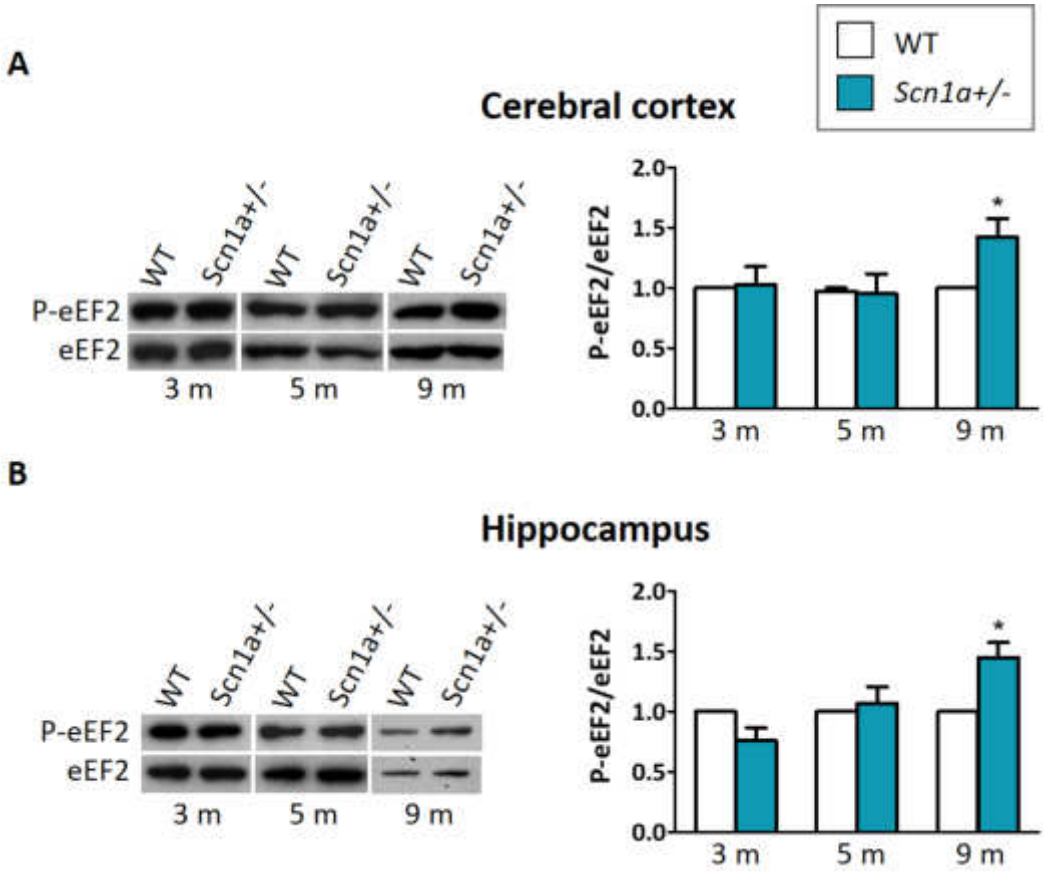

Figure 11. P-eEF2 level in total homogenate of cerebral cortex and hippocampus in WT and *Scn1a*$^{+/-}$ mice. A representative western blot for eEF2 phosphorylation in samples from cerebral cortex (A) and hippocampus (B) in 3, 5, 9-months-old mice (m=months) and their relative quantification. *p< 0.05 versus corresponding WT; t-test.

On the contrary, in the P2 fraction the level of P-eEF2 is similar between WT and *Scn1a*$^{+/-}$ mice for all time-points considered, meaning that alteration of the level of P-eEF2 in these mice does not occur in excitatory synapse compartment (see Fig. 12).

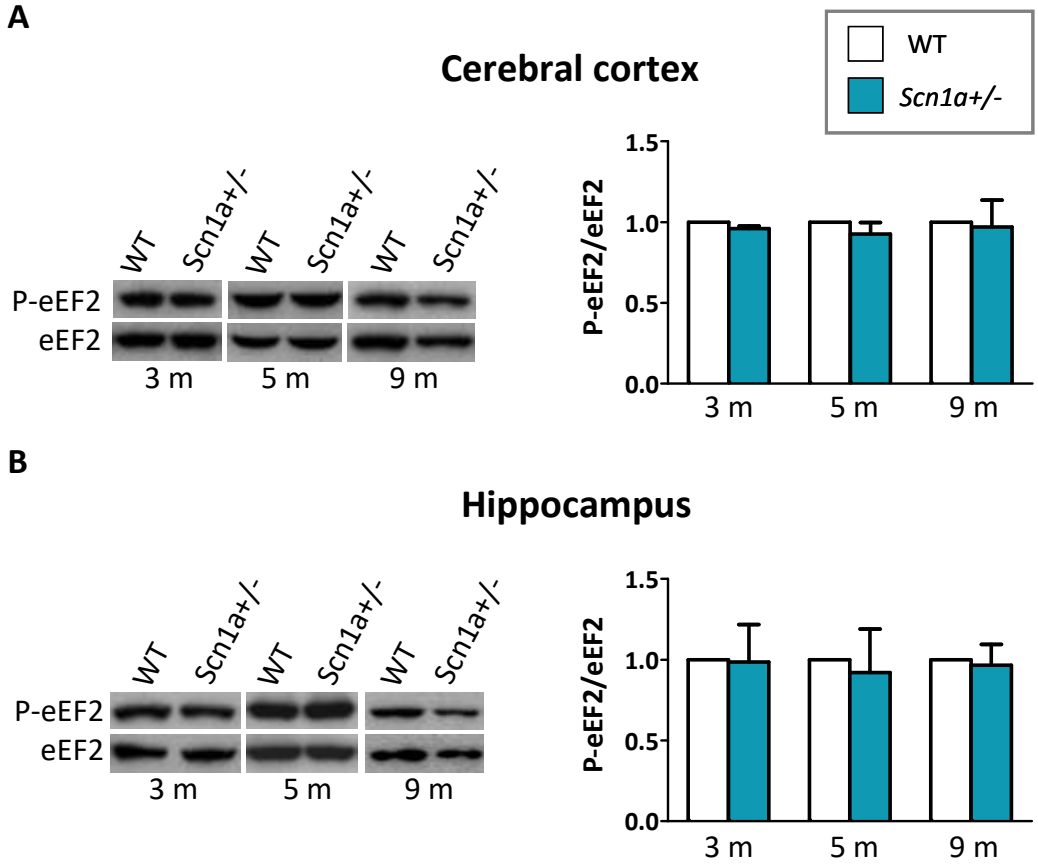

Figure 12. P-eEF2 level in P2 fraction of cerebral cortex and hippocampus in WT and *Scn1a*$^{+/-}$ mice. A representative western blot for eEF2 phosphorylation in samples from cerebral cortex (A) and hippocampus (B) in 3, 5, 9-months-old mice (m=months) and their relative quantification.

5.5. Pharmacological inhibition of eEF2K in Dravet syndrome mice

In the first part of the project, we demonstrated that eEF2K genetic deletion in the *Scn1a*$^{+/-}$ rescued the main symptoms (epileptic seizures, motor coordination impairments and cognitive deficits) of Dravet syndrome on this murine model of the pathology. However, we aimed to validate our findings by using selective eEF2K inhibitors. It was recently demonstrated that eEF2K inhibition by NH125, a well described eEF2K inhibitor (Autry et al., 2011) may be effective in inhibiting eEF2K activity and epileptic behaviour in *Synapsin1*$^{-/-}$ mice (Heise et al., 2017). However the NH125 compound has a number of important adverse side effects in part caused by off-target inhibitory activity on other kinases (Chen et al., 2011).

For this reasons we decided to test the efficacy of a new eEF2K inhibitors, one called JAN–384 (Kenney et al., 2015), that we recently obtained by Janssen Research & Development and A-484954, a small molecule recently used in another genetic mouse model (Jan et al., 2017).

5.5.1. JAN-384 decreased eEF2 phosphorylation in cultured neurons, but couldn't be delivered to living mice brain

JAN-384 action was characterized on murine neuronal culture and his efficacy *in vitro* is well established (Kenney et al., 2015). On the contrary, little is known about the action of JAN-384 *in vivo* in brain. We administered JAN-384 to wild-type mice through intraperitoneal injections in order to define:

- the concentration (we tried 10 mg/kg, 25 mg/kg and 50 mg/kg on Janssen Pharmaceutical suggestion);
- the number of doses necessary to inhibit eEF2K catalytic activity;
- the drug capability to pass through the brain blood barrier.

We sacrificed the animals 5-7 hours after the last treatment with vehicle (10% DMSO, 90% PEG) or JAN-384 and we detected eEF2K activity by analysing eEF2 phosphorylation on liver and on brain samples.

For the first experiment, we injected mice with vehicle or one dose of JAN-384 (50 mg/kg). Mice were sacrificed 7 hours after the treatment, but this acute administration had no effect on reducing eEF2 phosphorylation neither in liver nor in brain (data not shown). We continued the trial by administering 2 doses of JAN-384 or vehicle once a day and eEF2 phosphorylation level was dramatically decreased in the liver samples of mice treated with JAN-384 (Fig. 13A), but nearly unchanged in the brain samples of the same mice (Fig. 13B). Thus, our data strongly indicate that JAN-384 could not pass the brain blood barrier and cannot be used in our mice model.

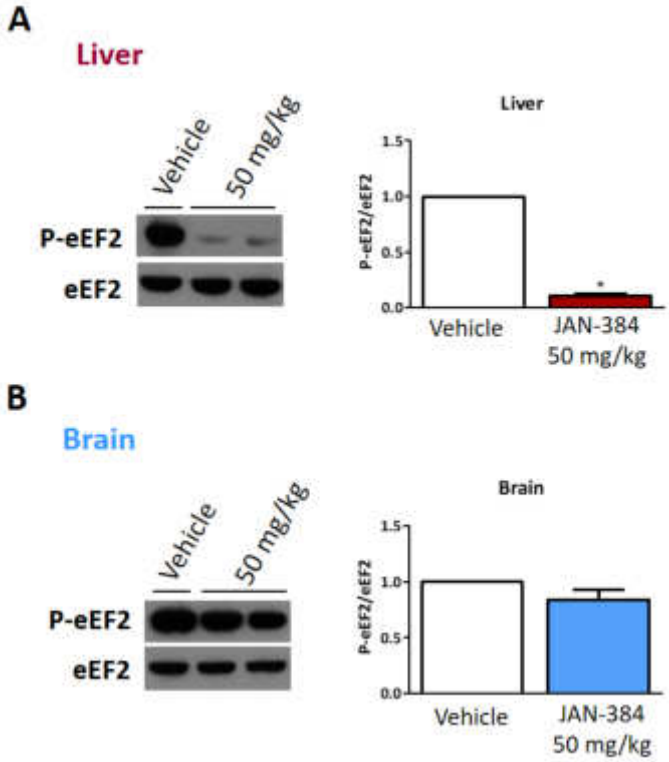

Figure 13. JAN-384 effect on eEF2 phosphorylation *in vivo*. (A,B) A representative western blot for eEF2 phosphorylation on liver (A) and brain (B) samples from mice treated with 2 doses of vehicle or JAN-384 50 mg/kg and the relative quantification. **p*< 0.05 versus corresponding vehicle; t-test.

However, we wanted to test the efficacy of JAN-384 in human neurons to eventually use the drug in an *in vitro* model of Dravet syndrome using iPSC (induced Pluripotent Stem Cell)-derived neurons obtained by Dravet patients in order to validate the use of eEF2K inhibitors also in a human model. We tested JAN-384 on a mixed population (both glutamatergic and GABAergic) of neurons derived from human iPSC of a healthy donor. iPSC-derived neurons were treated with 5µM JAN-384 and after 90 minutes were analysed for the level of eEF2 phosphorylation by western blot. Interestingly the phosphorylation of eEF2 was dramatically decreased in the treated cultures, indicating that this drug can be eventually used to study if inhibition of eEF2K rescue some of the alteration described in human neurons from Dravet syndrome patients recently described (Liu et al., 2013) (Fig. 14).

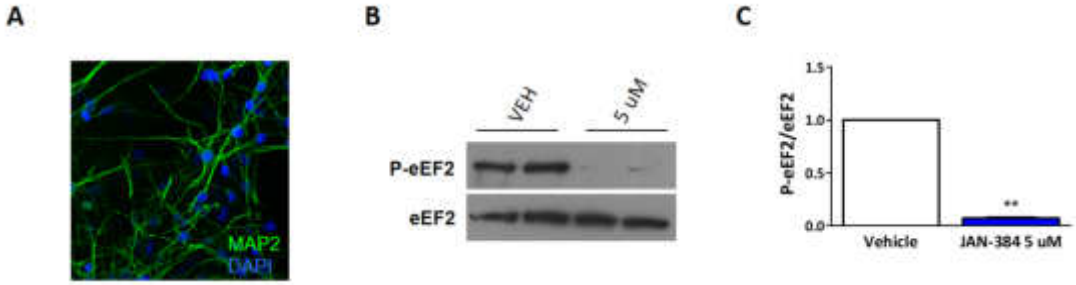

Figure 14. JAN-384 effect on eEF2 phosphorylation in neurons derived from human iPSC. (A) A representative image of neurons derived from human iPSC. (B) A representative western blot of eEF2 phosphorylation 90 minutes after treatment and its quantification (C). ***p*< 0.01 versus corresponding vehicle; t-test.

5.5.2. A-484954 efficiently reduced eEF2 phosphorylation in mice brain

We recently tested a new commercial compound, a small molecule called A-484954. This drug has been described as more selective eEF2K inhibitor than another widely used, the NH125, for the *in vitro* experiments (Chen et al., 2011). A-484954 has been demonstrated also to be efficient in inhibiting eEF2K activity *in vivo* in mouse brain (Jan et al., 2017).

As for mice treatment with JAN-384, we sacrificed the animals 5-7 hours after the last treatment with vehicle (10% DMSO, 90% PEG) or A-484954 and we evaluated the efficacy in eEF2K inhibition by analysing the level of eEF2 phosphorylation on brain samples.

We first administered one dose of vehicle or A-484954 20 mg/kg (the dose used in Jan et al., 2017) through an intraperitoneal injection in order to detect the efficacy of acute treatment in alter eEF2K activity. As shown in figure 15A, one-day treatment had no effect on eEF2 phosphorylation in brain samples.

We then proceeded administering vehicle or A-484954 once a day for 5 days. We tested the inhibitor at two different doses: 20 mg/kg, the one suggested from Jan and collaborators, and 10 mg/kg, a lower concentration in order to verify the minimum efficacious dose. A reiterative treatment with A-484954 was able to reduce P-eEF2 at a concentration of 20 mg/kg, suggesting that 20 mg/kg is the minimum concentration able to inhibit eEF2K activity in mice brain (Fig. 15B).

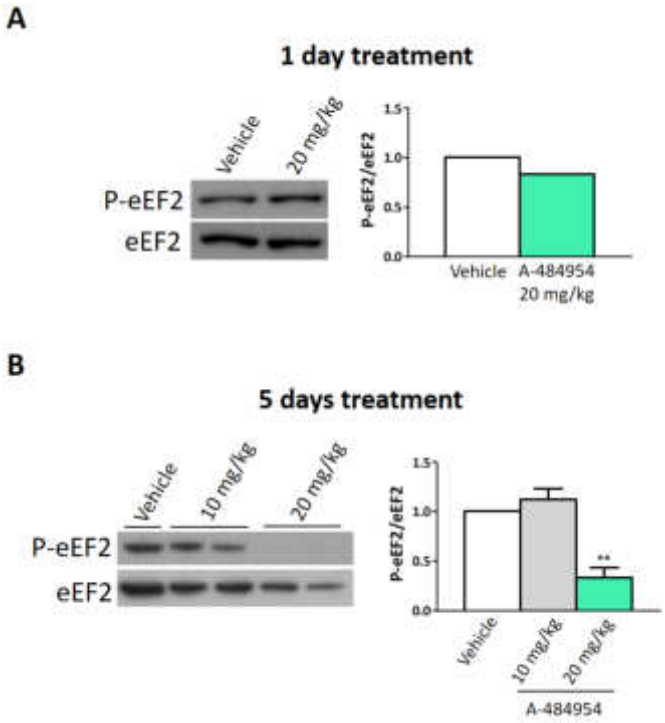

Figure 15. A-484954 effect on eEF2 phosphorylation *in vivo*. (A) A representative western blot of eEF2 phosphorylation after the acute treatment with vehicle and A-484954 (20 mg/kg) and its quantification. (B) A representative western blot of eEF2 phosphorylation after a 5 days treatment with vehicle and A-484954 (10 and 20 mg/kg) and its quantification. ***p*< 0.01 versus corresponding vehicle; t-test.

6. DISCUSSION

Epilepsy is one of common neurological disorders worldwide. Despite there are several drugs available to control epileptic seizures, 20% of the patients is not sufficiently controlled by the therapy. Patient with untreated seizures is more susceptible to develop neurological and psychiatric disorders (Laxer et al., 2014). Given the incidence of epilepsy, discovering novel targets for the development of new antiepileptic drugs can ameliorate the quality of life of patients. In this work, we attempted to identify and validate of a new pharmacological target for epilepsy treatment using a genetic model of human epilepsy, a murine model of Dravet syndrome.

Dravet syndrome is devastating infantile pathology characterized by pharmacoresistant epileptic seizures, ataxia, cognitive deficits and elevated mortality (Dravet et al., 2005; Jansen et al., 2006). 80% of Dravet syndrome patients carry a mutation in *Scn1a* gene that encodes for sodium channel subunit that is more expressed in parvalbumin-positive GABAergic interneurons (Claes et al., 2001; Mantegazza et al., 2005; Ogiwara et al., 2007). The loss of function mutations in $Na_V1.1$ leads to a hyperexcitable neuronal network in Dravet syndrome patients as well as in the mouse model of the pathology (Yu et al., 2006; Cheah et al., 2012; Liautard et al., 2013).

The therapy for Dravet syndrome aims to enhance the GABAergic transmission, mostly by the use of benzodiazepine such as clonazepam. Despite benzodiazepine are widely used in antiepileptic therapies, their high doses have an undesirable sedative effect. Interestingly, it has been demonstrated that a low dose of benzodiazepine, that has not antiepileptic and sedative effects, ameliorates other symptoms of Dravet syndrome such as ataxia and autism related behaviours (Kalume et al., 2007; Han et al., 2012).

Given the proven ameliorating effect of the potentiation of GABAergic synapses on Dravet syndrome symptomatology, we studied the effect of eEF2K inhibition, a condition known to potentiate GABAergic transmission (Heise et al., 2017), in another genetic mouse model of epileptic, the *Syn1*$^{-/-}$ mouse.

For this study, we first used a genetic approach by deleting eEF2K gene in *Scn1a*$^{+/-}$ mice, a model of Dravet syndrome that recapitulate all the major symptoms of the human pathology.

The generated double *Scn1a*$^{+/-}$/eEF2K$^{-/-}$ mice did not present alterations in development or in the survival rate (Fig. 5), indicating that the deletion of two genes did not affect mice survival and growth.

The first aim of the work was to verify if eEF2K deletion was able to reduce susceptibility to epileptic seizures in *Scn1a*$^{+/-}$ mice. Electroencephalographic analysis on freely moving mice revealed that double *Scn1a*$^{+/-}$/eEF2K$^{-/-}$ mice displayed a reduced number of both spontaneous and temperature induced epileptic discharges compared to *Scn1a*$^{+/-}$ mice and, interestingly, the number of epileptic discharges were similar between double *Scn1a*$^{+/-}$/eEF2K$^{-/-}$ mice and WT mice. Moreover, eEF2K deficiency gave resistance to *Scn1a*$^{+/-}$ mice in developing seizures under thermal stress (Fig. 6). These data suggested that eEF2K depletion could be an efficacious therapeutical approach for the treatment of epileptic seizures in Dravet syndrome. To better understand the molecular mechanism of the protecting effect of eEF2K deprivation, we recorded spontaneous inhibitory post-synaptic currents in hippocampus and we found that eEF2K deletion in *Scn1a*$^{+/-}$ mice enhanced both amplitude and instantaneous frequency (Fig. 7), indicating that the rescue of epileptic symptom may be mediated by the potentiation of GABAergic synapses, although amplitude and instantaneous frequency of sIPSC in *Scn1a*$^{+/-}$/eEF2K$^{-/-}$ mice was still lower compare to WT mice, suggesting there was only a partial rescue of the activity of GABAergic neurons in these mice.

As mentioned before, another important aspect of the project was investigating the effect of eEF2K deletion on motor and cognitive deficits observed in the *Scn1a*$^{+/-}$. Indeed the *Scn1a*$^{+/-}$ mice, as well as Dravet syndrome patients, are impaired in motor coordination, with no alteration in walking and climbing abilities nor in muscle strength (Fig. 8). *Scn1a*$^{+/-}$ mice are also compromised in both episodic and spatial memories as they showed impaired score in novel object recognition test and spatial

object recognition test in comparison with WT mice. This defect was confirmed by social novelty preference test, indeed, despite *Scn1a*$^{+/-}$ mice demonstrated a normal attitude in social interaction in the sociability test, this mice did not show a preference for the unfamiliar mouse as WT mice, suggesting a defect in memory and not in social interaction (Fig. 9). *Scn1a*$^{+/-}$ mice presented also stereotyped behaviours, tested by repetitive self-grooming test (Fig. 10A), but not anxiety-like or depression-like behaviours (Fig. 10 F-G). Interestingly, all the defects reported above, motor or cognitive, are rescued by eEF2K deletion, maybe through GABAergic synaptic potentiation, mimicking the effect of low-dose of benzodiazepine.

Taken together these data validate eEF2K inhibition as a possible therapy in Dravet syndrome for the more common symptoms (epileptic seizures, ataxia, cognitive impairments), by acting on GABAergic transmission, but avoiding the sedative effect typical of benzodiazepine (the spontaneous motor activity of double *Scn1a*$^{+/-}$/eEF2K$^{-/-}$ mice was not decreased compared to WT mice but, on the contrary, these mice exhibited an increased number of horizontal movements).

However, eEF2K deficiency was not able to rescue all the altered behavioral deficiency observed in the *Scn1a*$^{+/-}$ mice. Indeed the defect in aggressive/ dominant behavior exhibited by these mice was not reverted in double *Scn1a*$^{+/-}$*/eEF2K*$^{-/-}$ mice (Fig. 10 C-E). To our knowledge *eEF2K*$^{-/-}$ mice have never been tested for aggressive/ dominant behavior and we will perform behavioural test in order to define if it can be a phenotype these mice.

We previously demonstrated that in *Syn1*$^{-/-}$ mice, another mouse model of genetic epilepsy, that the level of P-eEF2 is higher in brain compare to WT mice. We thus characterized the level of P-eEF2 in the *Scn1a*$^{+/-}$ mice. We discovered that level of P-eEF2 was increased in both cerebral cortex and hippocampus of *Scn1a*$^{+/-}$ mice 9 months-old mice, in total homogenate (Fig. 11), but not in P2 fraction (Fig. 12), suggesting that alterations in eEF2K/eEF2 pathway can further contribute to the unbalance between excitation/inhibition developed in these mice and that this alteration does not specifically localize at synapse compartment.

In conclusion, the results from the genetic model suggest that eEF2K ablation can ameliorate the epileptic and behavioural alterations in the *Scn1a*$^{+/-}$ mice by acting on the GABAergic transmission. In addition, overactivation eEF2K/eEF2 pathway is also potentially involved in the progression of the pathology.

6.1. Future perspective

Data from genetic deletion of eEF2K in mice model of Dravet syndrome strongly indicate eEF2K inhibition as an antiepileptic therapy. In order to develop possible pharmacological treatment for epilepsy, we tested two selective inhibitors of eEF2K: JAN-384 and A-484954.

JAN-384 demonstrated a strong inhibitory effect on eEF2K both on neurons derived from human iPSC (Fig. 14) and in liver samples from treated mice (Fig. 13A), but not in brain samples, suggesting that this compound probably is unable to pass the blood brain barrier (Fig. 13B).

A-484954 was previously demonstrated efficacious on mouse brain (Jan et al., 20017) and we confirmed its inhibitory effect on eEF2K activity by a chronic treatment (5 days, 1 i.p. per die) at a concentration of 20mg/kg per dose (Fig. 15).

Thus, our data suggest the possibility to test the effect of A-484954 on rescue the epileptic, motor and cognitive symptoms displayed by *Scn1a*$^{+/-}$ mice in order to pharmacological confirm the data collected from the genetic model. Moreover, if testing A-484954 on *Scn1a*$^{+/-}$ mice will demonstrate an ameliorating effect on Dravet syndrome symptoms, I think that eEF2K inhibitors can be considered for the treatment of human patients.

Furthermore, it has been demonstrated that recurrent seizures leads to a protein changes in order to compensate and possibly prevent spreading epileptic episodes. Calbindin and NPY are proteins involved in these changes and, in particular, they have been described to be downregulated and upregulated, respectively, in another Dravet murine model and in epileptic patients (Gheyara et al., 2014). Thus, we are interested in understanding if these changes occurred also in our murine model and if eEF2K genetic and pharmacological inhibition can prevent alteration in the expression of calbindin and NPY.

7. REFERENCES

Audenaert, D, Schwartz, E, Claeys, KG *et al.* (2006) A novel GABRG2 mutation associated with febrile seizures. *Neurology* **67**: 687-90.

Autry, AE, Adachi, M, Nosyreva, E *et al.* (2011) NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* **475**: 91-5.

Bevins, RA and Besheer, J (2006) Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nat Protoc* **1**: 1306-11.

Braida, D and Sala, M (2000) Cannabinoid-induced working memory impairment is reversed by a second generation cholinesterase inhibitor in rats. *Neuroreport* **11**: 2025-9.

Breedlove SM, Rosenzweig MR, Watson NV (2007) Biological psychology : an introduction to behavioral, cognitive, and clinical neuroscience, 5th Edition. Sunderland, Mass.: Sinauer Associates.

Browne, GJ, Finn, SG, Proud, CG (2004) Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. *J Biol Chem* **279**: 12220-31.

Browne, GJ and Proud, CG (2002) Regulation of peptide-chain elongation in mammalian cells. *Eur J Biochem* **269**: 5360-8.

Cambiaghi, M, Cursi, M, Monzani, E *et al.* (2013) Temporal evolution of neurophysiological and behavioral features of synapsin I/II/III triple knock-out mice. *Epilepsy Res* **103**: 153-60.

Catterall, WA (2014) Sodium channels, inherited epilepsy, and antiepileptic drugs. *Annu Rev Pharmacol Toxicol* **54**: 317-38.

Cheah, CS, Yu, FH, Westenbroek, RE *et al.* (2012) Specific deletion of NaV1.1 sodium channels in inhibitory interneurons causes seizures and premature death in a mouse model of Dravet syndrome. *Proc Natl Acad Sci U S A* **109**: 14646-51.

Chen, Z, Gopalakrishnan, SM, Bui, MH *et al.* (2011) 1-Benzyl-3-cetyl-2-methylimidazolium iodide (NH125) induces phosphorylation of eukaryotic elongation factor-2 (eEF2): a cautionary note on the anticancer mechanism of an eEF2 kinase inhibitor. *J Biol Chem* **286**: 43951-8.

Chiappalone, M, Casagrande, S, Tedesco, M, Valtorta, F, Baldelli, P, Martinoia, S, Benfenati, F (2009) Opposite changes in glutamatergic and GABAergic transmission underlie the diffuse hyperexcitability of synapsin I-deficient cortical networks. *Cereb. Cortex* **9**: 1422–1439.

Chiron, C and Dulac, O (2011) The pharmacologic treatment of Dravet syndrome.

Epilepsia **52 Suppl 2**: 72-5.

Chuderland, D and Seger, R (2008) Calcium regulates ERK signaling by modulating its protein-protein interactions. *Commun Integr Biol* **1**: 4-5.

Claes, L, Del-Favero, J, Ceulemans, B, Lagae, L, Van Broeckhoven, C, De Jonghe, P (2001) De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. *Am J Hum Genet* **68**: 1327-32.

Clapham, DE (2007) Calcium signaling. *Cell* **131**: 1047-58.

Davidkova, G and Carroll, RC (2007) Characterization of the role of microtubule-associated protein 1B in metabotropic glutamate receptor-mediated endocytosis of AMPA receptors in hippocampus. *J Neurosci* **27**: 13273-8.

Depienne, C, Bouteiller, D, Keren, B *et al.* (2009) Sporadic infantile epileptic encephalopathy caused by mutations in PCDH19 resembles Dravet syndrome but mainly affects females. *PLoS Genet* **5**: e1000381.

Diggle, TA, Redpath, NT, Heesom, KJ, Denton, RM (1998) Regulation of protein-synthesis elongation-factor-2 kinase by cAMP in adipocytes. *Biochem J* **336 (Pt 3)**: 525-9.

Diggle, TA, Seehra, CK, Hase, S, Redpath, NT (1999) Analysis of the domain structure of elongation factor-2 kinase by mutagenesis. *FEBS Lett* **457**: 189-92.

Diggle, TA, Subkhankulova, T, Lilley, KS, Shikotra, N, Willis, AE, Redpath, NT (2001) Phosphorylation of elongation factor-2 kinase on serine 499 by cAMP-dependent protein kinase induces Ca2+/calmodulin-independent activity. *Biochem J* **353**: 621-6.

Dorovkov, MV, Pavur, KS, Petrov, AN, Ryazanov, AG (2002) Regulation of elongation factor-2 kinase by pH. *Biochemistry* **41**: 13444-50.

Dravet, C, Bureau, M, Oguni, H, Fukuyama, Y, Cokar, O (2005) Severe myoclonic epilepsy in infancy: Dravet syndrome. *Adv Neurol* **95**: 71-102.

Drennan, D and Ryazanov, AG (2004) Alpha-kinases: analysis of the family and comparison with conventional protein kinases. *Prog Biophys Mol Biol* **85**: 1-32.

Fisher, RS, Acevedo, C, Arzimanoglou, A *et al.* (2014) ILAE official report: a practical clinical definition of epilepsy. *Epilepsia* **55**: 475-82.

Franklin KBJ, Paxinos G (2008) The mouse brain in stereotaxic coordinates, 3rd Edition. Amsterdam ; Boston: Academic Press.

Genton, P, Velizarova, R, Dravet, C (2011) Dravet syndrome: the long-term outcome. *Epilepsia* **52 Suppl 2**: 44-9.

Gheyara, AL, Ponnusamy, R, Djukic, B *et al.* (2014) Tau reduction prevents disease in a mouse model of Dravet syndrome. *Ann Neurol* **76**: 443-56.

Goldin, AL (2001) Resurgence of sodium channel research. *Annu Rev Physiol* **63**: 871-94.

González-Terán, B, Cortés, JR, Manieri, E *et al.* (2013) Eukaryotic elongation factor 2 controls TNF-α translation in LPS-induced hepatitis. *J Clin Invest* **123**: 164-78.

Guerrini, R, Dravet, C, Genton, P, Belmonte, A, Kaminska, A, Dulac, O (1998) Lamotrigine and seizure aggravation in severe myoclonic epilepsy. *Epilepsia* **39**: 508-12.

Han, S, Tai, C, Westenbroek, RE *et al.* (2012) Autistic-like behaviour in Scn1a+/- mice and rescue by enhanced GABA-mediated neurotransmission. *Nature* **489**: 385-90.

Hartshorne, RP and Catterall, WA (1984) The sodium channel from rat brain. Purification and subunit composition. *J Biol Chem* **259**: 1667-75.

Heise, C, Taha, E, Murru, L *et al.* (2017) eEF2K/eEF2 Pathway Controls the Excitation/Inhibition Balance and Susceptibility to Epileptic Seizures. *Cereb Cortex* **27**: 2226-48.

Hermann, BP, Seidenberg, M, Bell, B (2002) The neurodevelopmental impact of childhood onset temporal lobe epilepsy on brain structure and function and the risk of progressive cognitive effects. *Prog Brain Res* **135**: 429-38.

Jan, A, Jansonius, B, Delaidelli, A *et al.* (2017) eEF2K inhibition blocks Aβ42 neurotoxicity by promoting an NRF2 antioxidant response. *Acta Neuropathol* **133**: 101-19.

Jansen, FE, Sadleir, LG, Harkin, LA *et al.* (2006) Severe myoclonic epilepsy of infancy (Dravet syndrome): recognition and diagnosis in adults. *Neurology* **67**: 2224-6.

Kalume, F, Yu, FH, Westenbroek, RE, Scheuer, T, Catterall, WA (2007) Reduced sodium current in Purkinje neurons from Nav1.1 mutant mice: implications for ataxia in severe myoclonic epilepsy in infancy. *J Neurosci* **27**: 11065-74.

Kaul, G, Pattan, G, Rafeequi, T (2011) Eukaryotic elongation factor-2 (eEF2): its regulation and peptide chain elongation. *Cell Biochem Funct* **29**: 227-34.

Kenney, JW, Adoff, MD, Wilkinson, DS, Gould, TJ (2011) The effects of acute, chronic, and withdrawal from chronic nicotine on novel and spatial object recognition in male C57BL/6J mice. *Psychopharmacology (Berl)* **217**: 353-65.

Kenney, JW, Genheden, M, Moon, KM, Wang, X, Foster, LJ, Proud, CG (2016) Eukaryotic elongation factor 2 kinase regulates the synthesis of microtubule-related proteins in neurons. *J Neurochem* **136**: 276-84.

Knebel, A, Haydon, CE, Morrice, N, Cohen, P (2002) Stress-induced regulation of eukaryotic elongation factor 2 kinase by SB 203580-sensitive and -insensitive

pathways. *Biochem J* **367**: 525-32.

Laxer, KD, Trinka, E, Hirsch, LJ *et al.* (2014) The consequences of refractory epilepsy and its treatment. *Epilepsy Behav* **37**: 59-70.

Leprivier, G, Remke, M, Rotblat, B *et al.* (2013) The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. *Cell* **153**: 1064-79.

Liautard, C, Scalmani, P, Carriero, G, de Curtis, M, Franceschetti, S, Mantegazza, M (2013) Hippocampal hyperexcitability and specific epileptiform activity in a mouse model of Dravet syndrome. *Epilepsia* **54**: 1251-61.

Lister, RG (1987) The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* **92**: 180-5.

Liu, Y, Lopez-Santiago, LF, Yuan, Y *et al.* (2013) Dravet syndrome patient-derived neurons suggest a novel epilepsy mechanism. *Ann Neurol* **74**: 128-39.

Manfredi, I, Zani, AD, Rampoldi, L *et al.* (2009) Expression of mutant beta2 nicotinic receptors during development is crucial for epileptogenesis. *Hum Mol Genet* **18**: 1075-88.

Mantegazza, M, Gambardella, A, Rusconi, R *et al.* (2005) Identification of an Nav1.1 sodium channel (SCN1A) loss-of-function mutation associated with familial simple febrile seizures. *Proc Natl Acad Sci U S A* **102**: 18177-82.

McFarlane, HG, Kusek, GK, Yang, M, Phoenix, JL, Bolivar, VJ, Crawley, JN (2008) Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav* **7**: 152-63.

Meldrum, BS (1989) GABAergic mechanisms in the pathogenesis and treatment of epilepsy. *Br J Clin Pharmacol* **27 Suppl 1**: 3S-11S.

Mitsui, K, Brady, M, Palfrey, HC, Nairn, AC (1993) Purification and characterization of calmodulin-dependent protein kinase III from rabbit reticulocytes and rat pancreas. *J Biol Chem* **268**: 13422-33.

Nairn, AC, Bhagat, B, Palfrey, HC (1985) Identification of calmodulin-dependent protein kinase III and its major Mr 100,000 substrate in mammalian tissues. *Proc Natl Acad Sci U S A* **82**: 7939-43.

Nairn, AC and Palfrey, HC (1987) Identification of the major Mr 100,000 substrate for calmodulin-dependent protein kinase III in mammalian cells as elongation factor-2. *J Biol Chem* **262**: 17299-303.

Oakley, JC, Cho, AR, Cheah, CS, Scheuer, T, Catterall, WA (2013) Synergistic GABA-enhancing therapy against seizures in a mouse model of Dravet syndrome. *J Pharmacol Exp Ther* **345**: 215-24.

Oakley, JC, Kalume, F, Yu, FH, Scheuer, T, Catterall, WA (2009) Temperature- and age-dependent seizures in a mouse model of severe myoclonic epilepsy in infancy.

Proc Natl Acad Sci U S A **106**: 3994-9.

Ogiwara, I, Miyamoto, H, Morita, N *et al.* (2007) Nav1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: a circuit basis for epileptic seizures in mice carrying an Scn1a gene mutation. *J Neurosci* **27**: 5903-14.

Oliván, S, Calvo, AC, Rando, A, Muñoz, MJ, Zaragoza, P, Osta, R (2015) Comparative study of behavioural tests in the SOD1G93A mouse model of amyotrophic lateral sclerosis. *Exp Anim* **64**: 147-53.

Pan, H and Xia, LZ (2008) Efficient object recognition using boundary representation and wavelet neural network. *IEEE Trans Neural Netw* **19**: 2132-49.

Park, S, Park, JM, Kim, S *et al.* (2008) Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3.1 essential for mGluR-LTD. *Neuron* **59**: 70-83.

Pavur, KS, Petrov, AN, Ryazanov, AG (2000) Mapping the functional domains of elongation factor-2 kinase. *Biochemistry* **39**: 12216-24.

Pinheiro, PS, and Mulle, C (2008) Presynaptic glutamate receptors: physiological functions and mechanisms of action. *Nature reviews. Neuroscience* **9**: 423-436.

Pigott, CR, Mikolajek, H, Moore, CE *et al.* (2012) Insights into the regulation of eukaryotic elongation factor 2 kinase and the interplay between its domains. *Biochem J* **442**: 105-18.

Proud, CG, Wang, X, Patel, JV *et al.* (2001) Interplay between insulin and nutrients in the regulation of translation factors. *Biochem Soc Trans* **29**: 541-7.

Rial, D, Castro, AA, Machado, N *et al.* (2014) Behavioral phenotyping of Parkin-deficient mice: looking for early preclinical features of Parkinson's disease. *PLoS One* **9**: e114216.

Ryazanov, AG (2002) Elongation factor-2 kinase and its newly discovered relatives. *FEBS Lett* **514**: 26-9.

Ryazanov, AG, Shestakova, EA, Natapov, PG (1988) Phosphorylation of elongation factor 2 by EF-2 kinase affects rate of translation. *Nature* **334**: 170-3.

Ryazanov, AG, Ward, MD, Mendola, CE *et al.* (1997) Identification of a new class of protein kinases represented by eukaryotic elongation factor-2 kinase. *Proc Natl Acad Sci U S A* **94**: 4884-9.

Sala, M, Braida, D, Lentini, D *et al.* (2011) Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism. *Biol Psychiatry* **69**: 875-82.

Scheetz, AJ, Nairn, AC, Constantine-Paton, M (1997) N-methyl-D-aspartate receptor

activation and visual activity induce elongation factor-2 phosphorylation in amphibian tecta: a role for N-methyl-D-aspartate receptors in controlling protein synthesis. *Proc Natl Acad Sci U S A* **94**: 14770-5.

Scheetz, AJ, Nairn, AC, Constantine-Paton, M (2000) NMDA receptor-mediated control of protein synthesis at developing synapses. *Nat Neurosci* **3**: 211-6.

Scheffer, IE and Berkovic, SF (1997) Generalized epilepsy with febrile seizures plus. A genetic disorder with heterogeneous clinical phenotypes. *Brain* **120 (Pt 3)**: 479-90.

Skluzacek, JV, Watts, KP, Parsy, O, Wical, B, Camfield, P (2011) Dravet syndrome and parent associations: the IDEA League experience with comorbid conditions, mortality, management, adaptation, and grief. *Epilepsia* **52 Suppl 2**: 95-101.

Steru, L, Chermat, R, Thierry, B, Simon, P (1985) The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* **85**: 367-70.

Sutton, MA, Taylor, AM, Ito, HT, Pham, A, Schuman, EM (2007) Postsynaptic decoding of neural activity: eEF2 as a biochemical sensor coupling miniature synaptic transmission to local protein synthesis. *Neuron* **55**: 648-61.

Taha, E, Gildish, I, Gal-Ben-Ari, S, Rosenblum, K (2013) The role of eEF2 pathway in learning and synaptic plasticity. *Neurobiol Learn Mem* **105**: 100-6.

Tavares, CD, O'Brien, JP, Abramczyk, O *et al.* (2012) Calcium/calmodulin stimulates the autophosphorylation of elongation factor 2 kinase on Thr-348 and Ser-500 to regulate its activity and calcium dependence. *Biochemistry* **51**: 2232-45.

Valtorta, F, Pozzi, D, Benfenati, F, Fornasiero, E.F (2011) The synapsins: multitask modulators of neuronal development. *Semin. Cell Dev. Biol.* **22**: 378–386.

Verpelli, C, Piccoli, G, Zibetti, C *et al.* (2010) Synaptic activity controls dendritic spine morphology by modulating eEF2-dependent BDNF synthesis. *J Neurosci* **30**: 5830-42.

Wallace, RH, Scheffer, IE, Barnett, S *et al.* (2001) Neuronal sodium-channel alpha1-subunit mutations in generalized epilepsy with febrile seizures plus. *Am J Hum Genet* **68**: 859-65.

Wang, X, Li, W, Williams, M, Terada, N, Alessi, DR, Proud, CG (2001) Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J* **20**: 4370-9.

World Health Organization (WHO). “Epilepsy”. WHO Factsheet, February 2017. Available at: http://www.who.int/mediacentre/factsheets/fs999/en/.

Yu, FH, Mantegazza, M, Westenbroek, RE *et al.* (2006) Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in

infancy. *Nat Neurosci* **9**: 1142-9.

Zhou, Y, Kaiser, T, Monteiro, P et al. (2016) Mice with Shank3 Mutations Associated with ASD and Schizophrenia Display Both Shared and Distinct Defects. Neuron 89: 147-62