



## A triheptanoin-supplemented diet rescues hippocampal hyperexcitability and seizure susceptibility in *FoxG1*<sup>+/-</sup> mice

Giovanna Testa<sup>a</sup>, Marco Mainardi<sup>a,b,\*\*</sup>, Francesco Olimpico<sup>a</sup>, Laura Pancrazi<sup>b</sup>, Antonino Cattaneo<sup>a</sup>, Matteo Caleo<sup>a,b</sup>, Mario Costa<sup>a,b,\*</sup>

<sup>a</sup> Laboratory of Biology “Bio@SNS”, Scuola Normale Superiore, Piazza dei Cavalieri, 7, 56124, Pisa, Italy

<sup>b</sup> Institute of Neuroscience, Italian National Research Council (CNR), Via Moruzzi, 1, 56124, Pisa, Italy



### HIGHLIGHTS

- *FoxG1*<sup>+/-</sup> mice show hippocampal hyperexcitability.
- *FoxG1*<sup>+/-</sup> mice are highly vulnerable to proconvulsant-induced seizures.
- *FoxG1*<sup>+/-</sup> mice have decreased expression of the chloride transporter KCC2.
- Triheptanoin rescues functional and molecular abnormalities of *FoxG1*<sup>+/-</sup> mice.

### ARTICLE INFO

#### Keywords:

FoxG1  
Anaplerotic diet  
Kainic acid  
Hippocampus  
KCC2  
vGAT

### ABSTRACT

The *Forkhead Box G1 (FOXP1)* gene encodes a transcription factor with an essential role in mammalian telencephalon development. *FOXP1*-related disorders, caused by deletions, intragenic mutations or duplications, are usually associated with severe intellectual disability, autistic features, and, in 87% of subjects, epileptiform manifestations. In a subset of patients with *FoxG1* mutations, seizures remain intractable, prompting the need for novel therapeutic options. To address this issue, we took advantage of a haploinsufficient animal model, the *FoxG1*<sup>+/-</sup> mouse. *In vivo* electrophysiological analyses of *FoxG1*<sup>+/-</sup> mice detected hippocampal hyperexcitability, which turned into overt seizures upon delivery of the proconvulsant kainic acid, as confirmed by behavioral observations. These alterations were associated with decreased expression of the chloride transporter KCC2.

Next, we tested whether a triheptanoin-based anaplerotic diet could have an impact on the pathological phenotype of *FoxG1*<sup>+/-</sup> mice. This manipulation abated altered neural activity and normalized enhanced susceptibility to proconvulsant-induced seizures, in addition to rescuing altered expression of KCC2 and increasing the levels of the GABA transporter vGAT. In conclusion, our data show that *FoxG1* haploinsufficiency causes dysfunction of hippocampal circuits and increases the susceptibility to a proconvulsant insult, and that these alterations are rescued by triheptanoin dietary treatment.

### 1. Introduction

Mutation or dysregulation of the gene encoding the transcription factor *Forkhead box g1 (FoxG1 for Mus musculus and FOXP1 for Homo sapiens)* have been identified in several important human diseases, including different types of cancer (Verginelli et al., 2013) and neurodevelopmental disorders, such as Rett syndrome (RTT), an autism spectrum disorder (Ariani et al., 2008). RTT affects approximately 1 in 10,000 live female births; besides the classical type, due to *MECP2*

mutations, a significant fraction of the congenital RTT variant is due to *FOXP1* mutations (OMIM#164874) (Seltzer et al., 2014), and is characterized by earlier onset (first months of life), cognitive impairment, autism-like features, stereotypic hand movements, hypotonia, microcephaly and epilepsy (Ariani et al., 2008).

FOXP1 is a well-conserved, DNA-binding transcription factor, whose gene is located on the 14q12 chromosome (De Filippis et al., 2012). This protein is organized into four functional domains: (i) DNA-binding forkhead (FHD), (ii) GROUCHO-binding, (iii) JARID1B-binding

\* Corresponding author. Institute of Neuroscience, Italian National Research Council (CNR), Via Moruzzi, 1, 56124, Pisa, Italy.

\*\* Corresponding author. Laboratory of Biology “Bio@SNS”. Scuola Normale Superiore, Piazza dei Cavalieri, 7, 56124, Pisa, Italy.

E-mail addresses: [m.mainardi@sns.it](mailto:m.mainardi@sns.it) (M. Mainardi), [costa@in.cnr.it](mailto:costa@in.cnr.it) (M. Costa).

and (iv) mitochondrial localization (Pancrazi et al., 2015). Indeed, overexpression of full-length FoxG1 increases mitochondrial fission; conversely, restriction of FoxG1 localization to mitochondria (via deletion of the N-terminal domains) enhances fusion of these organelles. This corresponds to a shift in the equilibrium of metabolic reactions towards glycolysis or oxidative phosphorylation, respectively. In addition, the balance between cell proliferation and differentiation is altered (Pancrazi et al., 2015). This finding shed new light on the aetiology of FOXG1-RTT and on the original mitochondrial dysfunction hypothesis for RTT pathogenesis (Shulyakova et al., 2017).

FoxG1 is abundantly expressed since early embryonic life and persists at lower levels in adulthood (Eagleson et al., 2007). Altered FoxG1 dosage, such as in FoxG1-null mice (FoxG1<sup>-/-</sup>) results in a dramatic reduction in the size of cerebral hemispheres and perinatal lethality. On the other hand, heterozygous FoxG1<sup>+/-</sup> mice are viable and fertile, but show microcephaly, hyperlocomotion, impaired habituation in the open field test and a severe deficit in contextual fear conditioning, thus recapitulating some features of disorders caused by dysregulation of FoxG1, e.g., the congenital variant of RTT and West syndrome (Eagleson et al., 2007; Shen et al., 2006; Striano et al., 2011). Despite epilepsy being a hallmark of FOXG1-related disorders, the propensity to seizures of FoxG1<sup>+/-</sup> mice and the molecular underpinnings of this important contributor to the severity of RTT had not been studied so far. Recent evidence indicates the involvement of an excitation/inhibition imbalance (Patriarchi et al., 2016), which appears to be a general pathogenetic mechanism (Rakhade and Jensen, 2009) in common with non-genetic forms of epilepsy (Mainardi et al., 2012). Moreover, altered chloride homeostasis can affect neuronal excitability, thus determining the propensity to seizures (Ben-Ari et al., 2012). A crucial regulator of chloride intracellular concentration in the adult brain is the K<sup>+</sup>/Cl<sup>-</sup> cotransporter KCC2 (Kahle et al., 2013). Indeed, experimental downregulation of KCC2 expression contributes to seizure generation (Chen et al., 2017). Given the role of KCC2 in regulating the inhibitory tone of neural circuits, an alteration in the expression of this cotransporter could correlate with the excitation/inhibition imbalance observed in FoxG1<sup>+/-</sup> mice.

The mitochondrial localization of FoxG1, as well as the metabolic dysregulation observed in Mecp2-null mice (Panighini et al., 2013), suggests that a dietary or metabolic approach could be exploited to ameliorate the symptoms caused by FOXG1 mutation or imbalance. In this regard, oral treatment with triheptanoin (a C7 triglyceride) has been demonstrated to enhance life span, metabolism and sociability in a Mecp2-null animal model (Park et al., 2014). Triheptanoin is a tasteless oil, whose cleavage into heptanoate and subsequent metabolism to propionyl-CoA can result in anaplerosis, i.e., the refilling of tri-carboxylic acid cycle (TCA) with intermediate products (Tefera et al., 2017).

The lack of knowledge on the electrophysiological phenotype of FoxG1<sup>+/-</sup> mice prompted us to analyse, in this RTT model, the possible alterations in hippocampal activity and the accompanying biochemical changes. We found that FoxG1<sup>+/-</sup> mice display a seizure-prone phenotype and altered expression of KCC2. Then, we tested a triheptanoin-supplemented anaplerotic diet, which was able to ameliorate the electrophysiological and biochemical alterations of FoxG1<sup>+/-</sup> mice, thus demonstrating the potential therapeutic value of this approach.

## 2. Materials and methods

Detailed experimental procedures are described in the Supplementary Materials and Methods.

### 2.1. Animals and diets

Experiments were carried out on FoxG1<sup>+/-</sup> mice and C57BL/6J wild type controls by taking into account the “Animal Research: Reporting of In Vivo Experiments” (ARRIVE) guidelines and the

principles of the Basel Declaration.

Diets were prepared as described in Park et al. (2014). Mice were ad libitum fed a standard maintenance chow food (Mucedola, Italy) until weaning, on postnatal day (P) 28. Mice were, then, fed either a diet supplemented with triheptanoin (TRI, kindly provided by Ultragenyx Pharmaceutical, Inc., USA) or an isocaloric control diet (CD) (see Supplementary Table S1). The TRI diet did not significantly affect the survival rate of both wild type and FoxG1<sup>+/-</sup> mice, which were also not significantly different from each other (data not shown) and there were no significant differences between the body weights of wild type and FoxG1<sup>+/-</sup> mice measured at P60, either under TRI or CD (Supplementary Fig. 1).

### 2.2. Recording of local field potentials

Electrode implants and data analysis were performed at P55 by adapting the techniques described in Mainardi et al. (2014, 2012). After recovery, Local Field Potential (LFP) recording sessions lasting 60 min or, in a subset of animals, 4 h, were performed.

### 2.3. Behavioral assessment of seizure susceptibility

Mice were individually caged and habituated to the new cage for 1 h, then the proconvulsant kainic acid (Sigma-Aldrich K0250) was i.p. injected (10 mg/kg; 2 mg/ml in PBS), as described in Corradini et al. (2014).

### 2.4. Western blotting

Hippocampal protein extracts were prepared according to the protocol of Mainardi et al. (2010a). The rabbit polyclonal primary antibodies used were: anti-KCC2 (1:1,000, Millipore 07–432), anti-vGAT (1:1,000, Synaptic Systems 131003); GAPDH was revealed using a mouse monoclonal antibody (1:10,000, Fitzgerald Industries Int. 10R-G109a).

### 2.5. Statistical analyses

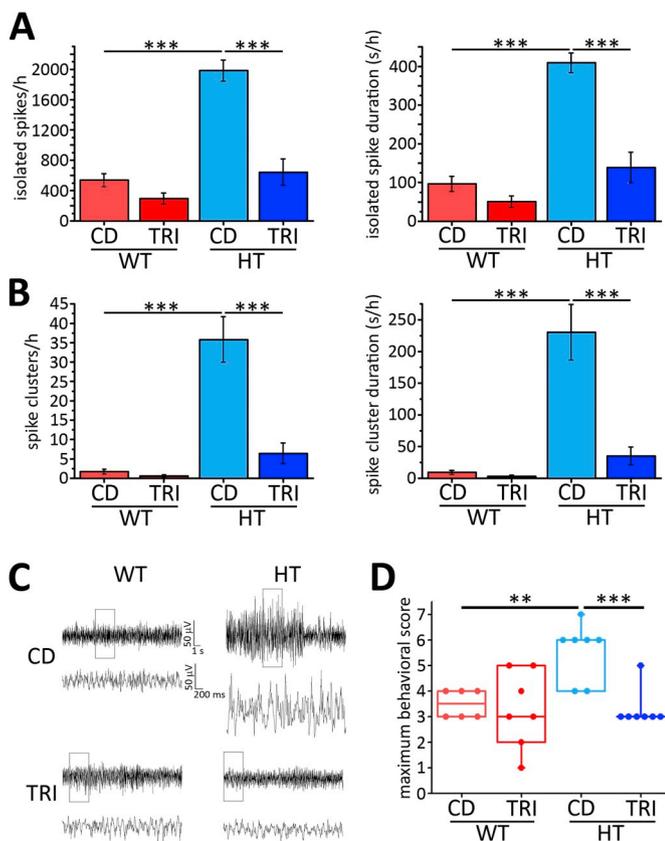
Statistical significance was assessed using SigmaStat 12 (Systat Software, USA), using ANOVA-2, with “genotype” (i.e., FoxG1<sup>+/-</sup> or wild-type) and diet (i.e., control or triheptanoin) as main factors. Data are expressed as mean ± SEM.

## 3. Results

### 3.1. Hippocampal hyperexcitability in FoxG1<sup>+/-</sup> mice is rescued by triheptanoin

To investigate the possible consequences of FoxG1 haploinsufficiency on the function of neural circuits *in vivo*, we employed electrodes chronically implanted into the hippocampus for Local Field Potential (LFP) recording. FoxG1<sup>+/-</sup> mice fed a control diet (HT-CD group) displayed electrophysiological alterations, which were not observed in WT mice fed the same diet (WT-CD group). In particular, HT-CD mice displayed, in comparison to WT-CD mice, a higher number of large-amplitude spikes (i.e., larger than 4.5 times the standard deviation of the baseline activity), either in the form of isolated spikes (Fig. 1A and C), or as clusters (i.e., groups of spikes extending for more than 4 s; see Supplementary Materials and Methods) (Fig. 1B and C). Accordingly, HT-CD mice also showed a significantly longer duration of both isolated and clustered spiking events (with an average inter-spike interval of 0.63 ± 0.12 s) than WT-CD mice (Fig. 1A and B). Spike clusters detected at the EEG level did not correspond to generalized behavioral manifestations (e.g., tonic-clonic events), but were accompanied by immobility (Fig. 1C and Supplementary Movies 1–2).

Supplementary videos related to this article can be found at <https://>



**Fig. 1. Hippocampal hyperexcitability and higher severity of kainic acid-induced seizures in *FoxG1*<sup>+/-</sup> mice are rescued by triheptanoin.** A) *left*, HT-CD mice show a higher number of isolated spikes/hour, compared to WT-CD mice, and this alteration is ameliorated in HT-TRI mice (WT-CD, *n* = 9; WT-TRI, *n* = 4; HT-CD, *n* = 9; HT-TRI, *n* = 9; ANOVA-2 *p*<sub>interaction</sub> = 0.001, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001); *right*, HT-CD mice show a longer total duration of isolated spiking events, compared to WT-CD mice, and this alteration is ameliorated in HT-TRI mice (WT-CD, *n* = 9; WT-TRI, *n* = 4; HT-CD, *n* = 9; HT-TRI, *n* = 9; ANOVA-2 *p*<sub>interaction</sub> = 0.001, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001). B) *left*, HT-CD mice show a higher number of clustered spikes/hour compared to WT-CD mice and this alteration is ameliorated in HT-TRI mice (WT-CD, *n* = 9; WT-TRI, *n* = 4; HT-CD, *n* = 9; HT-TRI, *n* = 9; ANOVA-2 *p*<sub>interaction</sub> = 0.002, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001); *right*, HT-CD mice show a longer total duration of clustered spiking events, compared to WT-CD mice, and this alteration is ameliorated in HT-TRI mice (WT-CD, *n* = 9; WT-TRI, *n* = 4; HT-CD, *n* = 9; HT-TRI, *n* = 9; ANOVA-2 *p*<sub>interaction</sub> = 0.003, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001). C) Representative traces showing LFP recordings obtained from the hippocampus of WT and HT mice subjected to either CD or TRI treatments. D) HT-CD mice display a higher behavioral seizure score after kainic acid (KA) injection in comparison to WT-CD mice, and this increased susceptibility to KA-induced seizures is absent in HT-TRI mice; on the other hand, triheptanoin treatment does not significantly affect seizure propensity in WT mice (WT-TRI group) (WT-CD, *n* = 6; WT-TRI, *n* = 7; HT-CD, *n* = 7; HT-TRI, *n* = 7; ANOVA-2, *p*<sub>interaction</sub> = 0.019, followed by Holm-Sidak *post hoc* test, \*\**p* = 0.002, \*\*\**p* < 0.001).

[doi.org/10.1016/j.neuropharm.2019.01.005](https://doi.org/10.1016/j.neuropharm.2019.01.005).

Taking into account these EEG alterations and the fact that hyperexcitability affects the susceptibility to seizures (Navidhamidi et al., 2017), we also tested the propensity of *FoxG1*<sup>+/-</sup> mice to develop behaviorally detectable seizures in response to a single i.p. administration of a proconvulsant, namely kainic acid (KA). Consistent with their EEG hyperexcitability phenotype, HT-CD mice injected with KA displayed a significantly higher behavioral seizure score in comparison to WT-CD mice (Fig. 1D).

To evaluate whether triheptanoin (TRI) treatment could impact the

phenotype described above, *FoxG1*<sup>+/-</sup> (HT-TRI) and WT mice (WT-TRI) were fed a TRI-supplemented diet for 3 weeks (see Supplementary Table 1 for composition). This treatment dramatically decreased the frequency and duration of isolated and clustered spiking events recorded in the hippocampus of *FoxG1*<sup>+/-</sup> mice (Fig. 1A–C). Even though the altered EEG phenotype of HT-TRI mice was not completely rescued (i.e., the parameters were not fully normalized to the values observed in WT-CD mice, a difference that was not statistically significant), TRI treatment resulted in a ~68% decrease of isolated spike frequency (HT-CD, 1982 ± 140 vs. HT-TRI 642 ± 174 isolated spike events/hour) and duration (HT-CD, 409 ± 25 s vs. HT-TRI 139 ± 39 s spent in isolated spikes/hour; Fig. 1A). The same trend, namely a ~84% drop, was observed for clustered spike frequency (HT-CD, 36 ± 6 vs. HT-TRI 6 ± 3 spike clusters/hour) and duration (HT-CD, 230 ± 44 s vs. HT-TRI 35 ± 14 s spike clusters duration/hour; Fig. 1B).

In agreement with electrophysiological data, TRI was also able to protect *FoxG1*<sup>+/-</sup> mice from the development of proconvulsant-induced behavioral seizures. Indeed, HT-TRI mice displayed a full rescue to the values observed in WT-CD mice, and their average maximum behavioral score shifted towards a lower value in comparison to HT-CD mice (Fig. 1D), thus indicating decreased propensity to KA-evoked seizures.

To verify whether TRI could influence the altered hippocampal activity of *FoxG1*<sup>+/-</sup> mice also after a proconvulsive insult, we performed EEG recordings after KA administration. Quantification of number and duration of both isolated and clustered spikes still showed significantly higher values for HT-CD mice, in comparison to WT-CD controls (Fig. 2A and B); notably, this trend was exacerbated by KA administration, as it can be appreciated by comparing Fig. 1A and B and 2A, B. These electrophysiological findings correlated with the development, in HT-CD mice, of behaviorally detectable, generalized seizures after a single dose of KA (Supplementary Movie 4), along with high-amplitude spike clusters (Fig. 2C). WT-CD mice also developed spontaneous seizure-like events; however, these displayed a lower severity (Fig. 2C and Supplementary Movie 3). These data are consistent with the higher score attained by HT-CD mice, in comparison to WT-CD controls, in the behavioral assessment of seizure susceptibility (Fig. 1D). On the other hand, these behavioral and electrophysiological alterations were controlled by TRI dietary supplementation; indeed, both number and duration of either isolated or clustered events were significantly decreased in HT-TRI mice, compared to HT-CD mice (Fig. 2 and Supplementary Movie 4).

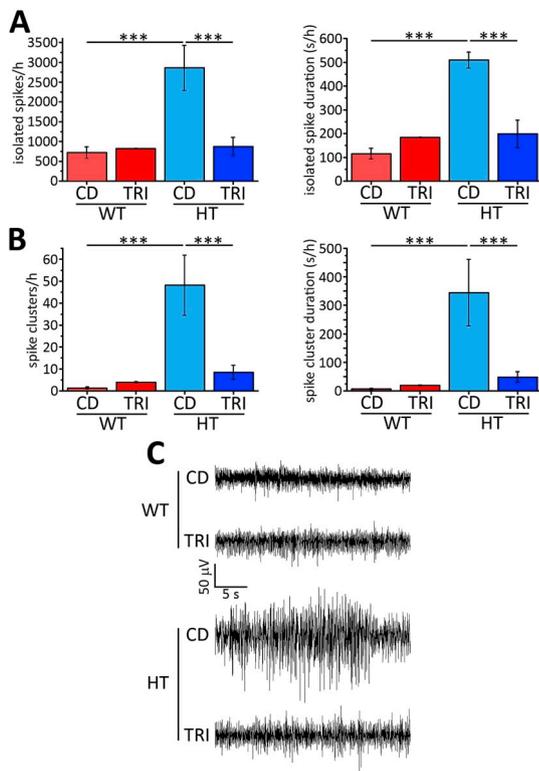
Supplementary videos related to this article can be found at <https://doi.org/10.1016/j.neuropharm.2019.01.005>.

These findings demonstrate that triheptanoin, in addition to ameliorating the alterations in hippocampal pathophysiology caused by *FoxG1* haploinsufficiency, can also exert a protective role on the increased sensitivity of *FoxG1*<sup>+/-</sup> mice to develop overt seizures in response to a single proconvulsive event.

### 3.2. Triheptanoin normalizes *KCC2* expression and increases *vGAT* levels

Seizures and neural circuit hyperexcitability are tightly associated to alterations in chloride homeostasis (Ben-Ari et al., 2012). In this regard, we quantified by Western blotting the expression of the chloride transporter *KCC2* and found a decrease in HT-CD mice, compared to WT-CD mice. Strikingly, TRI treatment corrected this alteration, and HT-TRI mice displayed *KCC2* levels that were not significantly different from WT-CD mice. On the other hand, *KCC2* expression in WT-TRI mice was not significantly affected (Fig. 3A).

In addition to homeostasis of chloride gradient across the neuronal membrane, the inhibitory tone also plays a role in regulating circuit excitability, thus affecting susceptibility to seizures (Mainardi et al., 2012). Based on this, we analyzed the expression of vesicular GABA transporter (*vGAT*), a reliable marker of the inhibitory system (Mainardi et al., 2010a, 2010b). We found that TRI was able to increase



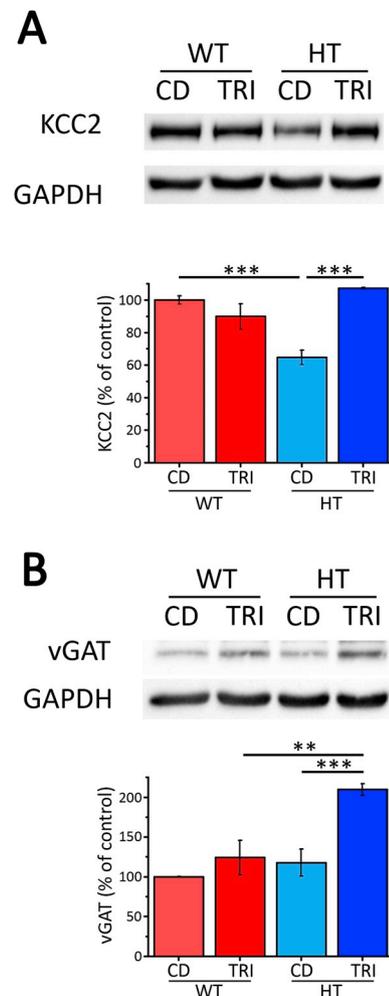
**Fig. 2.** Triheptanoin protects *FogG1*<sup>+/-</sup> mice from seizures caused by proconvulsant administration. **A) left**, triheptanoin reduces the number of isolated spiking events in kainic-acid (KA) injected HT-TRI mice (WT-CD, *n* = 4; WT-TRI, *n* = 2; HT-CD, *n* = 3; HT-TRI, *n* = 6; ANOVA-2 *p*<sub>interaction</sub> = 0.009, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001); **right**, triheptanoin reduces the duration of isolated spiking events in KA-injected HT-TRI mice (WT-CD, *n* = 4; WT-TRI, *n* = 2; HT-CD, *n* = 3; HT-TRI, *n* = 6; ANOVA-2 *p*<sub>interaction</sub> = 0.007, followed by Holm-Sidak *post hoc* test, \*\*\**p* ≤ 0.001). **B) left**, triheptanoin reduces the number of clustered spiking events in KA-injected HT-TRI mice (WT-CD, *n* = 4; WT-TRI, *n* = 2; HT-CD, *n* = 3; HT-TRI, *n* = 6; ANOVA-2 *p*<sub>interaction</sub> = 0.007, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001); **right**, triheptanoin reduces the duration of clustered spiking events in KA-injected HT-TRI mice (WT-CD, *n* = 4; WT-TRI, *n* = 2; HT-CD, *n* = 3; HT-TRI, *n* = 6; ANOVA-2 *p*<sub>interaction</sub> = 0.012, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001). **C)** Representative traces showing LFP recordings obtained from the hippocampus of WT and HT mice, treated with either CD or TRI, after KA injection.

vGAT expression specifically in HT mice, whereas no difference was present between WT and HT mice feeding on control diet; in addition, no effect of TRI was observed on WT mice (Fig. 3B).

Therefore, TRI is able to modulate the expression of key markers of inhibitory neurotransmission.

#### 4. Discussion

In this study, we analyzed brain pathophysiology of *FoxG1*<sup>+/-</sup> mice, and determined if it could be affected by triheptanoin (TRI) administration. These experiments demonstrated altered function of hippocampal circuits, which correlated with changes in the expression of key proteins for neuronal homeostasis. Specifically, we describe spontaneous hyperexcitability in the hippocampus of *FoxG1*-haploinsufficient mice, which could result in increased vulnerability to a proconvulsive insult. Indeed, after being treated with a single injection of kainic acid, *FoxG1*<sup>+/-</sup> mice developed overt behavioral seizures, that showed a higher severity in comparison to wild type mice receiving the same treatment. Thus, a single proconvulsive insult was able to unmask



**Fig. 3.** Triheptanoin modulates inhibitory system markers in *FoxG1*<sup>+/-</sup> mice. **A) top**, representative blots showing KCC2 and GAPDH expression; **bottom**, HT-CD mice show decreased levels of KCC2 in comparison to WT-CD mice, which are restored in HT-TRI mice (WT-CD, *n* = 5; WT-TRI, *n* = 4; HT-CD, *n* = 3; HT-TRI, *n* = 3; ANOVA-2, *p*<sub>interaction</sub> < 0.001, followed by Holm-Sidak *post hoc* test, \*\*\**p* < 0.001). **B) top**, representative blots showing vGAT and GAPDH expression; **bottom**, HT-TRI mice display increased expression of vGAT (WT-CD, *n* = 6; WT-TRI, *n* = 4; HT-CD, *n* = 6; HT-TRI, *n* = 3; ANOVA-2, *p*<sub>interaction</sub> = 0.039, followed by Holm-Sidak *post hoc* test, \*\**p* = 0.003, \*\*\**p* < 0.001).

a latent seizure-prone phenotype in *FoxG1*<sup>+/-</sup> mice, whereas this did not occur in control mice. These data reveal that the *FoxG1*<sup>+/-</sup> animal model shares some components with the clinical phenotype of patients with *FOXG1* dysregulation (Seltzer et al., 2014). In keeping with this, Patriarchi et al. (2016) found an excitatory/inhibitory imbalance in *FoxG1*<sup>+/-</sup> mice. Interestingly, EEG hyperexcitability has also been described in both *Mecp2*<sup>-/-</sup>, which have a non-cell type-specific gene deletion, and *Viaat-Mecp2*<sup>-/-</sup> mice, which harbor a GABAergic interneuron-specific deletion of *MeCP2* (Chao et al., 2010). In addition, enhanced sensitivity to KA-induced seizures has also been described in a further model of RTT, the *Cdk15*<sup>-/-</sup> mouse (Amendola et al., 2014). Thus, this evidence indicates a common phenotypic feature of RTT caused by different genetic aetiologies.

The finding of a seizure-prone phenotype, paralleled by EEG alterations, in *FoxG1*<sup>+/-</sup> mice prompted us to look for possible molecular correlates, also considering that impaired inhibitory synapses and circuitry are a hallmark of epilepsy (Ben-Ari et al., 2012; Mainardi et al.,

2012). To shed light on this point, we first quantified the expression of the potassium/chloride cotransporter KCC2, which (i) plays a key role in the maintenance of a proper inhibitory tone, (ii) shows altered expression in epilepsy, and (iii) had not yet been analyzed in the *FoxG1*<sup>+/-</sup> model. Our results show that *FoxG1*<sup>+/-</sup> mice display a significant reduction in hippocampal KCC2 expression, which could represent the substrate for both altered electrophysiological activity and increased propensity for and severity of KA-induced seizures. On the other hand, the expression of the GABA transporter vGAT was not affected by *FoxG1* haploinsufficiency, differently from the GABA-synthesizing enzyme GAD67, whose levels have been shown to be decreased in adult mice (Patriarchi et al., 2016).

The second finding of the present study regards a possible therapeutic approach based on a TRI-supplemented anaplerotic diet. TRI has already been successfully used in different mitochondrial pathologies, including MeCP2-dependent RTT (Park et al., 2014). TRI has also shown anticonvulsant effects in specific mouse models (Thomas et al., 2012; Willis et al., 2010), in addition to mitigating the effects of pilocarpine-induced status epilepticus in Wistar rats (Gama et al., 2015), suggesting that it could be employed as an adjuvant or alternative therapy for refractory epilepsy. This prompted us to evaluate the effectiveness of this treatment in *FoxG1*<sup>+/-</sup> mice. Strikingly, TRI treatment caused a dramatic improvement in altered EEG activity, not only by positively affecting basal hippocampal hyperexcitability, but also by increasing the resistance to the development of overt seizures in response to a proconvulsive insult. From a behavioral point of view, TRI was able to counteract the higher severity of seizure events induced in *FoxG1*<sup>+/-</sup> mice by KA injection. These improvements were accompanied by restored expression of KCC2. Moreover, TRI increased vGAT specifically in HT mice, with no significant effect on WT mice. This indicates a specific effect of TRI in the context of the *FoxG1*<sup>+/-</sup> genotype, which can correlate with the positive effects on electrophysiological alterations and on behavioral manifestations of seizures.

TRI is known to exert a neuroprotective role by preserving mitochondrial metabolism in both epilepsy and stroke models (Schwarzkopf et al., 2015; Tan et al., 2018b), although it should be pointed out that, for the latter case, treatment after an ischemic insult is not effective (Tan et al., 2018a). At the cellular level, anaplerotic diets appear to counteract the decrease in the abundance of TCA cycle intermediates and GABA (Tefera et al., 2017). On the other hand, it remains to be determined if neurotransmitter receptor function can be affected, as it has been shown for ketogenic diets (Boison, 2017). It is tempting to speculate that the effects on mitochondrial metabolism could explain neuroprotection, whereas the action on GABA levels or on neurotransmitter receptors might more directly contribute to modulate neuronal excitability, a point that deserves specific investigation in the future.

Ketogenic and anaplerotic diets, in general, appear to be effective in ameliorating an expanding list of disorders (Boison, 2017; Tefera et al., 2017) but, strikingly, an anaplerotic approach based on TRI seems to be more effective on seizures. Indeed, our control diet, based on soybean oil, did not display any effect on the hyperexcitability and behavioral seizure phenotype of *FoxG1*<sup>+/-</sup> mice, in agreement with Gama et al. (2015). Combined with our biochemical findings, this may suggest novel specific targets and new actions of TRI on brain physiopathology. Thus, modifications of gene transcription profile and mitochondrial function in *FoxG1*<sup>+/-</sup> mice, along with the possible effect of triheptanoin on them, represent a future research effort.

Seizures can be triggered by a variety of external insults (Rakhade and Jensen, 2009), and our data indicate that mutations in *FoxG1* can impinge on this process. Since epilepsy associated to *FOXG1* mutations is often difficult to treat pharmacologically (Seltzer et al., 2014), and considering that promising results have been obtained by oral administration of TRI to children with pharmacoresistant epilepsy (Calvert et al., 2018), our results highlight the potential of triheptanoin-supplemented diets as a possible support to drug therapy in individuals

with brain dysfunction caused by *FOXG1* mutations.

## Acknowledgements

We wish to thank Francesca Biondi, MSc, for excellent assistance in breeding and care of mouse colonies.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2019.01.005>.

## Funding and disclosure

This research was funded by grants “AIRC IG 2016-18925” from AIRC and “NanoBioMarker 2016” from Fondazione Pisa.

The authors declare that no competing financial interests exist in relation to the work described.

## References

- Amendola, E., Zhan, Y., Mattucci, C., Castroflorio, E., Calcagno, E., Fuchs, C., Lonetti, G., Silingardi, D., Vysotski, A.L., Farley, D., Ciani, E., Pizzorusso, T., Giustetto, M., Gross, C.T., 2014. Mapping pathological phenotypes in a mouse model of CDKL5 disorder. *PLoS One* 9, e91613.
- Ariani, F., Hayek, G., Rondinella, D., Artuso, R., Mencarelli, M.A., Spanhol-Rosseto, A., Pollazzon, M., Buoni, S., Spiga, O., Ricciardi, S., Meloni, I., Longo, I., Mari, F., Broccoli, V., Zappella, M., Renieri, A., 2008. *FOXG1* is responsible for the congenital variant of Rett syndrome. *Am. J. Hum. Genet.* 83, 89–93.
- Ben-Ari, Y., Khalilov, I., Kahle, K.T., Cherubini, E., 2012. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. *Neuroscientist* 18, 467–486.
- Boison, D., 2017. New insights into the mechanisms of the ketogenic diet. *Curr. Opin. Neurol.* 30, 187–192.
- Calvert, S., Barwick, K., Par, M., Ni Tan, K., Borges, K., 2018. A pilot study of add-on oral triheptanoin treatment for children with medically refractory epilepsy. *Eur. J. Paediatr. Neurol.* 22 (6), 1074–1080.
- Chao, H.T., Chen, H., Samaco, R.C., Xue, M., Chahrouh, M., Yoo, J., Neul, J.L., Gong, S., Lu, H.C., Heintz, N., Ekker, M., Rubenstein, J.L., Noebels, J.L., Rosenmund, C., Zoghbi, H.Y., 2010. Dysfunction in *GABA* signalling mediates autism-like stereotypies and Rett syndrome phenotypes. *Nature* 468, 263–269.
- Chen, L., Wan, L., Wu, Z., Ren, W., Huang, Y., Qian, B., Wang, Y., 2017. *KCC2* down-regulation facilitates epileptic seizures. *Sci. Rep.* 7, 156.
- Corradini, L., Donzelli, A., Antonucci, F., Welzl, H., Loos, M., Martucci, R., De Astis, S., Pattini, L., Inverardi, F., Wolfer, D., Caleo, M., Bozzi, Y., Verderio, C., Frassoni, C., Braidà, D., Clerici, M., Lipp, H.P., Sala, M., Matteoli, M., 2014. Epileptiform activity and cognitive deficits in *SNAP-25* (+/-) mice are normalized by antiepileptic drugs. *Cerebr. Cortex* 24, 364–376.
- De Filippis, R., Pancrazi, L., Bjorgo, K., Rosseto, A., Kleefstra, T., Grillo, E., Panighini, A., Cardarelli, F., Meloni, I., Ariani, F., Mencarelli, M.A., Hayek, J., Renieri, A., Costa, M., Mari, F., 2012. Expanding the phenotype associated with *FOXG1* mutations and in vivo *FoxG1* chromatin-binding dynamics. *Clin. Genet.* 82, 395–403.
- Eagleson, K.L., Schlueter McFadyen-Ketchum, L.J., Ahrens, E.T., Mills, P.H., Does, M.D., Nickols, J., Levitt, P., 2007. Disruption of *Foxg1* expression by knock-in of *cre* recombinationase: effects on the development of the mouse telencephalon. *Neuroscience* 148, 385–399.
- Gama, I.R., Trindade-Filho, E.M., Oliveira, S.L., Bueno, N.B., Melo, I.T., Cabral-Junior, C.R., Barros, E.M., Galvao, J.A., Pereira, W.S., Ferreira, R.C., Domingos, B.R., da Rocha Ataíde, T., 2015. Effects of ketogenic diets on the occurrence of pilocarpine-induced status epilepticus of rats. *Metab. Brain Dis.* 30, 93–98.
- Kahle, K.T., Deeb, T.Z., Puskarjov, M., Silayeva, L., Liang, B., Kaila, K., Moss, S.J., 2013. Modulation of neuronal activity by phosphorylation of the K-Cl cotransporter *KCC2*. *Trends Neurosci.* 36, 726–737.
- Mainardi, M., Di Garbo, A., Caleo, M., Berardi, N., Sale, A., Maffei, L., 2014. Environmental enrichment strengthens corticocortical interactions and reduces amyloid-beta oligomers in aged mice. *Front. Aging Neurosci.* 6, 1.
- Mainardi, M., Landi, S., Gianfranceschi, L., Baldini, S., De Pasquale, R., Berardi, N., Maffei, L., Caleo, M., 2010a. Environmental enrichment potentiates thalamocortical transmission and plasticity in the adult rat visual cortex. *J. Neurosci. Res.* 88, 3048–3059.
- Mainardi, M., Pietrasanta, M., Vannini, E., Rossetto, O., Caleo, M., 2012. Tetanus neurotoxin-induced epilepsy in mouse visual cortex. *Epilepsia* 53, e132–136.
- Mainardi, M., Scabia, G., Vottari, T., Santini, F., Pinchera, A., Maffei, L., Pizzorusso, T., Maffei, M., 2010b. A sensitive period for environmental regulation of eating behavior and leptin sensitivity. *Proc. Natl. Acad. Sci. U. S. A.* 107, 16673–16678.
- Navidhamidi, M., Ghaseini, M., Mehranfard, N., 2017. Epilepsy-associated alterations in hippocampal excitability. *Rev. Neurosci.* 28, 307–334.
- Pancrazi, L., Di Benedetto, G., Colombaioni, L., Della Sala, G., Testa, G., Olimpico, F., Reyes, A., Zeviani, M., Pozzan, T., Costa, M., 2015. *Foxg1* localizes to mitochondria and coordinates cell differentiation and bioenergetics. *Proc. Natl. Acad. Sci. U. S. A.*

- 112, 13910–13915.
- Panighini, A., Duranti, E., Santini, F., Maffei, M., Pizzorusso, T., Funel, N., Taddei, S., Bernardini, N., Ippolito, C., Virdis, A., Costa, M., 2013. Vascular dysfunction in a mouse model of Rett syndrome and effects of curcumin treatment. *PLoS One* 8, e64863.
- Park, M.J., Aja, S., Li, Q., Degano, A.L., Penati, J., Zhuo, J., Roe, C.R., Ronnett, G.V., 2014. Anaplerotic triheptanoin diet enhances mitochondrial substrate use to remodel the metabolome and improve lifespan, motor function, and sociability in MeCP2-null mice. *PLoS One* 9, e109527.
- Patriarchi, T., Amabile, S., Frullanti, E., Landucci, E., Lo Rizzo, C., Ariani, F., Costa, M., Olimpico, F., J. W.H., F. M.V., Renieri, A., Meloni, I., 2016. Imbalance of excitatory/inhibitory synaptic protein expression in iPSC-derived neurons from FOXG1(+/-) patients and in foxg1(+/-) mice. *Eur. J. Hum. Genet.* 24, 871–880.
- Rakhade, S.N., Jensen, F.E., 2009. Epileptogenesis in the immature brain: emerging mechanisms. *Nat. Rev. Neurol.* 5, 380–391.
- Schwarzkopf, T.M., Koch, K., Klein, J., 2015. Reduced severity of ischemic stroke and improvement of mitochondrial function after dietary treatment with the anaplerotic substance triheptanoin. *Neuroscience* 300, 201–209.
- Seltzer, L.E., Ma, M., Ahmed, S., Bertrand, M., Dobyns, W.B., Wheless, J., Paciorkowski, A.R., 2014. Epilepsy and outcome in FOXG1-related disorders. *Epilepsia* 55, 1292–1300.
- Shen, L., Nam, H.S., Song, P., Moore, H., Anderson, S.A., 2006. FoxG1 haploinsufficiency results in impaired neurogenesis in the postnatal hippocampus and contextual memory deficits. *Hippocampus* 16, 875–890.
- Shulyakova, N., Andreatza, A.C., Mills, L.R., Eubanks, J.H., 2017. Mitochondrial dysfunction in the pathogenesis of Rett syndrome: implications for mitochondria-targeted therapies. *Front. Cell. Neurosci.* 11, 58.
- Striano, P., Paravidino, R., Sicca, F., Chiurazzi, P., Gimelli, S., Coppola, A., Robbiano, A., Traverso, M., Pintaudi, M., Giovannini, S., Operto, F., Vigliano, P., Granata, T., Coppola, G., Romeo, A., Specchio, N., Giordano, L., Osborne, L.R., Gimelli, G., Minetti, C., Zara, F., 2011. West syndrome associated with 14q12 duplications harboring FOXG1. *Neurology* 76, 1600–1602.
- Tan, K.N., Hood, R., Warren, K., Pepperall, D., Carrasco-Pozo, C., Manzanero, S., Borges, K., Spratt, N.J., 2018a. Heptanoate is neuroprotective in vitro but triheptanoin post-treatment did not protect against middle cerebral artery occlusion in rats. *Neurosci. Lett.* 683, 207–214.
- Tan, K.N., Simmons, D., Carrasco-Pozo, C., Borges, K., 2018b. Triheptanoin protects against status epilepticus-induced hippocampal mitochondrial dysfunctions, oxidative stress and neuronal degeneration. *J. Neurochem.* 144, 431–442.
- Tefera, T.W., Tan, K.N., McDonald, T.S., Borges, K., 2017. Alternative fuels in epilepsy and amyotrophic lateral sclerosis. *Neurochem. Res.* 42, 1610–1620.
- Thomas, N.K., Willis, S., Sweetman, L., Borges, K., 2012. Triheptanoin in acute mouse seizure models. *Epilepsy Res.* 99, 312–317.
- Verginelli, F., Perin, A., Dali, R., Fung, K.H., Lo, R., Longatti, P., Guiot, M.C., Del Maestro, R.F., Rossi, S., di Porzio, U., Stechishin, O., Weiss, S., Stifani, S., 2013. Transcription factors FOXG1 and Groucho/TLE promote glioblastoma growth. *Nat. Commun.* 4, 2956.
- Willis, S., Stoll, J., Sweetman, L., Borges, K., 2010. Anticonvulsant effects of a triheptanoin diet in two mouse chronic seizure models. *Neurobiol. Dis.* 40, 565–572.