HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

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RESEARCH THESIS PROJECT

THE SEPARATE AND SYNERGISTIC EFFECTS OF AMPAR ANTAGONISTS ON THE TRANSITION OF CORTICAL NETWORKS TO EPILEPTIFORM ACTIVITY

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The separate and synergistic effects of AMPAR antagonists on the transition of cortical networks to epileptiform activity

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Summary

The incidence of drug-resistant seizures and epilepsy is considerably high in children, but the underlying etiology for their refractoriness to antiepileptic treatment remains elusive. As primary contributors to the inherent seizure susceptiveness of the immature brain, AMPA receptors represent unique targets for the development of novel antiepileptic drugs for intractable pediatric syndromes. Specifically, decanoic acid, a main constituent of the medium chain triglyceride ketogenic diet, and perampanel, a recently approved anticonvulsant, has been shown to provide strong separate and synergistic antiseizure action through sufficient AMPA receptor inhibition. However, it is unknown yet whether these compounds block AMPA receptors globally across the brain and during critical developmental transitions. Here, we present an *ex vivo* model of endogenous neocortical network activity from brain slice preparations of pre- and post-wining mice to explore the impact of developmental changes on seizure generation and spread, as well as on the pharmacosensitivity of paroxysmal activity to these two pharmacological agents. Our data supported the existence of age-specific patterns of epileptiform activity in the developmental transitions from early to late childhood. Decanoic acid displayed a higher potency compared to perampanel, which failed to terminate 0-Mg^{2+} induced epileptiform discharges at clinically relevant concentrations. These results denote that the development of resistance to certain drugs may be an intrinsic characteristic of immature tissue or specific brain networks.

Highlights

- Under zero-Mg^{2+} bathing conditions, neocortex exhibits age-dependent patterns of epileptiform activity
- Decanoic acid is more potent against zero-Mg^{2+} induced epileptiform discharges compared to perampanel
- There are marks of a synergistic relationship between the two drugs that is expressed only to one of our proposed quantification metrics of epileptiform discharges

Keywords

neocortex, drug-resistant epilepsy, epileptiform activity, zero Mg^{2+}, S1BF, Up state, decanoic acid, perampanel, childhood epilepsy

Abbreviations

ACSF – artificial cerebrospinal fluid

AED – antiepileptic drug

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

DA – decanoic acid

DMSO – dimethyl-sulfoxide

GABA – γ-aminobutyric acid

LFP – local field potential

LSE – late stage event

MCT – Medium Chain Triglyceride

NREM – non-rapid eye movement

PER – perampanel

PTZ – pentylenetetrazole

SLE – seizure-like event

S1BF – primary somatosensory cortex barrel field

SWD – spike-wave discharge

Introduction

Epilepsy is a serious medical trouble and accounts for approximately 2.5 million of the worldwide population (Devinsky *et al.*, 2018). Standard therapeutic approaches for epileptic patients involve clinically available anticonvulsant drugs in an effort to fully prevent or reduce seizure incidence and propagation and also minimize the potential of cognitive and psychosocial impacts (French *et al.*, 2013; Schmidt and Schachter, 2014). Despite the elevated availability of clinically approved pharmacological agents the last two decades, only two third of the adults and approximately 75% of the children under pharmacotherapy can secure a seizure free life without long-term relapse. Drug-resistant epilepsy, in particular, refers to inadequate or complete lack of seizure management by drugs, forcing patients to resort to alternative treatment options, including neurosurgery, polytherapy or stringent dietary plans (Pati and Alexopoulos, 2010; Wahab *et al.*, 2010). Moreover, it is assumed that immature brain

inclines towards excitatory paths due to incomplete entrenchment of excitatory-inhibitory interplay during these critical developmental periods, making it extremely prone to epileptogenic inputs and new-onset seizures incidences (Rakhade and Jensen, 2009). There are several reports substantiating the increased predisposition to seizures in neonatal and childhood ages compared to adulthood, often arising without an apparent cause (Sanchez *et al.*, 2001; Wong and Yamada, 2001). This means that failure of immediate seizure handling in early pediatric period due to emerging pharmacoresistance could place children to a poor health-related quality of life, along with an increased morbidity and mortality risk (Lagae, 2009; Berg *et al.*, 2012). At the same time, as biological mechanisms underlying drug refractoriness still lack a cohort explanation, the need for further efficacy scrutiny and mechanistic specificity of existing or under development pharmacological components in the developing brain will rise.

Glutamatergic neurotransmission plays a pivotal role in shaping the balance between excitation and inhibition (Wang, Gilbert and Man, 2012). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are heterotetrameric postsynaptic structures that act as chief mediators of the fast-excitatory synaptic transmission through their ubiquitous expression in the central nervous system (CNS) (Kondo, Sumino and Okado, 1997; Lalanne *et al.*, 2016). Excessive activation of AMPARs is a common phenomenon often encountered in normal neuronal processes, like synaptic potentiation and developmental changes, and also in pathological conditions, including neuronal hyperexcitability and neurotoxicity (Charsouei, Jabalameli and Karimi-Moghadam, 2020). It is reported that overactivation of AMPARs, in addition to their established role in several epilepsy syndromes, is considerably implicated in the pathophysiology of seizures generation, disease progression, as well as drug refractoriness prevalence (Rogawski, 2011). This pathological outcome has often been associated with maladjusted functional and dynamic properties of AMPA receptors, defined by dysregulated subunit arrangements and splice variants, and thus disturbed desensitization kinetics, trafficking and signal transduction processes (Chater and Goda, 2014; Charsouei, Jabalameli and Karimi-Moghadam, 2020). Following this, AMPARs received marked attention as potential targets in the treatment of epilepsy and the development of new AEDs (Rogawski, 2011, 2013; Hanada and Hanada, 2014). Of note, several studies in both animal and human models demonstrated the anticonvulsant prospective of AMPARs antagonists in providing broader spectrum antiepileptic efficacy over other conventional antiepileptic therapeutics (Russo *et al.*, 2012; Hanada and Hanada, 2014; Barygin, 2016;).

DA is a 10-carbon unbranched fatty acid and one of the primary intermediate-chained constituents of the MCT diet, after 8-carbon octanoic acid, that can be provided by natural sources, like coconut oil and palm kernel oil (Chang *et al.*, 2013). MCT diet, a

more tolerable version of the classic high-fat, low carbohydrate-intake ketogenic diet, represents an effective alternative for the treatment and seizure control of pediatric epilepsy and drug-resistant epilepsy in children (Sills *et al.*, 1986; Winesett, Bessone and Kossoff, 2015). Recent relevant studies found a positive correlation between anticonvulsant effectiveness of the diet and the increase of DA levels in patients' serum (Haidukewych, Forsythe and Sills, 1982; Sills *et al.*, 1986; Dean, Bonser and Gent, 1989; Shrestha *et al.*, 2015). Interestingly, DA, which non-competitively acts as an AMPAR antagonist, shows a better anticonvulsant potential than its quantitively superior MCT relatives; octanoic acid and ketones, and other widely used non-competitive AMPAR antagonists, such as GYKI-52446 (NAKAMURA *et al.*, 1990; Chang *et al.*, 2013, 2016; Wlaź *et al.*, 2015; Williams, 2017). In addition, Chang et al. further validated DA's unique antiseizure profile by showing its inhibitory dose-dependent effect against both PTZ and 0-Mg^{2+} induced epileptiform activity in CA1 hippocampal rat slices and its direct, voltage-dependent activity by blocking AMPAR-mediated excitatory postsynaptic currents (Chang *et al.*, 2016). DA constitutes, therefore, a promising non-pharmacological candidate with a remarked clinical potential in the future for the treatment of intractable children epilepsy.

PER, is another negative allosteric antagonistic modulator of AMPARs that has been recently approved as a clinical adjunctive treatment for intractable partial-onset seizures with or without secondarily generalized seizures, and is speculated to exhibit a comparable antiseizure effect with DA (Hanada *et al.*, 2011; Russo *et al.*, 2012; Frampton, 2015; Di Bonaventura *et al.*, 2017). Among other recognized potent AEDs, PER provides a broader spectrum activity against seizures, evidenced by its efficacy in minimizing seizure frequency and associated behaviors in preclinical animal models of varied epileptiform types (Rogawski and Hanada, 2013; Hanada and Hanada, 2014; Potschka and Trinka, 2019). Additional *in vitro* studies validated PER's selectivity against AMPAR-induced excitatory postsynaptic field potentials in neocortical single neurons (Ceolin *et al.*, 2012a; Rogawski and Hanada, 2013; Chen *et al.*, 2014) and its extensive applicability against AMPARs of native rat and human hippocampus and cerebellum reconstituted into Xenopus oocytes (Zwart *et al.*, 2014). It has been reported recently that co-administration of PER and DA reinforces seizure inhibition in rat hippocampal slices and human resected neocortical tissue indicating their synergistic action (Augustin *et al.*, 2018). In an attempt to fully understand PER's inhibitory mode of action and its suggested region-inclusive anticonvulsant response and in order to propose a clinically safer dosage profile of both agents with less associated side-effects, it would be useful to characterize PER's single and synergistic action under differing experimental settings and various epileptiform induction conditions.

Divergent regional, developmental and pathophysiological aspects of epileptic syndromes often engender conflicting outcomes across similar experimental models,

rendering the study of seizures, a rather unpredictable process. Brain animal slices preparations are particularly useful in the management of heterogeneous epileptic environments and in the classification of seizures, allowing for more targeted pharmacological and neurophysiological interventions under more specific developmental and localization conditions (Raimondo *et al.*, 2017). Another problem obscuring the elucidation of a consistent mechanistic path underlying epilepsy, concerns current related literature, which usually involves a large animal age range, thus withholding the contribution of the critical phase factor that distinguishes younger from older ages. On top of that, despite the well-documented role of cortical areas in seizure initiation and spread in other epileptiform foci, research emphasis still prevails in the study of hippocampus as the predominant contributor in neuronal hypersynchronization (Codadu, Parrish and Trevelyan, 2019). However hippocampal slices, are marked by complete inactivity under input-free circumstances, compared to neocortical, which are endogenously very active, by constantly oscillating between highly synchronous up and down states to serve higher brain functions (Hablitz, 1987; Sanchez-Vives and McCormick, 2000; Chauvette, Volgushev and Timofeev, 2010). It becomes clear that an *in vitro* neocortical model of increased vulnerability towards hyperactivity would be a more appropriate approach to assess epileptiform activity and perform pharmacological manipulations in the developing brain.

In the present study, we introduce an *ex vivo* model of acute mouse neocortical slices that transitions from spontaneous synchronous Up state events to evoked hypersynchronous discharges using a Mg^{2+}-free bathing solution. Since most pediatric epileptic syndromes are thought to have a neocortical origin (Hablitz, 1987; Bourgeois, 1998; Wong and Yamada, 2001), we employed two early life developmental stages, resembling human ages of early and late childhood, in an effort to examine differing patterns of epileptiform activity in the immature brain. Effect comparisons and synergistic evaluation of two novel anticonvulsants, with a direct AMPAR blocking effect, DA and PER, were tested in our proposed model of refractory neocortical epilepsy, with an intend to suggest a new quantification metric that is destined to facilitate analytical consistency in the identification process of epileptiform events. Our results reveal distinct pharmacological profiles of the two agents confirming different inhibitory responses across the CNS.

STAR★Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE
Experimental models: Organisms/Strains	
Mouse: C57BL/6	Jackson Laboratory
Software and Algorithms	
Axograph X (version 1.3.5)	https://axograph.com
LFP Analyzer	Tsakanikas et. al, 2017 doi: 10.1038/s41598-017-03269-9
SPSS (version 25)	https://www.ibm.com/analytics/spss-statistics-software
Prism	GraphPad https://www.graphpad.com

Experimental Model and Subject Details

Animals C57Bl/6J mice were handled and bred in the animal facility of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. This animal and experimental facility is registered according to the Presidential Decree of the Greek Democracy 160/91, which harmonizes the Greek national legislation with the European Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes. This study was approved by the Regional Veterinary Service, in accordance to the National legal framework for the protection of animals used for scientific purposes (reference number 3372/05-07-2018). Mice were weaned at 21 days postnatally (P21), housed in groups of 2–8, in 267 mm × 483 mm × 203 mm cages supplied with bedding material and kept at a 12–12 dark-light schedule. Food and water were provided *ad libitum*.

Neocortical slice preparation Young male mice from two different age groups; P16-20 and P27-31, were sacrificed with cervical dislocation, and the brain was rapidly extracted and placed in oxygenated (95% O2– 5% CO2) ice-cold dissection buffer containing, in mM: KCl 2.14; $NaH_2PO_4.H_2O$ 1.47; $NaHCO_3$ 27; $MgSO_4$ 2.2; D-Glucose 10; Sucrose 200; and $CaCl_2.2H_2O$ 2; osmolarity (mean ± SD): 298 ± 5 mOsm, pH: 7.4±0.2. Coronal brain slices (400 μm) of neocortex were obtained from the level of primary somatosensory cortex of the whiskers [i.e., barrel cortex, S1BF; Anterior-Posterior from Bregma (A/P): 0.38 to -1.94 mm, Medial-Lateral (M/L): 2.50 to 4.00 mm] of the right hemisphere using a vibratome (VT 1000S, Leica) and placed in a holding chamber with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126; KCl 3.53; $NaH_2PO_4.H_2O$ 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; D-Glucose 10 and $CaCl_2.2H_2O$ 2 [osmolarity (mean ± SD): 317 ± 4 mOsm, pH: 7.4±0.2], where they were

incubated for recovery at room temperature (RT: 24–26°C) for at least 1 h prior to recording.

***Ex vivo* electrophysiology** Following recovery, slices were transferred to a submerged chamber (Luigs & Neumann) coated with a layer of transparent silicone onto which up to four slices could be pinned, in order to maximize yield, lower the number of animals used and compare different experimental groups under identical conditions. The slices were constantly perfused at high flow rates (10–15 ml/min) to ensure optimal oxygenation of the cortical tissue (Hájos *et al.*, 2009).

Recordings were initially performed at room temperature using an "*in vivo*-like" ACSF (i.e. same composition as the above mentioned, but with 1mM $CaCl_2$). This ionic buffer more closely resembles the *in vivo* cerebrospinal fluid (Harris *et al.*, 1984; Jones and Keep, 1988) and favors the development and maintenance of cortical spontaneous activity in the form of alternating Up and Down states (Sanchez-Vives and McCormick, 2000; Hájos *et al.*, 2009; Rigas *et al.*, 2015; Sigalas *et al.*, 2015).

Following recording of endogenous cortical activity for 20-30 mins, the temperature was increased to 33-35^{o}C and the extracellular solution was switched to the Mg^{2+} free ACSF, a standard protocol for the induction of epileptiform activity (Mody, Lambert and Heinemann, 1987; Tancredi *et al.*, 1990). The transition to epileptiform activity was recorded for up to 120 minutes in Mg^{2+} free conditions in order to ensure the establishment of a stable pattern of epileptiform activity for at least 20 minutes prior to pharmacological interventions.

Compounds and pharmacology The compounds that were employed for the purposes of this study were decanoic acid (10, 100, 500 and 1000μM, Sigma-Aldrich) and perampanel (0.1, 0.3, 1, 3 and 10μM, provided as a generous gift by Eisai Co., Ltd). DA and PER were diluted in DMSO (1%, Sigma-Aldrich) and stocked as 1M and 10mM aliquots respectively and their final concentrations were adjusted with appropriate dilutions in ACSF. Each drug concentration was applied for a total of 40 min and epileptiform activity was quantified for the second 20 min-period, in order to ensure adequate drug penetration and stability of antiepileptic effect. In experiments examining DA's effect and synergy between the two drugs, an additional 20 min of wash-out was recorded to ensure specificity of drug action.

Data Analysis Network activity was assessed by local field potential (LFP) recordings (sampled at 15 kHz, low-pass filtered at 200 Hz), obtained from cortical layers II/III using low impedance (∼0.5 MΩ) glass pipettes filled with ACSF. Signals were continuously acquired (AxoGraph X, version 1.3.5), amplified (MultiClamp 700B; Molecular Devices) and digitized (InstruTech; ITC-18). LFP traces were exported to

MATLAB format and analyzed offline with a custom-made MATLAB software (LFP Analyzer; Tsakanikas et al., 2017). Each recording session lasted 340-360 minutes and included: 20-minutes recording at room temperature to establish baseline Upstate activity; 20-minutes transition at 33-35^{o}C; 120-minutes recording in 0-Mg^{2+} conditions; 180-200 minutes recording in the presence of drugs; and 20-minutes washout period (**Figure 1A**).

Epileptiform activity was quantified using the following parameters: a) mean event duration (seconds), b) mean event occurrence (events/min), c) mean event rectified area, which is a measure of the overall size of epileptiform events reflecting both amplitude and duration, d) percent time in epileptiform activity, i.e. the proportion of time spent in epileptiform activity within a given recording period. In addition, we calculated the latency to both the first epileptiform discharge and to the onset SLEs, expressed in min after the 0-Mg^{2+} switch. The latency to the first LSE was not measured due to the high variability among slices of the same or different animal.

Statistical Analysis Data were analyzed using SPSS (IBM) and GraphPad Prism software. Sample size was defined based on the number of individual slices. Data were tested for normality (Shapiro-Wilk's test of normality), outliers (assessed by inspection of a boxplot), homogeneity of variances (Levene's test of homogeneity of variances) and sphericity (Mauchly's test of sphericity). Data that represented extreme outliers (defined as any data points that lie more than 3.0 times the interquartile range below the first quartile or above the third quartile) were discarded. In cases of departure from sphericity, the *p values* reported were corrected using the Greenhouse-Geisser estimate of sphericity. For cases of double comparisons, we used Mann-Whitney or Student's *t*-test. For multiple comparisons, we performed ANOVA followed by *post hoc* Bonferroni test. One-way repeated ANOVA test was performed for the investigation of the response to increasing concentrations of each drug (DA and PER). Synergistic effect of the two drugs was analyzed using a two-way mixed ANOVA model. Statistical significance was set at $p < 0.05$. All data are expressed as mean values ± SEM, and *n* indicates the number of brain slices.

Results

Perfusion with zero Mg^{2+} ACSF leads to age-dependent patterns of neuronal hyperexcitation

When active neocortical slices were subjected to zero Mg^{2+} conditions, a characteristic progressive pattern of distinct types of epileptiform events emerged in both age groups: Upstate activity was progressively dysregulated until it eventually disappeared giving rise to epileptiform discharges. For the purpose of clarity and quantification

accuracy we divided the response to Mg^{2+} - free conditions into three time periods: (i) the early stage activity (0-40 minutes), (ii) the intermediate stage activity (40-90 minutes) and (iii) the late stage activity (90-120 minutes) (**Figure 2**).

Early stage activity contained some remaining Up states at the beginning of the 0-Mg^{2+} transition (**Figure 2a**) that quickly disappeared to give rise to short (<1 second), irregularly occurring spike-and-wave discharges (SWDs, **Figure 2b**). **Intermediate stage activity** was marked by sustained seizure-like events (SLEs), which were long ictal discharges (≥ 20 seconds) comprising of a high frequency tonic wavefront, followed by an evolving tonic-clonic phase of periodically occurring afterdischarges (**Figure 2c**). **Late stage activity** was characterized by late stage events (LSEs) that either manifested recurring tonic discharges (**Figure 2d**), or had SLE-like morphology but with considerably shorter tonic-clonic components than those found in the earliest SLEs (not shown).

To the best of our knowledge, the impact of age on the transition to paroxysmal activity and on the pharmacosensitivity of epileptiform discharges has not been systematically examined. However, previous studies using animals of different ages have reached conflicting conclusions on epileptiform activity measures (e.g. frequency, duration) during the first three postnatal weeks of rats (Wong and Yamada, 2001; Wahab, Albus and Heinemann, 2011). Therefore, we decided to investigate the progression to the epileptiform state and the response to AEDs in two distinct developmental stages, under identical experimental conditions. To do this we used pre-weaning (P16-20) and post-weaning (P27-31) animals, and we measured the onset of both SWDs and SLEs, as well as the characteristics of steady-state activity during the final 20 min before drug application (occurrence, duration and rectified area of late stage events; and percent (%) time in epileptiform activity). The latency to either the first SWD (P16-20: 26.10 ± 7.13 min, n = 9; P27-31: 21.68 ± 4.58 min, n = 9; **Figure 3A**) or to the first SLE (P16-20: 46.75 ± 6.82 min, n=9; P27-31: 49.06 ± 3.98 min, n=9; **Figure 3B**) did not reveal statistically significant differences between the two age groups, indicating that the initiation of both interictal and ictal epileptiform activity is similar throughout these early developmental stages.

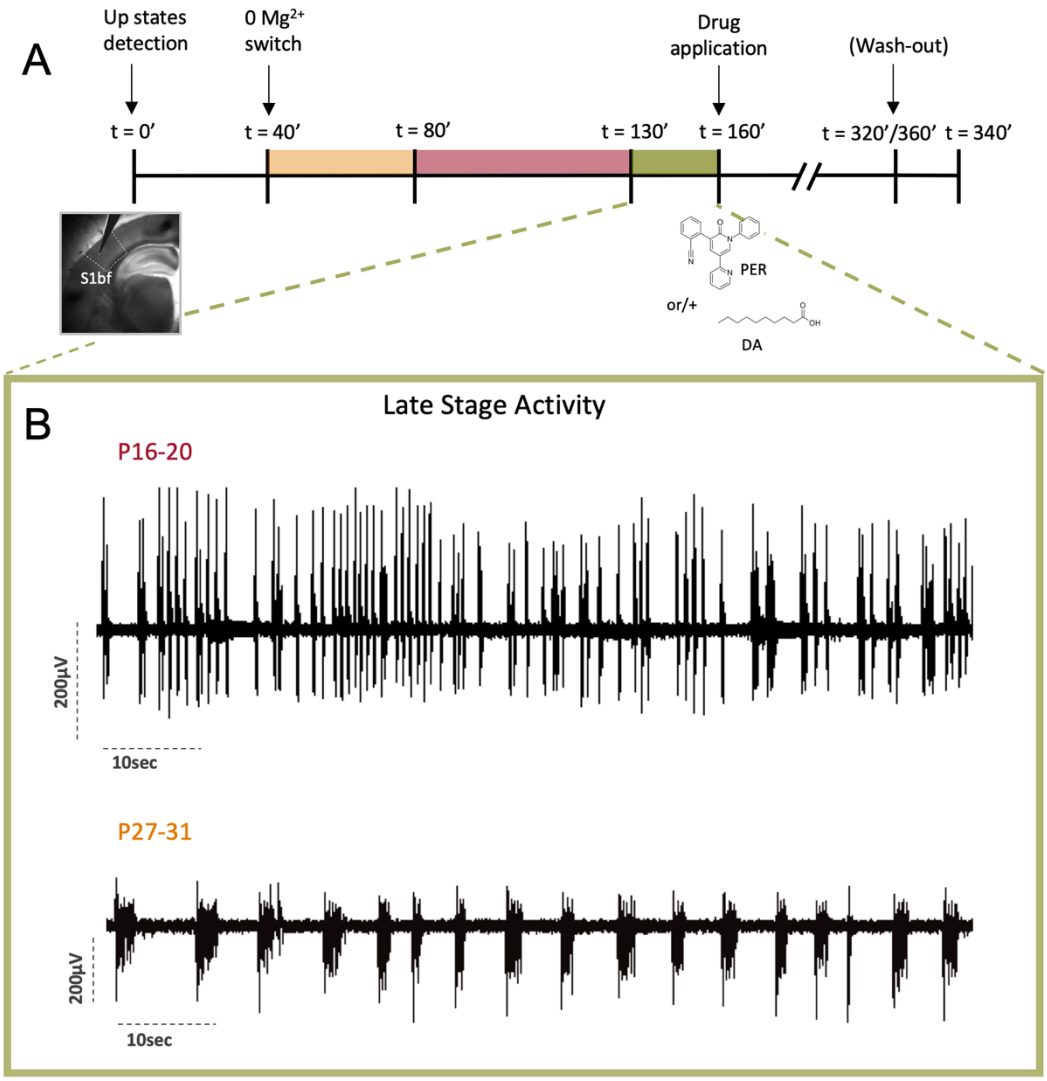

Figure 1. Experimental protocol and example of late stage epileptiform activity in the age groups tested in our model of pharmacoresistant epilepsy employing active cortical mouse brain slices. **A.** Timeline indicating the switch to Mg^{2+}- free ACSF and application of anti-epileptic compounds. The time values indicate an approximation of the onset to each stage. The total duration of drug perfusion depended on the number of different concentrations tested for each drug. In the case of DA and synergistic effect experiments, an additional 20 min wash-out period was recorded. **B.** Representative LFP traces of late stage activity prior to pharmacological interventions, illustrating the prevailing patterns of epileptiform events observed in the two tested age groups; P16-20 (top) and P27-33 (bottom).

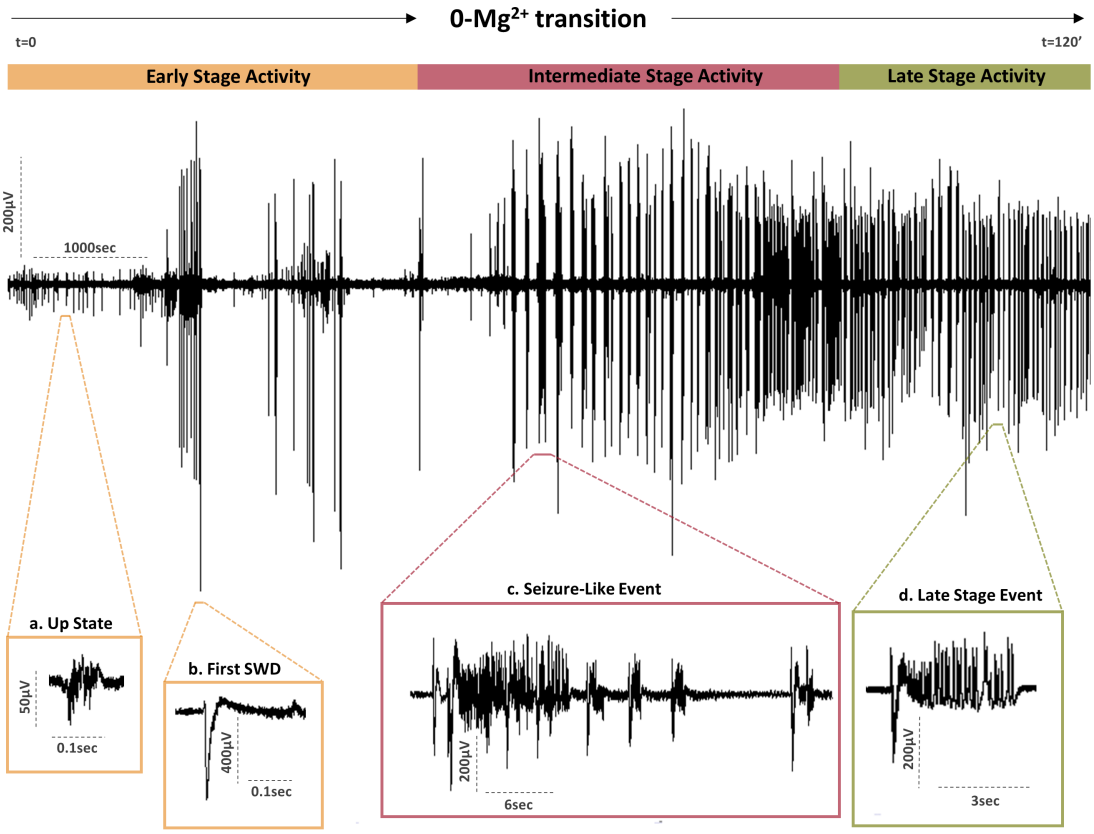

Figure 2. Cortical slices develop characteristic patterns of evolving epileptiform activity following perfusion with 0-Mg^{2+}ACSF**.** Epileptiform activity is divided into three distinct transitional stages, based on characteristic features in the pattern of epileptiform events observed throughout 0-Mg^{2+} conditions. Expanded views of LFP event traces of each transitional stage are depicted in the boxes; Early stage activity: a) Up state event and b) first spike-wave discharge; Intermediate stage activity: c) seizure-like event; Late stage activity: d) late stage seizure-like event.

In contrast, there were significant differences in the pattern of late stage activity between the two age groups (**Figure 1B, 3C-F**). Specifically, the occurrence of epileptiform events in pre-weaning animals was 47% higher compared to the older group (P16-20: 3.72 ± 0.50 event/min, n = 17; P27-31: 1.96 ± 0.30 event/min, n = 17; p = 0.0049; **Figure 3C**); while both indices/metrics of event size were smaller: the duration was 75% shorter (P16-20 : 7.06 ± 0.92 sec, n = 17; P27-31: 28.47 ± 9.47 sec, n = 17; p < 0.0001; **Figure 3D**) and the rectified area was 58% smaller (3.36 ± 0.77 V*sec, n = 17 vs. 1.41 ± 0.25 V*sec, n = 17; p = 0.0376; **Figure 3E**). Interestingly, the percent time in epileptiform activity, a measure of the total duration of epileptiform events within a given time interval, was not statistically different between the two groups (P16-20: 19.84 ± 2.30, n = 17; P27-31: 12 ± 2.28, n = 17; p = 0.1165; **Figure 3F**).

These data show that although the commonly used metrics of epileptiform activity, such as duration and occurrence of individual events, revealed significant differences between the two age groups, a more integrated metric such as the total duration of epileptiform activity highlights an overall similarity in the state of the network. In addition, this metric is not dependent on the often-arbitrary definition of distinct

types of events and more accurately reflects the %time that the network is generating paroxysmal discharges.

Taken together, these findings indicate that even small age differences can play a critical role in the evolution and establishment of epileptiform activity and that the use of complementary metrics for seizure parametrization and quantification is advantageous.

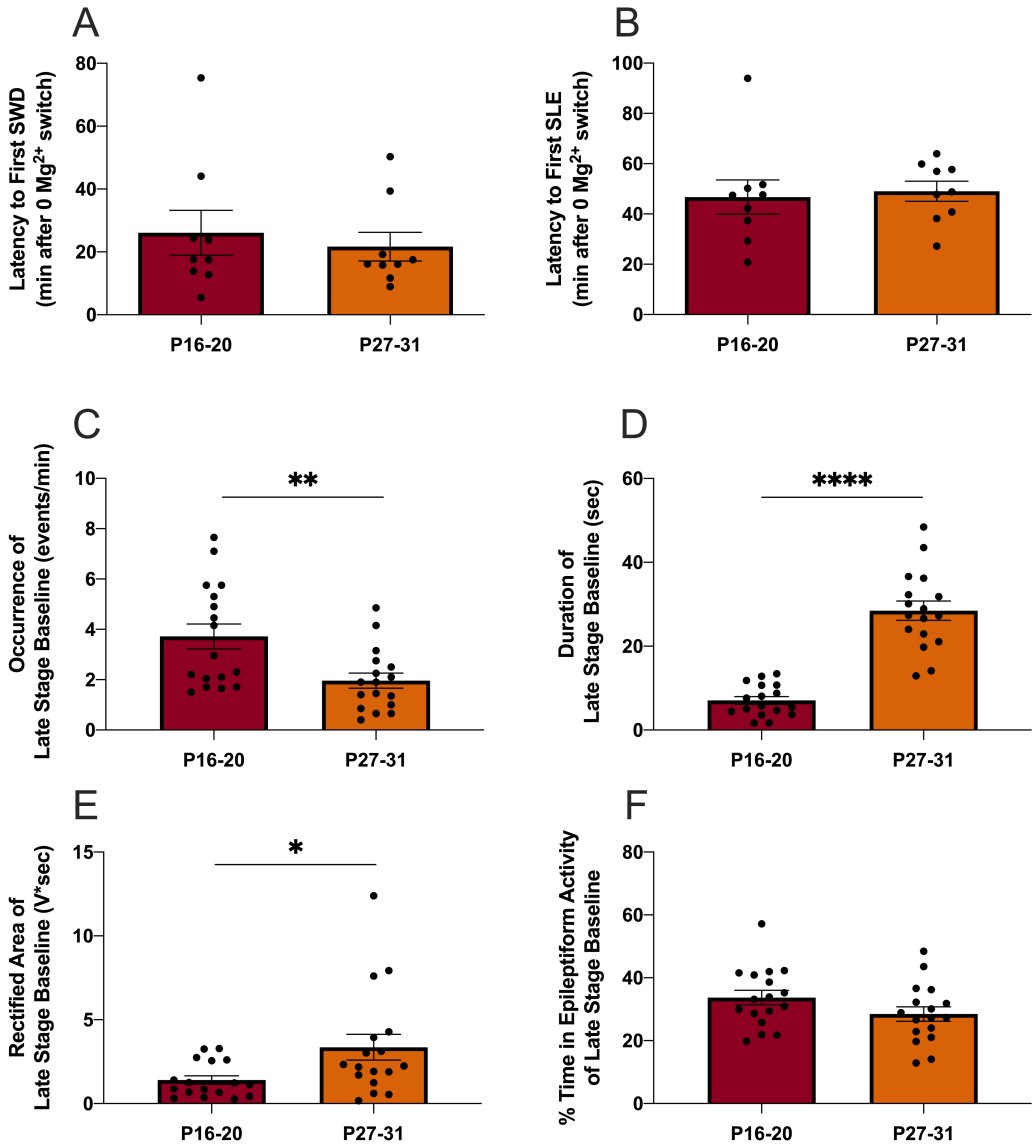

Figure 3. Developmental differences in epileptiform activity metrics A. and **B.** Latency to SWDs and LSEs does not differentiate early life stages **C- F.** Late stage activity manifests as distinct patterns of epileptiform events during early development, as quantified using four different parameters: occurrence (events/min) (**C**), duration (sec) (**D**) and rectified area (V*sec) (**E**) of individual events; as well as % time in epileptiform activity (**E**). Data are acquired from 17 slices for each age group and graphs show mean ± SEM values; unpaired *t* test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Decanoic acid attenuates epileptiform discharges in a concentration-dependent manner

The efficacy of DA, a fatty acid with reported antiseizure effect (Chang *et al.*, 2013) was tested in our Mg^{2+}-free model of epileptiform activity. The age factor is not included as a separate parameter in this set of experiments, as there were no significant changes in pharmacosensitivity between the two age groups in any of the tested metrics (two-way interaction in occurrence: $F_{(4,64)} = 2.694$, $p = 0.039$, partial $\eta^2 = 0.144$; two-way interaction in duration: $F_{(4,64)} = 1.617$, $p = 0.1807$, partial $\eta^2 = 0.092$; two-way interaction in rectified area: $F_{(4,64)} = 1.586$, $p = 0.1888$, partial $\eta^2 = 0.090$; two-way interaction in % time in epileptiform activity: $F_{(4,64)} = 1.095$, $p = 0.3665$, partial $\eta^2 = 0.064$).

To examine DA's anticonvulsant potential, we used four different concentrations: 10μM, 100μM, 500μM and 1000μM and found that attenuation of spontaneous epileptiform activity begins at 500μM for the 3 out of the 4 metrics used (**Figure 4**). DA produced a concentration-dependent response with almost full abolition of epileptiform discharges at 1mM, the final and higher concentration tested. Increasing DA concentrations reduced both mean duration ($F_{(1.374, 19.24)} = 17.08$, $p = 0.0002$, partial $\eta^2 = 0.5496$; **Figure 4C**) and mean rectified area ($F_{(2.171, 30.40)} = 16.66$, $p < 0.0001$, partial $\eta^2 = 0.5433$; **Figure 4D**) of epileptiform events with a statistical significant change observed at 500μM (mean duration: baseline vs 500μM, $p = 0.0073$; mean rectified area: baseline vs 500μM, $p = 0.0097$) and at 1mM (mean duration: baseline vs 1000μM, $p = 0.0065$; mean rectified area: baseline vs 1000μM, $p = 0.0015$). In contrast, the effect of DA on the occurrence of epileptiform events was biphasic ($F_{(2.129, 29.80)} = 23.73$, $p < 0.0001$, partial $\eta^2 = 0.6289$; **Figure 4B**), with an initial increase at 100μM (baseline vs 100μM, $p = 0.0035$) and a subsequent decrease leading to an almost complete block at 1mM (baseline vs 1000μM, $p = 0.0005$). This initial (and apparently paradoxical) increase in epileptiform events reflects the drug-induced changes in the *pattern* of activity: lower doses gave rise to shorter but more frequent epileptiform events, or isolated afterdischarges; whereas higher concentrations lead to more scattered, usually isolated spike wave discharges without tonic afterdischarges (bursting characteristics) (**Figure 4A**). Hence, the increase in event occurrence induced by DA does not indicate an increase in overall epileptiform activity, but rather a change in the pattern of this activity which became dominated by briefer events. For this reason, we include the additional metric of "percent time in epileptiform activity" as a more accurate and reliable indicator of anti-epileptic efficacy among discharge patterns with different morphological characteristics. Based on this metric, DA was found to significantly decrease the % time in epileptiform activity ($F_{(2.856, 39.98)} = 131.5$, $p < 0.0001$, partial $\eta^2 = 0.9038$; **Figure 4E**), which was expressed as a 85% decrease at 500μM (baseline vs 500μM, $p < 0.0001$) and a 99.5% decrease at 1000μM (baseline vs 1000μM, $p < 0.0001$), respectively. DA produced statistically significant differences

between 500μM and 1000μM in all the metrics measured (500μM vs 1000μM: mean duration, p = 0.0162; mean occurrence, p = 0.0008; mean % time in epileptiform activity, p = 0.0129) except from rectified area. Epileptiform discharges reappeared almost instantly after the perfusate was switched back to the regular ACSF (**Figure 4A**), further confirming the specificity of the anticonvulsant effect. These data confirm and extend previous findings and show that, at high concentrations, DA displays marked efficacy against 0-Mg^{2+} induced epileptiform activity.

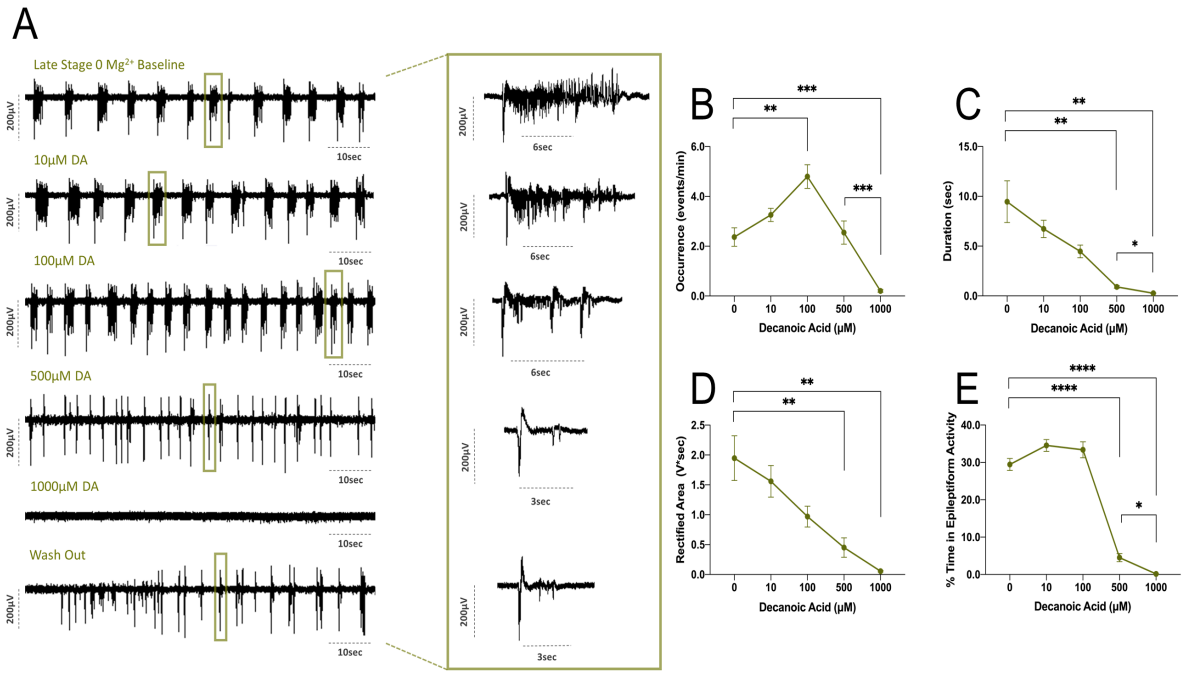

Figure 4. Decanoic acid successfully abolishes 0-Mg^{2+} induced epileptiform discharges in cortical slices. **A.** Example recording traces of a slice subjected to increasing concentrations of DA. Note the changes in the pattern of epileptiform activity induced by pharmacological transitions to higher DA concentrations. The effect of DA is quantified using four measures of epileptiform activity: **B**. occurrence (events/min), **C.** duration (sec) and **D.** rectified area (V*sec) of epileptiform events; and **E.** % time in epileptiform activity. Data are from 15 slices and graphs show mean ± SEM values. Statistical analysis was done using one-way ANOVA for repeated measures (*post hoc* Bonferroni test). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

Perampanel alters network dynamics but does not abolish epileptiform activity

We next investigated the anticonvulsant potential of PER, a clinically approved AED with antagonistic AMPA receptor action. As previous studies, using different protocols, brain areas and seizure induction methods, have reported highly variable IC_{50} values (Ceolin *et al.*, 2012b; Rogawski and Hanada, 2013; Chen *et al.*, 2014; Zwart *et al.*, 2014; Barygin, 2016), we decided to examine five different concentrations (0.1μM, 0.3μM, 1μM, 3μM and 10μM) in order to cover the entire IC_{50} spectrum. Again, the age factor was not considered as a separate parameter, due to non-significant differences in PER pharmacosensitivity between the two age groups (two-

way interaction in occurrence: $F_{(5,70)} = 1.105$, $p = 0.3659$, partial $\eta^2 = 0.073$; two-way interaction in duration: $F_{(5,70)} = 0.7500$, $p = 0.5888$, partial $\eta^2 = 0.51$; two-way interaction in rectified area: $F_{(5,70)} = 0.3683$, $p = 0.8687$, partial $\eta^2 = 0.26$; two-way interaction in % time in epileptiform activity: $F_{(5,70)} = 1.698$, $p = 0.1466$, partial $\eta^2 = 0.108$).

These experiments revealed a distinctively different action of PER compared to DA, as even the highest PER concentrations failed to abolish epileptiform activity. Nevertheless, PER caused notable changes in the *pattern* of activity (**Figure 5A**). Particularly, the duration of epileptiform activity was reduced ($F_{(1.339, 18.74)} = 31.20$, $p < 0.0001$ and partial $\eta^2 = 0.1393$), with all concentrations displaying significant difference compared to the initial baseline activity (baseline vs 0.1µM: $p = 0.0132$; baseline vs 0.3µM: $p = 0.0021$; baseline vs 1µM: $p = 0.0034$; baseline vs 3µM: $p = 0.0003$; baseline vs 10µM: $p = 0.0003$; **Figure 5C**). Statistically significant in-between differences were also detected among the higher concentrations administered (1µM vs 10µM: $p < 0.0001$; 1µM vs 3µM: $p = 0.0169$). The rectified area metric displayed a highly similar pattern ($F_{(1.213, 16.98)} = 27.95$, $p < 0.0001$ and partial $\eta^2 = 0.6663$; baseline vs 0.1µM: $p = 0.0057$; baseline vs 0.3µM: $p = 0.0039$; baseline vs 1µM: $p = 0.0020$; baseline vs 3µM: $p = 0.0008$; baseline vs 10µM: $p = 0.0007$; **Figure 5D**). In contrast, PER caused a gradual increase in the occurrence of epileptiform ($F_{(2.933, 41.06)} = 12.79$, $p < 0.0001$ and partial $\eta^2 = 0.4774$; baseline vs 0.3µM: $p = 0.0018$; baseline vs 1µM: $p = 0.0099$; baseline vs 3µM: $p = 0.0017$; baseline vs 10µM: $p = 0.0051$), which, unlike DA, did not reverse it even at the highest concentrations (**Figure 5B)**. Also unlike DA, PER caused only a modest decrease in the % time in epileptiform activity and only at the highest concentrations ($F_{(3.384, 47.37)} = 21.91$, $p < 0.0001$ and partial $\eta^2 = 0.6101$; baseline vs 3µM: $p = 0.0005$; baseline vs 10µM: $p = 0.0007$; **Figure 5E**).

These data demonstrate that PER is inefficacious at blocking 0-Mg^{2+} induced epileptiform discharges in cortical slices. Nevertheless, an attenuation of some epileptiform features was observed at the highest PER concentrations, affecting the pattern and morphology of seizure-like events.

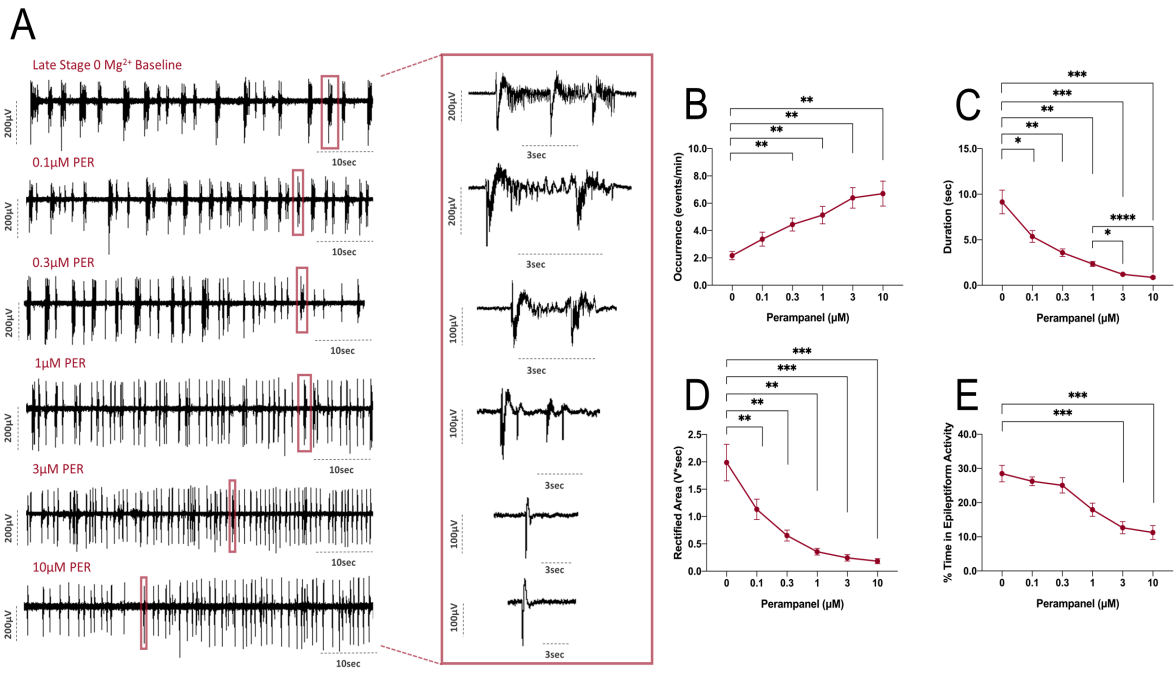

Figure 5. Perampanel does not abolish 0-Mg^{2+} induced epileptiform activity in neocortical slices. **A.** Example recording traces of a slice subjected to increasing concentrations of the drug. Even at the highest concentrations tested PER does not block epileptiform activity but it induces morphological changes in the discharge pattern. Summary data showing the effect of PER in four features of epileptiform activity; **B.** occurrence (events/min); **C.** duration (sec); **D.** rectified area (V*sec) and **E.** % time in epileptiform activity. Data are from 15 slices for each group and graphs show mean ± SEM values. Statistical analysis was realized using one-way ANOVA for repeated measures (*post hoc* Bonferroni test). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

Decanoic acid and perampanel act synergistically to reduce epileptiform activity in cortical slices

Our results thus far indicated that 0 Mg^{2+}-induced epileptiform activity in cortical slices displays different sensitivity to DA and PER, with DA showing a marked efficacy at suppressing and eventually abolishing epileptiform activity, compared to PER, which has a much smaller effect and fails to prevent epileptiform discharges even at the highest doses. This prompted us to investigate a possible synergistic interaction of the two compounds, as has been suggested in a previous study (Augustin *et al.*, 2018), by applying increasing concentrations of DA while keeping PER concentration constant at either 0.3 or 1µM. In this comparison we used the percent time in epileptiform activity as the most reliable metric to assess anti-epileptic efficacy.

The data revealed a clear synergistic effect of DA and PER compared to either drug acting on its own. This effect was most prominent at 100µM of DA, which on its own had no anti-epileptic effect (**Figure 4**), as documented by the significant two-way interaction between drug condition and drug concentrations (drug condition*concentration effect: $F_{(6,72)} = 7.640$, $p < 0.0001$, partial $\eta^2 = 0.389$; drug condition effect: $F_{(2,24)} = 10.51$, $p = 0.0005$, partial $\eta^2 = 0.467$; concentration effect: $F_{(2.127,51.05)} = 99.50$, $p < 0.0001$, partial $\eta^2 = 0.806$; **Figure 6A**). Specifically, while the

presence of DA alone did not seem to influence the % time in epileptiform activity, addition of PER to the perfusate led to a significant reduction of this metric reaching up to 62% ($F_{(3,31)}$ = 30.9, p < 0.0001, partial η^2 = 0.7497; control vs 100μM DA + 0.3μM Per: p < 0.0001; **Figure 6B**). The presence of PER alone did not have an effect in the % time in epileptiform activity. Combining higher DA concentrations (500μM) with PER showed significant differences from both baseline activity ($F_{(3,32)}$ = 97.6, p < 0.0001, partial η^2 = 0.9015; control vs 500μM DA: p < 0.0001; control vs 500μM DA + 0.3μM Per: p < 0.0001) and PER alone (0.3μM Per vs 500μM DA + 0.3μM Per: p < 0.0001; **Figure C**). Even this combined effect though, was not sufficient to block epileptiform discharges in any of the tested slices. It is also notable that higher PER concentrations did not augment the synergistic effect of the two drugs, suggesting that the effect of PER reaches a plateau after a certain drug concentration.

Collectively, our experimental evidence supports the synergistic action between DA and PER, expressed as a reduction in the % time of epileptiform activity. The synergistic effect did not depend on the applied PER dose, meaning that higher PER concentrations are not necessarily followed by a greater anticonvulsant response.

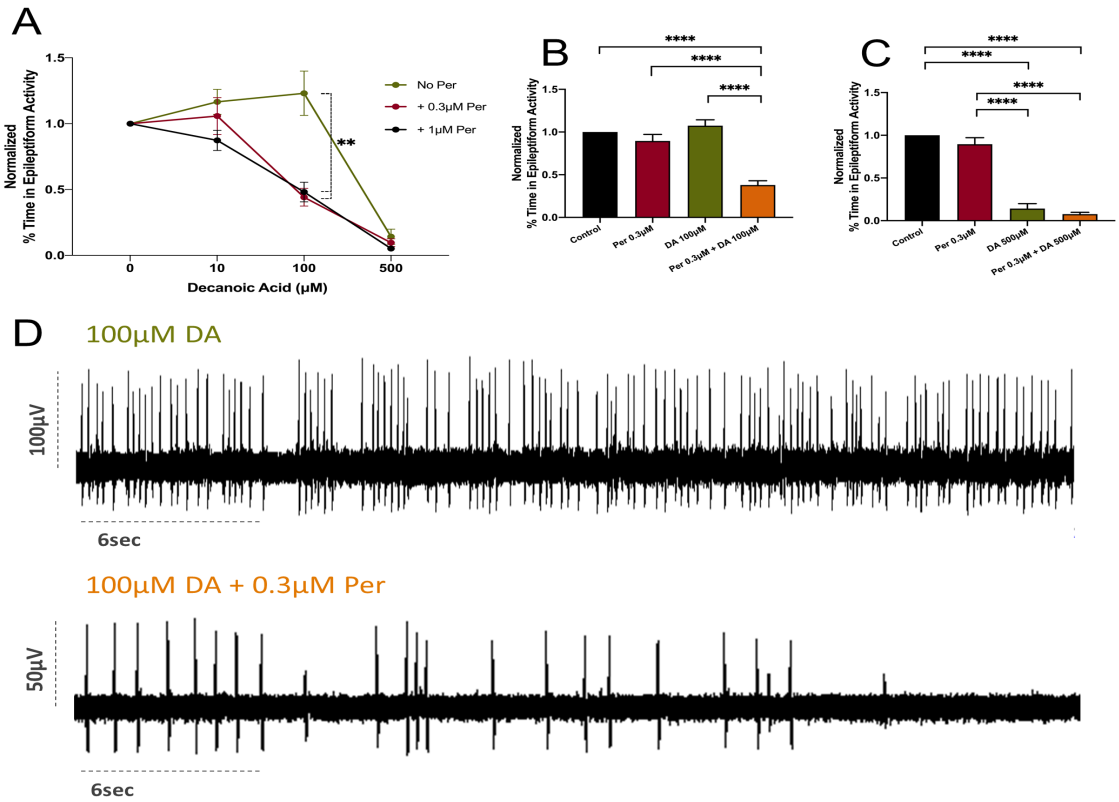

Figure 6. Decanoic acid and perampanel act synergistically to reduce epileptiform activity in cortical slices. Synergistic action of the two drugs was assessed by applying two fixed PER concentrations, while simultaneously increasing DA concentrations. **A.** Normalized epileptiform activity as a function of separate or combined

application of the two drugs. Data were collected from 9 slices for each group and normalized either to baseline (for DA only conditions), or to the PER-only condition (for combined drug conditions). Graph shows mean ± SEM values. Statistical analysis was performed using two-way ANOVA (*post hoc* Bonferroni test). ** $p < 0.01$. **B., and C.** Bar plots depicting synergistic efficacy of the two drugs against normalized % time in epileptiform activity. Data were collected from 9 slices for each group and normalized to baseline. Graphs show mean ± SEM values. Statistical analysis was realized using one-way ANOVA (*post hoc* Bonferroni test). **** $p < 0.0001$. **D.** Example recording traces illustrating evolving epileptiform activity of a slice subjected to DA alone (100μM) and a slice subjected to combination of the two drugs (100μM DA + 0.3μM Per).

Decanoic acid shows cortical specific antiseizure efficacy at high concentrations

An unexpected finding of our experiments was that anticonvulsant potency of DA (with or without PER) varied significantly between slices of the same animal. Specifically, we noticed that, at 500μM, the first concentration that caused a significant reduction in all tested metrics, DA had a stronger effect in more anterior cortical slices compared to more posterior ones (**Figure 7A, B**). In a typical experiment we use 2-4 consecutive 400μm sections corresponding to a bregma distance of roughly 0.70mm to 1.94mm, that cover the entire S1BF area (Paxinos and Keith, 2001). Our analysis revealed that the more anterior slices (bregma distance 0.7-1.2 mm) showed a greater sensitivity to DA, significantly affecting all metrics of epileptiform activity (anterior *vs* posterior slices: occurrence = 1.259 $\pm$ 0.2578 *vs* 3.491 $\pm$ 0.4747 events/min, $p = 0.0005$, $n = 11$; duration = 0.4872 $\pm$ 0.06 *vs* 0.6981 $\pm$ 0.258 sec, $p = 0.0473$, $n = 11$; rectified area = 0.038 $\pm$ 0.0165 *vs* 0.2054 $\pm$ 0.086 V*sec, $p = 0.0002$, $n = 11$; % time in epileptiform activity = 1.148 $\pm$ 0.28 *vs* 4.670 $\pm$ 0.9961, $p = 0.0028$, $n = 11$, **Figure 7D**). After DA application, these slices exhibited either almost complete abolition of epileptiform activity, or displayed very brief, small-amplitude, barely above the baseline discharges (mean occurrence < 0.5 events/min, 27.3%). In contrast, the more posterior slices (bregma distance 1.3-1.9 mm) displayed stronger, more enduring discharges against DA, in the form of disorganized SLEs rather than a noticeable reduction of the discharges. It should be noted that this behavior was consistent in all tested sets of anterior/posterior slices of the same animal.

An emerging question was whether this discrepancy concerning the DA effect between different sections in the anterior-posterior brain axis reflected a regional difference in the epileptiform activity itself. To test this, we performed a two-way repeated ANOVA to reveal a possible interaction between brain region and drug effect. The analysis revealed/confirmed a main effect of brain region in the efficacy of DA in terms of both occurrence ($F_{(1,18)} = 6.285$, $p = 0.0220$, partial $\eta^2 = 0.259$; control anterior slices vs control posterior slices: $p > 0.9999$; 500μM DA anterior slices vs 500μM DA posterior slices: $p = 0.0036$) and percent time in epileptiform activity ($F_{(1,18)} = 12.55$, $p = 0.0023$, partial $\eta^2 = 0.497$; control anterior slices vs control posterior slices: $p > 0.9999$; 500μM DA anterior slices vs 500μM DA posterior slices: $p < 0.0001$) (**Figure 7E**). However, there was no two-way interaction between region and drug in terms of

the duration and rectified area of the epileptiform discharges (two-way interaction in duration: $F_{(1,18)} = 0.702$, $p = 0.413$, partial $\eta^2 = 0.038$; two-way interaction in rectified area: $F_{(1,18)} = 0.5382$, $p = 0.476$, partial $\eta^2 = 0.029$). This regional specificity was not observed in the pattern of epileptiform discharges, meaning that DA's efficacy is specific to the cortical section, with the more posterior S1BF sections exhibiting greater drug resistance compared to more anterior ones.

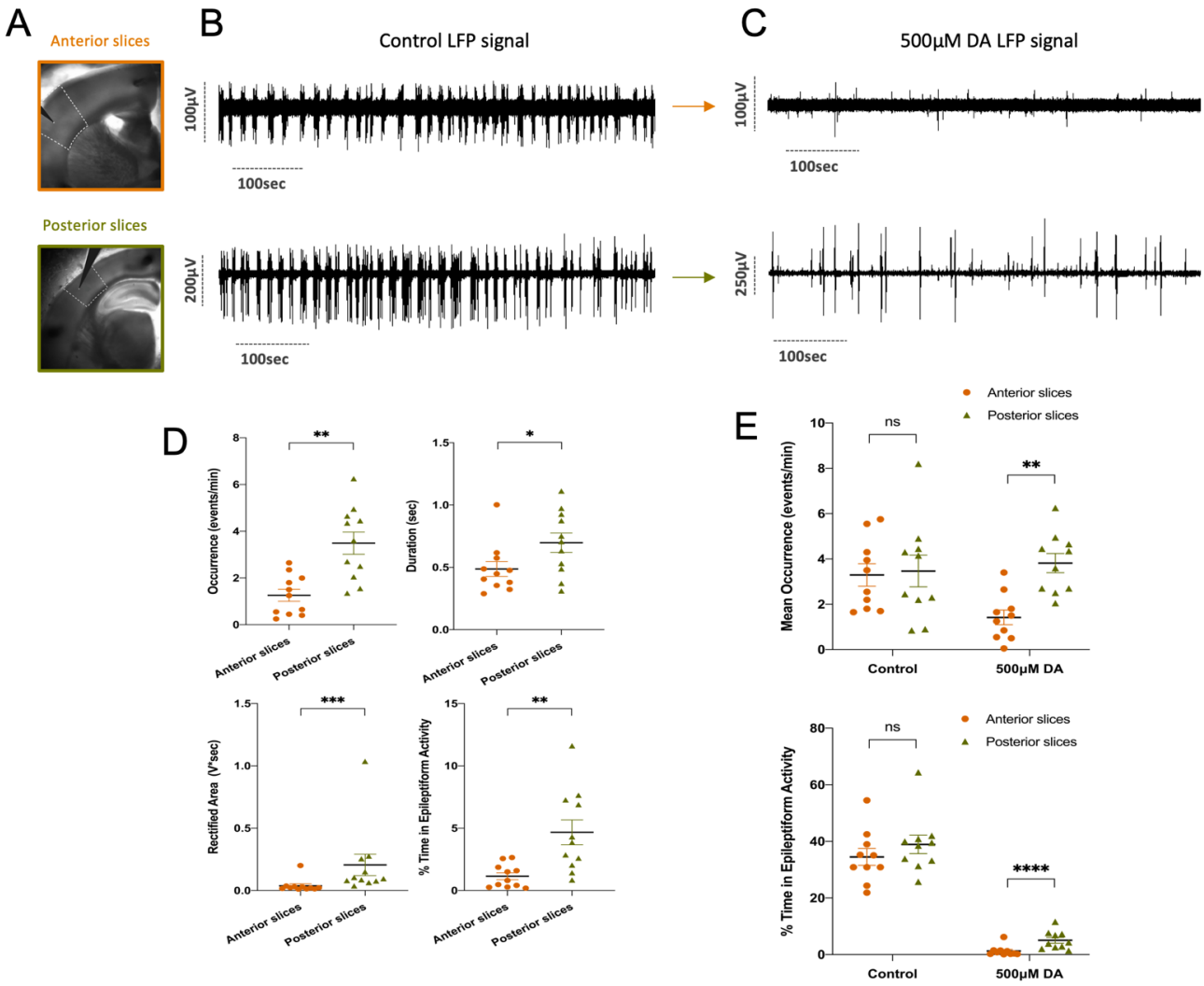

Figure 7. Decanoic acid exhibits variable efficacy across different S1BF cortical slices **A.** Illustration of an anterior (upper; Bregma distance: 0.70mm) and a posterior (down; Bregma distance: 1.70mm) slice with electrodes positioned in the area of S1BF. Dotted white lines represent the area of S1BF in each slice. Note the clear presence of hippocampus in the posterior slice, which is usually absent or very small in anterior slices. **B.** Representative LFP recordings of the anterior (upper trace) and posterior (lower trace) slice at control conditions, during the last 20-min segment of 0-Mg^{2+} transition. **C.** Representative LFP recordings of the anterior (upper trace) and posterior (lower trace) slice after administration of 500µM DA. **D.** Bar plots showing the different effect of 500µM DA in anterior vs. posterior slices with respect to occurrence (upper left), duration (upper right), rectified area (lower left) and % time in epileptiform activity (lower right). Data are from 10 slices for each group and graphs show mean ± SEM values; unpaired *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$. The effect of age was not examined in this set of experiments and data represent results from the younger mice (P16-20). **E.** Graphs illustrate the effect of cortical region in the drug's efficacy in terms of occurrence (upper graph) and % time percent in epileptiform

activity (down graph). Data were collected from 10 slices for each group. Graphs show mean ± SEM values. Statistical analysis was realized using two-way repeated ANOVA (*post hoc* Bonferroni test). ** $p < 0.01$, **** $p < 0.0001$.

Discussion

The incidence of seizures is predominantly high in early and late pediatric ages and often difficult to handle with common antiepileptic agents. This is mainly ascribed to poorly understood developmental and regional discrepancies involved in the cellular and synaptic mechanisms, regulating physiological network activity, seizure susceptibility, as well as drug resistance. In line with this concept, we aimed to reproduce here a modified *ex vivo* mouse model of neocortical pediatric epilepsy that accommodates for different aspects of seizure vulnerability; 1) neocortex as the target of seizure induction and spread, 2) transition to epileptiform hypersynchronicity from physiological synchronous activity and 3) immature brain network. Our previous results, in tandem with related studies, investigating the role of isolated neocortical areas as targets of intractable epilepsy, support the earlier onset of paroxysmal activity in neocortex compared to other epilepsy-relevant areas, including hippocampus (Wong and Yamada, 2001; Timofeev and Steriade, 2004; Sigalas *et al.*, 2018; Codadu, Parrish and Trevelyan, 2019; Breton *et al.*, 2020). When studying seizure susceptibility, special attention should be also given at the endogenous slow oscillatory properties of neocortical slices, resembling those observed *in vivo* during NREM sleep and certain types of anesthesia (Sanchez-Vives and McCormick, 2000; Chauvette, Volgushev and Timofeev, 2010; Favero and Castro-Alamancos, 2013; Rigas *et al.*, 2015), as they can lower seizure threshold and account for the cognitive risks of certain types of childhood epilepsies (Timofeev and Steriade, 2004; Ujma *et al.*, 2017; Halász *et al.*, 2019). This reductionistic model could provide particular insight into the circuit and cellular components underlying transitions from pharmacosensitive to pharmacoresistant states. To test this, our model preparations were implemented for the characterization of progressing epileptiform activity at a given developmental context of two early life stages and for the anticonvulsant assessment of two AMPAR antagonists, DA and PER.

As a means of hyperexcitability induction, we adopted the well-known magnesium-free protocol for its marked utility in manifesting stable and gradual transitions to various types of evolving, pharmacoresistant epileptiform activity without directly influencing inhibitory transmission and ion conductance properties (Quilichini *et al.*, 2003; Campos *et al.*, 2018; Rodrigues *et al.*, 2020), thus allowing for more controlled pharmacological interventions and comparisons between age- and region-specific variations in terms of seizures and drug sensitivity (Wahab *et al.*, 2010; Antonio *et al.*, 2016). Previous literature has repeatedly described the transiently progressing pattern of distinct epileptiform events promoted under zero Mg^{2+} conditions in

neocortical areas, with the most dominant ones being interictal discharges, seizure-like events and late recurrent discharges (Dreier and Heinemann, 1991; Gloveli, Albrecht and Heinemann, 1995; Wong and Yamada, 2001; Quilichini *et al.*, 2003; Codadu *et al.*, 2019). However, our model did not express this late rhythmic switch and instead, prolonged perfusions with the zero-Mg^{2+} medium increased the propagation rate of SLEs by mostly shortening tonic-clonic afterdischarges or depressing most of the clonic components. Based on prior works, this transition to shorter SLE activity is thought to arise primarily from intracellular changes leading to protracted network disinhibition (Dzhala *et al.*, 2010; Trevelyan and Schevon, 2013; Ellender *et al.*, 2014).

A major problem concerning the quantification process of SLEs, is the accurate identification of their onset and offset parameters. One proposed method for the objective detection of SLEs has been the calculation of extracellular potassium concentrations (Fröhlich, Bazhenov, *et al.*, 2008; Fröhlich, Timofeev, *et al.*, 2008). Although this method is useful in terms of onset and tonic phase determination, still it can lead to offset detection defects and clonic phase misinterpretations, since multiple ionic elements (e.g. intracellular sodium, chloride and calcium), in addition to extracellular potassium can shape the termination of the seizure (Krishnan and Bazhenov, 2011). Automated seizure detection programs also have been constructed in an attempt to minimize subjective quantification of SLEs (Rasekhi *et al.*, 2013; Breton, Bardakjian and Carlen, 2019), but these deterministic programs are mainly dependent on the context of each experimental setting, implying limited applicability to other experimental systems, while also may suffer from activity or inactivity misjudgments and lead to false predictions. Here, SLEs/seizures quantification was realized in a manual way. The onset of each SLE was visually defined by the start of the fast-negative tonic spike and the clonic offset was determined by setting an in-between clonic events threshold after appropriate single-event distributional analysis of a total recording (data not shown).

In the present study we introduce an alternative metric for SLEs/seizures calculation, in an effort to compensate for arising biases of subjective analysis and account for the activity-modifying effect of the administered drugs. This metric, termed "percentage time in epileptiform activity", quantifies epileptiform activity by relating duration and occurrence of paroxysmal events, to yield a measure that does not depend on the type of events encountered during pharmacological transitions. We found that this metric is particularly valuable in the context of concentration evaluations of pharmacological agents, in respect of other commonly used metrics of epileptiform activity, including duration and frequency, which may reveal conflicting response results at subeffective drug doses. Despite the usefulness of our metric in providing objective validity in seizures analysis, it cannot be used as an index of seizure severity. Yet, to our knowledge, neither duration, nor frequency and seizure sensitivity can accurately

reflect seizure severity in *in vitro* animal studies, as they lack essential information about the behavioral and cognitive impacts of different seizure types (Cramer and French, 2001; Wahab, Albus and Heinemann, 2011).

Although it is reported that seizures and drug sensitivity change considerably with age, with children being the most affected age group displaying a high variety of epileptic syndromes, few studies incorporate the developmental criterion to examine epileptiform transitions and drug efficacy in neocortex, especially after infantile stages (Wong and Yamada, 2001; Quilichini *et al.*, 2003; Wahab, Albus and Heinemann, 2011; Chapman, Raol and Brooks-Kayal, 2012). Herein, we attempted to compare seizure patterns and anticonvulsant sensitivity employing two young mice ages, P16-P20 and P27-32, that are equivalent to approximately early and late human childhood (Romijn, Hofman and Gramsbergen, 1991; Dutta and Sengupta, 2016). Both ages exhibited comparable latency to the time of seizure initiation and first SLE appearance meaning that they are both equally prone at inducing and spreading epileptiform activity under 0-Mg^{2+} conditions. Furthermore, while the two young ages displayed similar epileptiform activity patterns, both expressing interictal and tonic-clonic transitions, there were several seizure parameters that varied according to this age range. Younger ages tented to produce shorter SLEs with increased incidence rate over time, whereas ages corresponding to children manifested less frequent and more protracted SLEs, marked by larger tonic fast spikes and persisting clonic elements. These differences in terms of SLE parametrization may be indicative of the distinct synaptic functions regulating seizures sustainment and rate of propagation in the developing network, even within short age ranges. Prolonged SLEs with strong tonic features and roughly intact afterdischarges in older ages may be assigned to more established ion homeostasis and stable ion channels expression favoring slower sodium influx and more controlled inhibitory response against seizure termination and propagation, thus extending seizure duration (Kriegstein, Suppes and Prince, 1987; Ben-Ari and Holmes, 2006; Krishnan and Bazhenov, 2011; Baram, 2012).

A link between the effectiveness of MCT diet and the accumulation of DA, an AMPAR antagonist, in the serum of children with refractory epilepsy has been already reported (Haidukewych, Forsythe and Sills, 1982; Sills *et al.*, 1986; Dean, Bonser and Gent, 1989; Chang *et al.*, 2013, 2015; Shrestha *et al.*, 2015). Consistent with previous findings (Chang *et al.*, 2016), our results, using an acute model of neocortical children epilepsy, demonstrate DA's efficacy at blocking 0-Mg^{2+} induced epileptiform discharges in a dose-dependent fashion. Interestingly, DA showed greater anticonvulsant potency compared to a clinically validated non-competitive AMPAR antagonist, PER, which failed to positively affect the incidence of epileptiform discharges. This further substantiates the key hypothesis of the discrete physiological effects of the two compounds. The anticonvulsant superiority of DA in our model may

be indicative of its extensive action at hyperexcitable synapses. Indeed, its action is not limited at postsynaptic AMPARs as it is thought to affect intrinsic excitability and the number of arising action potentials by decreasing persistent currents of voltage-gated sodium channels (I_{NaP}) (Williams, 2017), which are involved at several types of acquired and inherited epileptic syndromes (Köhling, 2002; George, 2005; Meisler, 2005; Stafstrom, 2007), including intractable childhood epilepsy (Biervert *et al.*, 1998; Fujiwara *et al.*, 2003). Additionally, it has been suggested that DA is a positive modulator of the peroxisome-proliferator-activated receptor γ (PPARγ) and its anticonvulsant efficacy may be attributed to mitochondrial protection through increases in citrate synthase, and associated complex I and catalase activity (Malapaka *et al.*, 2012; Hughes *et al.*, 2014). Its speculated involvement in the restoring of phosphorylation of several protein kinases, including p38 mitogen-activated kinase (MAPK) and extracellular signal-regulated kinase (ERK) may explain its neuroprotective effect against permanent alterations of intrinsic excitability in chronic models of epilepsy (Jiang *et al.*, 2005; Jung *et al.*, 2010; Wlaź *et al.*, 2015). Of particular interest is also DA's potential role in the enhancement of the adenosine production, which is critical for the elimination of excessive presynaptic glutamate release and thus SLEs state transitions and termination through the activation of A1 receptors (Masino *et al.*, 2011; Klaft *et al.*, 2016; Warren, Walker and Williams, 2018).

Perampanel's unique pharmacological profile relies on its reported broad-spectrum anticonvulsant action against multiple types of seizures, including partial discharges, generalized tonic-clonic seizures and status epilepticus, as well as brain tumor related epilepsies and refractory pediatric syndromes, such as Lennox-Gastaut and Dravet syndromes (Hsu *et al.*, 2013; Schmidt and Schachter, 2014; Frampton, 2015; Oi *et al.*, 2019; Potschka and Trinka, 2019). However, PER's exact mode of AMPAergic inhibition at the circuitry level remains inconclusive. We were at odds with most of the previous *in vitro* and *in vivo* findings (Hanada *et al.*, 2011; Rogawski and Hanada, 2013; Chen *et al.*, 2014; Zwart *et al.*, 2014), showing that PER did not abolish 0-Mg^{2+} induced epileptiform discharges at clinically relevant concentrations, yet it significantly modified some parameters of the SLEs with increasing doses. PER notably aggravated the frequency of epileptiform discharges and shortened the total SLEs duration through gradual elimination of the afterdischarges. Other studies also support the increase of afterdischarges threshold produced by PER, highlighting its potential at the disruption of hypersynchronous networks (Wu *et al.*, 2019; Hanada, 2020; Yang *et al.*, 2020). We believe, however, that this modifying effect of PER is very doubtful to be translated to seizures improvement at a clinical setting. AMPARs transmission undergoes constant changes and their activity is dependent on several factors including their subunit conformation, the number of AMPARs at synapses, their localization, the degree of phosphorylation and the synaptic functions that they serve

in response to external inputs (Szczurowska and Mareš, 2013; Chater and Goda, 2014; Di Bonaventura *et al.*, 2017). In addition, the conflicting IC_{50} values estimations of PER obtained from several animal and human studies, may be the outcome of the different experimental properties and the associated differences in the AMPAR distribution of the epileptic source. For instance, immature somatosensory cortex predominantly expresses Ca^{2+} permeable AMPARs (not containing GluA2 subunits) and a limited number of Ca^{2+} impermeable AMPARs (containing GluA2 subunits) (Hsu *et al.*, 2010). Although it has been reported that PER equally inhibits both subunit AMPAR categories under normal synaptic conditions (Barygin, 2016), maybe it cannot prevent the excessive amount of GluA2-lacking AMPARs present at the immature neocortical tissue, in combination with the synaptic changes triggered under 0-Mg^{2+} hyperexcitable conditions. Indeed, incorporation of calcium permeable AMPARs are thought to be implicated in the progression of hyperexcitable networks (Rajasekaran *et al.*, 2013; Whitehead *et al.*, 2013; Hanada and Hanada, 2014; Hanley, 2014; Charsouei, Jabalameli and Karimi-Moghadam, 2020), but the precise magnesium-free effect on AMPARs distribution has not been sufficiently defined. Another aspect that may account for PER's anticonvulsant inefficacy in our model relates the preferential expression of GluA2-lacking AMPARs on neocortical interneurons of thalamo-cortical origin with its proconvulsant potential (Jonas, 1994; Cruikshank, Lewis and Connors, 2007; Szczurowska and Mareš, 2015; Lalanne *et al.*, 2016). A recent study confirms this hypothesis by describing PER's inhibitory response against 4-AP-induced interneuronal recurrent activity and concomitant reduction of pyramidal IPSPs (Yang *et al.*, 2020). PER, therefore, can promote worsening of seizures through interneuronal disinhibition.

Similar to valproic acid, DA has been shown to inhibit histone deacetylase activity, which correlates with increased risk of teratogenicity in pregnant women (Gurvich *et al.*, 2004; Eikel, Lampen and Nau, 2006; Chang *et al.*, 2013). Likewise, some common side-effects of PER involve somnolence, dizziness and psychotomimetic symptoms in humans (Rogawski, 2011; Lattanzi and Striano, 2019), and motor coordination dysfunctions in animals (Yamaguchi, Donevan and Rogawski, 1993). While these undesirable effects may significantly limit their preclinical evaluation and subsequent use at effective doses, most importantly, they do not appear to affect normal synaptic plasticity and developing cognitions (Pulsifer *et al.*, 2001; Pan *et al.*, 2010; Meador *et al.*, 2016; Kim *et al.*, 2019). Based upon the distinct binding sites of DA and PER, Augustin et al proposed synergistic action of the two agents as a means to provide an additive seizure therapy combining maximum efficacy of both agents with smaller doses and minimum side effects (Augustin *et al.*, 2018). Synergistic action of PER has also been described before with several other anticonvulsants, including valproate, carbamazepine and zonisamide (Hanada *et al.*, 2011; Russmann *et al.*, 2016). Aiming

at expanding this synergistic potential in our model, we showed that co-administration of the two AMPAR antagonists produce a significant additive inhibitory effect only in one of our total epileptiform metrics (% time in epileptiform activity) and in one tested DA concentration (100μM). Though there is an evident synergistic action in terms of time percentage in epileptiform activity, we believe that this effect is not general for other metrics, like epileptiform occurrence due to small sample size. Age- and region- specific variances are also possible to affect the synergy of the two compounds, yet further studies including adult ages and other brain areas are necessary to validate this scenario.

In conclusion, the main finding of the present study is that DA shows a better anticonvulsant potential compared to PER, a clinically approved AMPAR antagonist with similar pharmacological profile. PER modified without abolishing 0-Mg^{2+} induced epileptiform activity in neocortical slices of young mice, meaning that it does not influence all brain regions and ages the same way. The two compounds display great synergistic potential in our model. However, in order this combinatory therapy to have a clinical prospect, additional studies should assess this synergy under different experimental conditions, as well as evaluate its therapeutic impact on epileptic patients following an MCT-oil based regime with added PER.

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