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Experimental insights into absence seizures: Focus on their correlation with comorbid anxiety and their modulation by cannabinoid system in a genetic animal model of absence epilepsy

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"Do everything for Love.

Thus there will be no little things: everything will be big.

Perseverance in little things for Love is

heroism."

St. Josemaría Escrivá

The Way, 813

To My Family

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Abstract

Childhood absence epilepsy is a pediatric epilepsy syndrome, characterized by frequent absence seizures and neuropsychiatric comorbidities, which have a great impact on the quality of life in these children and their families. Neuropsychiatric comorbidities together with the high rates of pharmacoresistance call for a better understanding of the correlation between absence seizures and associated-comorbidities, the effect of currently available antiepileptic drugs on these comorbidities and identification of possible new therapeutic targets. The results of this thesis showed that seizure severity and anxiety level are correlated in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) rats, an animal model of absence epilepsy. Moreover, it is demonstrated that two gold-standard antiepileptic drugs to treat absence seizures, ethosuximide and valproate, affect anxiety-related behavior in GAERS and also in two control strains, Non-Epileptic Control (NEC) and Wistar rats.

Furthermore, based on growing evidence about cannabinoid effects in epilepsy, the effect of systematically administered cannabinoids on absence seizures has been investigated, by using electroencephalographic recordings in freely moving GAERS rats, providing a comprehensive overview of the effects of synthetic cannabinoids, phytocannabinoids and endogenous cannabinoids on absence seizures. These experiments demonstrated that direct activation of the cannabinoid (CB) system by systemic administration of the CB1 receptor agonist, aggravated spike and wave discharges (SWDs) in GAERS. On the other hand, cannabidiol (CBD), a very well-known and extensively studied phytocannabinoid, did not have an effect on seizures in GAERS rats. In addition, results showed that enhancement of anandamide (AEA) tone, did not have an effect on SWDs, while enhancement of 2-arachidonoyl glycerol (2-AG) tone reduced seizure length in GAERS rats.

In conclusion, this thesis shows that anxiety-like behavior in GAERS is correlated with seizure severity and it contributes to further our knowledge of cannabinoid modulation in absence epilepsy.

List of abbreviations

2-AG – 2-arachidonoyl glycerol
5-HT_{1A} – serotonin receptor 1A
AA – arachidonic acid
ABHD – alpha-beta hydrolase
ADHD – attention deficit hyperactivity disorder
AEA – anandamide
ANOVA – analysis of variance
AS – absence seizure
AED – antiepileptic drug
BOLD – blood-oxygen-level-dependent
CAE – childhood absence epilepsy
CB – cannabinoid
CBD – cannabidiol
CG – cingulate
CIN – cortical initiation network
COX-2 – cyclo-oxygenase 2
CPS – complex partial seizures
DAG – diacylglycerol
DGL – diacylglycerol lipase
DNMT – DNA methyltransferase enzyme
DREADD – Designer Receptor Exclusively Activated by Designer Drug
EA – ethanolamine
eCB – endocannabinoid
EEG – electroencephalogram
ES – Edge Sniff
ETX – ethosuximide
FAAH – fatty acid amide hydrolase
fMRI – functional magnetic resonance imaging
FP – frontal polar
GABA – gamma-Aminobutyric acid
GAERS – Genetic Absence Epilepsy Rat from Strasbourg
GAT-1 – GABA transporter 1
Glyc – glycerol
HB – Hole board
HD – Head Dip
Hz – Hertz
i.c.v. – intracerebroventricular
ILAE – International League Against Epilepsy
In/FO – insula/frontal operculum
i.p. – intraperitoneally
IPSP – inhibitory postsynaptic potentials
IQ – intelligence quotient
LF – lateral frontal
LO – lateral occipital
LP – lateral parietal

LT – temporal
LTG – lamotrigine
MAGL – monoacylglycerol lipase
Max – maximal
MFC – medial frontal cortex
Min – minimum
MO – medial occipital
mRNA – messenger RNA
NAPE – N-arachidonoyl phosphatidylethanolamine
NAT – N-acyltransferase
NEC – Non-Epileptic Control
NRT – *nucleus reticularis thalami*
OF – orbital frontal
PC – precuneus
PCB – printed circuit boards
PE – phosphatidylethanolamine
PEG – polyethylenegycol
PI – phosphatidylinositol
PLC – phospholipase C
PLD – phospholipase D
PoC – peri-oral region of cortex
PPARγ – peroxisome proliferator-activated receptor-gamma
RM – repeated measures
S1 – primary somatosensory cortex
SCB – synthetic cannabinoid
SEM – standard error of the mean
SLQ – spoken language quotient
SN – substantia nigra
SNr – substantia nigra, *pars reticulata*
SWD – spike and wave discharge
TC – thalamocortical
Th – thalamus
THC – 9-Δ- tetrahydrocannabinol
TRPV1 – transient receptor potential vanilloid type 1
VB – ventrobasal
VPA – valproate, valproic acid
VPM – ventroposteromedial thalamic nucleus
WAG/Rij – Wistar albino Glaxo from Rijswijk

Rationale, aims and contributions

This thesis aims i) to provide further validation of the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) rat model of absence epilepsy and ii) to investigate how the modulation of the cannabinoid system affects absence seizures (ASs).

In order to achieve the first aim, I evaluated absence seizures correlation with comorbid anxiety performing a behavioral test in EEG implanted GAERS rats, providing valuable data on how the behavior is changing in respect to ictal and interictal periods. Moreover, I evaluated the effect of two gold standard anti-absence drugs, i.e., ethosuximide and valproate on anxiety-like behavior in GAERS as well as in non-epileptic control (NEC) rats and importantly in normal Wistars rats, to gain a better understanding of the psychiatric effects of antiepileptic drugs in normal animals.

In order to achieve the second aim, I modulated the cannabinoid system by administering exogenous cannabinoids (synthetic or phytocannabinoids) or by modulating the endocannabinoid tone pharmacologically. These results provide a valuable contribution to the scarce knowledge about cannabinoid modulation in absence epilepsy.

Introduction

Over 300 years have passed from the first documented description of an absence seizure (AS) reported in the records of *Académie Royale des Sciences* (Brigo et al., 2018):

"In that occasion Mr. Poupart added that he was aware of a case of a young female child with epilepsy, who at the onset of the seizure sits down in a chair, and there remains immobile, without speaking and senseless. Her eyes are open, and after the episode she does not absolutely remember having fallen into this state. If she had previously begun a talk that the seizure interrupted, she takes it up again exactly at the same point at which she stopped talking, and she thinks she has talked without interruption." (Brigo et al., 2018; Poupart, 1705).

This description from 1705, highlights the key features of a typical AS (Brigo et al., 2018). However, the term "absence" was introduced over a hundred years later, it was mentioned for the first time in the doctoral thesis of a French physician Calmeil in 1824 (Brigo et al., 2018; Calmeil, 1824).

The term "*petit mal*" (in French "little illness") was introduced in 1838, as a distinction from "*grand mal*" seizures (in French "big illness" i.e., tonic – clonic seizures) (Brigo et al., 2018). In the early 20th century, the term "pyknolepsy" (Greek word πυκνός, *pyknos*, meaning "dense", "closely packed", "aggregated") was introduced, describing the frequent occurrences of seizures (Brigo et al., 2018; Buchhalter, 2011). The first electroclinical description of ASs was published in 1935 using the recently invented electroencephalogram technique (EEG; allowing the recording of brain activity thanks to electrodes positioned on the scalp): American neurologist Frederic Gibbs and his colleagues reported a 3 Hz generalized Spike and Wave Discharges (SWDs) pattern in the EEG of a patient with "pyknolepsy" (Brigo et al., 2018; Gibbs FA, 1935).

Presently, ASs are defined by the International League Against Epilepsy (ILAE) as *generalized onset, non-motor seizures which are characterized by sudden behavioral arrest and distinctive SWDs in the EEG* (Fisher, 2017). ILAE differentiates between four different types of ASs, commonly present in different types of pediatric and adult epilepsies: typical ASs, atypical ASs, myoclonic ASs and eyelid myoclonia (Fisher, 2017; Tenney & Glauser, 2013).

The emphasis of this thesis will be on typical ASs, which are the hallmark of childhood absence epilepsy (Tenney & Glauser, 2013).

Childhood absence epilepsy

Clinical presentation

Childhood absence epilepsy (CAE) is a common pediatric epileptic syndrome, diagnosed in 10-17 % of all school-aged children with epilepsy (Berg, Shinnar, Levy, and Testa (1999); Matricardi, Verrotti, Chiarelli, Cerminara, and Curatolo (2014)). The prevalence of CAE in children population is estimated to be 0.4-0.7 per 1000 persons, with generally a higher frequency in girls (Matricardi et al., 2014). It has been shown that CAE is genetically determined, as positive family history is found in 16-45 % of cases, however the genetic etiology is multifactorial, complex and not yet completely elucidated (V. Crunelli & Leresche, 2002). ASs arise early in life between the age of 3 to 8 years (with a peak around the age of 6-7 years) and occur as frequent as 200 times/day with a substantial disruption of normal daily life (H. Blumenfeld, 2005; V. Crunelli & Leresche, 2002). ASs lasts in average 10 seconds (ranging from 4 to 20 seconds) and the duration is affected by different factors, such as sleep deprivation,

state of arousal, provocation (e.g., by hyperventilation or by photic stimulation) and therapy (V. Crunelli & Leresche, 2002; Matricardi et al., 2014).

Apart the characteristic SWDs in the EEG, the concomitant lack of consciousness is the second main feature of ASs, presenting as general loss of awareness and unresponsiveness to external acoustic or visual stimuli (H. Blumenfeld, 2005; Matricardi et al., 2014). Recent studies performing EEG and functional magnetic resonance imaging (fMRI) with simultaneous behavioral testing showed that the degree of impairment is variable, depending on the different attention tasks used for testing (Berman et al., 2010; Guo et al., 2016). These variations are present among different ASs, even in the same child (Berman et al., 2010; Guo et al., 2016).

Other clinical manifestations associated with ASs are staring spells, blinking and automatisms, involuntary actions which are mostly oral and associated with longer seizures (V. Crunelli & Leresche, 2002; Matricardi et al., 2014).

EEG presentation

The characteristic EEG recording during an AS shows generalized, symmetrical and bilaterally synchronous SWDs at 3 Hz (1 Hz = 1 cycle per second (range between 2.5-4 Hz) (Hal Blumenfeld, 2005; Matricardi et al., 2014) (**Figure 1**).

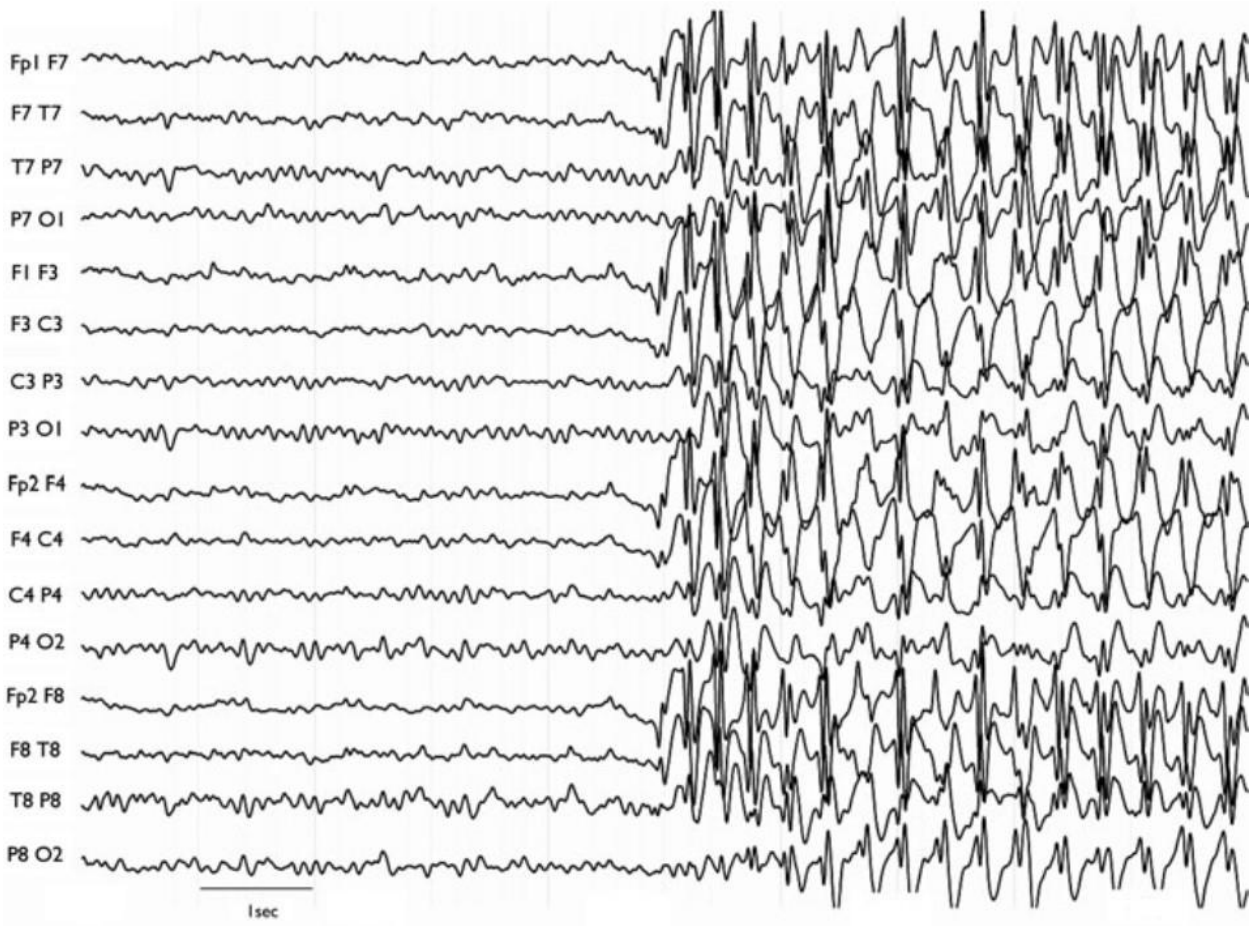

Figure 1. Example of a typical AS recorded in EEG. An AS is characterized by 3 Hz generalized SWDs arising from normal background activity in the EEG. Adapted from (Moeller et al., 2008).

Generally, SWDs arise and end abruptly from a normal background activity (Matricardi et al., 2014). However, SWDs are not uniform, in fact they can vary both in duration and amplitude, the latter being usually highest in the midline of the frontal regions (Hal Blumenfeld, 2005; Matricardi et al., 2014). Other variabilities include single spikes, polyspikes and irregularities at the end of the seizure (Matricardi et al., 2014).

Treatment and pharmacoresistance

Treatment of ASs relies on three established AEDs (ethosuximide, valproate and lamotrigine), but for years there were no randomized controlled trials comparing the efficacy of these drugs in children with CAE (Penovich & Willmore, 2009).

Ethosuximide (ETX; 2-ethyl-2-methyl-succinamide) is an antiepileptic drug with a narrow clinical spectrum, used since 1958 (Kessler & McGinnis, 2019; Penovich & Willmore, 2009). It is effective in suppressing ASs, but not in suppressing other types of seizures (Kessler & McGinnis, 2019). The proposed mechanism of action is the blockage of T-type calcium channels in thalamic neurons, thereby preventing the synchronized firing of TC neurons (Kessler & McGinnis, 2019). However, ETX at therapeutic concentrations also reduces both the non-inactivating Na^+ current, and the calcium-activated K^+ current in thalamic and cortical neurons and these effects might also be important in its therapeutic mechanism (V. Crunelli & Leresche, 2002). The most common possible side effects are gastrointestinal side effects, such as abdominal discomfort, vomiting, diarrhea, and hiccups (Kessler & McGinnis, 2019; Penovich & Willmore, 2009). Moreover, different neurological side effects such as headaches, sedation, fatigue, insomnia, ataxia, or extrapyramidal symptoms may occur, and rarely also behavioral side effects, like nervousness, irritability, depression, hallucinations, and even psychosis (Kessler & McGinnis, 2019; Penovich & Willmore, 2009). Serious side effects, such as blood dyscrasias, ETX-induced lupus and allergic rashes occur rarely (Kessler & McGinnis, 2019).

Valproate (VPA), in clinical use since 1978, is a broad-spectrum antiepileptic medication (Kessler & McGinnis, 2019). It is not clear how VPA blocks ASs, although multiple mechanism

of actions are known, such as elevation of GABA levels in the brain, blockage of voltage-sensitive sodium channels and activation of calcium-dependent potassium conductance (Kessler & McGinnis, 2019). There are many potential side effects of VPA treatment, that may occur in relation to the dose used or as idiosyncratic reactions (Kessler & McGinnis, 2019). These include high-frequency tremor, altered mental status, increased appetite and weight gain, pancreatitis, hepatic failure, thrombocytopenia, platelet dysfunction, fibrinogen depletions, coagulation factor deficiencies, carnitine depletion, hyperammonemia, hyperinsulinemia, polycystic ovarian syndrome, vitamin D deficiency, decreased bone mineral density, hypothyroidism and teratogenicity (Kessler & McGinnis, 2019; Penovich & Willmore, 2009).

Lamotrigine (LTG; 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) is a newer antiepileptic drug, a phenyltriazine derivate in use since the mid-90s (Kessler & McGinnis, 2019; Penovich & Willmore, 2009). It is known that LTG is a voltage-dependent sodium channel blocker, although not all of its mechanisms of action have been completely described (Kessler & McGinnis, 2019). It is well tolerated and has fewer cognitive side effects compared to ETX and VPA, however a very slow initial titration of LTG is required to lower the risk of developing the Stevens-Johnson syndrome (T. A. Glauser et al., 2010; Kessler & McGinnis, 2019).

Efficacy, tolerability and neuropsychological effects of ETX, VPA and LTG were finally well studied in a double-blind randomized controlled clinical trial that was conducted on a large cohort of children with newly diagnosed CAE (446 subjects) to determine the optimal initial monotherapy (Tracy A. Glauser et al., 2013; T. A. Glauser et al., 2010). The outcomes were verified at 16-20 week and after 12 months from treatment initiation (Tracy A. Glauser et al., 2013; T. A. Glauser et al., 2010). At the 12 month visit i) ETX and VPA showed similar freedom-from-failure rates (45% and 44%), which were higher in comparison to LTG (21%);

however, ii) VPA was associated with negative effects on attention and with higher rates of intolerable adverse events in comparison to ETX; so, iii) these results affirmed ETX as the optimal initial monotherapy for CAE (Tracy A. Glauser et al., 2013). Even though ETX is the optimal initial choice, it fails to achieve freedom from treatment in 55 % of children with CAE after 12 months of treatment (Tracy A. Glauser et al., 2013). Moreover, this study shows that overall, 63 % of enrolled children with CAE experienced treatment failure during the first 12 months of initial therapy, mostly due to lack of seizure control and intolerable side effects, which clearly highlights the need to improve the treatment options in children with CAE (Tracy A. Glauser et al., 2013).

Pharmacoresistance of ASs

From the original cohort of children with CAE in the study conducted by Glauser and colleagues, 80 % (357/446) were further included in a pharmacogenetic analysis to understand whether the genetic variations might play a role in the drug response (T. A. Glauser et al., 2017). This pharmacogenetic analysis was focused on variants of T-type calcium channel, coded by three genes (*CACNA1G*, *CACNA1H*, and *CACNA1I*) and involved in the thalamocortical pathways, and variants of the *ABCB1* transporter gene, coding for the P-glycoprotein, a drug efflux transporter, which could also affect the drug response (T. A. Glauser et al., 2017). The analysis confirmed that genetic variations play a role in the drug response in children with CAE, as it showed that there are four T-type calcium channel variants and one ABCB1 transporter variant associated with a differential drug response in children with CAE (T. A. Glauser et al., 2017).

Children that experienced treatment failure with the initial treatment during the double-blind phase were randomized to an open-label second monotherapy trial. the results were similar to the first double-blind phase, that is i) ETX and VPA were more efficient in seizure control than

LTG, and ii) VPA was associated with more attentional dysfunction; interestingly, iii) response to the second monotherapy was not affected by the initial treatment (Cnaan et al., 2017). However, when the results of the two trials are combined, it can be seen that one-third of children with CAE do not respond to initial and second monotherapy, which meets the ILAE criteria for drug-resistant epilepsy (**Figure 4**) (Cnaan et al., 2017; Vincenzo Crunelli et al., 2020; Masur et al., 2013).

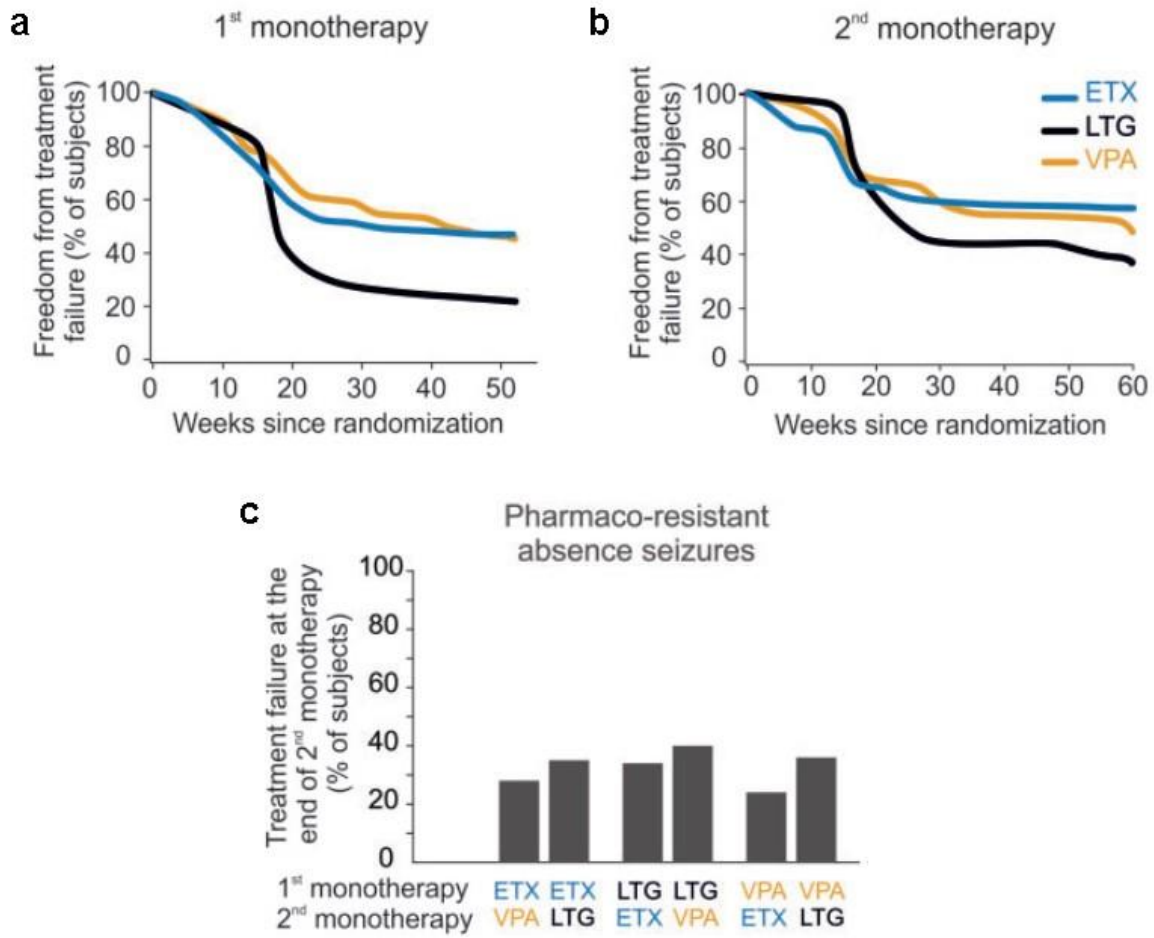

Figure 4. Pharmacoresistance of ASs. (**a**) Freedom from treatment failure rates during the 12 months treatment with the first monotherapy with either ETX (*blue*), VPA (*yellow*) or LTG (*black*), based on the results from the double-blind, randomized controlled clinical trial on a cohort of 446 children with CAE. Results show that ETX and VPA are more effective in controlling absence seizures than LTG. (**b**) Freedom from treatment failure rates during the following12 months of treatment in 208 children with CAE that experienced treatment failure during the double-blind phase (*see a*) and were randomized to the open-label second monotherapy trial with either ETX (*blue*), VPA (*yellow*) or LTG (*black*). Results show that as second monotherapy ETX and VPA were more effective than LTG in controlling absence seizures; moreover, the choice of initial monotherapy did not affect the response rate to the second monotherapy. (**c**) Combined treatment failure results at the end of the 12-month treatment period with the second monotherapy show that approximately 30 % of children with CAE have pharmaco-resistant absence seizures, as they fail to achieve seizure freedom with two tolerated and appropriately chosen antiepileptic drugs. Modified from (Vincenzo Crunelli et al., 2020) (graphs based on data from (Tracy A. Glauser et al., 2013) and (Cnaan et al., 2017)).

The proposed treatment algorithm of CAE is to initially start with ETX monotherapy and if the seizures persist to switch to VPA monotherapy (Kessler & McGinnis, 2019). If VPA treatment also fails, LTG monotherapy is proposed (Kessler & McGinnis, 2019). Other treatment options are combination of VPA and LTG, or other AEDs, such as clobazam, topiramate, zonisamide, levetiracetam, or even ketogenic diet (Kessler & McGinnis, 2019; Penovich & Willmore, 2009). It has been shown that the duration of individual ASs affects the treatment response regardless of the choice of treatment, that is, patients with longer seizures respond better to the initial treatment (Dlugos et al., 2013).

The data from Glauser and Cnaan confirm ETX as the first-line treatment for CAE because it shows better efficacy in seizure control than LTG and has fewer cognitive and behavioral adverse effects than VPA (Cnaan et al., 2017; Tracy A. Glauser et al., 2013; Shinnar et al., 2017). Moreover, Berg and colleagues (Berg, Levy, Testa, & Blumenfeld, 2014) found that initial ETX treatment is associated with better long-term seizure outcome. They conducted a community-based prospective study to investigate the association between long-term seizure outcome and the initial treatment choice in children with CAE. The authors found that the use of ETX as the initial treatment is associated with a higher rate of complete remission in comparison to valproate, both at 5 years and 10 years follow-up (seizure freedom and medication freedom). These findings show that ETX has an impact on the long-term course of CAE and that it might have disease-modifying properties (Berg et al., 2014).

CAE was often seen as benign epilepsy, with a high rate of long-term seizure remission (ranging from 56 % to 95 %), however the risk of lasting psychosocial comorbidities remains (Kessler & McGinnis, 2019).The pharmacoresistance of absence seizures and the high incidence of neuropsychological comorbidities demands the identification of new therapeutic targets (Tracy A. Glauser et al., 2013; Masur et al., 2013).

Neuropsychiatric comorbidities

ASs were considered relatively benign in the past (hence "*petit mal*"), thereby the classic historical definition of ASs included only SWDs and the concomitant lack of consciousness, but more recently this vision has been broadening, including also a general *neuropsychological impairment*, as ~ 60 % of children with CAE show cognitive deficits (including attentional deficits, lower academic performance) and ASs-associated comorbidities (anxiety and depression) (Vincenzo Crunelli et al., 2020; Gruenbaum et al., 2021). It is important to recognize psychiatric comorbidities in epilepsy, as they have an enormous impact on the quality of life, even greater than seizures themselves (Munger Clary, 2014).

25% of children with CAE show subtle *cognitive deficits* and it has been shown that frequent ASs are a negative prognostic factor for cognitive functioning (Cheng et al., 2017; Verrotti, Matricardi, Rinaldi, Prezioso, & Coppola, 2015). Cognitive deficits are specifically related to the frontal-lobe dysfunction, possibly related to neuronal network abnormalities in this area, as: i) untreated children with CAE show deficits in reasoning, visual attention and executive function, while other cognitive abilities, not directly related to the frontal lobe are unaffected; ii) the cognitive deficit in visual attention persists even in treated children (Cheng et al., 2017). Moreover, studies have shown that children with CAE have a lower intelligence quotient (IQ)

and difficulties with language skills, while studies reporting memory functioning are not consistent, however visual memory is particularly impaired in children with CAE (Verrotti et al., 2015). A cross-sectional study including 69 children with CAE and their matched healthy controls (Caplan et al., 2008) found cognitive, linguistic and behavioral comorbidities. In particular: i) almost two-thirds of children with CAE included in this study had psychiatric diagnoses, from which attention deficit hyperactivity disorder (ADHD) and affective/anxiety disorder diagnoses were the most frequent ones; ii) interestingly, girls were more likely to have comorbid anxiety than boys; iii) seizure variables, such as longer duration of the illness and a higher seizure frequency were associated with a greater chance of having a psychiatric diagnosis; iv) unfortunately, only 23 % of these children have received an intervention for these comorbidities (Caplan et al., 2008).

Attention deficits are also a core feature of CAE (Caplan et al., 2008; Killory et al., 2011; Masur et al., 2013) as they persist despite successful treatment of ASs and EEG normalization. Neuropsychological testing of children with newly diagnosed untreated CAE found that: i) 36 % of the cohort exhibited attention deficits, which is approximately up to 4-fold higher than in the pediatric population; ii) attention deficits are associated more with an inattentive form rather than a hyperactive form, that is, children with CAE have a higher tendency to lose focus on the task, which affects their executive functioning and school performance; and iii) these attention deficits persist despite ASs freedom obtained by AEDs (Masur et al., 2013).

Epidemiological data show that *anxiety* symptoms can precede absence epilepsy onset or follow it, which indicates that there is a bidirectional relation between absence epilepsy and anxiety

(Munger Clary, 2014). Moreover, data from families affected both from epilepsy and anxiety support the possibility of a common pathophysiological mechanism (Munger Clary, 2014).

A recently published meta-analysis conducted by Gruenbaum and colleagues comprised the available literature that investigated mood disorders, *anxiety* and *depression,* in people with AS and found that they had greater odds of developing depression and anxiety when compared to age-matched controls (odds ratio = 4.93, 95% confidence interval = 2.91– 8.35, $p < 0.01$) (Gruenbaum et al., 2021).

A study by Caplan and colleagues (Caplan et al., 2005) examined affective disorders, anxiety disorders and suicidality in children with complex partial seizures (CPS) and children with CAE compared to healthy children and found i) a high rate of affective and anxiety disorders (33 %), as well as suicidal ideation (20 %) in children with CPS and CAE compared with healthy children; moreover, ii) children with CAE had a higher rate of anxiety disorders in comparison to children with CPS; finally iii) children with epilepsy and comorbid affective and anxiety disorders had lower IQ and spoken language quotient (SLQ) scores and more school difficulties, compared with both the normal children without a psychiatric diagnosis and with the epilepsy patients without a psychiatric diagnosis.

Likewise, Shinnar and colleagues (Shinnar et al., 2017) reported pretreatment behavior problems and behavioral effect of initial therapy from a double-blind randomized clinical trial which examined the efficacy and tolerability of ethosuximide, valproate and lamotrigine in children with newly diagnosed CAE: from a total of 382 participants included in this study, 8% of children with CAE had elevated total problems scores at baseline, which is higher than what would be expected in healthy children (2.1%). These findings in newly diagnosed CAE confirm that behavioral problems are present at baseline, in untreated children with CAE; and

interestingly they found that older age at seizure onset is associated with more behavioral comorbidities (Shinnar et al., 2017).

Hence, the high occurrence of neuropsychiatric comorbidities in children with ASs has raised a question of a shared pathophysiology between the two (Vincenzo Crunelli et al., 2020; Gruenbaum et al., 2021; Killory et al., 2011; Munger Clary, 2014) and which drugs might be candidates for a comprehensive tackling of absence epilepsy.

The evidence of a possible bidirectional relationship between absence epilepsy and behavioral disorders – anxiety and depression, may be found on different levels (Gruenbaum et al., 2021): i) similar genetic predisposition, as mutation in the gamma-aminobutyric acid ionotropic ($GABA_A$) receptor has been associated with CAE (Wallace et al., 2001), while deficits in $GABA_A$ergic neurotransmission has been associated with major depressive disorder (Luscher & Fuchs, 2015) and anxiety spectrum disorders (Pham et al., 2009); ii) impaired functioning of glutamatergic and GABAergic neurotransmitter systems has been found both in ASs models (Cope et al., 2009; Touret, Parrot, Denoroy, Belin, & Didier-Bazes, 2007), and in depression and anxiety (Duman, Sanacora, & Krystal, 2019); iii) involvement of the thalamocortical circuit both in ASs generation (Meeren, Pijn, Van Luijtelaar, Coenen, & Lopes da Silva, 2002) and in depression (Bora, Harrison, Davey, Yücel, & Pantelis, 2012); iv) the involvement of limbic structures in both disorders (Onat, van Luijtelaar, Nehlig, & Snead, 2013); and finally, v) a distinct effect of AEDs, found to ameliorate those behavioral comorbidities, in children with CAE (Salpekar, 2018; Shinnar et al., 2017), as well as in animal models of absence epilepsy (Huang, Lee, Chen, & Shaw, 2012; K. Y. Sarkisova, Kuznetsova, Kulikov, & van Luijtelaar, 2010; van Luijtelaar et al., 2013).

Control of neuropsychiatric comorbidities by AEDs

The question that arises is whether AEDs, apart from ASs control, have also an effect on neuropsychological impairments characterizing CAE, and whether they could be the elective drug candidates for a comprehensive treatment of CAE symptoms as a whole, thereby reducing the need of different drugs to treat different symptoms (i.e., polytherapy, for example ASs and anxiety/depression), hence reducing the side effects.

It has been shown that attention deficits persisted despite seizure freedom obtained by AEDs and VPA affected attention more than ETX and LTG (Masur et al., 2013). Furthermore, i) a small improvement in the behavioral problems was found in the first year of the study; however, ii) the behavioral outcomes were affected by the treatment and by the seizure outcomes and; iii) VPA was associated with worse behavioral outcomes than ETX and LTG, while there were no differences between ETX and LTG (Shinnar et al., 2017).

In other words, these data show that ETX improves both seizures and behavior in children with CAE, which is surprising since ETX is not being used in the treatment of any psychiatric disease, in contrast to VPA and LTG (Salpekar, 2018; Shinnar et al., 2017). This evidence suggests that ASs and neuropsychological comorbidities might share the same pathophysiology in children with CAE (Salpekar, 2018).

Finally, it is necessary to gain more knowledge about the effects of AEDs on the ASs related neuropsychological comorbidities, not only of the currently available AEDs, but also of the potential new therapies (Vincenzo Crunelli et al., 2020).

Pathophysiology of absence seizures

The thalamocortical loop

Studies in children with ASs and animal models demonstrated that ASs have a strong genetic component and that SWDs originate from aberrant neuronal oscillations within the thalamo-cortical loop (Vincenzo Crunelli et al., 2020; Leresche, Lambert, Errington, & Crunelli, 2012; Matricardi et al., 2014) that becomes susceptible to generate ASs, by a trigger area that becomes a part of the oscillating network during ASs (Leresche et al., 2012; Matricardi et al., 2014).

The thalamocortical loop includes cortical pyramidal neurons, thalamocortical (TC) relay neurons and GABAergic neurons in the *nucleus reticularis thalami* (NRT) (**Figure 2**) (Huguenard, 2019; Leresche et al., 2012): TC neurons i) receive sensory input from the periphery through excitatory synapses, and transform them in spikes conveyed to the cortex *via* excitatory synapses, and ii) send excitatory collaterals to NRT neurons that increase their spiking activity; iii) NRT neurons (in return) send feedback inhibition *via* GABA-mediated inhibitory postsynaptic potentials (IPSPs) to TC neurons; and iv) cortical neurons close the circuit sending excitatory signals to NRT and TC neurons.

The NRT cells fire in two different modes; i) they rhythmically oscillate during sleep (*burst firing mode*); and ii) tonically fire during wakefulness (*tonic firing mode*), thus modifying the flow of information between the thalamus and the cortex; this effect is possible because of the expression of low-threshold transient calcium channels (T-type calcium channels) (Lambert, Bessaïh, Crunelli, & Leresche, 2014). During ASs, the thalamocortical circuit becomes synchronized and multiple NRT cells send strong IPSPs to the TC neurons leading to rebound burst firing of the TC neurons, due to the activation of T-type calcium channels, which then

activate the NRT and cortical neurons; the latter strongly activate NRT, which generates bursts of action potentials (Vincenzo Crunelli et al., 2020).

All the neuronal populations in the thalamocortical circuitry are in a different degree synchronized during ASs, and the NRT inhibitory output to the TC neurons is pivotal for the pacing of the circuit (Vincenzo Crunelli et al., 2020). Although sleep spindles and SWDs originate from the thalamocortical circuit, their initiation site is different; the sleep spindles originate from the thalamus, while the SWDs originate as aberrant thalamocortical oscillations from an initiation site in the cortex (Leresche et al., 2012).

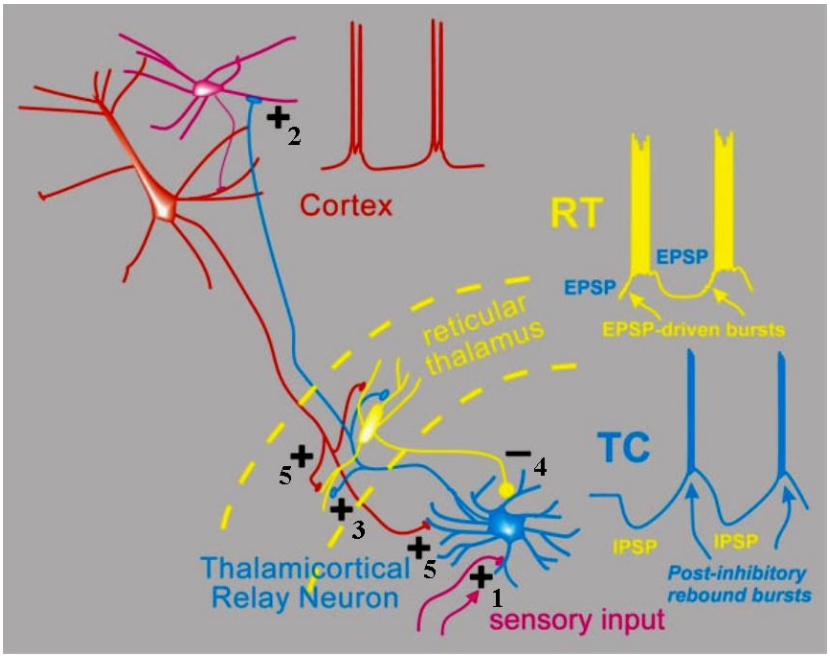

Figure 2. The thalamocortical loop. The thalamocortical loop includes cortical pyramidal neurons (*red*), thalamocortical relay neurons (TC; *blue*) and the *nucleus reticularis thalami* (NRT; *yellow*). (*1*) TC relay neurons receive sensory input (*purple*) from the periphery through excitatory synapses. (*2*) TC relay neurons signals are transmitted to the cortex via excitatory synapses and (*3*) to the *nucleus reticularis thalami* (NRT) via collaterals. (*4*) NRT sends feedback inhibition (inhibitory post-synaptic potentials - IPSPs) to TC neurons. (*5*) Cortical neurons that send excitatory signals to the NRT and the TC neurons. Adapted from: (Huguenard, 2019).

The Cortical Initiation Network

High density EEG and fMRI studies in humans have demonstrated that both the neocortex and thalamus are necessary for the full expression of the ASs (Bai et al., 2010; V. Crunelli & Leresche, 2002; Hamandi et al., 2006), and that the initiation site of ASs is located in the cortex. Indeed, this cortical network or a region is referred to as a *cortical initiation network* (CIN), which may be different between children, but it is usually consistent in the same child and it is possibly correlated to different genotypes or treatment response (Bai et al., 2010; Vincenzo Crunelli et al., 2020; Moeller et al., 2010). From the CIN, ASs spreads to other brain areas. Bai

and co-workers (Bai et al., 2010) analyzed the dynamic time course of typical ASs by using simultaneous EEG–fMRI and behavioral testing (**Figure 3**) and found out: i) a complex sequences of hemodynamic and metabolic fMRI changes are recorded in children with CAE preceding the seizure onset, during the seizure, and after the seizure termination; ii) positive fMRI changes are observed 8–14 seconds before clinical or EEG signs of an AS in cortical regions including the medial orbital frontal, frontal polar, cingulate, lateral parietal, precuneus, and lateral occipital cortex; iii) as the AS begins, the positive fMRI changes progress to the lateral frontal and temporal cortex, and following the end of a AS, also in the medial occipital cortex and the thalamus; and iv) these positive fMRI changes are followed by negative fMRI changes in the same areas and in the basal ganglia, beginning approximately 10 seconds after the AS onset and continue for over 20 seconds after the seizure termination.

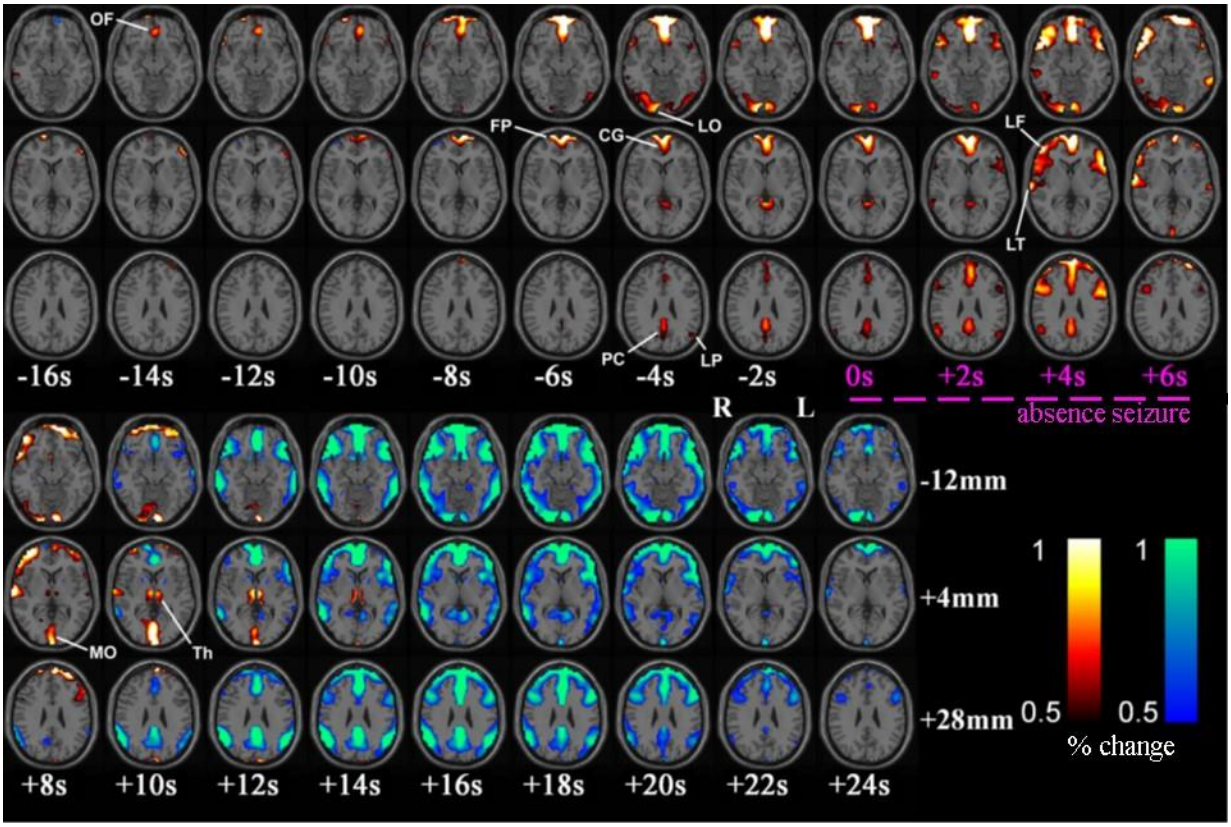

Figure 3. fMRI signal changes in children with CAE. Changes in the fMRI signal are shown as a percentage from a group analysis of 51 seizures from 8 children (increases are shown with warm colors and decreases with cool colors; display threshold= 0.5 %). Mean seizure duration is 6.6 seconds, the preictal and postictal periods were aligned across all seizures to match the ictal period scaled to the mean seizure duration. Positive fMRI changes are observed 8–14 seconds before clinical or EEG signs of a seizure in cortical regions including the medial orbital frontal (*OF*), frontal polar (*FP*), cingulate (*CG*), lateral parietal (*LP*), precuneus (*PC*), and lateral occipital (*LO*) cortex. As the seizure begins, the positive fMRI changes progress to the lateral frontal (*LF*) and temporal (*LT*) cortex, and following the end of a seizure, also in the medial occipital (*MO*) cortex and the thalamus (Th). These positive fMRI changes are followed by negative fMRI changes in the same areas and the basal ganglia, beginning approximately 10 seconds after the seizure onset. Reproduced from (Bai et al., 2010).

Widespread changes in brain networks important for attention (that is, thalamus and frontoparietal association cortex) and in primary processing regions (that's is visual, auditory, somatosensory, and motor cortex) result in the impairment of consciousness during ASs. The magnitude of blood-oxygen-level-dependent (BOLD) imaging changes was found to correlate

with the degree of behavioral impairments (Berman et al., 2010; Guo et al., 2016). A study conducted by Killory and colleagues (Killory et al., 2011) provided anatomical and functional evidence for the impaired interictal attention in children with CAE: by using simultaneous EEG-fMRI-behavioral testing, they found i) decreased medial frontal cortex (MFC) activation during a behavioral task; and ii) impaired connectivity between the right anterior insula/frontal operculum (In/FO) and MFC at rest, suggesting a primary deficit in attention network function in children with CAE. In another study, Tangwiriyasakul and colleagues found that the cortical sensorimotor network is altered in people with absence seizures, both during ictal and interictal periods (Tangwiriyasakul et al., 2018).

Animal models of absence epilepsy

Animal models of absence epilepsy are essential to provide electrophysiological, pharmacological and histological data that can be translated in the clinical practice (Depaulis, David, & Charpier, 2016); they portray the characteristics of ASs in humans, such as correlation of the EEG findings, behavioral features and the pharmacological response to AEDs (Manning, Richards, & Bowery, 2003). There are different animal models of absence epilepsy, which can be divided into two main categories – *pharmacological* and *genetic* models (see **Table 1**) (Manning et al., 2003).

Chemically induced models of absence epilepsy		
Model	*Species*	*SWD freq. (Hz)*
Penicillin	Cat	3
Low-dose pentylenetetrazol	Rat	7-9
THIP (4,5,6,7 tetrahydroisoxazolo[4,5,-c]pyridine-3-ol)	Rat	4-6
γ-Hydroxy-butyrate	Rat, cat, monkey	7-9
AY9944	Rat	4-6
Genetic mouse models (single gene mutations)		
Model		*SWD freq. (Hz)*
Lethargic, Stargazer, Tottering, Leaner, Mocha, Ducky		5-7
Genetic rat models (polygenetic)		
Model		*SWD freq. (Hz)*
GAERS		7-10
WAG/Rij		7-11

Table 1. Pharmacological and genetic animal models of absence epilepsy. Adapted from (Manning et al., 2003).

Chemically induced ASs can be provoked by injecting different noxious substances, which produce bilateral, synchronous SWDs of various duration and various frequencies depending on the drug used (**Table 1**) (Manning et al., 2003).

In comparison to the pharmacological models, genetic models of absence epilepsy show spontaneous SWDs (Manning et al., 2003). There are genetic mouse models and genetic rat models of absence epilepsy (**Table 1**) (V. Crunelli & Leresche, 2002; Manning et al., 2003). Genetic *mouse* models arise from spontaneous point mutations on single-genes, encoding subunits of voltage-gated Ca^{2+} channels (V. Crunelli & Leresche, 2002; Manning et al., 2003).

These genetic mouse models develop SWDs of a higher frequency (5-7 Hz) than in humans, accompanied by behavioral arrest (phenocopying the lack of consciousness in humans) in the first few weeks of life (V. Crunelli & Leresche, 2002; Manning et al., 2003). However, their usefulness to study ASs behavioral and psychiatric comorbidities is limited, as they also develop gait/ataxic deficits and cerebellar abnormalities (Fisher, 2017).

The best-characterized and most extensively studied models of absence epilepsy are genetic *rat models* Genetic Absence Epilepsy Rats from Strasbourg (GAERS) and Wistar albino Glaxo from Rijswijk rats (WAG/Rij) both derived from the normal Wistar strain (Depaulis et al., 2016). GAERS and WAG/Rij rats are polygenic models of absence epilepsy and genetic transmission of seizure in these strains is autosomal dominant. The genetic background that leads to ASs expression in these strains has not been elucidated, but some mutations of *Cacna1h* gene controlling the low threshold T-type calcium channel Cav3.2 have been identified (Cope et al., 2009; V. Crunelli & Leresche, 2002; Depaulis et al., 2016; Powell et al., 2009). These rat models show spontaneous SWDs that are associated with behavioral arrest, unresponsiveness to mild stimuli and minor orofacial disturbances, which are all similar to typical ASs in humans (V. Crunelli & Leresche, 2002). Moreover, these rats do not show the neurological abnormalities observed in genetic mouse models (i.e., in tottering, lethargic, stargazer mouse) which makes them suitable for behavioral testing (V. Crunelli & Leresche, 2002). As seen in humans, SWDs start and end abruptly from a normal EEG background, mostly during a state of passive wakefulness or drowsiness and sporadically during active arousal slow-wave and paradoxical sleep (Coenen & Van Luijtelaar, 2003; Depaulis et al., 2016). Both GAERS and WAG/Rij strains respond to the AEDs used to treat ASs in humans, which further supports their validity as

a good animal model (Depaulis et al., 2016; Kandratavicius et al., 2014). In contrast to human AS, rat models show a higher SWDs frequency, complete lack of polyspikes, late onset of seizures and persistence into adulthood (V. Crunelli & Leresche, 2002; Depaulis et al., 2016).

There are differences between GAERS and WAG/Rij rats in different parameters of SWDs:, SWDs frequency which is higher in WAG/Rij rats; SWDs number, total duration and mean length is higher in GAERS (Depaulis et al., 2016). Moreover, ASs are detected earlier in GAERS than in WAG/Rij rats (around postnatal day 30 in GAERS; postnatal day 75 in WAG/Rij rats) (V. Crunelli & Leresche, 2002; Depaulis et al., 2016).

GAERS model of absence epilepsy was outbred over several generations from a Wistar colony, to obtain a strain in which 100 % of animals show ASs, and from the same Wistar colony, another strain was obtained – Non-Epileptic Control strain (NEC) rats which do not display ASs, and are used in experiments as the control strain for GAERS rats (Marescaux, Vergnes, & Depaulis, 1992). As seen in human EEG, SWDs in GAERS start and end abruptly from a normal EEG background, they occur mostly during a state of passive wakefulness or drowsiness (Depaulis et al., 2016). Moreover, like children with CAE, genetic rat models of absence epilepsy also show ASs associated behavioral comorbidities, such as anxiety-like behaviors and depressive-like behaviors (Rita Citraro, Leo, De Fazio, De Sarro, & Russo, 2015; Dezsi et al., 2016; K. Sarkisova & van Luijtelaar, 2011; K. Y. Sarkisova et al., 2010). In particular, anxiety-like behavior has been extensively studied, by applying various interventions and by using different behavioral tests, such as open field, elevated plus maze and light–dark box; the main finding from those studies are listed in the table below (**Table 2**).

Reference	*Model*	*Test*	*Intervention*	*Main findings*
(Bouilleret et al., 2009)	GAERS	OF	/	↑ anxious behavior compared to control strain
(Jones et al., 2008)	GAERS	OF, EPM	/	↑ anxious behavior compared to control strain
(Marks et al., 2016)	GAERS	OF, EPM	/	↑ anxious behavior compared to control strain
(Marques-Carneiro et al., 2014)	GAERS	OF, EPM	/	↑ anxious behavior compared to control strains
(K. Y. Sarkisova, Midzianovskaia, & Kulikov, 2003)	WAG/Rij	OF, EPM, LDB	Imipramine, subchronic (15 days)	No effect on anxious behavior
(K. Y. Sarkisova, Kulikov, Midzyanovskaya, & Folomkina, 2008)	WAG/Rij	OF, LDB	Imipramine, parlodel, subchronic (15 days)	No effect on anxious behavior
(Shaw, Chuang, Shieh, & Wang, 2009)	Long–Evans	EPM, OF	Ethosuximide, acute	↓ number of seizures, no effect on anxious behavior
(K. Y. Sarkisova et al., 2010)	WAG/Rij	OF, LDB	Ethosuximide, early chronic (P21, 17 days), prolonged early chronic (P21, 152 days)	↓ number of seizures, no effect on anxious behavior
(Huang et al., 2012)	Long–Evans	OF, EPM	Lamotrigine, chronic (35 days)	↓ number and duration of SWDs, ↓ anxious behavior
(Russo et al., 2013)	WAG/Rij	OF, EPM	Aripiprazole, subchronic (>14 days)	↓ number and duration of SWDs, ↓ anxious behavior
(R. Citraro et al., 2014)	WAG/Rij	OF	Statins, acute, chronic (119 days), early chronic (<P45, 119 days)	↓ development of seizures with early chronic; no effect with acute or chronic, ↓ anxious behavior with early chronic; no effect with acute or chronic
(Ari et al., 2016)	WAG/Rij	EPM	Ketone supplements, subchronic (7 days), chronic (83 days)	↓ anxious behavior
(R. Citraro et al., 2017)	WAG/Rij	OF	Perampanel, acute, subchronic (7 days), early chronic (<P30, 152 days)	No effect on seizures with acute and subchronic; ↓ seizure development with chronic, no effect on anxious behavior
(Aygun, 2020)	WAG/Rij	OF	Trazodone, acute	↑ number and duration of SWDs, ↑ anxious behavior
(R. Citraro et al., 2020)	WAG/Rij	EPM	Valproic acid, sodium butyrate, early chronic (P30, 152 days), prolonged early chronic (P30, 212 days)	↓ number and duration of SWDs, no effect on anxious behavior
(Dezsi et al., 2013)	GAERS	OF	Ethosuximide, early chronic (P21, 154 days)	↓ frequency of seizures, ↓ anxious behavior
(Dezsi et al., 2016)	GAERS	OF, EPM, LDB	Enriched vs. standard environment chronic (42 days), early chronic (P21, 77 days) prolonged early chronic (P30, 140 days)	↓ development and frequency of seizures, ↓ anxious behavior
(Marks, Zabder, Cain, Snutch, & Howland, 2019)	GAERS	OF, EPM	Z944, pan-T-type calcium channel antagonist, acute	↑ anxious behavior

Table 2. Animal studies reporting an association between absence seizures and anxiety with or without an intervention in different behavioral tests. Note: Imipramine: antidepressant; parlodel, dopamine agonist; ethosuximide, antiepileptic; lamotrigine: antiepileptic; aripiprazole: antipsychotic; statins: cholesterol synthesis inhibitor; perampanel: antiepileptic; trazodone: antidepressant; valproic acid: antiepileptic. Abbreviations: OF (Open field), EPM (Elevated Plus Maze), LDB (Light–Dark Box). Adapted from: (Gruenbaum et al., 2021).

Insights from studies in ASs models

In animal models of absence epilepsy, the CIN location is different, it is localized in the peri-oral region (PoC) of the primary somatosensory cortex (S1), from which the seizure further spreads to the thalamus and other cortical sites (Meeren et al., 2002; Polack et al., 2007). Newer studies have shown different cortical initiation sites in different animal models, which is in line with the findings in humans (Vincenzo Crunelli et al., 2020).

Recent studies in animal models demonstrated that the degree of *synchrony* during ASs might be lower than it was expected in the past (Vincenzo Crunelli et al., 2020). Meyer and colleagues have found decreased neuronal firing in the occipital visual cortex during AS (Meyer, Maheshwari, Noebels, & Smirnakis, 2018). Sorokin and colleagues used optogenetic tools to modify the ability of TC neurons in the somatosensory part of the ventrobasal (VB) thalamus to generate burst responses and found that hyperpolarization of TC neurons, which generates burst firing, is sufficient to initiate ASs, while the depolarization of TC neuron membrane is able to block a naturally occurring AS (Sorokin et al., 2017). In contrast, a study conducted by McCafferty and colleagues, demonstrated that during ASs, NRT neurons increase their burst firing while TC neurons decrease their firing, indicating that TC neurons are less involved in the seizure generation. In line with these results, these authors showed that the pharmacological blockade of low threshold T-type calcium channels in TC neurons had no effect on ASs, while the blockage in cortical and NRT neurons suppressed ASs (McCafferty et al., 2018).

Using the Designer Receptor Exclusively Activated by Designer Drug (DREADD) technology, Panthi and Leitch recently showed the involvement of cortical interneurons in ASs control, as

they found that the selective inhibition of cortical parvalbumin-positive interneurons by DREADDs elicits absence-like seizures in normal mice (Panthi & Leitch, 2019).

In the animal models of absence epilepsy, the normal function of the thalamo-cortical loop is dysregulated by an altered ionotropic *$GABA_A$ receptor-mediated inhibition* (V. Crunelli, Leresche, & Cope, 2012). A study conducted by Cope and colleagues (Cope et al., 2009) showed that an enhanced GABA tonic current mediated by extrasynaptic δ-containing $GABA_A$ receptors is necessary and sufficient for the full expression of ASs: increasing GABA tonic current in normal Wistar rats elicits SWDs, while decreasing the aberrant tonic current in GAERS reduces SWDs. The enhanced tonic GABA current had been related to dysfunction in the astrocytic GABA transporter 1 (GAT-1) (Cope et al., 2009; Vincenzo Crunelli et al., 2020). These results show that the enhanced tonic current mediated by extrasynaptic δ-containing $GABA_A$ receptors is necessary and sufficient for the full expression of ASs and suggest that the normalization of the aberrant tonic GABA current could have an important therapeutic value. However, there are no direct antagonists of the $GABA_A$ receptors available, so an indirect modulation of GABA tonic current could be a target for new therapeutic strategies (Deidda, Crunelli, & Di Giovanni, 2021).

Important ASs modulation occurs also via the *basal ganglia* (Vincenzo Crunelli et al., 2020). Changes in the firing of GABAergic neurons of the substantia nigra (SN) modulate ASs both via the direct and the indirect pathway, and it has been shown that the firing of GABAergic SN *pars reticulata* (SNr) neurons is increased during the ASs (Deransart & Depaulis, 2002; Deransart et al., 2003; Paz, Chavez, Saillet, Deniau, & Charpier, 2007).

In summary, the increase in the inhibition of TC neurons, produced by two key sources – NRT and SNr, is the key feature of the thalamic ictogenesis and results in an overall decrease in the

total firing of TC neurons and few single action potential firings; and an increased burst firing mediated by T-channels during ASs (Vincenzo Crunelli et al., 2020).

Cannabinoid system in absence epilepsy

Cannabis-based treatment of epilepsy has received a lot of attention in recent years, as almost miraculous responses to marijuana extracts have been reported in few refractory cases of pediatric epilepsy (Detyniecki & Hirsch, 2015).

The *endocannabinoid* (eCB) *system* is a neuromodulatory system that has an important role in various aspects of neuronal functioning, including cognition, learning and memory, motor behavior, regulation of appetite, temperature regulation, pain perception and various psychiatric and neurological disorders (Katona & Freund, 2012; Morena, Patel, Bains, & Hill, 2016; Soltesz et al., 2015).

It consists of two cannabinoid (CB) receptors, type 1 and type 2, and two endogenous ligands, anandamide (AEA; N-arachidonyl ethanolamine) and 2-arachidonoyl glycerol (2-AG) (Katona & Freund, 2012) (**Figure 5**).

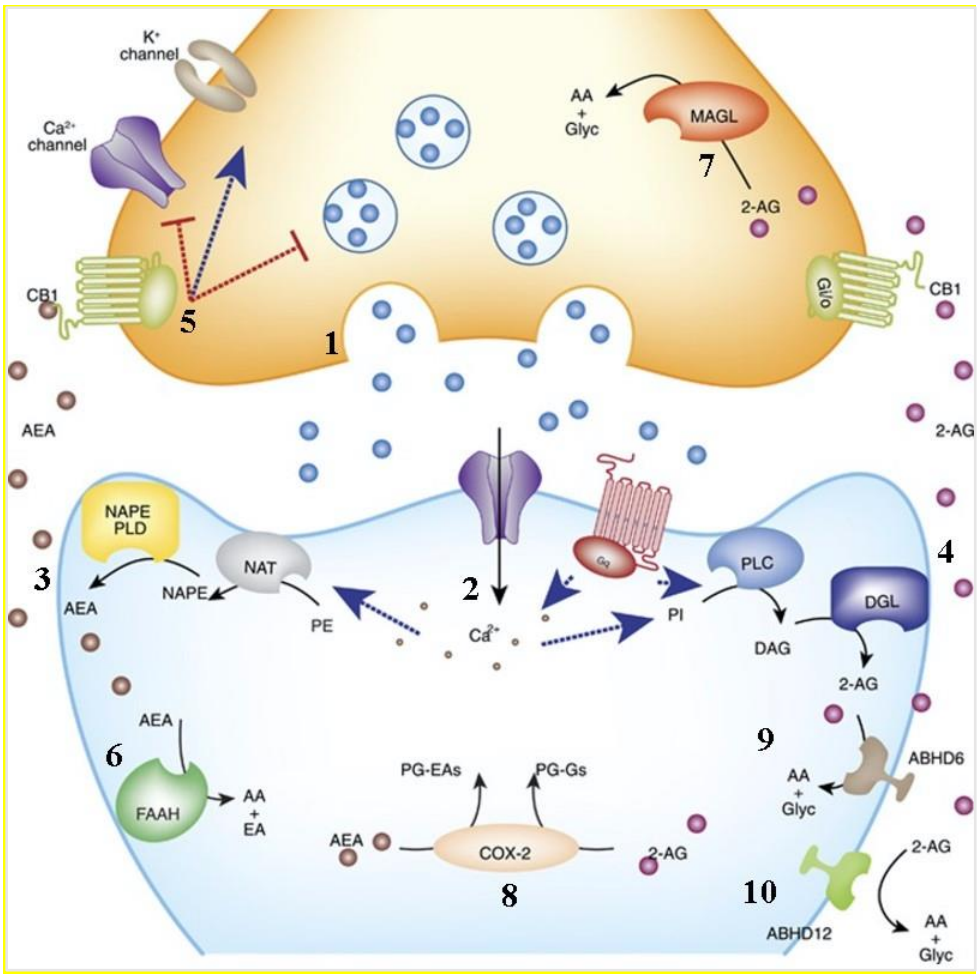

Figure 5. The endocannabinoid retrograde signaling.

Release of neurotransmitters (*blue circles*; e.g., glutamate) from the presynaptic neuron (**1**) causes postsynaptic depolarization and an increase of intracellular Ca2+ levels and increases endocannabinoid biosynthesis (**2**). Anandamide (AEA; *brown circles*) is synthesized from phospholipid precursors, phosphatidylethanolamine (PE) in the following steps: from PE, N-acyltransferase (NAT; a Ca2+-dependent transacylase) produces N-arachidonoyl PE (NAPE), which is hydrolyzed by a phospholipase D (NAPE-PLD) to AEA (**3**). 2-arachidonoyl glycerol (2-AG; *purple circles*) is synthetized in the following steps: phospholipase C (PLC) hydrolyses phosphatidylinositol (PI) into diacylglycerol (DAG), which is converted to 2-AG by diacylglycerol lipase (DGL) (**4**). From the postsynaptic neuron, AEA and 2-AG migrate to bind presynaptic cannabinoid type 1(CB1) receptor, which couples to Gi/o proteins to inhibit adenylyl cyclase activity and regulate ion channels (activate potassium channels, inhibit voltage-gated calcium channels) to suppress the neurotransmitter release at the synapse (**5**). The two main metabolic enzymes responsible for eCB degradation are two hydrolytic enzymes. Fatty acid amide hydrolase (FAAH), localized on intracellular membranes in postsynaptic cells, hydrolyzes AEA to arachidonic acid (AA) and ethanolamine (EA) (**6**). Monoacylglycerol lipase (MAGL), localized in presynaptic terminals, hydrolizes 2-AG to AA and glycerol (Glyc) (**7**). Small proportions of eCBs are metabolized by cyclo-oxygenase 2 (COX-2) and alpha-beta hydrolase (ABHD) class of enzymes, specifically ABHD6 and ABHD12 (*8;9;10*). Modified from: (Morena et al., 2016).

CB1 receptors are the most abundant class of G-protein-coupled receptors in the central nervous system, located presynaptically, at axon terminals, and coupled to Gi/o proteins to inhibit adenylyl cyclase activity and regulate ion channels (activate potassium channels, inhibit voltage-gated calcium channels) and suppress the neurotransmitter release at in the synapse (Herkenham et al., 1990; Howlett et al., 2002; Morena et al., 2016; Soltesz et al., 2015). Although CB1 receptors are expressed at many different types of synapses, their eCB effects are mainly seen through the effects on GABAergic and glutamatergic synapses (Katona & Freund, 2012; Morena et al., 2016). CB2 receptors are mostly located in the peripheral tissue and immune cells (Katona & Freund, 2012).

The *endogenous cannabinoids* (eCBs; also called endocannabinoids) AEA and 2-AG are lipid molecules, that are synthesized 'on demand' from phospholipid precursors in the postsynaptic membrane and act as retrograde messengers activating the CB receptors (Katona & Freund, 2012; Wilson & Nicoll, 2002). The two main metabolic enzymes responsible for eCB degradation are two hydrolytic enzymes, fatty acid amide hydrolase (FAAH) for AEA degradation and monoacylglycerol lipase (MAGL) for 2-AG degradation (Cravatt et al., 2001; Dinh et al., 2002; Katona & Freund, 2012; Long, Nomura, & Cravatt, 2009). Studies have shown that FAAH and MAGL enzymes are localized at different cellular sites in the brain: FAAH on intracellular membranes in postsynaptic cells, while MAGL is localized in presynaptic terminals, close to the CB1 receptors (Cristino et al., 2008; Gulyas et al., 2004; Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). The inactivation of FAAH or MAGL enzymes leads to an accumulation of AEA or 2-AG, proving their significance in eCB level regulation (Cravatt et al., 2001; Long et al., 2009). Additionally, small proportions of eCBs are

metabolized by cyclo-oxygenase 2 (COX-2), alpha-beta hydrolase (ABHD) class of enzymes, specifically ABHD6 and ABHD12 (Katona & Freund, 2012; Morena et al., 2016). It is thought that eCB system operates in phasic and tonic mode, where AEA conducts tonic signals, regulating the basal synaptic transmission, while 2-AG conducts phasic signals and plays a role in synaptic plasticity (Katona & Freund, 2012). To conclude, the endogenous ligands of cannabinoid receptors are (AEA and 2-AG) retrograde signaling molecules synthesized postsynaptically, which activate presynaptic CB1 receptors to inhibit neurotransmitter release (Katona & Freund, 2012; Wilson & Nicoll, 2002).

The eCB system can also be modified by *phytocannabinoids* (Tai & Fantegrossi, 2014). These are the cannabinoids naturally found in the *Cannabis sativa* plant, known for their psychoactive effect (subjective euphoria, relaxation, elevated mood) and widely used as a recreational drug (Peres et al., 2018; Perucca, 2017; Tai & Fantegrossi, 2014). Over 100 phytocannabinoids have been identified in *Cannabis sativa* (Perucca, 2017; Tai & Fantegrossi, 2014) including 9-Δ-tetrahydrocannabinol (THC) and cannabidiol (CBD) being the two most abundantly represented and best-characterized phytocannabinoids (Perucca, 2017).

Cannabinoid system can also be modified by the administration of *synthetic cannabinoids* (SCBs); after the discovery of THC, many different synthetic cannabinoids have been synthesized as pharmacological tools in research. However, SCBs have also emerged as drugs of abuse, since they produce psychoactive effects similar to THC, are easily accessible and difficult to detect in drug screenings (Auwärter et al., 2009; Tai & Fantegrossi, 2014). There are 4 major

classes of SBCs: classic cannabinoids (tricyclic terpenoid derivatives bearing a benzopyran moiety; i.e., Δ9-THC, AM2389, cannabinol, nabilone, HU-210), nonclassical cannabinoids (bicyclic and tricyclic analogs of Δ9-THC lacking the pyran ring of classical cannabinoids; i.e., CP55,940, HU-308), aminoalkylindoles (i.e., WIN55,212-2, JWH-018, JWH-073, AM1241) and 1,5-biarylpyrazole ligands (i.e., AM251, rimonabant, SR144528); 1,5-biarylpyrazole ligands act as cannabinoid receptor antagonists, rimonabant and AM251 as selective CB1 receptor antagonists and SR144528 as selective CB2 receptor antagonist (Tai & Fantegrossi, 2014).

THC is a partial agonist of the CB1 receptor and is responsible for the psychoactive effect of the *Cannabis* plant (Perucca, 2017). THC has shown variable effects in different seizure models (Perucca, 2017). Regarding absence epilepsy, a recent study conducted by Roebuck and colleagues has shown that THC dramatically increases SWDs in GAERS rats (Roebuck et al., 2020). Due to adverse psychotropic effects variable effects on seizures, THC is undesirable for development as a treatment for epilepsy (Perucca, 2017).

Cannabidiol (CBD) is the second most prevalent cannabinoid found in the *Cannabis sativa* plant and it does not produce a psychoactive effect (Peres et al., 2018). CBD acts via multiple mechanisms, antagonizing the effects of CB receptor agonists and it shows little affinity for CB receptors; it is suggested that CBD acts as an inverse agonist or as a negative allosteric modulator of CB receptors (Bisogno et al., 2001; Peres et al., 2018; Silvestro, Mammana, Cavalli, Bramanti, & Mazzon, 2019). Also, CBD inhibits the FAAH enzyme, leading to an increase in AEA levels (Bisogno et al., 2001; Peres et al., 2018). Moreover, CBD acts via multiple other receptors, such as transient receptor potential vanilloid type 1 (TRPV1),

peroxisome proliferator-activated receptor-gamma (PPARγ), serotonin receptor 1A (5-HT_{1A}) and directly in the mitochondria and possibly on the T-type Ca^{2+} channels (Bisogno et al., 2001; Peres et al., 2018). It has been shown that CBD can act directly at the extrasynaptic $GABA_A$ receptors (Bakas et al., 2017). CBD showed an antiepileptic effect in different clinical trials, when used alone or as adjunctive therapy to conventional antiepileptic drugs (Silvestro et al., 2019). Moreover, CBD is approved by the FDA as an adjunctive treatment for Lennox-Gastaut Syndrome and Dravet syndrome, severe forms of early-onset encephalopathic epilepsies (Devinsky et al., 2017; Thiele et al., 2018).

Moreover, two phase 2 clinical trials were initiated in 2017 to assess the efficacy (ClinicalTrials.gov #NCT03336242) and long-term safety and tolerability (ClinicalTrials.gov #NCT03355300) of CBD oral solution in the treatment of pediatric participants with treatment-resistant childhood ASs, however, those trials have been terminated early due to a lack of a clinically meaningful reduction of seizure count.

In contrast, a recent study conducted on the GAERS rats has shown that CBD produces a reduction in SWDs (Roebuck et al., 2020).

In different animal models of epilepsy, the cannabinoid system activation has been shown to attenuate several types of seizures, to reduce status epilepticus and it showed a seizure protective role in animal models of brain injuries (Aghaei et al., 2015; Suleymanova, Shangaraeva, van Rijn, & Vinogradova, 2016). CB1 receptor activation by WIN 55,212-2 has been shown to revert pilocarpine-induced status epilepticus (Colangeli et al., 2019; Di Maio, Colangeli, & Di Giovanni, 2019). In contrast, cannabinoid receptor antagonists have been found to induce and increase the occurrence of convulsive seizures (M. F. Perescis et al., 2017).

However, only a few studies investigated the involvement of cannabinoid system in animal models of absence seizures, and showed contrasting results (Roebuck et al., 2020; Sales-Carbonell et al., 2013; Clementina M. Van Rijn et al., 2010).

The aim of my thesis is to modulate the cannabinoid system by administering exogenous cannabinoids (synthetic or phytocannabinoids) or by modulating the endocannabinoid tone pharmacologically and provide contribution to the knowledge about cannabinoid modulation in absence epilepsy.

Material and methods

Animals

Adult (3 months old) male Wistar (Charles River, Margate, UK), NEC and GAERS rats (originally obtained from Strasbourg, France) were used in the experiments. Rats were housed in the animal facility of the Department of Physiology and Biochemistry of the University of Malta. Rats were housed in a room maintained at a temperature of 21±1 °C, while the humidity was kept at the range of 60±5 %. The circadian rhythm was adjusted to a 12-hour day-light cycle, with the lights being switched on at 7 am and switched off at 7 pm. Food and water was provided to the animals ad libitum.

Institutional guidelines on experimental animals were adhered to and all procedures were carried out in conformity to European directives (EU Directive, 2010/63/EU) and local regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Hole board test

Experimental apparatus

HB test is an exploration based, unconditioned test to study anxiety related behaviors in rodents (Brown & Nemes, 2008). The HB apparatus (**Figure 6**) consists of a white Plexiglas arena (dimensions: 50 x 50 cm), raised 5 cm above the surface and surrounded by Plexiglas walls (height: 50 cm), 3 opaque white walls and front transparent wall. The floor contains 4 holes (diameter: 4 cm), symmetrically placed near the corners of the arena.

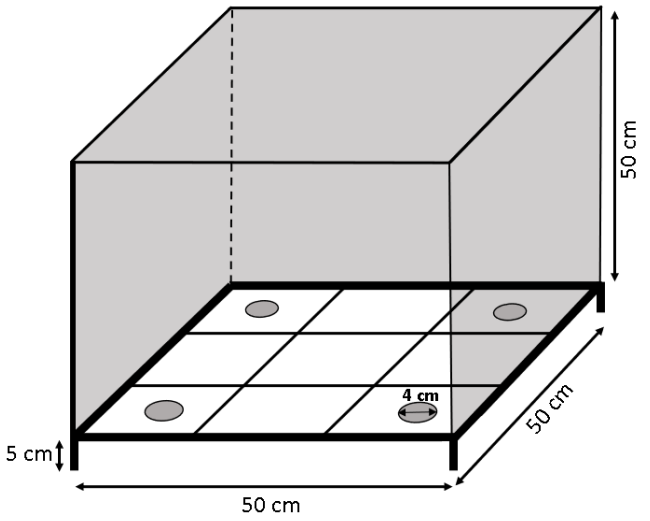

Figure 6. A schematic diagram representing the hole bord apparatus.
The HB apparatus is a square arena (50 cm x 50 cm), surrounded by 3 white, opaque walls and a front transparent wall (height: 50 cm). It has 4 holes located in each corner of the square, measuring a diameter of 4 cm. It is 5 cm elevated from the surface.

Test procedure

All rats tested in hole board were experimentally naïve and tested only once. On the day of the experiment, rats were moved to the testing room inside their home cages and left there to acclimate for 30 minutes.

If the experiment did not require a drug injection, the experiment started by placing rats one by one in the middle of the hole board apparatus. Whereas, if the experiment included a drug injection rats were injected intraperitoneally (i.p.) with the drug of interest or vehicle, one after the other, the subsequent rat was injected 15 minutes after the preceding rat. After injection, a rat was placed back in its home cage and it was recorded in the hole board apparatus 40 minutes later.

Each rat was left to explore in the hole board apparatus for 10 minutes (**Figure 7**). All behaviors were recorded by a digital camera (Toshiba Camileox200 S, 720p 8 Megapixel camera, Minato City, Tokyo, Japan). The camera was placed in front of the apparatus, allowing a good angle for visualization of all behaviors. After each rat, the apparatus was carefully cleaned with 70 % ethyl alcohol. Recordings were saved and analyzed offline.

Figure 7. HB test.
Snapshot from the recording camera showing a rat exploring the hole board arena.

Data analysis

Video recordings were analyzed in an observation software (The Observer, Noldus IT, The Netherlands) and annotated on the basis of an ethogram containing 11 behaviors, divided in 4 main categories (**Figure 8**): general exploration, focused exploration, grooming activity and immobility, as described before (Casarrubea, Sorbera, & Crescimanno, 2009). The first category is general exploration: immobile sniffing (rat sniffs the environment while standing in the same place), walking (rat walks around the arena), rearing (rat stands with erect posture without leaning on the wall) and climbing (rat stands with erect posture while leaning on the wall). The second category is grooming activity: front paw licking (rat licks its front paws), hind paw licking (rat licks it hind paws), face grooming (rat grooms its face with its front paws) and body grooming (rat licks and grooms its body). The third category is focused exploration: edge sniff (rats sniffs the border of a hole) and head dip (rat puts its head in a hole). The fourth category is immobility (rat is not moving).

The observational software enabled frame by frame video playback (frame rate: 30 frames per second); hence it was possible to mark the start of the behaviors very precisely. Observational data were exported from the software and used for further analysis. To provide a quantitative description of the behavior in HB, mean occurrence and mean duration for each annotated behavior were calculated.

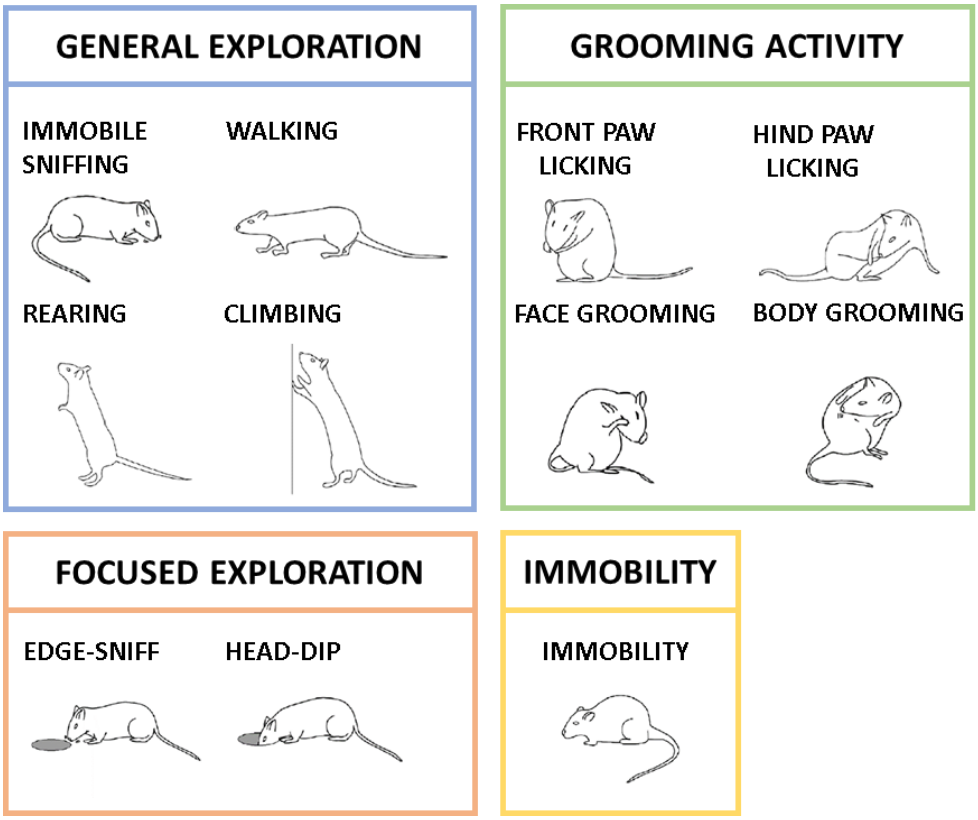

Figure 8. Ethogram of rat behavior in the hole board apparatus.
General exploration: Immobile Sniffing (rat sniffs the environment while standing in the same place), Walking (rat walks around the arena), Rearing (rat stands with erect posture without leaning on the wall), Climbing (rat stands with erect posture while leaning on the wall). Grooming activity: Front Paw Licking (rat licks its front paws), Hind Paw Licking (rat licks it hind paws), Face Grooming (rat grooms its face with its front paws), Body Grooming (rat licks and grooms its body). Adapted from (Casarrubea et al., 2017).

Electroencephalographic recordings

GAERS rats underwent chronic implantation of frontal-parietal electroencephalographic (EEG) electrodes to record and mark spike-and-wave discharges (SWDs, as previously described (Venzi et al., 2016). It enabled tracking SWDs in control conditions as well as tracking the changes in SWDs while treating the rats with different drugs of interest.

Anesthesia

EEG electrodes implantation was performed under general anesthesia using the inhalant anesthetic isoflurane (IsoFlo, Zoetis, UK; Iso-Vet, Chanelle Pharma, Ireland). Firstly, the rat was placed in an induction box and general anesthesia was induced by delivering 5 % isoflurane in 2 L/min 100 % oxygen. After induction, the rat was transferred to a stereotaxic frame and continued to receive the isoflurane/oxygen mixture through a facemask fitted on the nose bar. The concentration was gradually reduced to a final concentration of 2 % isoflurane in 0.5 L/min 100 % oxygen. The absence of the pedal withdrawal reflex was assessed regularly to ensure that the anesthetic delivery is sufficient to maintain the surgical plane of anesthesia. Respiratory rate and pattern were monitored at regular intervals. The temperature was monitored with a rectal probe and it was maintained at 37.5 °C with a heating pad.

Surgical procedure

EEG electrodes implantation surgery was conducted in a designated surgical room. All surfaces used for surgery were disinfected with a 70 % alcohol solution. All surgical instruments and materials were autoclaved before the beginning of the surgery. EEG electrodes were kept in a 70 % alcohol solution for at least 20 minutes prior to the implantation.

After the rat was anesthetized in the induction box, the head was shaved in the area from the line between the eyes to the line between the ears with an electric razor. The rat was transferred to the stereotaxic frame and its snout was secured in the nose bar. It was placed on the heating pad and the rectal thermometer probe was inserted in the rectum. Non-rupture ear bars were used to fix the rat to the stereotaxic frame. Eye cream (Ocry-Gel, TVM, UK) was applied to both eyes to avoid dehydration. Skin in the shaved area was scrubbed three times using an iodine disinfectant (Betadine, Mundipharma, Switzerland). A sterile drape was put over the rat, exposing only the surgical site. A mixture of lidocaine and adrenaline (Xylocaine 2% with adrenaline (Epinephrine) 1:200,000, AstraZeneca, UK) was injected subcutaneously in the area around the midline, for vasoconstriction and local anesthesia. An anterior-posterior skin incision was made in the midline going from the eyes to the ears. Four bulldog clamps were used to hold the skin and keep the incision open. Connective tissue was removed using a spatula and the skull surface was exposed. The skull surface was cleaned with cotton buds dipped in a 15 % hydrogen peroxide solution. Next, the skull surface was cleaned with a sterile saline solution and dried. Any bleeding was stopped by cauterizing the blood vessel using a metal spatula heated in the sterilizer (Germinator 500, BiosebLab, France). At this point, the skull surface was clean and dry and the frontal, parietal and occipital bones were visible and exposed.

Using an electric micro drill, a hole was drilled on the left and right frontal, parietal and occipital bone, in total six holes were drilled. Holes were not penetrating through the bone but thinning the bone to facilitate the insertion of EEG electrodes.

EEG electrodes were made of an eight millimeters long gold-plated screw post (No. 4402 S1, Swedish Dental Supplies AB, Sweden) soldered to a two centimeters long copper wire. A metal pin, taken from a PCB (printed circuit boards) socket (RS Stock No.702-3117, Preci-Dip,

Switzerland) was soldered on the other end of the copper wire. The copper insulation was removed from the tips of the wire, before soldering it to the screw and the metal pin. The screws were screwed in the holes drilled on the skull, making sure that only the tip of the screw penetrates the bone, leaving the dura mater intact. After, all screws were positioned, the skull surface and the screws were covered with a thin layer of self-curing dental adhesive resin cement (Super-Bond C&B Kit, Prestige Dental, UK). Metal pins from each electrode were connected to a ten-contact PCB socket (RS Stock No.702-3085, Preci-Dip, Switzerland). The ten-contact PCB socket consists of 5 rows of pins placed in 2 columns. Metal pins of the electrodes were connected to the 10-pin connector in the following way: left and right frontal electrode to the anterior left and right pin, left and right parietal electrode to the middle left and right pin, and finally left and right occipital electrode to the posterior left and right pin. In this way, between each row of connection, there was an empty row.

Acrylic cement (Simplex rapid, Kemdent, Swindon, England) was used to coat the screws, wires, and the sides of the ten contact PCB socket connector, ensuring the stability of the implant. After the cement dried completely, the four bulldog clamps were removed and the skin was sutured with a simple interrupted stitch, anterior and posterior from the implant, with surgical suture (PROLENE® Polypropylene Suture W8881T, Ethicon, USA).

Postoperative care

At the end of the surgery rats received a subcutaneous injection of warm, sterile saline (3% of body weight (in grams) in ml) and a subcutaneous injection of painkiller meloxicam (meloxicam 5 mg/ml, 0.1 mg/kg body weight; Meloxidolor 5 mg/ml solution, La Vet Pharma, The Netherlands). They were removed from the stereotaxic frame and the rectal thermometer probe was removed. They were placed in a recovery cage and kept warm with a red-light lamp. For the

consecutive two days, they received one subcutaneous injection of painkiller per day. They were housed individually and allowed to recover for at least seven days before the start of experiments.

Test procedure

All EEG recordings started in the morning, from 9 a.m. On the day of the experiment, rats were moved from the housing room to the recording room inside their home cages. They were left to acclimate to the recording room for half an hour. Then, they were connected to the EEG recording system and placed in an individual recording cage (dimensions: 30 cm × 58 cm × 60 cm). EEG recording system consists of a cable ending with a PCB Header (RS Stock No.702-0262, Preci-Dip, Switzerland). It was connected to the PCB Socket on the rats' implant. On the other end, the cable is connected to a pre-amplifier (0.08 Hz high-pass filter, 500 Hz low-pass filter, impedance 10 MOhm) and in turn to an analogue EEG amplifier (4-channel BioAmp, SuperTech Inc., Hungary) with a 1000 gain and low-pass filter set at 500 Hz. The signal is digitalized at 500 Hz using a CED 1401 Micro3 analog-digital converter (Cambridge Electronic Design Ltd., Cambridge, UK), stored on a computer and analyzed offline using Spike2 7.04 software (Cambridge Electronic Design Ltd., Cambridge, UK).

Once connected to the EEG recording system, rats were left undisturbed to habituate for half an hour. After the habituation time, the control period started and lasted for 60 minutes. Then, rats were intraperitoneally injected (i.p.) with a drug or matching vehicle. The recording continued for the following 180 minutes (**Figure 9**). During the recording, it was made sure that the rats were not sleeping, by gently holding them in the hand for a few seconds or by tapping on the recording cage walls. Each rat was recorded 3 times, each time with a different drug dose in a randomized order. In between each recording there was a 7 days washout period.

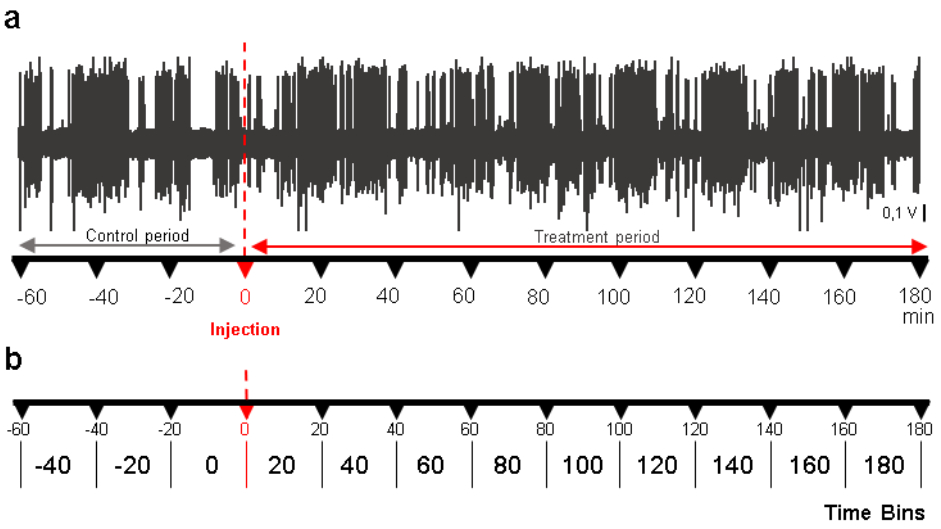

Figure 9. Example of EEG traces in GAERS rats.
(a) After one hour of a control period GAERS rats were injected with the drug of interest and recorded for the following three hours. (b) Scheme illustrating the 4-hour recording period, divided into 20 min time bins, based on which the time course of seizures was represented in the results section.

Data analysis

EEG recordings were analyzed using the SeizDetect8.s2s script in the Spike2 7.04 software. The script differentiates between SWDs and sleep spindles, by detecting peaks/troughs in the recording that are above/below the baseline level. The analysis started by choosing one channel to analyze for each rat, since EEG recordings were done from the left and right channels for each rat. Both channels were recording the same general brain activity, however, the quality of the signal was sometimes different, depending on the artefacts and surrounding noise. The chosen channel was processed by applying a high pass filter, DC remove filter, time constant 0.1 seconds. The following steps were required to detect the seizures: first, to find peaks, standard deviations for peak detection were set on 7 or 8 (used as threshold to detect the crossings above or below the interictal period), activity detection was based on peaks and cursors had to be set in an interictal period (awake and desynchronized EEG signal containing no seizures). Second, to find bursts, the following parameters were applied: Onset = 0.2 sec, Maximal (Max) interval =

0.75 sec, Minimum (Min) spikes = 5, Min burst interval = 1 sec, Min duration = 0.6 sec. Third, the frequency range for seizure activity was chosen, the set-up range was 5 to 12 Hz. Time was an additional criterion, all seizures shorter than 1 second were not included in the data analysis. Finally, all SWDs were marked, and the results were checked manually. The log file containing the number of seizures, start, end and duration of each seizure was exported from the software and imported in Excel (MS Excel, Microsoft Corporation, Redmond, Washington, USA) for further analysis.

The analysis continued by dividing the recordings into 20 minutes time bins. The control period was divided into three-time bins and the treatment period was divided into nine-time bins. For each time bin, three parameters were calculated: time spent in seizures, the number of seizures, average seizure length. Time spent in seizures is the sum of the duration of all absence seizures occurring in a time bin. The number of seizures is the sum of the number of all absence seizures occurring in a time bin. Average seizure length is the average duration of a seizure and it is calculated by dividing time spent in seizures by the number of seizures in a time bin.

Differences between treatment groups were compared based on those three parameters, expressed in the 20 minutes time bins. To normalize the data in a group, for each parameter the value in a time bin was divided by the average of the three control time bins and expressed as a percentage. All statistical analysis has been done on normalized data. Data were additionally expressed as the percentage of the control group average to simplify the graph appearance.

Hole board test and simultaneous EEG recording,

Test procedure

GAERS rats, implanted with EEG electrodes, underwent a behavioral test in hole board paired with simultaneous EEG recording. This experiment was preformed after the 1-week postsurgical recovery period and one day before the start of EEG recording experiments. On the day of the experiment, implanted GAERS rats were transferred to the testing room and left undisturbed for 30 minutes. After this 30 min habituation period, rats were put in the hole board apparatus and left to explore for 10 min, as explained earlier (see Hole board – test procedure). Just before being placed in the HB apparatus, implanted GAERS rats were connected to the EEG recording system. EEG signal was recorded throughout the HB test, while the rat's behavior was simultaneously recorded by a digital camera. EEG recordings and video recordings of the behavior were saved for analysis.

Data analysis

EEG recordings were analyzed as explained above (see EEG recordings – data analysis) and three parameters were calculated: time spent in seizures, the number of seizures and average seizure length. Rats were divided into two groups, based on the occurrences of SWDs during hole board – GAERS rats with SWDs during HB ("HB-SWDs^{+}") and GAERS rats with no SWDs during HB ("HB-SWDs^{-}").

Hole board video recordings were annotated using the observational software Observer (The Observer, Noldus IT, The Netherlands). The following behaviors were annotated: Walking, Immobility, Head Dip and Edge-Sniff. For Walking and Immobility, mean occurrences and

mean durations have been calculated. For Head Dip and Edge Sniff, mean occurrences and the ratio between Head Dip and Edge Sniff has been calculated.

All the comparisons have been done based on the two groups, HB-SWDs^{+} - GAERS rats with SWDs during HB and HB-SWDs^{-} - GAERS rats with no SWDs during HB. In addition, for the HB-SWDs^{+} group correlations of the EEG and HB parameters have been done. Namely correlation between the number of seizures, time spent in seizures and average seizure length with Head Dip occurrences and with the ratio between Head Dip and Edge Sniff.

Statistical analysis

Statistical analysis was performed with Prism version 7 (GraphPad, San Diego, CA). Comparisons of quantitative features between rats of different groups were assessed by an Unpaired two-tailed Student's t-test for normally distributed data or by Mann-Whitney test for not normally distributed data. When more than two groups/factors were analyzed, one/two-way analysis of variance (ANOVA) followed by Holm-Sidak test were used. For correlations, Spearman correlation was used. For the EEG recordings, Two-Way ANOVA repeated measures (RM) was used. Data are expressed as means ± standard error of the mean (SEM). The level of significance was $p< 0.05$.

Drugs and treatments

ETX and VPA, purchased from Sigma-Aldrich (Sigma-Aldrich Company, St.Louis Mo, USA), were dissolved in saline solution and injected i.p., in a volume of 1 ml/kg.

WIN 55,212-2 and AM 251 were purchased from TOCRIS (TOCRIS Bioscience, Bristol, UK). Cannabidiol was purchased from THC Pharm (THC Pharm Frankfurt, Germany). MJN110 was

purchased from MedChemExpress (MedChemExpress, New Jersey, USA). URB 597 was kindly gifted by Prof. Stefania Butini, Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Italy.

WIN 55,212-2, AM 251, cannabidiol, MJN110, URB 597 were dissolved in a vehicle composed of a mixture of 5 % polyethylene glycol (PEG), 5 % Tween80 and 90 % saline solution and injected i.p. in a volume of 2 ml/kg.

PEG was purchased from Acros organics (Acros organics, Geel, Belgium). TWEEN-80 was purchased from Sigma-Aldrich (Sigma-Aldrich, Sigma-Aldrich Company, St.Louis Mo, USA). Sodium chloride was purchased from Acros organics (Acros organics, Geel, Belgium).

Results

GAERS rats display ethosuximide- and valproate-sensitive SWDs

GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system before and after acute i.p. injection of vehicle (n= 10), ETX 200 mg/kg (n= 9) and VPA 200 mg/kg (n= 9) (**Figure 12a**), to investigate the effect of the two most commonly used anti-ASs drugs in GAERS rats. Two-way ANOVA RM with treatment x time as factors revealed a significant main effect of drug treatments on time spent in seizures (treatment $F_{2,25}$= 28.17; p< 0.0001). The time spent in seizures varied significantly over time ($F_{11,275}$= 11.11 p< 0.0001) and time-course of the seizures changed (treatment x time interaction $F_{22,275}$= 4.701; p< 0.0001) (**Figure 12b** *top*). Two-way ANOVA RM showed a significant main effect of drug treatments on number of seizures (**Figure 12b** *middle*) (treatment $F_{2,25}$= 35.86, p< 0.0001; time $F_{11,275}$= 11.23, p< 0.0001; interaction $F_{22,275}$= 4.859, p< 0.0001). Similarly, two-way ANOVA RM revealed a significant main effect of drug treatments on average seizure length (**Figure 12b** *bottom*) (treatment $F_{2,25}$= 183.1, p< 0.0001; time $F_{11,275}$= 17.69, p< 0.0001; interaction $F_{22,275}$= 10.26, p< 0.0001). Holm-Sidak multiple comparison test showed that the acute i.p. treatment of ETX (200 mg/kg), induced a significant decrease of time spent in seizures (**Figure. 12b** *top*), number of seizures (**Figure 12b** *middle*) and average seizure length (**Figure. 12b** *bottom*) in comparison to the vehicle during all three hours after injection.

In fact, the time spent in seizures parameter shows that ETX completely stopped SWDs in all time bins (ETX 200 mg/kg = 0 ± 0 %; vehicle vs ETX 200 mg/kg, p< 0.0001), except two time bins (20 min time bin: ETX 200 mg/kg = 1.1 ± 0.9 %; vehicle vs ETX 200 mg/kg, p< 0.0001; 80 min time bin: ETX 200 mg/kg = 0.05 ± 0.05 %; vehicle vs ETX 200 mg/kg, p< 0.0001) (**Figure**

12b *top*). This effect of ETX treatment has been observed also in the other two seizure parameters: number of seizures (all time bins: ETX 200 mg/kg = 0 ± 0 %; vehicle vs ETX 200 mg/kg, p< 0.0001; except: 20 min time bin: ETX 200 mg/kg = 2.4 ± 2.1 %; vehicle vs ETX 200 mg/kg, p< 0.0001; 80 min time bin: ETX 200 mg/kg = 0.4 ± 0.4 %; vehicle vs ETX 200 mg/kg, p< 0.0001) (**Figure 12b** *middle*) and average seizure length (all time bins: ETX 200 mg/kg = 0 ± 0 %; vehicle vs ETX 200 mg/kg, p< 0.0001; except: 20 min time bin: ETX 200 mg/kg = 12.5 ± 8.4 %; vehicle vs ETX 200 mg/kg, p< 0.0001; 80 min time bin: ETX 200 mg/kg = 1.8 ± 1.8 %; vehicle vs ETX 200 mg/kg, p< 0.0001) (**Figure 12b** *bottom*).

Acute i.p. treatment of valproate (VPA; 200 mg/kg), induced a significant decrease of time spent in seizures (**Figure 12b** *top*) in comparison to the vehicle that lasted during the first 80 minutes after VPA injection (20 min time bin: VPA 200 mg/kg = 27 ± 11 %; vehicle vs e VPA 200 mg/kg, p= 0.0072 ; 40 min time bin: VPA 200 mg/kg = 11 ± 8 %; vehicle vs VPA 200 mg/kg, p= 0.0003; 60 min time bin: VPA 200 mg/kg = 10 ± 7 %; vehicle vs VPA 200 mg/kg, p= 0.0003; 80 min time bin: VPA 200 mg/kg = 25 ± 10 %; vehicle vs VPA 200 mg/kg, p=0.0056). In addition, VPA injection had this decreasing effect of same duration on number of seizures (20 min time bin: VPA 200 mg/kg = 41 ± 16 %; vehicle vs VPA 200 mg/kg, p= 0.0417; 40 min time bin: VPA 200 mg/kg = 15 ± 8 %; vehicle vs VPA 200 mg/kg, p= 0.0003; 60 min time bin: VPA 200 mg/kg = 21 ± 9 %; vehicle vs VPA 200 mg/kg, p= 0.0009; 80 min time bin: VPA 200 mg/kg = 34 ± 12 %; vehicle vs VPA 200 mg/kg, p= 0.0129) (**Figure 12b** *middle*). Moreover, the average seizure length was significantly decreased during the first hour after the VPA injection (20 min time bin: VPA 200 mg/kg = 57 ± 13 %; vehicle vs VPA 200 mg/kg, p= 0.0134 ; 40 min time bin: VPA 200 mg/kg = 20 ± 11 %; vehicle vs VPA 200 mg/kg, p< 0.0001; 60 min time bin: VPA 200 mg/kg = 25 ± 9 %; vehicle vs VPA 200 mg/kg, p< 0.0001) (**Figure 12b** *bottom*).

Furthermore, ETX induced a decrease in the time spent in seizure that was significantly lower in comparison to VPA in the 120 min (ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 107 ± 25 %; p< 0.0001) and the 180 min (ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 72 ± 23 %; p= 0.0114) time bin (**Figure 12b** *top*). Similarly, ETX induced a decrease in number of seizures that was significantly lower in comparison to VPA in the 120 min (ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 110 ± 29 %; p< 0.0001), 160 min (ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 60 ± 11 %; p= 0.0417) and 180 min (ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 73 ± 23 %; p= 0.0045) time bin (**Figure 12b** *middle*). Moreover, ETX induced a decrease in average seizure length that was significantly lower in comparison to VPA during the first 20 min (20 min time bin: ETX 200 mg/kg mg/kg = 13 ± 8 % vs VPA 200 mg/kg = 57 ± 13 %; p= 0.0106) after injection and during the second (80 min time bin: ETX 200 mg/kg mg/kg = 1.8 ± 1.8 % vs VPA 200 mg/kg = 66 ± 17 %; p< 0.0001; 100 min time bin: ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 82 ± 15 %; p< 0.0001; 120 min time bin: ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 123 ± 20 %; p< 0.0001) and third hour (140 min time bin: ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 82 ± 17 %; p< 0.0001; 160 min time bin: ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 87 ± 13 %; p< 0.0001; 180 min time bin: ETX 200 mg/kg mg/kg = 0 ± 0 % vs VPA 200 mg/kg = 97 ± 5 %; p< 0.0001) after injection (**Figure 12b** *bottom*).

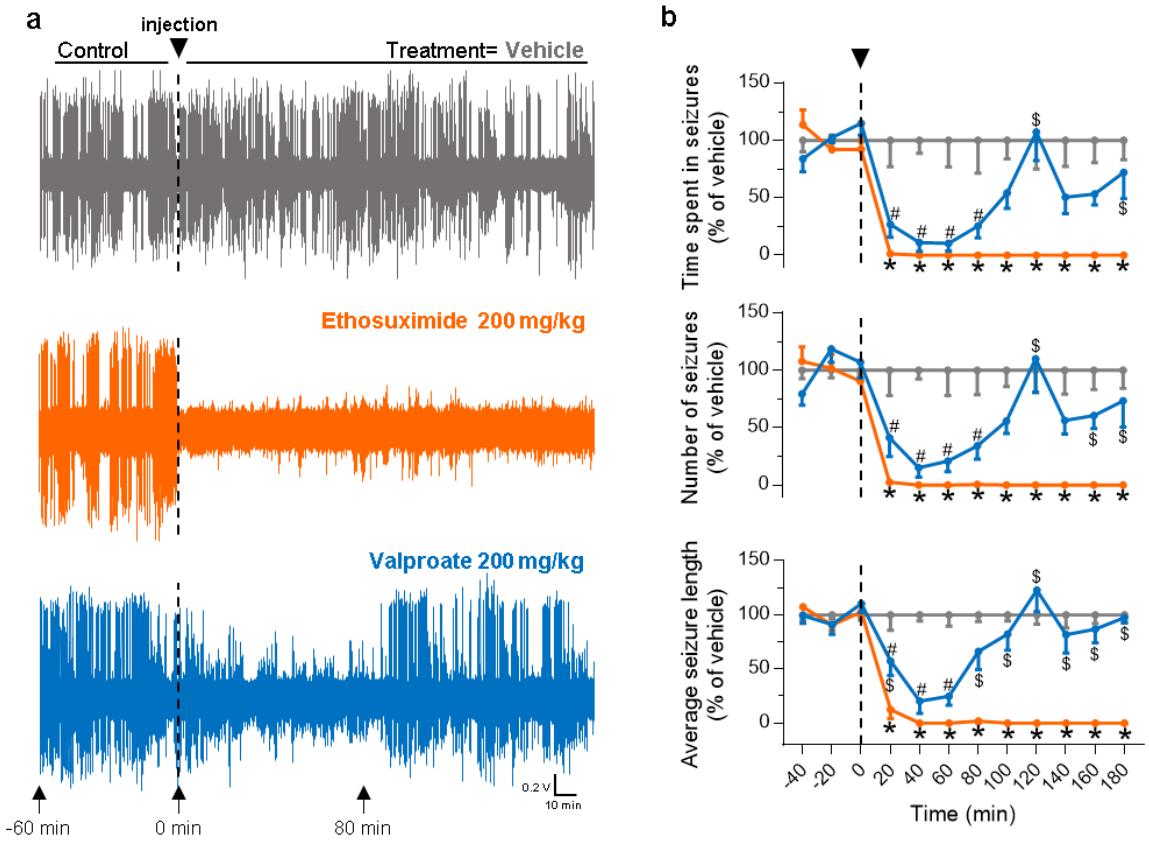

Figure 12. GAERS rats display ethosuximide- and valproate-sensitive SWDs.
(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after acute i.p. injection (arrow/dashed line) of vehicle (grey), ETX 200 mg/kg (orange) and VPA 200 mg/kg (blue). (**b**) Time course (mean ± SEM) of the time spent in seizures (*top*), number of seizures (*middle*) and length of a seizure (*bottom*) in vehicle (grey; n=10), ETX 200 mg/kg (orange; n=9) and VPA 200 mg/kg (blue; n=9) treated GAERS before and after the injection time (dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. * - $p<0.05$, vehicle vs ETX 200 mg/kg. # - $p<0.05$, vehicle vs VPA 200 mg/kg, $ - $p<0.05$, ETX 200 mg/kg vs VPA 200 mg/kg.

In conclusion, acute i.p. injection of ETX (200 mg/kg) and VPA (200 mg/kg) decreased the severity of SWDs in GAERS rats. Moreover, ETX induced a longer-lasting effect, which lasted during all three hours of recording, while VPA induced an effect that lasted during the first 80 min after injection.

Ethosuximide and valproate affect anxiety-like behavior in GAERS, NEC and Wistar rats

GAERS rats, and two control strains, NEC (Non Epileptic Control) and Wistar rats (original strain from which both GAERS and NEC originate from), were tested in the hole board (HB) test, 40 minutes after receiving an i.p. injection of anti-absence drugs, ETX 200 mg/kg, VPA 200 mg/kg or their respective vehicle (n=12 per group; except Wistar-VPA, n= 13). Their behavior was annotated based on the ethogram showed in the methods section (Hole Board – Data Analysis) and quantitative analysis was performed to investigate the effect of the anti-absence drugs on anxiety-like behavior in the HB test.

For the frequency of the focused hole exploration behavior, Head Dip, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 4.943; p= 0.009) and no significant difference between the strains (strain $F_{2,100}$= 1.034; p= 0.3595). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 6.622; p< 0.0001) (**Figure 13a** *left*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced a decrease in the Head Dip frequency (Wistar: vehicle vs ETX 200 mg/kg, p< 0.0001), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p= 0.0014). In NEC rats, VPA treatment induced a significant increase in the Head Dip frequency (NEC: vehicle vs VPA 200 mg/kg, p= 0.0222). In GAERS rats, ETX and VPA treatments did not induce any significant changes in the Head Dip frequency. Moreover, for the Head Dip frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC, between Wistar and GAERS and between NEC and GAERS (saline: Wistar vs NEC, p< 0.0001; Wistar vs GAERS, p= 0.0274; NEC vs GAERS, p= 0.0274). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.3347; Wistar vs GAERS, p=

0.3855; NEC vs GAERS, p= 0.7694). Also, in the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.315; Wistar vs GAERS, p= 0.315; NEC vs GAERS, p= 0.8767) (**Figure 13a** *left*).

For the frequency of the general vertical exploration behavior, Rearing, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 4.1; p= 0.0194) and a significant difference between the strains (strain $F_{2,100}$= 8.499; p= 0.0004). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 3.763; p= 0.0068) (**Figure 13a** *middle*). Holm-Sidak's multiple comparison test showed that in Wistar and NEC rats, ETX and VPA treatments did not induce any significant changes in the Rearing frequency. For GAERS rats VPA treatment induced an increase in the Rearing frequency (GAERS: vehicle vs VPA 200 mg/kg, p= 0.0064) and this increase was also significantly higher in comparison to the ETX group (GAERS: ETX 200 mg/kg vs VPA 200 mg/kg, p= 0.0003). Moreover, for the Rearing frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.2253; Wistar vs GAERS, p= 0.6672; NEC vs GAERS, p= 0.3393). In the ETX 200 mg/kg treatment group there were significant differences between Wistar and NEC rats and between NEC and GAERS rats (ETX: Wistar vs NEC, p= 0.0006; Wistar vs GAERS, p= 0.2368; NEC vs GAERS, p= 0.0183). In the valproate (VPA) 200 mg/kg treatment group there were significant differences between Wistar and GAERS rats and between NEC and GAERS rats (VPA: Wistar vs NEC, p= 0.2313; Wistar vs GAERS, p= 0.0015; NEC vs GAERS, p= 0.0415) (**Figure 13a** *middle*).

For the frequency of the grooming behavior, Face Grooming, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 4.244; p= 0.017) and there was no significant difference between the strains (strain $F_{2,100}$= 9.828; p= 0.0001). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 1.701; p= 0.1557) (**Figure 13a** *right*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX treatment induced a decrease in the Face Grooming frequency (Wistar: vehicle vs ETX 200 mg/kg, p= 0.0013), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p= 0.0152). In NEC and GAERS rats, ETX and VPA treatment did not induce any significant changes in Face Grooming frequency. Moreover, for the Face Grooming frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and between Wistar and GAERS (saline: Wistar vs NEC, p= 0.0004; Wistar vs GAERS, p= 0.0003; NEC vs GAERS, p= 0.8209). In the ETX treatment group there were no significant difference the three strains (ETX: Wistar vs NEC, p= 0.5982; Wistar vs GAERS, p= 0.5934; NEC vs GAERS, p= 0.8209). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.4054; Wistar vs GAERS, p= 0.1685; NEC vs GAERS, p= 0.4975) (**Figure 13a** *right*).

For the duration of the general horizontal exploration behavior, Immobile Sniffing, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 40.23; p< 0.0001) and a significant difference between the strains (strain $F_{2,100}$= 5.167; p= 0.0073). The interaction between the treatment and the strain was also significant (treatment x strain interaction $F_{4,100}$= 10.71; p< 0.0001) (**Figure 13b** *left*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced an increase in the

Immobile Sniffing duration (Wistar: vehicle vs ETX 200 mg/kg, $p< 0.0001$), and this increase was significantly higher also in comparison to the VPA treatment group (Wistar: ETX 200 mg/kg vs VPA 200 mg/kg, $p< 0.0001$). In NEC rats, ETX induced a decrease in the Immobile Sniffing duration (NEC: vehicle vs ETX 200 mg/kg, $p= 0.0124$), as well as VPA treatment (NEC: vehicle vs VPA 200 mg/kg, $p< 0.0001$). Also, the decrease in the Immobile Sniffing duration in NEC, induced by VPA, was significantly lower in comparison to the ETX group (NEC: ETX 200 mg/kg vs VPA 200 mg/kg, $p= 0.0038$). In GAERS rats VPA treatment induced a decrease in the Immobile Sniffing duration (GAERS: vehicle vs VPA 200 mg/kg, $p< 0.0001$) and this decrease was also significant in comparison to the ETX group (GAERS: ETX 200 mg/kg vs VPA 200 mg/kg, $p< 0.0001$). Moreover, for the Immobile Sniffing duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and Wistar and GAERS (saline: Wistar vs NEC, $p< 0.0001$; Wistar vs GAERS, $p< 0.0001$; NEC vs GAERS, $p= 0.4139$). In the ETX treatment group there were significant differences between the Wistar and NEC rats and between NEC and GAERS rats (ETX: Wistar vs NEC, $p= 0.0162$; Wistar vs GAERS, $p= 0.5943$; NEC vs GAERS, $p= 0.0456$). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, $p= 0.8611$; Wistar vs GAERS, $p= 0.8607$; NEC vs GAERS, $p= 0.8611$) (**Figure 13b***left*).

For the duration of the general horizontal exploration behavior, Walking, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}= 15.65$; $p< 0.0001$) and a significant difference between the strains (strain $F_{2,100}= 7.164$; $p= 0.0012$). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}= 10.76$; $p< 0.0001$) (**Figure 13b** *middle*). Holm-Sidak's multiple comparison

test showed that in Wistar rats there was no significant effect of the ETX or VPA treatment. In NEC rats ETX treatment induced an increase in the Walking duration (NEC: vehicle vs ETX 200 mg/kg, $p< 0.0001$), as well as VPA treatment (NEC: vehicle vs VPA 200 mg/kg, $p= 0.0054$). Moreover, the increase in Walking duration induced by ETX was significantly higher than the increase induced by VPA in NEC rats (NEC: ETX 200 mg/kg vs VPA 200 mg/kg, $p= 0.0001$). In addition, for GAERS rats ETX treatment induced an increase in the Walking duration (GAERS: vehicle vs ETX 200 mg/kg, $p= 0.0006$) and VPA treatment induced an increase as well (GAERS: vehicle vs VPA 200 mg/kg, $p= 0.0003$). Moreover, for the Waking duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, $p= 0.2550$; Wistar vs GAERS, $p= 0.1367$; NEC vs GAERS, $p= 0.6154$). In the ETX treatment group there were significant differences between all three strains (ETX: Wistar vs NEC, $p< 0.0001$; Wistar vs GAERS, $p= 0.0011$; NEC vs GAERS, $p= 0.0007$). In the VPA treatment group there were no significant differences between the strains (VPA: Wistar vs NEC, $p= 0.5644$; Wistar vs GAERS, $p= 0.2504$; NEC vs GAERS, $p= 0.5644$) (**Figure 13b** *middle*).

For the duration of Immobility, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}= 13.77$; $p< 0.0001$) and no significant difference between the strains (strain $F_{2,100}= 2.233$; $p= 0.1126$). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}= 1.12$; $p= 0.3513$) (**Figure 13b** *right*). Holm-Sidak's multiple comparison test showed that in Wistar rats, VPA treatment induced an increase in the Immobility duration in comparison to the vehicle group (Wistar: vehicle vs VPA 200 mg/kg, $p< 0.0001$) and this increase was also significantly higher in comparison to the ETX group (Wistar: ETX 200 mg/kg vs VPA 200 mg/kg, $p=$

0.0217). Similarly, in NEC rats, VPA treatment induced an increase in the Immobility duration in comparison to the vehicle group (NEC: vehicle vs VPA 200 mg/kg, p= 0.0172) and this increase was also significantly higher in comparison to the ETX group (NEC: ETX 200 mg/kg vs VPA 200 mg/kg, p= 0.0422). In GAERS rats, ETX and VPA treatment did not induce any significant changes in the Immobility duration. Moreover, for the Immobility duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.6653; Wistar vs GAERS, p= 0.8356; NEC vs GAERS, p= 0.8356). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.7244; Wistar vs GAERS, p= 0.5435; NEC vs GAERS, p= 0.6341). In the VPA treatment group there was a significant difference between Wistar and GAERS rats (VPA: Wistar vs NEC, p= 0.5702; Wistar vs GAERS, p= 0.0487; NEC vs GAERS, p= 0.1348) (**Figure 13b** *right*).

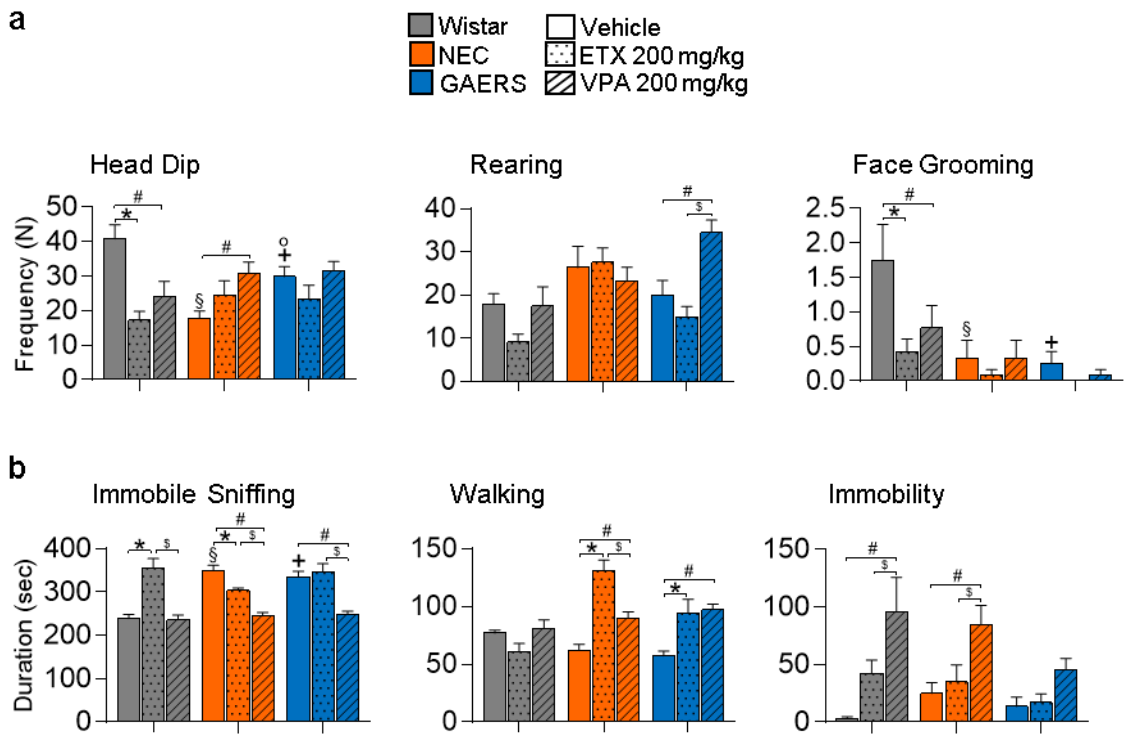

Figure 13. Ethosuximide and valproate affect anxiety-like behavior in GAERS, NEC and Wistar rats. Wistar (*grey*), NEC (*orange*) and GAERS rats (*blue*) were treated with an acute i.p. injection of vehicle (*no fill*), ETX 200 mg/kg (*dotted fill*) and VPA 200 mg/kg (*dashed fill*); 12 rats per group (except Wistar VPA, n= 13). (**a**) Frequency of Head Dip (*left*), Rearing (*middle*) and Face Grooming (*right*). (**b**) Duration of Immobile Sniffing (*left*), Walking (*middle*) and Immobility (*right*). Data are expressed as Mean ± SEM. Two-way ANOVA (treatment x strain interaction) was performed. * - $p<0.05$, vehicle vs ETX 200 mg/kg. # - $p<0.05$, vehicle vs VPA 200 mg/kg. $ - $p<0.05$, ETX 200 mg/kg vs VPA 200 mg/kg. + - $p<0.05$, Wistar vehicle vs GAERS vehicle. ° - $p<0.05$, NEC vehicle vs GAERS vehicle. § - $p<0.05$, Wistar vehicle vs NEC vehicle.

For the duration of the focused hole exploration behavior, Head Dip, two-way ANOVA with treatment x strain as factors revealed no significant main effect of drug treatment (treatment $F_{2,100}= 1.914$; $p= 0.1528$) and no significant difference between the strains (strain $F_{2,100}= 1.484$; $p= 0.2318$). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}= 1.604$; $p= 0.1792$) (**Figure 14** *left*).

For the duration of the general vertical exploration behavior, Rearing, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment

F2,100= 9.822; p= 0.0001) and a significant difference between the strains (strain F2,100= 3.536; p= 0.0328). The interaction between the treatment and the strain was not significant (treatment x strain interaction F4,100= 2.441; p= 0.0517) (**Figure 14** *middle*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX and VPA treatments did not induce any significant changes in the Rearing duration. In NEC rats, ETX induced a decrease in the Rearing duration (NEC: vehicle vs ETX 200 mg/kg, p= 0.0157), as well as VPA treatment (NEC: vehicle vs VPA 200 mg/kg, p= 0.0371). In GAERS rats ETX treatment induced a decrease in the Rearing duration (GAERS: vehicle vs ETX 200 mg/kg, p= 0.017) and this decrease was also significant in comparison to the VPA group (GAERS: ETX 200 mg/kg vs VPA 200 mg/kg, p= 0.0003). Moreover, for the Rearing duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there was a significant difference between Wistar and NEC rats (saline: Wistar vs NEC, p= 0.0354; Wistar vs GAERS, p= 0.3530; NEC vs GAERS, p= 0.2021). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.3823; Wistar vs GAERS, p= 0.9290; NEC vs GAERS, p= 0.3823). In the VPA treatment group there was a significant difference between Wistar and GAERS rats (VPA: Wistar vs NEC, p= 0.6644; Wistar vs GAERS, p= 0.0337; NEC vs GAERS, p= 0.0746) (**Figure 14** *middle*).

For the duration of the grooming behavior, Face Grooming, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 7.317; p= 0.0011) and a significant difference between the strains (strain $F_{2,100}$= 10.27; p< 0.0001). The interaction between the treatment and the strain was also significant (treatment x strain interaction $F_{4,100}$= 6.673; p< 0.0001) (**Figure 14** *right*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX treatment induced a decrease in the Face Grooming duration

(Wistar: vehicle vs ETX 200 mg/kg, $p < 0.0001$), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, $p < 0.0001$). In NEC and GAERS rats, ETX and VPA treatment did not induce any significant changes in Face Grooming duration. Moreover, for the Face Grooming duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and between Wistar and GAERS (saline: Wistar vs NEC, $p < 0.0001$; Wistar vs GAERS, $p < 0.0001$; NEC vs GAERS, $p = 0.889$). In the ETX treatment group there were no significant difference between the three strains (ETX: Wistar vs NEC, $p = 0.9736$; Wistar vs GAERS, $p = 0.9736$; NEC vs GAERS, $p = 0.9736$). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, $p = 0.8851$; Wistar vs GAERS, $p = 0.8851$; NEC vs GAERS, $p = 0.8851$) (**Figure 14** *right*).

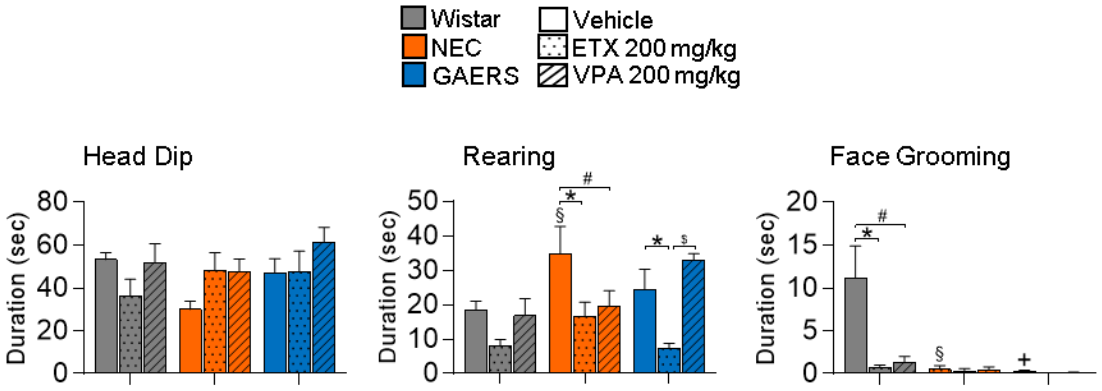

Figure 14. Ethosuximide and valproate affect anxiety-like behavior in GAERS, NEC and Wistar rats – Duration of Head Dip, Rearing and Face Grooming.
Wistar (*grey*), NEC (*orange*) and GAERS rats (*blue*) were treated with an acute i.p. injection of vehicle (*no fill*), ETX 200 mg/kg (*dotted fill*) and VPA 200 mg/kg (*dashed fill*); 12 rats per group (except Wistar VPA, n= 13). Duration of Head Dip (*left*), Rearing (*middle*) and Face Grooming (*right*). Data are expressed as Mean ± SEM. Two-way ANOVA (treatment x strain interaction) was performed. * - $p<0.05$, vehicle vs ETX 200 mg/kg. # - $p<0.05$, vehicle vs VPA 200 mg/kg. $ - $p<0.05$, ETX 200 mg/kg vs VPA 200 mg/kg. + - $p<0.05$, Wistar vehicle vs GAERS vehicle. ° - $p<0.05$, NEC vehicle vs GAERS vehicle. § - $p<0.05$, Wistar vehicle vs NEC vehicle.

For the frequency of the general horizontal exploration behavior, Immobile Sniffing, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}= 21.5$; $p< 0.0001$) and a significant difference between the strains (strain $F_{2,100}= 12.56$; $p< 0.0001$). The interaction between the treatment and the strain was also significant (treatment x strain interaction $F_{4,100}= 4.169$; $p= 0.0036$) (**Figure 15** *left*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced a decrease in the Immobile Sniffing frequency (Wistar: vehicle vs ETX 200 mg/kg, $p< 0.0001$), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, $p= 0.0015$). In NEC rats, ETX and VPA treatments did not induce any significant changes in the Immobile Sniffing frequency. In GAERS rats ETX treatment induced a decrease in the Immobile Sniffing frequency (GAERS: vehicle vs ETX 200 mg/kg, $p< 0.0001$) and this decrease was also significant in comparison to the VPA group (GAERS: ETX

200 mg/kg vs VPA 200 mg/kg, p= 0.0002). Moreover, for the Immobile Sniffing frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.6973; Wistar vs GAERS, p= 0.2766; NEC vs GAERS, p= 0.3775). In the ETX treatment group there were significant differences between the Wistar and NEC rats and between NEC and GAERS rats (ETX: Wistar vs NEC, p< 0.0001; Wistar vs GAERS, p= 0.1023; NEC vs GAERS, p= 0.0054). In the VPA treatment group there were significant differences between Wistar and NEC rats and between Wistar and GAERS rats (VPA: Wistar vs NEC, p= 0.0172; Wistar vs GAERS, p= 0.0005; NEC vs GAERS, p= 0.2308) (**Figure 15** *left*).

For the frequency of the general horizontal exploration behavior, Walking, two-way ANOVA with treatment x strain as factors revealed no significant main effect of drug treatment (treatment $F_{2,100}$= 0.7677; p= 0.4668). and a significant difference between the strains (strain $F_{2,100}$= 6.957; p= 0.0015). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 4.674; p< 0.0017) (**Figure 15** *middle*). Holm-Sidak's multiple comparison test showed that ETX treatment (ETX 200 mg/kg) induced a decrease in the Walking frequency in Wistar rats (Wistar: vehicle vs ETX 200 mg/kg, p= 0.0192) and an increase in NEC rats (NEC: vehicle vs ETX 200 mg/kg, p= 0.0342). In GAERS rats ETX (ETX 200 mg/kg) did not induce any significant changes in the Walking frequency. There was no significant effect of the valproate treatment (VPA 200 mg/kg) on Walking frequency in any of the three strains. Moreover, for the Walking frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.9909; Wistar vs GAERS, p= 0.9909; NEC vs GAERS, p= 0.9909). In the ETX treatment group there were significant differences between all three strains

(ETX: Wistar vs NEC, p< 0.0001; Wistar vs GAERS, p= 0.0170; NEC vs GAERS, p= 0.0179). In the VPA treatment group there were significant differences between Wistar and GAERS (VPA: Wistar vs NEC, p= 0.2810; Wistar vs GAERS, p= 0.0355; NEC vs GAERS, p= 0.2793) (**Figure 15** *middle*).

For the frequency of Immobility, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 4.725; p= 0.0109) and no significant difference between the strains (strain $F_{2,100}$= 1.081; p= 0.3433). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}$= 1.006; p= 0.4082) (**Figure 15** *right*). Holm-Sidak's multiple comparison test showed that ETX treatment did not induce any significant changes in Immobility frequency in any of the three strains. In Wistar strain, VPA treatment induced an increase in the Immobility frequency in comparison to the vehicle group (Wistar: vehicle vs VPA 200 mg/kg, p= 0.0295). Moreover, for the Immobility frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.1514; Wistar vs GAERS, p= 0.1514; NEC vs GAERS, p= 0.9017). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.9213; Wistar vs GAERS, p= 0.8672; NEC vs GAERS, p= 0.8672). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.7949; Wistar vs GAERS, p= 0.7949; NEC vs GAERS, p= 0.7711) (**Figure 15** *right*).

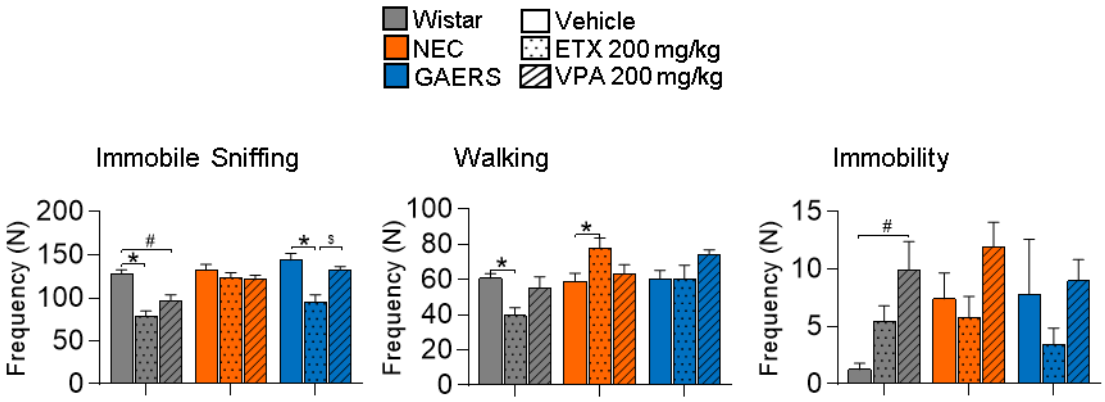

Figure 15. Ethosuximide and valproate affect anxiety-like behavior in GAERS, NEC and Wistar rats – Frequency of Immobile Sniffing, Walking and Immobility.
Wistar (*grey*), NEC (*orange*) and GAERS rats (*blue*) were treated with an acute i.p. injection of vehicle (*no fill*), ETX 200 mg/kg (*dotted fill*) and VPA 200 mg/kg (*dashed fill*); 12 rats per group (except Wistar VPA, n= 13). Frequency of Immobile Sniffing (*left*), Walking (*middle*) and Immobility (*right*). Data are expressed as Mean ± SEM. Two-way ANOVA (treatment x strain interaction) was performed. * - $p<0.05$, vehicle vs ETX 200 mg/kg. # - $p<0.05$, vehicle vs VPA 200 mg/kg. $ - $p<0.05$, ETX 200 mg/kg vs VPA 200 mg/kg. + - $p<0.05$, Wistar vehicle vs GAERS vehicle. ° - $p<0.05$, NEC vehicle vs GAERS vehicle. § - $p<0.05$, Wistar vehicle vs NEC vehicle.

For the frequency of the vertical exploration behavior, Climbing, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}= 41.75$; $p< 0.0001$). and no significant difference between the strains (strain $F_{2,100}= 2.894$; $p= 0.06$). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}= 2.973$; $p= 0.0229$) (**Figure 16a** *top*). Holm-Sidak's multiple comparison test showed that in Wistar rats ETX treatment induced a decrease in the Climbing frequency (Wistar: vehicle vs ETX 200 mg/kg, $p< 0.0001$), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, $p= 0.0002$). Moreover, the decrease in Climbing frequency induced by ETX was significantly lower than the decrease induced by VPA in Wistar rats (Wistar: ETX 200 mg/kg vs VPA 200 mg/kg, $p= 0.0044$). In NEC rats ETX treatment induced a decrease in the Climbing frequency (NEC: vehicle vs ETX 200 mg/kg, $p= 0.0006$). Moreover, this decrease in the

Climbing frequency induced by ETX was significantly lower in comparison to the VPA group in NEC rats (NEC: ETX 200 mg/kg vs VPA 200 mg/kg, p= 0.0006). In addition, for GAERS rats ETX treatment induced a decrease in the Climbing frequency (GAERS: vehicle vs ETX 200 mg/kg, p< 0.0001) and this decrease was also significantly lower in comparison to the VPA group (GAERS: ETX 200 mg/kg vs VPA 200 mg/kg, p< 0.0001). Moreover, for the Climbing frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.6525; Wistar vs GAERS, p= 0.6525; NEC vs GAERS, p= 0.9836). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.1395; Wistar vs GAERS, p= 0.4665; NEC vs GAERS, p= 0.4665). In the VPA treatment group there were significant differences between the Wistar strain in comparison to both NEC and GAERS (VPA: Wistar vs NEC, p= 0.0085; Wistar vs GAERS, p= 0.0085; NEC vs GAERS, p= 0.9836) (**Figure 16a** *top*).

For the duration of the vertical exploration behavior, Climbing, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 8.301; p= 0.0005). and no significant difference between the strains (strain $F_{2,100}$= 1.315; p= 0.2731). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}$= 2.36; p= 0.0584) (**Figure 16a** *bottom*). Holm-Sidak's multiple comparison test showed that in Wistar rats ETX treatment induced a decrease in the Climbing duration (Wistar: vehicle vs ETX 200 mg/kg, p< 0.0001), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p= 0.0184). In NEC and GAERS rats, ETX and VPA treatments did not induce any significant changes in the Climbing duration. Moreover, for the Climbing duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no

significant differences between the three strains (saline: Wistar vs NEC, p= 0.1206; Wistar vs GAERS, p= 0.4241; NEC vs GAERS, p= 0.4241). Also, in the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.3543; Wistar vs GAERS, p= 0.0617; NEC vs GAERS, p= 0.2950). Finally, in the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.4584; Wistar vs GAERS, p= 0.4584; NEC vs GAERS, p= 0.9869) (**Figure 16a** *bottom*).

For the frequency of the focused hole exploration, Edge Sniff, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 22.09; p< 0.0001) and a significant difference between the strains (strain $F_{2,100}$= 44.73; p< 0.0001). The interaction between the treatment and the strain was also significant (treatment x strain interaction $F_{4,100}$= 28.42; p< 0.0001) (**Figure 16b** *top*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced a decrease in the Edge Sniff frequency (Wistar: vehicle vs ETX 200 mg/kg, p< 0.0001), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p< 0.0001). In NEC and GAERS rats, ETX and VPA treatments did not induce any significant changes. Moreover, for the Edge Sniff frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and Wistar and GAERS (saline: Wistar vs NEC, p< 0.0001; Wistar vs GAERS, p< 0.0001; NEC vs GAERS, p= 0.4896). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.7425; Wistar vs GAERS, p= 0.6107; NEC vs GAERS, p= 0.6832). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.5330; Wistar vs GAERS, p= 0.4088; NEC vs GAERS, p= 0.6909) (**Figure 16b** *top*).

For the duration of the focused hole exploration, Edge Sniff, two-way ANOVA with treatment x strain as factors revealed a significant main effect of drug treatment (treatment $F_{2,100}$= 6.513; p= 0.0022) and a significant difference between the strains (strain $F_{2,100}$= 48.59; p< 0.0001). The interaction between the treatment and the strain was also significant (treatment x strain interaction $F_{4,100}$= 11.61; p< 0.0001) (**Figure 16b** *bottom*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced a decrease in the Edge Sniff duration (Wistar: vehicle vs ETX 200 mg/kg, p< 0.0001), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p< 0.0001). In NEC and GAERS rats, ETX and VPA treatments did not induce any significant changes. Moreover, for the Edge Sniff duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and Wistar and GAERS (saline: Wistar vs NEC, p< 0.0001; Wistar vs GAERS, p< 0.0001; NEC vs GAERS, p= 0.1927). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.0502; Wistar vs GAERS, p= 0.0502; NEC vs GAERS, p= 0.9602). In the VPA treatment group there were significant differences between Wistar and NEC and Wistar and GAERS (VPA: Wistar vs NEC, p= 0.0126; Wistar vs GAERS, p= 0.047; NEC vs GAERS, p= 0.5359) (**Figure 16b** *bottom*).

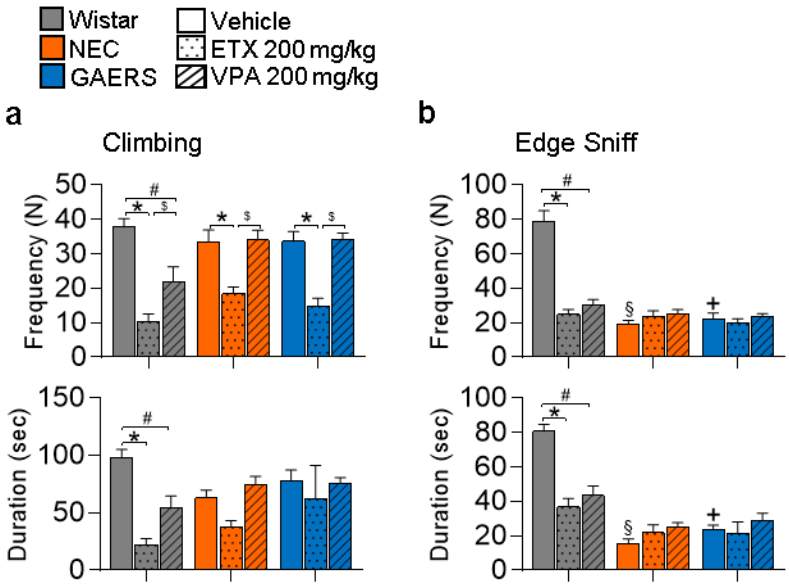

Figure 16. Ethosuximide and valproate affect anxiety-like behavior in GAERS, NEC and Wistar rats – Frequency and Duration of Climbing and Edge Sniff.
Wistar (*grey*), NEC (*orange*) and GAERS rats (*blue*) were treated with an acute i.p. injection of vehicle (*no fill*), ETX 200 mg/kg (*dotted fill*) and VPA 200 mg/kg (*dashed fill*); 12 rats per group (except Wistar VPA, n= 13). (**a**) Frequency (*top*) and duration (*bottom*) of Climbing. (**b**) Frequency (*top*) and duration (*bottom*) of Edge Sniff. Data are expressed as Mean ± SEM. Two-way ANOVA (treatment x strain interaction) was performed. * - $p<0.05$, vehicle vs ETX 200 mg/kg. # - $p<0.05$, vehicle vs VPA 200 mg/kg. $ - $p<0.05$, ETX 200 mg/kg vs VPA 200 mg/kg. + - $p<0.05$, Wistar vehicle vs GAERS vehicle. ° - $p<0.05$, NEC vehicle vs GAERS vehicle. § - $p<0.05$, Wistar vehicle vs NEC vehicle.

For the frequency of the grooming behavior, Body Grooming, two-way ANOVA with treatment x strain as factors revealed no significant main effect of drug treatment (treatment $F_{2,100}= 1.657$; $p= 0.196$) and a significant difference between the strains (strain $F_{2,100}= 8.104$; $p= 0.0005$). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}= 0.8884$; $p= 0.4738$) (**Figure 17a** *top*). Holm-Sidak's multiple comparison test showed that ETX and VPA treatment did not induce any significant changes in Body Grooming frequency in any of the three strains. Moreover, for the Body Grooming frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences

between the three strains (saline: Wistar vs NEC, p= 0.7221; Wistar vs GAERS, p= 0.581; NEC vs GAERS, p= 0.7221). In the ETX treatment group there were no significant differences between the three strains (ETX: Wistar vs NEC, p= 0.2187; Wistar vs GAERS, p= 0.0959; NEC vs GAERS, p= 0.5656). In the VPA treatment group there were significant differences between Wistar and NEC rats and between Wistar and GAERS rats (VPA: Wistar vs NEC, p= 0.0138; Wistar vs GAERS, p= 0.0021; NEC vs GAERS, p= 0.4729) (**Figure 17a** *top*).

For the duration of the grooming behavior, Body Grooming, two-way ANOVA with treatment x strain as factors revealed no significant main effect of drug treatment (treatment $F_{2,100}$= 0.5655; p= 0.5699) and a significant difference between the strains (strain $F_{2,100}$= 4.049; p= 0.0204). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}$= 0.7425; p= 0.5653) (**Figure 17a** *bottom*). Holm-Sidak's multiple comparison test showed that ETX and VPA treatment did not induce any significant changes in Body Grooming duration in any of the three strains. Moreover, for the Body Grooming duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.8829; Wistar vs GAERS, p= 0.8829; NEC vs GAERS, p= 0.9295). In the ETX treatment group there was a significant difference between Wistar and GAERS rats (ETX: Wistar vs NEC, p= 0.0566; Wistar vs GAERS, p= 0.0345; NEC vs GAERS, p= 0.7263). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.5894; Wistar vs GAERS, p= 0.2862; NEC vs GAERS, p= 0.4937) (**Figure 17a** *bottom*).

For the frequency of the grooming behavior, Front Paw Licking, two-way ANOVA with treatment x strain as factors revealed that the main effect of drug treatment was not significant (treatment $F_{2,100}$= 1.676; p= 0.1923) and there was no significant difference between the strains

(strain $F_{2,100}$= 0.6858; p= 0.5061). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 8.55; p< 0.0001) (**Figure 17b** *top*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced an increase in the Front Paw Licking frequency (Wistar: vehicle vs ETX 200 mg/kg, p= 0.0041), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p= 0.0016). In NEC rats, ETX treatment induced a significant decrease in the Front Paw Licking frequency (NEC: vehicle vs ETX 200 mg/kg, p= 0.01), as well as VPA treatment (NEC: vehicle vs VPA 200 mg/kg, p= 0.0167). And finally, in GAERS rats, ETX treatment induced a significant decrease in the Front Paw Licking frequency (GAERS: vehicle vs ETX 200 mg/kg, p= 0.0037). Moreover, for the Front Paw Licking frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and Wistar and GAERS (saline: Wistar vs NEC, p= 0.0002; Wistar vs GAERS, p= 0.0015; NEC vs GAERS, p= 0.5283). In the ETX treatment group there was a significant difference between Wistar and GAERS rats (ETX: Wistar vs NEC, p= 0.0828; Wistar vs GAERS, p= 0.01; NEC vs GAERS, p= 0.3447). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.0717; Wistar vs GAERS, p= 0.0717; NEC vs GAERS, p= 0.8746) (**Figure 17b** *top*).

For the duration of the grooming behavior, Front Paw Licking, two-way ANOVA with treatment x strain as factors revealed that the main effect of drug treatment was not significant (treatment $F_{2,100}$= 0.4286; p= 0.6526) and there was no significant difference between the strains (strain $F_{2,100}$= 2.922; p= 0.0584). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 8.677; p< 0.0001) (**Figure 17b** *bottom*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX induced an increase in the Front Paw

Licking duration (Wistar: vehicle vs ETX 200 mg/kg, p< 0.0001), as well as VPA treatment (Wistar: vehicle vs VPA 200 mg/kg, p= 0.0255). The increase in Front Paw Licking duration in Wistar rats induced by ETX was also significantly higher in comparison to the VPA group (Wistar: ETX 200 mg/kg vs VPA 200 mg/kg, p= 0.0255). In NEC rats, ETX and VPA treatment did not induce any significant changes in Front Paw Licking duration. And finally, in GAERS rats, ETX treatment induced a significant decrease in the Front Paw Licking duration (GAERS: vehicle vs ETX 200 mg/kg, p= 0.023). Moreover, for the Front Paw Licking duration, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were significant differences between Wistar and NEC and between Wistar and GAERS (saline: Wistar vs NEC, p= 0.0355; Wistar vs GAERS, p= 0.0221; NEC vs GAERS, p= 0.7458). In the ETX treatment group there was a significant difference between Wistar and NEC and between Wistar and GAERS rats (ETX: Wistar vs NEC, p< 0.0001; Wistar vs GAERS, p< 0.0001; NEC vs GAERS, p= 0.6718). In the VPA treatment group there were no significant differences between the three strains (VPA: Wistar vs NEC, p= 0.2568; Wistar vs GAERS, p= 0.2568; NEC vs GAERS, p= 0.8681) (**Figure 17b** *bottom*).

For the frequency of the grooming behavior, Hind Paw Licking, two-way ANOVA with treatment x strain as factors revealed that the main effect of drug treatment was not significant (treatment $F_{2,100}$= 1.687; p= 0.1904) and there was no significant difference between the strains (strain $F_{2,100}$= 1.663; p= 0.1948). The interaction between the treatment and the strain was significant (treatment x strain interaction $F_{4,100}$= 3.013; p= 0.0216) (**Figure 17c** *top*). Holm-Sidak's multiple comparison test showed that, in Wistar rats, ETX and VPA treatment did not induce any significant changes in the Hind Paw Licking frequency. In NEC rats, VPA treatment induced a significant increase in the Hind Paw Licking frequency (NEC: vehicle vs VPA 200

mg/kg, p= 0.0067). This increase was also significant in comparison to ETX treatment (NEC: ETX 200 mg/kg vs VPA 200 mg/kg, p= 0.0067). Finally, in GAERS rats, ETX and VPA treatment did not induce any significant changes in Hind Paw Licking frequency. Moreover, for Hind Paw Licking frequency, Holm-Sidak's multiple comparison test showed that in the saline treatment group there were no significant differences between the three strains (saline: Wistar vs NEC, p= 0.5115; Wistar vs GAERS, p= 0.5115; NEC vs GAERS, p> 0.9999). In the ETX treatment group there were no significant difference the three strains (ETX: Wistar vs NEC, p> 0.9999; Wistar vs GAERS, p> 0.9999; NEC vs GAERS, p> 0.9999). In the VPA treatment group there were significant differences between Wistar and NEC and between NEC and GAERS (VPA: Wistar vs NEC, p= 0.0055; Wistar vs GAERS, p> 0.9999; NEC vs GAERS, p= 0.0055) (**Figure 17c** *top*).

For the duration of the grooming behavior, Hind Paw Licking, two-way ANOVA with treatment x strain as factors revealed no significant main effect of drug treatment (treatment $F_{2,100}$= 2.114; p= 0.1261) and no significant difference between the strains (strain $F_{2,100}$= 2.086; p= 0.1296). The interaction between the treatment and the strain was not significant (treatment x strain interaction $F_{4,100}$= 2.26; p= 0.068) (**Figure 17c** *bottom*).

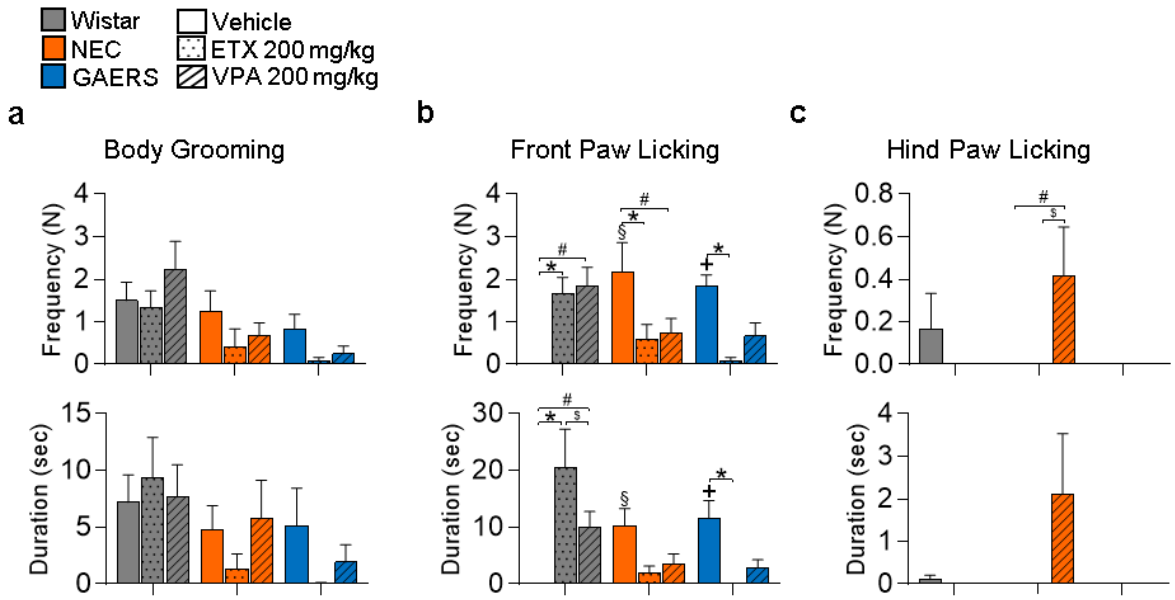

Figure 17. Ethosuximide and valproate affect anxiety-like behavior in GAERS, NEC and Wistar rats – Frequency and Duration of Body Grooming, Front Paw Licking and Hind Paw Licking.
Wistar (*grey*), NEC (*orange*) and GAERS rats (*blue*) were treated with an acute i.p. injection of vehicle (*no fill*), ETX 200 mg/kg (*dotted fill*) and VPA 200 mg/kg (*dashed fill*); 12 rats per group (except Wistar VPA, n= 13). (**a**) Frequency (*top*) and duration (*bottom*) of Body Grooming. (**b**) Frequency (*top*) and duration (*bottom*) of Front Paw Licking. (**c**) Frequency (*top*) and duration (*bottom*) of Hind Paw Licking. Data are expressed as Mean ± SEM. Two-way ANOVA (treatment x strain interaction) was performed. * - $p<0.05$, vehicle vs ETX 200 mg/kg. # - $p<0.05$, vehicle vs VPA 200 mg/kg. $ - $p<0.05$, ETX 200 mg/kg vs VPA 200 mg/kg. + - $p<0.05$, Wistar vehicle vs GAERS vehicle. ° - $p<0.05$, NEC vehicle vs GAERS vehicle. § - $p<0.05$, Wistar vehicle vs NEC vehicle.

Anxiety-like behavior in HB is in correlation with SWD severity in GAERS rats

GAERS rats, implanted with EEG electrodes, were tested in the HB, while their EEG was simultaneously recorded. From the total number of 55 GAERS rats recorded, 33 rats had SWDs during HB (60 %), while 22 rats (40 %) did not have SWDs during HB (**Figure 10a** *top*, **b**). Rats were divided into two groups based on the occurrence of SWDs during HB: GAERS rats with SWDs during HB ("HB-SWDs^{+}") and GAERS rats with no SWDs during HB ("HB-SWDs^{-}"). Between these two groups, the following HB parameters were compared: frequency and duration of Immobility and Walking (**Figure 10c**), frequency of Head-Dip and Edge-Sniff (**Figure 10d** *top*) and the ratio between Head-Dip and Edge-Sniff (**Figure 10d** *bottom*).

HB-SWDs^{+} rats were more immobile (Immobility frequency: p= 0.0129; Immobility duration: p= 0.0001; Mann-Whitney test) and were walking less (Walking frequency: p= 0.0107; Unpaired t test; Walking duration: p= 0.0377; Mann-Whitney test) than HB-SWDs^{-} rats (**Figure 10c**).

Analysis of hole exploration behaviors, Head-Dip and Edge-Sniff, revealed that HB-SWDs^{+} rats did fewer Head-Dips than HB-SWDs^{-} rats (Head-Dip frequency: p= 0.0137; Unpaired t test), while the Edge-Sniff frequency did not vary between the two groups (Edge-Sniff frequency: p= 0.4830; Unpaired t test) (**Figure 10d** *top*). However, when comparing the difference of the ratio between Head-Dip and Edge-Sniff, it was not significantly different between the two groups (Ratio: HD/ES, p= 0.1833; Mann-Whitney test) (**Figure 10d** *bottom*).

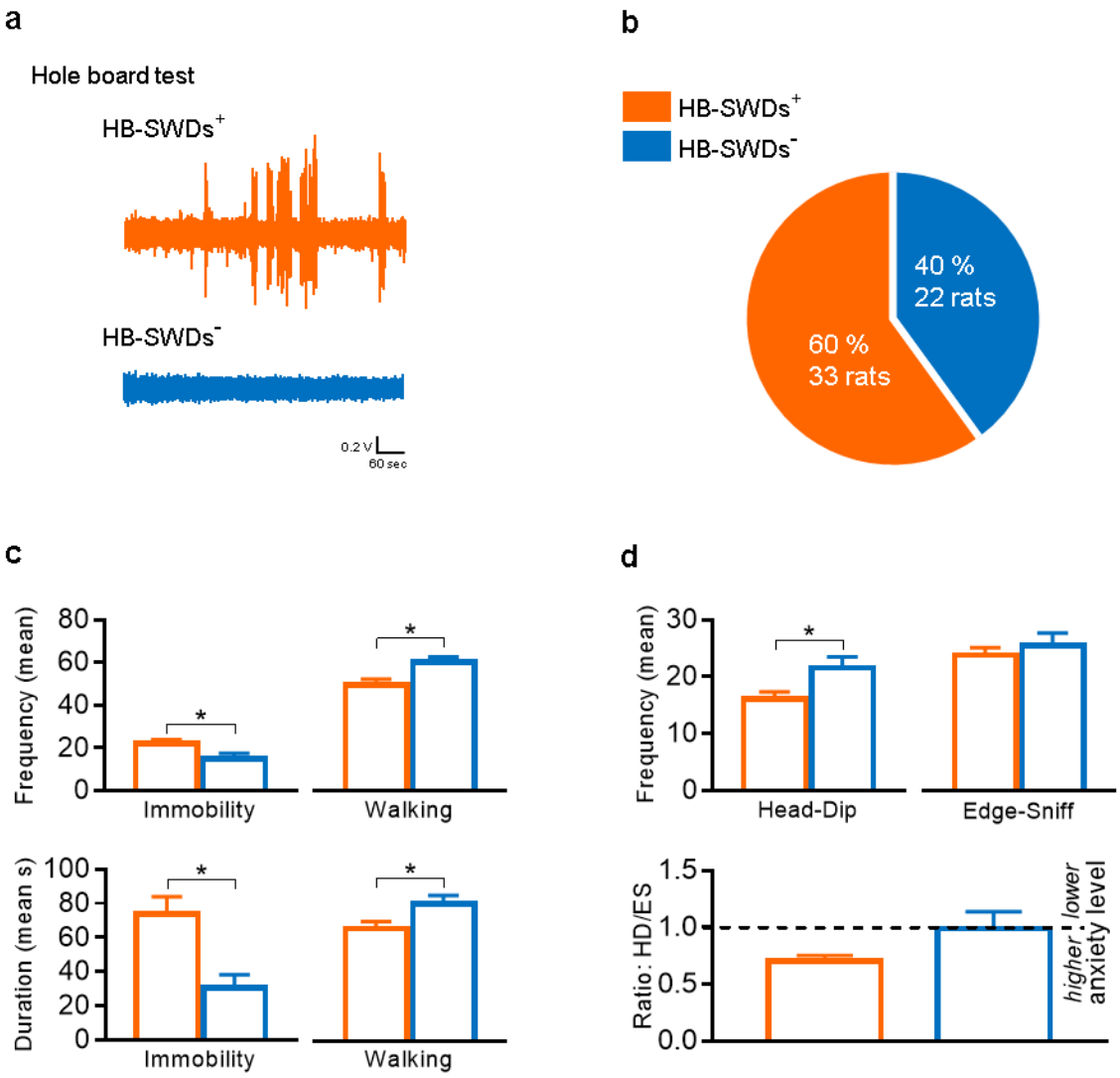

Figure 10. Anxiety-like behavior is aggravated by SWDs in GAERS rats.
(**a**) Example traces of EEG recorded in GAERS rats during 10 minutes of HB test. (**b**) Pie chart showing the percentage of GAERS rats with SWDs during HB (HB-SWDs^{+}, 60 %; 33 rats; *orange*) and GAERS rats with no SWDs during HB (HB-SWDs^{-}, 40 %; 22 rats; *blue*); total number of GAERS rats tested= 55. (**c**) Frequency (*top*; mean ± SEM) and duration (*bottom*; mean ± SEM), of Immobility and Walking of HB-SWDs^{+} rats (*orange*) and HB-SWDs^{-} rats (*blue*) (Mann-Whitney test for frequency and duration of Immobility and duration of Walking; Unpaired t test for frequency of Walking). (**d**, ***top***) Frequency (mean ± SEM) of Head-Dip and Edge-Sniff of HB-SWDs^{+} rats (*orange*) and HB-SWDs^{-} rats (*blue*); Unpaired t test was performed. (**d**, ***bottom***) Ratio: HD/ES (mean ± SEM) of HB-SWDs^{+} rats (*orange*) and HB-SWDs^{-} GAERS rats (*blue*); Mann Whitney test was performed. * - $p<0.05$, HB-SWDs^{+} vs HB-SWDs^{-}.

Further analysis was done for the 33 GAERS rats that showed SWDs during HB test (HB-SWDs^{+}), to investigate if there is a correlation between the annotated HB behaviors and SWD severity in GAERS rats. Head-Dip frequency, Edge-Sniff frequency, the HD/ES ratio, Immobility frequency and duration and Walking frequency and duration were correlated with the SWD parameters of EEG, namely time spent in seizures, number of seizures and average seizure length.

Spearman correlation between the Head-Dip frequency and time spent in seizures revealed a negative correlation (r= -0.3542, p= 0.0431; Spearman correlation) (**Figure 2.1a** *top*). The same negative correlation was found between HD/ES ratio and time spent in seizures (r= -0.377, p= 0.0306; Spearman correlation) (**Figure 2.1c** *top*). Edge Sniff frequency did not correlate with time spent in seizures (r= -0.00201, p= 0.9911; Spearman correlation) (**Figure 2.1b** *top*).

However, there were no correlations between the three HB parameters and number of seizures: Head-Dip frequency (r= -0.2213; p= 0.2159; Spearman correlation) (**Figure 2.1a** *middle*), Edge Sniff frequency (r= -0.0262; p= 0.8849; Spearman correlation) (**Figure 2.1b** *middle*); HD/ES ratio (r= -0.2289; p= 0.2; Spearman correlation) (**Figure 2.1c** *middle*).

Conversely, Head-Dip frequency negatively correlated with the average seizure length (r= -0.387; p= 0.0261; Spearman correlation) (**Figure 2.1a** *bottom*). However, there was no correlation between Edge Sniff frequency (r=-0.02529 ; p= 0.8889; Spearman correlation) (**Figure 2.1b** *bottom*) and HD/ES ratio (r= -0.3339; p= 0.0576; Spearman correlation) (**Figure 2.1c** *bottom*) with the average seizure length.

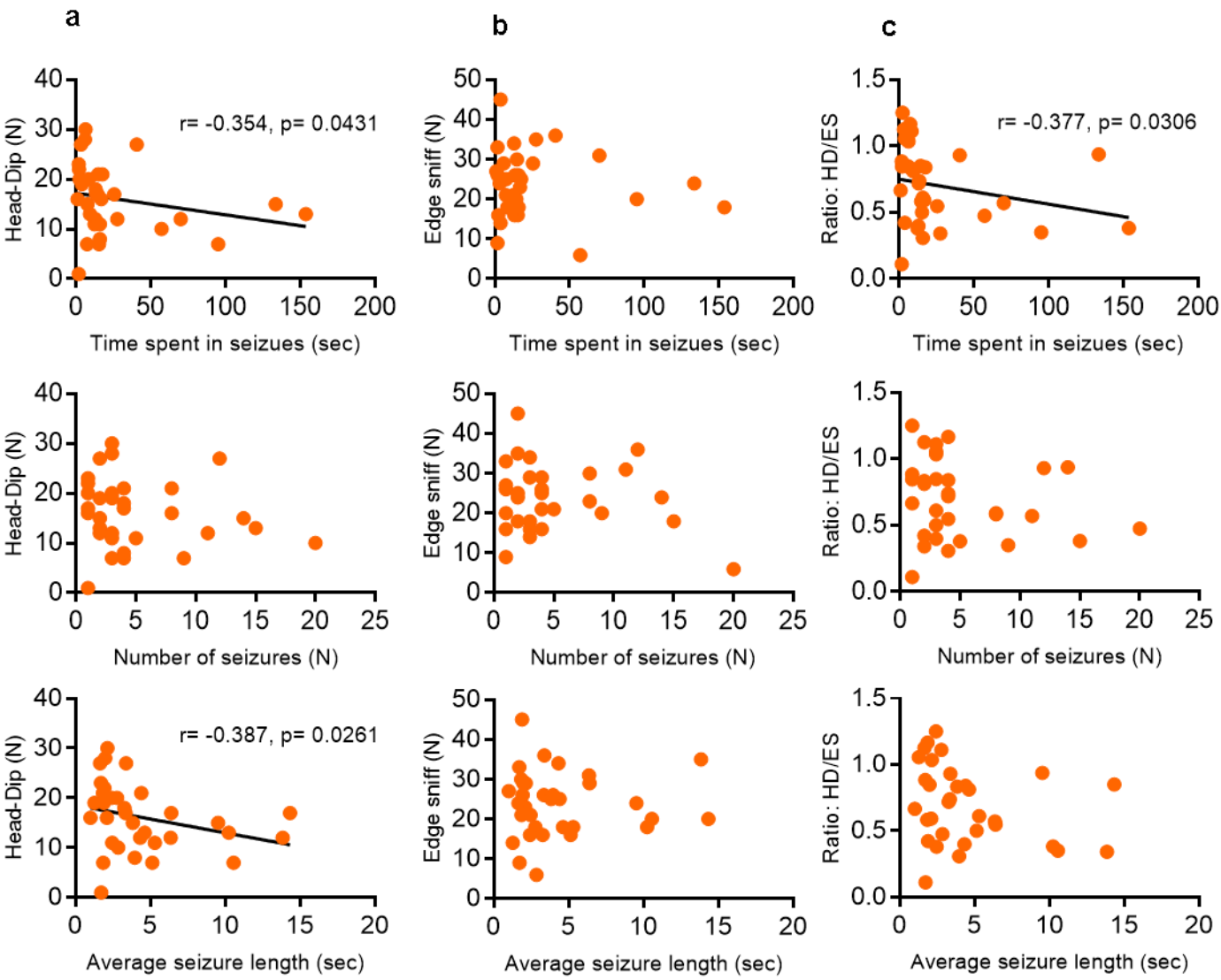

Figure 2.1. Hole exploration behaviors of HB are in correlation with SWD severity in GAERS rats. (a) Spearman correlations between the Head-Dip frequency and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). **(b)** Spearman correlations between the Edge Sniff frequency and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). **(c)** Spearman correlations between the HD/ES ratio and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). Significant Spearman's correlations are shown by the line and labeled by r-coefficients and p's.

Walking frequency did not correlate with time spent in seizures (r= 0.1627, p= 0.3656; Spearman correlation), number of seizures (r= 0.1381, p= 0.4435; Spearman correlation) and average seizure length (r= 0.07726, p= 0.6691; Spearman correlation) (**Figure 2.2a**). Similarly, Walking duration did not correlate with time spent in seizures (r= 0.1638, p= 0.3625; Spearman correlation), number of seizures (r= 0.1892, p=0.2917 ; Spearman correlation) and average seizure length (r= 0.1003, p=0.5788 ; Spearman correlation) (**Figure 2.2b**).

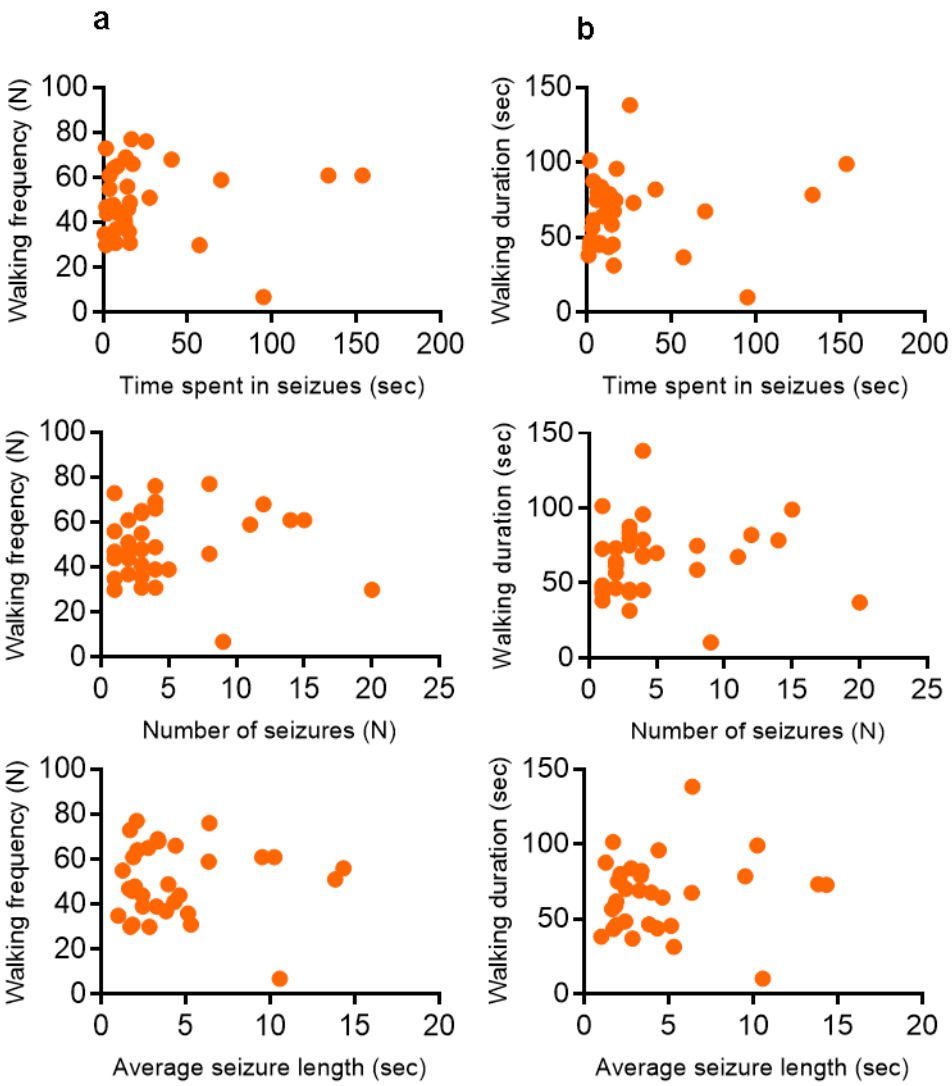

Figure 2.2. Walking frequency and duration in correlation with SWD severity in GAERS rats. (a) Spearman correlations between the Walking frequency and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). **(b)** Spearman correlations between the Walking duration and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). Significant Spearman's correlations are shown by the line and labeled by r-coefficients and p's.

Immobility frequency did not correlate with time spent in seizures (r= -0.002677, p= 0.9882; Spearman correlation), number of seizures (r= 0.1387, p= 0.4415; Spearman correlation) and average seizure length (r= -0.2018, p= 0.2601; Spearman correlation) (**Figure 2.3a**). Similarly, Immobility duration did not correlate with time spent in seizures (r= 0.2216, p= 0.2152; Spearman correlation), number of seizures (r= 0.3328, p= 0.0584; Spearman correlation) and average seizure length (r= 0.05816, p=0.7479; Spearman correlation) (**Figure 2.3b**).

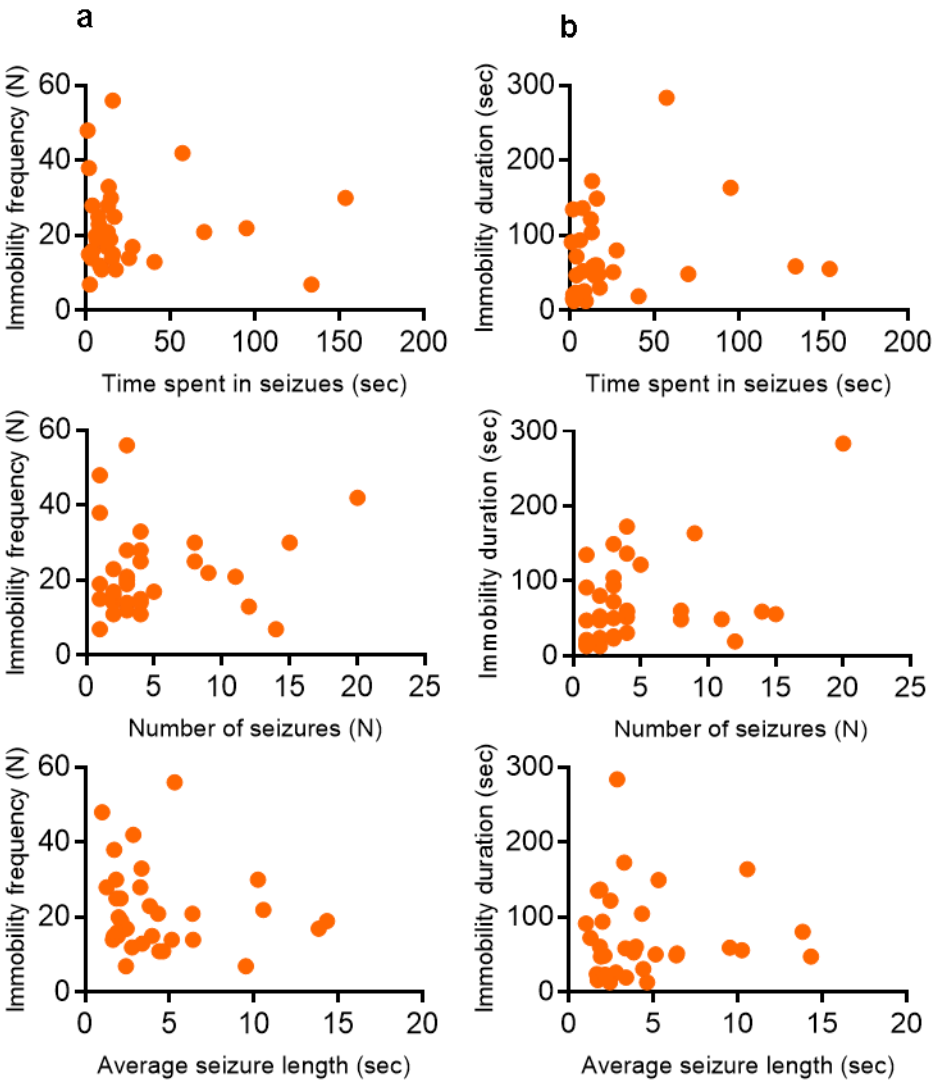

Figure 2.3. Immobility frequency and duration in correlation with SWD severity in GAERS rats. (a) Spearman correlations between the Immobility frequency and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). **(b)** Spearman correlations between the Immobility duration and time spent in seizures (*top*), number of seizures (*middle*) and average seizure length (*bottom*) in GAERS rats with SWDs during HB (n=33). Significant Spearman's correlations are shown by the line and labeled by r-coefficients and p's.

Acute CB1 receptor activation exacerbates SWDs in GAERS rats

GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system before and after acute i.p. injection of a synthetic CB1 receptor agonist WIN55,212-2 in three different doses (1 mg/kg: n=11; 2 mg/kg: n=9; 5 mg/kg: n=34) and the respective vehicle (n=33) (**Figure 18a**), to investigate the effect of the cannabinoid receptor activation on absence seizures in GAERS rats.

Two-way ANOVA RM with treatment x time as factors revealed no significant main effect of treatment WIN55,212-2 on time spent in seizures (treatment $F_{3,83}$= 2.291; p= 0.0843). The time spent in seizures varied significantly over time ($F_{11,913}$= 4.758; p<0.0001) and time-course of the seizures changed (treatment x time interaction $F_{33,913}$= 3.572; p< 0.0001) (**Figure 18b** *top*).

Two-way ANOVA RM showed a significant main effect of treatment WIN55,212-2 on number of seizures (**Figure 18b** *middle*) (treatment $F_{3,83}$ = 6.15; p= 0.0008; time $F_{11,913}$= 5.533; p< 0.0001; interaction $F_{33,913}$= 5.147, p= 0.0001).

Similarly, two-way ANOVA RM revealed a significant main effect of WIN55,212-2 on average seizure length (**Figure 18b** *bottom*) (treatment $F_{3,83}$ = 4.339; p= 0.0068; time $F_{11,913}$= 3.764; p< 0.0001; interaction $F_{33,913}$= 2.392, p< 0.0001).

Holm-Sidak multiple comparison test showed that the acute i.p. treatment of two lower doses of WIN55,212-2 (1 mg/kg and 2 mg/kg) used, did not induce any significant changes of time spent in seizures (**Figure 18b** *top*), number of seizures (**Figure 18b** *middle*) or average seizure length (**Figure 18b** *bottom*) in comparison to the vehicle during the three hours after injection.

In contrast, treatment with 5 mg/kg of WIN55,212-2 (highest dose used) induced a decrease of average seizure length, which lasted throughout the first hour after injection (20 min time bin:

WIN 5 mg/kg = 66 ± 8 %; vehicle vs WIN 5 mg/kg, p< 0.0001; 40 min time bin: WIN 5 mg/kg = 65 ± 6 %; vehicle vs WIN 5 mg/kg, p< 0.0001; 60 min time bin: WIN 5 mg/kg = 70 ± 6 %; vehicle vs WIN 5 mg/kg, p= 0.0001) and during first 20 min of the third hour after injection (140 min time bin: WIN 5 mg/kg = 81 ± 5 %; vehicle vs WIN 5 mg/kg, p= 0.0390) in comparison to the vehicle group (**Figure 18b** *bottom*).

Moreover, highest dose of WIN55,212-2 (5 mg/kg) induced a decrease of average seizure length that was significantly lower in comparison to 2 mg/kg (WIN 2 mg/kg = 95 ± 10 % vs WIN 5 mg/kg = 66 ± 8 %; p= 0.0236) and to 1 mg/kg of WIN55,212-2 (WIN 1 mg/kg = 96 ± 7 % vs WIN 5 mg/kg = 66 ± 8 %; p= 0.0102) in the 20 min time bin, and significantly lower in comparison to 2 mg/kg of WIN55,212-2 (WIN 2 mg/kg = 110 ± 13 % vs WIN 5 mg/kg = 81 ± 5 %; p= 0.039) in the 140 min time bin (**Figure 18b** *bottom*).

In addition, highest dose of WIN55,212-2 (5 mg/kg) induced a decrease of time spent in seizures in comparison to 2 mg/kg of WIN55,212-2 during the first 20 minutes after injection (20 min time bin: WIN 2 mg/kg = 163 ± 50 % vs WIN 5 mg/kg = 49 ± 15 %; p= 0.004) (**Figure 18b** *top*). Also, the same effect was seen for the number of seizures (20 min time bin: WIN 2 mg/kg = 162 ± 31 % vs WIN 5 mg/kg = 63 ± 10 %; p= 0.0164) (**Figure 18b** *middle*).

Starting from the second hour after injection, highest dose of WIN55,212-2 (5 mg/kg) induced a significant increase of the number of seizures in comparison to the vehicle group in all 20 min time bins until the end of the recording (80 min time bin: WIN 5 mg/kg = 166 ± 20 %; vehicle vs WIN 5 mg/kg, p= 0.0136; 100 min time bin: WIN 5 mg/kg = 190 ± 21 %; vehicle vs WIN 5 mg/kg, p= 0.0002; 140 min time bin: WIN 5 mg/kg = 243 ± 35 %; vehicle vs WIN 5 mg/kg, p< 0.0001 ; 160 min time bin: WIN 5 mg/kg = 214 ± 23 %; vehicle vs WIN 5 mg/kg, p< 0.0001; 180 min time bin: WIN 5 mg/kg = 269 ± 23 %; vehicle vs WIN 5 mg/kg, p< 0.0001), except the

120 min time bin, where the increase was not significant (120 min time bin: WIN 5 mg/kg = 151 ± 15 %; vehicle vs WIN 5 mg/kg, p= 0.1026) (**Figure 18a, 18b** *middle*).

Moreover, the increase of the number of seizures induced by the highest dose, was also significantly different from the two lower doses. The differences have been observed in the following time bins: 100 min time bin (WIN 2 mg/kg = 87 ± 20 % vs WIN 5 mg/kg = 190 ± 21 %; p= 0.0096), 140 min time bin (WIN 2 mg/kg = 157 ± 42 % vs WIN 5 mg/kg = 243 ± 35 %; p= 0.0392; WIN 1 mg/kg = 133 ± 30 % vs WIN 5 mg/kg = 243 ± 35 %; p= 0.0019), 180 min time bin (WIN 2 mg/kg = 113 ± 32 % vs WIN 5 mg/kg = 269 ± 38 %; p< 0.0001; WIN 1 mg/kg = 130 ± 26 % vs WIN 5 mg/kg = 269 ± 38 %; p< 0.0001).

During the second hour after injection, highest dose of WIN55,212-2 (5 mg/kg) induced a significant increase of time spent in seizures in comparison to the vehicle group in all 20 min time, starting from the 100 min time bin, until the end of the recording (100 min time bin: WIN 5 mg/kg = 163 ± 22 %; vehicle vs WIN 5 mg/kg, p= 0.0236; 140 min time bin: WIN 5 mg/kg = 188 ± 26 %; vehicle vs WIN 5 mg/kg, p= 0.0004; 160 min time bin: WIN 5 mg/kg = 178 ± 22 %; vehicle vs WIN 5 mg/kg, p= 0.0019; 180 min time bin: WIN 5 mg/kg = 236 ± 32 %; vehicle vs WIN 5 mg/kg, p< 0.0001), except for the 120 min time bin, where the increase was not significant (120 min time bin: WIN 5 mg/kg = 133 ± 18 %; vehicle vs WIN 5 mg/kg, p= 0.5605) (**Figure 18a, 18b** *top*).

Moreover, the increase of time spent in seizures induced by the highest dose, was also significantly different from the two lower doses during the last 20 min of recording (180 min time bin: WIN 2 mg/kg = 126 ± 43 % vs WIN 5 mg/kg = 236 ± 32 %; p= 0.0039; WIN 1 mg/kg = 113 ± 28 % vs WIN 5 mg/kg = 236 ± 32 %; p= 0.0003).

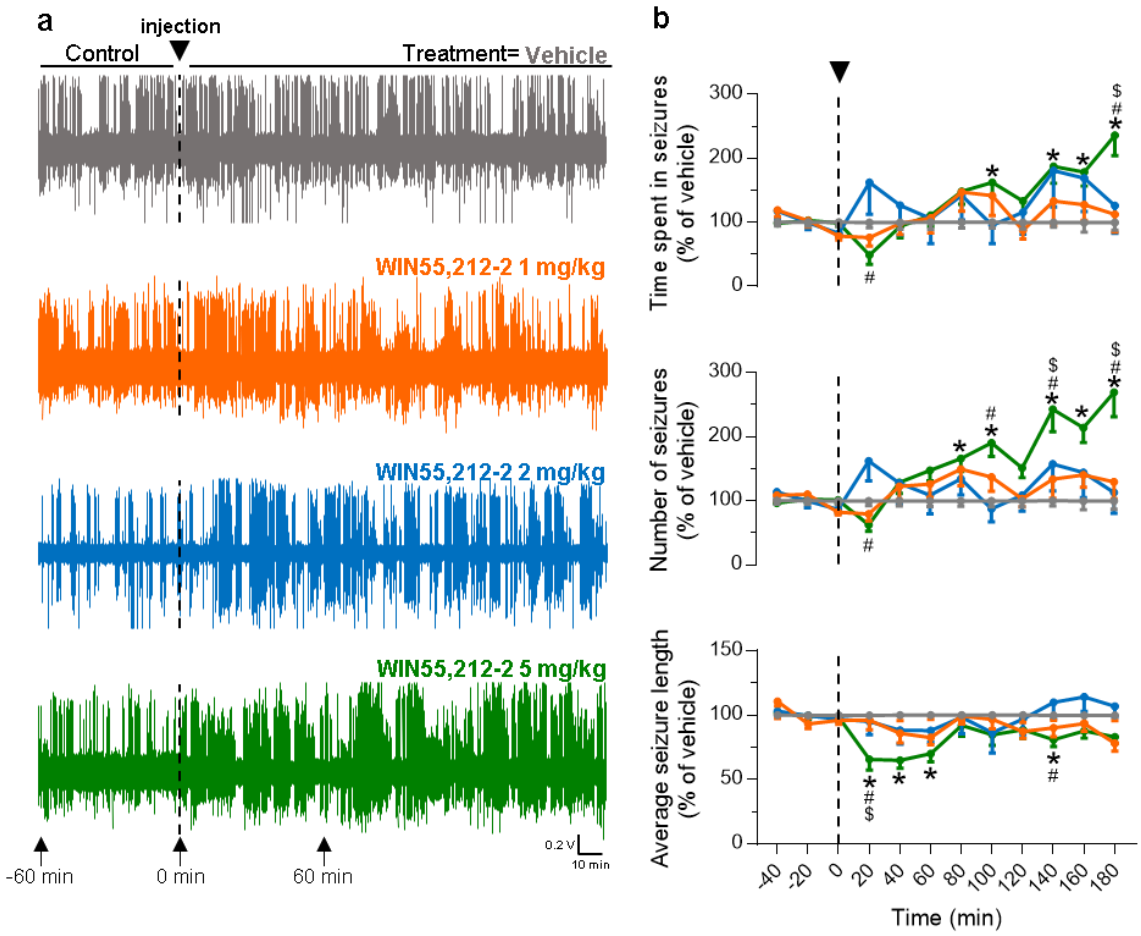

Figure 18. Acute CB1 receptor activation exacerbates SWDs in GAERS rats.
(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after acute i.p. injection (arrow/dashed line) of vehicle (grey), WIN55,212-2 at 1 mg/kg (orange), 2 mg/kg (blue) or 5 mg/kg (green). (**b**) Time course (mean ± SEM) of the time spent in seizures (top), number of seizures (middle) and length of a seizure (bottom) in vehicle (grey; n=33), WIN55,212-2 at 1 mg/kg (orange; n=11), 2 mg/kg (blue; n=9) or 5 mg/kg (green; n=34) treated GAERS before and after the injection time (dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. * - $p<0.05$, vehicle vs WIN55,212-2 5 mg/kg. # - $p<0.05$, WIN55,212-2 5 mg/kg vs WIN55,212-2 2 mg/kg, $ - $p<0.05$, WIN55,212-2 5 mg/kg vs WIN55,212-2 1 mg/kg.

In conclusion, acute i.p. injection of 5 mg/kg of the CB1 receptor agonist WIN55,212-2 in GAERS rats, induces a decrease of the average seizure length during the first hour after

injection. From the second hour after injection, the severity of seizures is aggravated, which is shown by the increase of time spent in seizures and the number of seizures.

Moreover, GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system before and after acute i.p. injection of CB1 receptor antagonist AM251 2 mg/kg (n=17) to investigate the effect of the CB1 receptor blockage on absence seizures in GAERS rats. Also, another group received an acute injection of AM251 2 mg/kg followed by an injection of WIN55,212-2 5 mg/kg after 15 minutes (n=6) (**Figure 19a**), to investigate if pretreatment with AM251 is able to prevent the seizure aggravation induced by CB-1 receptor agonist by WIN55,212-2 5 mg/kg.

Two-way ANOVA RM with treatment x time as factors revealed a significant main effect of drug treatment on time spent in seizures (treatment $F_{3,86}$= 3.908; p= 0.0114). The time spent in seizures varied significantly over time ($F_{11,946}$= 4.258; p<0.0001) and time-course of the seizures changed (treatment x time interaction $F_{33, 946}$= 3.937; p< 0.0001) (**Figure 19b** *top*).

Also, two-way ANOVA RM showed a significant main effect of drug treatment on number of seizures (**Figure 19b** *middle*) (treatment $F_{3,86}$= 8.128; p< 0.0001; time $F_{11,946}$= 5.018; p< 0.0001; interaction $F_{33, 946}$= 5.49, p< 0.0001).

Similarly, two-way ANOVA RM revealed an effect of drug treatment on average seizure length (**Figure 19b** *bottom*) (treatment $F_{3,86}$= 8.466; p< 0.0001; time $F_{11,946}$= 5.746; p< 0.0001; interaction $F_{33, 946}$= 2.775, p< 0.0001).

Holm-Sidak multiple comparison test showed that the acute i.p. treatment of CB-1 receptor antagonist AM251 (2 mg/kg), did not induce any significant changes of time spent in seizures

(**Figure 19b** *top*), number of seizures (**Figure 19b** *middle*) or average seizure length (**Figure 19b** *bottom*) in comparison to the vehicle during the three hours after injection.

As shown in the previous figure (**Figure 18**), acute i.p. treatment with 5 mg/kg of WIN55,212-2 (**Figure 19b**; *green, dashed line*) induced a decrease of average seizure length, which lasted throughout the first hour after injection and during first 20 min of the third hour after injection (**Figure 18b** *bottom* and **Figure 19b** *bottom*) in comparison to the vehicle group (**Figure 19b**; vehicle, dashed line). The observed average seizure length decrease induced by 5 mg/kg of WIN55,212-2 was also significant in comparison to AM251 2 mg/kg (20 min time bin: WIN 5 mg/kg = 66 ± 8 % vs AM 2 mg/kg = 103 ± 5 %; WIN 5 mg/kg vs AM 2 mg/kg, $p< 0.0001$; 40 min time bin: WIN 5 mg/kg = 65 ± 6 % vs AM 2 mg/kg = 101 ± 8 %; WIN 5 mg/kg vs AM 2 mg/kg, $p= 0.0002$; 60 min time bin: WIN 5 mg/kg = 70 ± 6 % vs AM 2 mg/kg = 102 ± 6 %; WIN 5 mg/kg vs AM 2 mg/kg, $p= 0.001$; 80 min time bin: WIN 5 mg/kg = 92 ± 8 % vs AM 2 mg/kg = 115 ± 6 %; WIN 5 mg/kg vs AM 2 mg/kg, $p= 0.036$; 140 min time bin: WIN 5 mg/kg = 81 ± 5 % vs AM 2 mg/kg = 103 ± 5 %; WIN 5 mg/kg vs AM 2 mg/kg, $p= 0.0491$) (**Figure 19b** *bottom*).

Moreover, as shown above (**Figure 18**), from the second hour after injection till the end of the recording, WIN55,212-2 5 mg/kg induced seizure aggravation, presenting as an increase of time spent in seizures (**Figure 18b** *top* and **Figure 19b** *top*) and number of seizures (**Figure 18b** *middle* and **Figure 19b** *middle*). The observed increase of time spent in seizures induced by WIN55,212-2 5 mg/kg was also significant in comparison to AM251 2 mg/kg during the last 40 minutes of recording (160 time bin: WIN 5 mg/kg = 178 ± 22 % vs AM 2 mg/kg = 82 ± 22 %; WIN 5 mg/kg vs AM 2 mg/kg, $p= 0.0007$; 180 time bin: WIN 5 mg/kg = 236 ± 32 % vs AM 2 mg/kg = 124 ± 19 %; WIN 5 mg/kg vs AM 2 mg/kg, $p< 0.0001$) (**Figure 19b** *top*). Also, the

observed increase of number of seizures induced by WIN55,212-2 5 mg/kg was significant in comparison to AM251 2 mg/kg as well (80 time bin: WIN 5 mg/kg = 166 ± 20 % vs AM 2 mg/kg = 89 ± 11 %; WIN 5 mg/kg vs AM 2 mg/kg, p= 0.0133; 120 time bin: WIN 5 mg/kg = 151 ± 15 % vs AM 2 mg/kg = 75 ± 15 %; WIN 5 mg/kg vs AM 2 mg/kg, p= 0.0189; 140 time bin: WIN 5 mg/kg = 243 ± 35 % vs AM 2 mg/kg = 150 ± 26 %; WIN 5 mg/kg vs AM 2 mg/kg, p= 0.0012; 160 time bin: WIN 5 mg/kg = 214 ± 23 % vs AM 2 mg/kg = 82 ± 20 %; WIN 5 mg/kg vs AM 2 mg/kg, p< 0.0001 ; 180 time bin: WIN 5 mg/kg = 269 ± 38 % vs AM 2 mg/kg = 108 ± 14 %; WIN 5 mg/kg vs AM 2 mg/kg, p< 0.0001) (**Figure 19b** *middle*).

Interestingly, pretreatment with CB-1 receptor antagonist AM-251 (2 mg/kg, i.p.), 15 minutes before an injection of CB-1 receptor agonist WIN55,212-2 (5 mg/kg, i.p.) did not block the average seizure length decrease induced by WIN55,212-2 treatment alone (**Figure 19b** *bottom*). In comparison to vehicle, the decrease was significant during the first hour after injection (20 min time bin: AM 2 mg/kg + WIN 5 mg/kg= 67 ± 17 %; vehicle vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0380; 40 min time bin: AM 2 mg/kg + WIN 5 mg/kg= 54 ± 14 %; vehicle vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0012; 60 min time bin: AM 2 mg/kg + WIN 5 mg/kg= 52 ± 14 %; vehicle vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.001) and during 40 minutes of the third hour of recording (140 min time bin: AM 2 mg/kg + WIN 5 mg/kg= 68 ± 9 %; vehicle vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0491; 180 min time bin: AM 2 mg/kg + AM 2 mg/kg= 59 ± 10 %; vehicle vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0072). This average seizure length decrease was also significantly lower in comparison to the AM251 (2 mg/kg) treatment group during the first 80 minutes after injection (20 min time bin: AM 2 mg/kg= 103 ± 5 vs AM 2 mg/kg + WIN 5 mg/kg= 67 ± 17 %; AM 2 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.038; 40 min time bin: AM 2 mg/kg= 101 ± 8 vs AM 2 mg/kg + WIN 5 mg/kg= 54 ± 14 %; AM 2 mg/kg vs AM 2

mg/kg + WIN 5 mg/kg, p= 0.0018; 60 min time bin: AM 2 mg/kg= 102 ± 6 vs AM 2 mg/kg + WIN 5 mg/kg= 52 ± 14 %; AM 2 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.001; 80 min time bin: AM 2 mg/kg= 115 ± 6 vs AM 2 mg/kg + WIN 5 mg/kg= 73 ± 13 %; AM 2 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0116), last 20 minutes of the second hour of recording (120 min time bin: AM 2 mg/kg= 105 ± 8 vs AM 2 mg/kg + WIN 5 mg/kg= 67 ± 14 %; AM 2 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0323) and during 40 minutes of the third hour of recording (140 min time bin: AM 2 mg/kg= 103 ± 5 vs AM 2 mg/kg + WIN 5 mg/kg= 68 ± 9 %; AM 2 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0491; 180 min time bin: AM 2 mg/kg= 103 ± 8 vs AM 2 mg/kg + WIN 5 mg/kg= 59 ± 10 %; AM 2 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0072) (**Figure 19b** *bottom*).

In contrast, pretreatment with CB-1 receptor antagonist AM-251 (2 mg/kg, i.p.), 15 minutes before an injection of CB-1 receptor agonist WIN55,212-2 (5 mg/kg, i.p.), blocked the WIN55,212-2 5 mg/kg induced seizure aggravation, presenting as an increase of time spent in seizures and number of seizures. The blocking effect on time spent in seizures increase was observed during the third hour after injection (140 time bin: WIN 5 mg/kg = 188 ± 26 % vs AM 2 mg/kg + WIN 5 mg/kg = 85 ± 43 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0292; 160 time bin: WIN 5 mg/kg = 178 ± 22 % vs AM 2 mg/kg + WIN 5 mg/kg = 67 ± 18 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0104; 180 time bin: WIN 5 mg/kg = 236 ± 32 % vs AM 2 + WIN 5 mg/kg= 81 ± 37 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0001) (**Figure 19b** *top*), while the blocking effect on the number of seizures increase was observed during 20 minutes of the second hour after injection (100 min time bin: WIN 5 mg/kg = 190 ± 21 % vs AM 2 mg/kg + WIN 5 mg/kg = 87± 20 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0347) and during the third hour after injection (140 time bin: WIN 5 mg/kg = 243

± 35 % vs AM 2 mg/kg + WIN 5 mg/kg = 99 ± 39 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0009 ; 160 time bin: WIN 5 mg/kg = 214 ± 23 % vs AM 2 mg/kg + WIN 5 mg/kg = 76 ± 23 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0013; 180 time bin: WIN 5 mg/kg = 269 ± 38 % vs AM 2 + WIN 5 mg/kg= 111 ± 37 %; WIN 5 mg/kg vs AM 2 mg/kg + WIN 5 mg/kg, p= 0.0002) (**Figure 19b** *middle*).

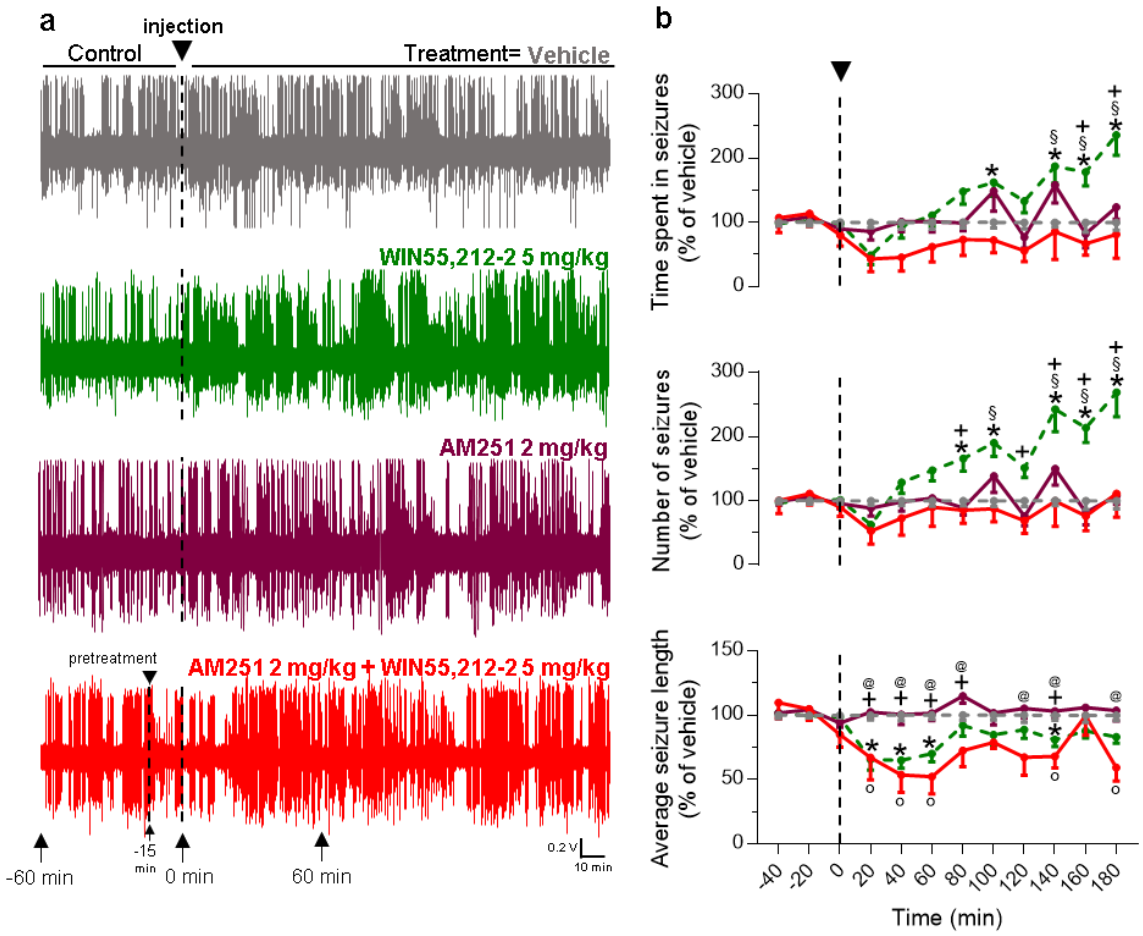

Figure 19. Blockage of CB-1 receptors does not affect SWDs in GAERS rats, but it prevents the seizure aggravation induced by CB-1 receptor agonist WIN55,212-2.

(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after acute i.p. injection (arrow/dashed line) of vehicle (grey, dashed), WIN55,212-2 at 5 mg/kg (green, dashed), AM251 at 2 mg/kg (purple), AM251 2 mg/kg + WIN55,212-2 at 5 mg/kg (red). (**b**) Time course (mean ± SEM) of the time spent in seizures (top), number of seizures (middle) and length of a seizure (bottom) in vehicle (grey; n=33), WIN55,212-2 at 5 mg/kg (green; n=34), AM251 at 2 mg/kg (purple; n=17), AM251 2 mg/kg + WIN55,212-2 at 5 mg/kg (red; n=6) treated GAERS before and after the injection time (dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. Vehicle and WIN55,212-2 5 mg/kg groups are the same as represented in the Figure 18 and represented with a dashed line in this figure. * - $p<0.05$, vehicle vs WIN55,212-2 5 mg/kg. ° - $p<0.05$, vehicle vs AM251 2 mg/kg + WIN55,212-2 5 mg/kg. + - $p<0.05$, WIN55,212-2 5 mg/kg vs AM251 2 mg/kg. § - $p<0.05$, WIN55,212-2 5 mg/kg vs AM251 2 mg/kg + WIN55,212-2 5 mg/kg. @ - $p<0.05$, AM251 2 mg/kg vs AM251 2 mg/kg + WIN55,212-2 5 mg/kg.

In conclusion, acute i.p. injection of 2 mg/kg of CB-1 antagonist AM251 in GAERS rats, does not affect the SWDs. And even though the pretreatment with AM251 (2 mg/kg) does not prevent the decrease of average seizure length induced by 5 mg/kg of CB-1 receptor agonist WIN55,212-2, it does prevent the induced seizure aggravation.

Acute cannabidiol treatment has no effect on SWDs in GAERS rats

GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system before and after acute i.p. injection of phytocannabinoid CBD in three different doses (50 mg/kg: n=6; 100 mg/kg: n=16; 200 mg/kg: n=10) and the respective vehicle (n=10) (**Figure 20a**), to investigate the acute effect of CBD treatment on absence seizures in GAERS rats.

For acute treatment, two-way ANOVA RM with treatment x time as factors revealed no significant main effect of treatment CBD on time spent in seizures (treatment $F_{3,38}$= 1.858; p= 0.1531). The time spent in seizures varied instead significantly over time ($F_{11,418}$= 2.813; p= 0.0015). Time-course of the seizures did not change significantly (treatment x time interaction $F_{33,418}$= 1.097; p=0.3301) (**Figure 20b** *top*).

Similarly, two-way ANOVA RM showed a similar effect of acute CBD treatment on number of seizures (**Figure 20b** *middle*) (treatment $F_{3,38}$= 2.44; p= 0.0793; time $F_{11,418}$= 3.139; p= 0.0004; interaction $F_{33,418}$= 1.149, p= 0.2654).

In addition, two-way ANOVA RM revealed no significant main effect of acute CBD treatment on average seizure length (treatment $F_{3,38}$= 0.7639; p= 0.5214). The average seizure length varied significantly over time ($F_{11,418}$= 1.988; p= 0.0282). Time-course of the seizure length did not change significantly (treatment x time interaction $F_{33,418}$= 1.011; p= 0.4541) (**Figure 20b** *bottom*).

Holm-Sidak's multiple comparison test showed that acute i.p. injection of CBD 50 mg/kg and 100 mg/kg induced an increase in the time spent in seizures in comparison to the vehicle group during the last 20 minutes of the second hour of recording (120 min time bin, for CBD 50 mg/kg:

CBD 50 mg/kg = 202 ± 52 %; vehicle vs CBD 50 mg/kg, p= 0.0217; for CBD 100 mg/kg: CBD 100 mg/kg = 197 ± 42 %; vehicle vs CBD 100 mg/kg, p= 0.0055) (**Figure 20b** *top*).

This increase of time spent in seizures was also significant in comparison to the CBD 200 mg/kg treatment group (120 min time bin, for CBD 50: CBD 50 mg/kg= 202 ± 52 % vs CBD 200 mg/kg = 95 ± 15 %; CBD 50 mg/kg vs CBD 200 mg/kg, p= 0.0187; for CBD 100: CBD 100 mg/kg= 197 ± 42 % vs CBD 200 mg/kg = 95 ± 15 %; CBD 100 mg/kg vs CBD 200 mg/kg, p= 0.0034) (**Figure 20b** *top*).

Also, CBD 50 mg/kg and 100 mg/kg induced an increase in the number of seizures in comparison to the vehicle group during the last 20 minutes of the second hour of recording (120 min time bin, for CBD 50 mg/kg: CBD 50 mg/kg = 180 ± 39 %; vehicle vs CBD 50 mg/kg, p= 0.0418; for CBD 100 mg/kg: CBD 100 mg/kg = 186 ± 29 %; vehicle vs CBD 100 mg/kg, p= 0.0029) (**Figure 20b** *middle*).

The increase of the number of seizures induced by CBD 100 mg/kg was also significant in comparison to the CBD 200 mg/kg treatment group (120 min time bin: CBD 100 mg/kg= 186 ± 29 % vs CBD 200 mg/kg = 110 ± 16 %; CBD 100 mg/kg vs CBD 200 mg/kg, p= 0.0094) (**Figure 20b** *middle*).

Moreover, during the first 20 minutes of the third hour of recording, average seizure length of rats in the CBD 100 mg/kg treatment group was significantly lower compared to the CBD 200 mg/kg treatment group (140 min time bin: CBD 100 mg/kg= 99 ± 7 % vs CBD 200 mg/kg = 125 ± 9 %; CBD 100 mg/kg vs CBD 200 mg/kg, p= 0.0412) (**Figure 20b** *bottom*).

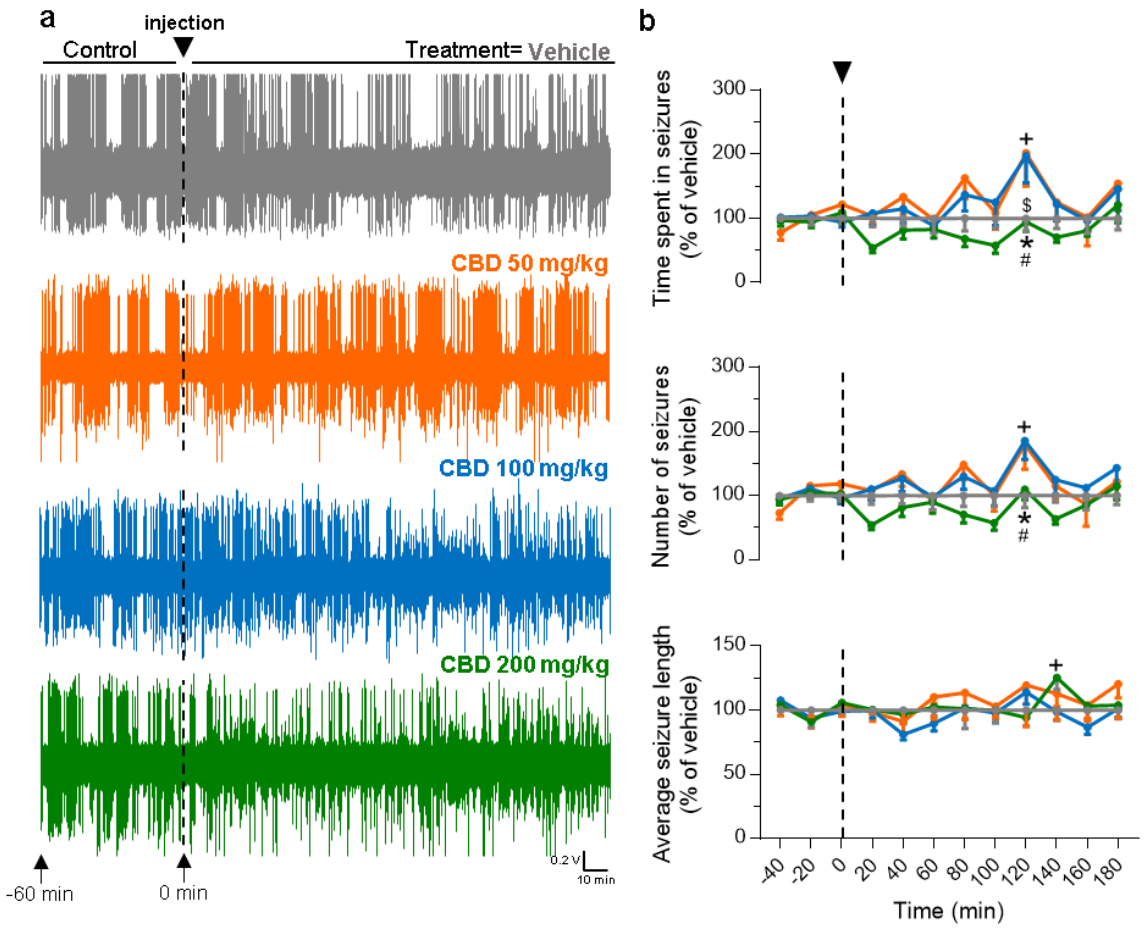

Figure 20. Acute cannabidiol treatment transiently exacerbates SWDs at lower doses (50-100 mg/kg) while it is ineffective at higher doses (200 mg/kg) in GAERS rats.
(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after acute i.p. injection (arrow/dashed line) of vehicle (grey), CBD at 50 mg/kg (orange), 100 mg/kg (blue) or 200 mg/kg (green). (**b**) Time course (mean ± SEM) of the time spent in seizures (top), number of seizures (middle) and average seizure length (bottom) in GAERS acutely treated with vehicle (n=10), CBD at 50 mg/kg (n=6), 100 mg/kg (n=16), or 200 mg/kg (n= 10) before and after the injection time (arrow/dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. * - $p<0.05$, vehicle vs CBD 50 mg/kg, # - $p<0.05$, vehicle vs CBD 100 mg/kg, $ - CBD 50 mg/kg vs CBD 200 mg/kg, + - CBD 100 vs CBD 200 mg/kg.

In conclusion, acute CBD treatment transiently exacerbated SWDs in GAERS rats at lower doses (50-100 mg/kg), while at higher doses (200 mg/kg) did not show an effect. However, the

transient exacerbation observed at lower doses, was observed only in one time bin, hence it cannot be excluded that it possibly occurred by chance.

Subchronic cannabidiol treatment has no effect on SWDs in GAERS rats

Another group of GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system received a subchronic treatment (for 7 days, one injection per day) of CBD 100 mg/kg (n=10) or respective vehicle (n=10) to investigate the effect of CBD after a longer treatment period (**Figure 21a**).

For subchronic treatment (one i.p. injection per day, for seven days), two-way ANOVA RM with treatment x time as factors revealed no significant effect of the CBD 100 mg/kg treatment on time spent in seizures (**Figure 21b** *top*) (treatment: $F_{1,18}$= 0.07838; p= 0.7827; time: $F_{11,198}$= 0.4139, p= 0.9489; interaction: $F_{11,198}$= 0.4139, p= 0.9489).

Similarly, two-way ANOVA RM showed that there is no effect of CBD 100 mg/kg on number of seizures (**Figure 21b** *middle*) (treatment $F_{1,18}$= 0.1173, p= 0.7360; time $F_{11,198}$= 0.5087; p= 0.8961; interaction $F_{11,198}$= 0.5087, p= =0.8961).

In addition, two-way ANOVA RM revealed no significant main effect of CBD 100 mg/kg on the average seizure length (treatment $F_{1,18}$= 0.874; p= 0.3622). The average seizure length varied significantly over time ($F_{11,198}$= 2.295; p= 0.0116). Time-course of the seizure length changed significantly (treatment x time interaction $F_{11,198}$= 2.295; p= 0.0116) (**Figure 21b** *bottom*).

Holm-Sidak's multiple comparison test showed that subchronic CBD treatment (CBD 100 mg/kg) during the third hour of recording transiently increased the average seizure length (CBD

100 mg/kg= 138 ± 6 %, vehicle vs CBD 100 mg/kg, p= 0.0102) in comparison to the vehicle group (**Figure 21b** *bottom*).

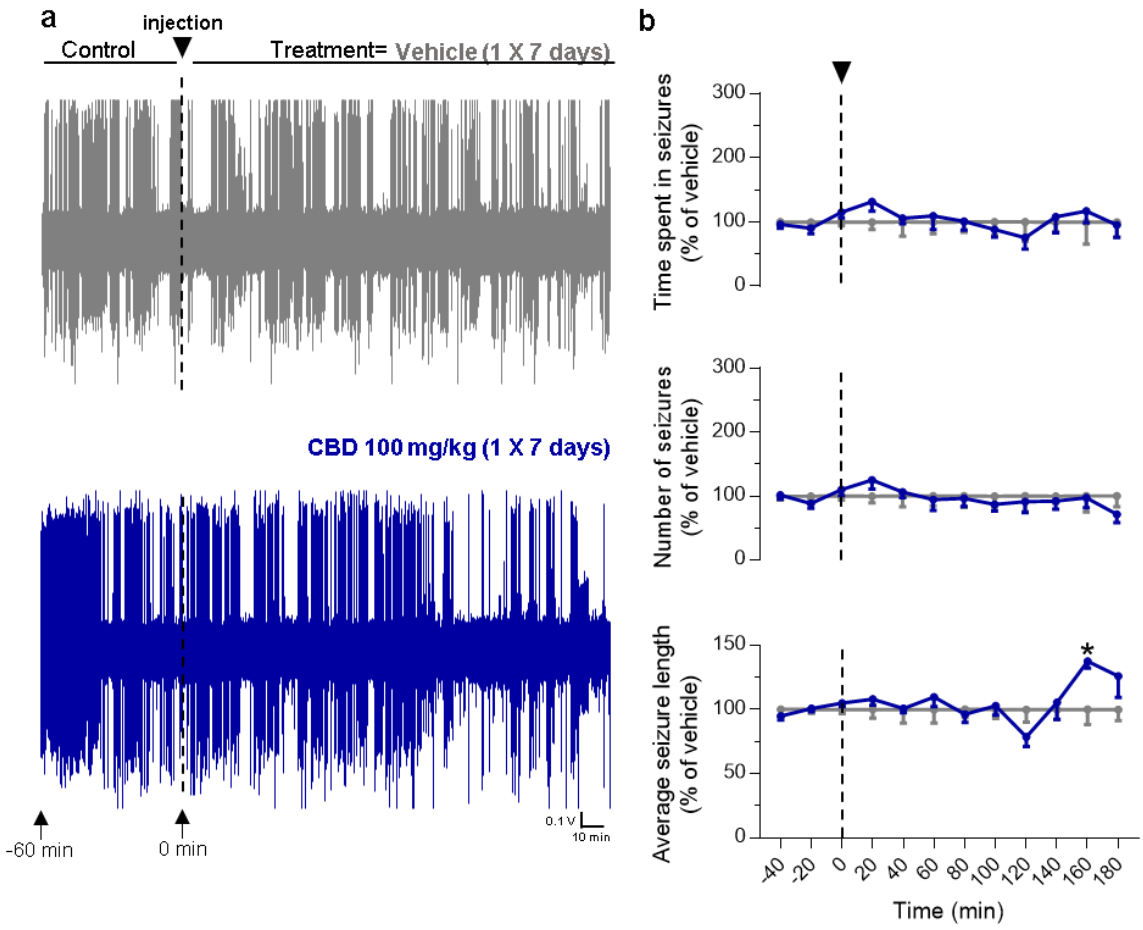

Figure 21. Subchronic cannabidiol treatment does not exacerbate SWDs in GAERS rats.
(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after the seventh i.p. injection (arrow/dashed line) of vehicle (grey), CBD at 100 mg/kg (dark blue); GAERS rats received one injection of CBD 100 mg/kg or vehicle per day, for 7 days, and were recorded in the EEG on the seventh day. (**b**) Time course (mean ± SEM) of the time spent in seizures (top), number of seizures (middle) and average seizure length (bottom) in GAERS subchronicaly (for 7 days) treated with vehicle (grey; n=10) or CBD at 100 mg/kg (dark blue; n=10) before and after the injection time (arrow/dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. * - $p<0.05$, vehicle vs CBD 100 mg/kg.

In conclusion, subchronic CBD treatment does affect the SWD severity, as there is no effect on time spent in seizures and the number of seizures in GAERS rats; the transient increase in

average seizure length observed might have possibly occurred by chance, as it was observed only in one time bin.

The block of the catabolism of the endocannabinoid anandamide has no effect on SWDs in GAERS rats

GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system before and after acute i.p. injection of the FAAH inhibitor URB597 in two different doses (1 mg/kg: n=18; 3 mg/kg: n=8) and the respective vehicle (n=8) (**Figure 22a**). FAAH enzyme is the primary degradation enzyme for the endocannabinoid anandamide and FAAH inhibition, produced by URB597, leads to an accumulation of anandamide (Katona & Freund, 2012), which was used in this experiment to investigate the acute effect of an increased anandamide tone on absence seizures in GAERS rats.

Two-way ANOVA RM with treatment x time as factors revealed no significant main effect of treatment URB597 on time spent in seizures (treatment $F_{2,31}$= 0.4116; p= 0.6661). The time spent in seizures varied instead significantly over time ($F_{11,341}$= 4.767; p< 0.0001) and time-course of the seizures changed (treatment x time interaction $F_{22,341}$= 1.614; p= 0.0411) (**Figure 22b** *top*).

Similarly, two-way ANOVA RM showed a similar effect of URB597 on number of seizures (**Figure 22b** *middle*) (treatment $F_{2,31}$= 0.2155; p= 0.8073; time $F_{11,341}$= 6.04; p< 0.0001; interaction $F_{22,341}$= 1.956, p= 0.0068).

And finally, two-way ANOVA RM revealed no significant main effect of treatment URB597 on average seizure length (treatment $F_{2,31}$= 0.2066; p= 0.8144). The average seizure length varied instead significantly over time (time $F_{11,341}$= 1.846; p= 0.0456) and time-course of the seizures did not change (treatment x time interaction $F_{22,341}$= 1.093; p= 0.3522) (**Figure 22b** *bottom*).

Holm-Sidak's multiple comparison test showed that acute i.p. treatment of FAAH inhibitor URB597 (1 mg/kg) induced a transient increase in the time spent in seizures one hour after the

drug injection (80 min time bin: URB597 1 mg/kg = 206 ± 25 %; vehicle vs URB597 1 mg/kg, p= 0.0003) and in the number of seizures (80 min time bin: URB597 1 mg/kg = 200 ± 20 %, vehicle vs URB597 1 mg/kg, p< 0.0001) in comparison to the vehicle treated group (**Figure 22a, b** *top*, *middle*). Moreover, URB597 3 mg/kg, the higher dose used, induced an increase in the number of seizures for 20 minutes during the first hour after injection (40 min time bin: URB597 3 mg/kg = 162 ± 42 %, vehicle vs URB 3 mg/kg, p= 0.0477) and during the first 20 minutes of the second hour after injection (80 min time bin: URB597 3 mg/kg = 168 ± 33 %, vehicle vs URB 3 mg/kg, p= 0.0167) in comparison to the vehicle treated group (**Figure 22b** *middle*). Also, during the last 20 minutes of the three hour EEG recording, higher dose used (URB597 3 mg/kg) induced a decrease in the average seizure length that was significantly lower in comparison to the lower dose (URB597 1 mg/kg) (180 min time bin: URB597 1 mg/kg = 111 ± 8 % vs URB597 3 mg/kg = 75 ± 18 %; URB597 1 mg/kg vs URB597 3 mg/kg, p= 0.0139).

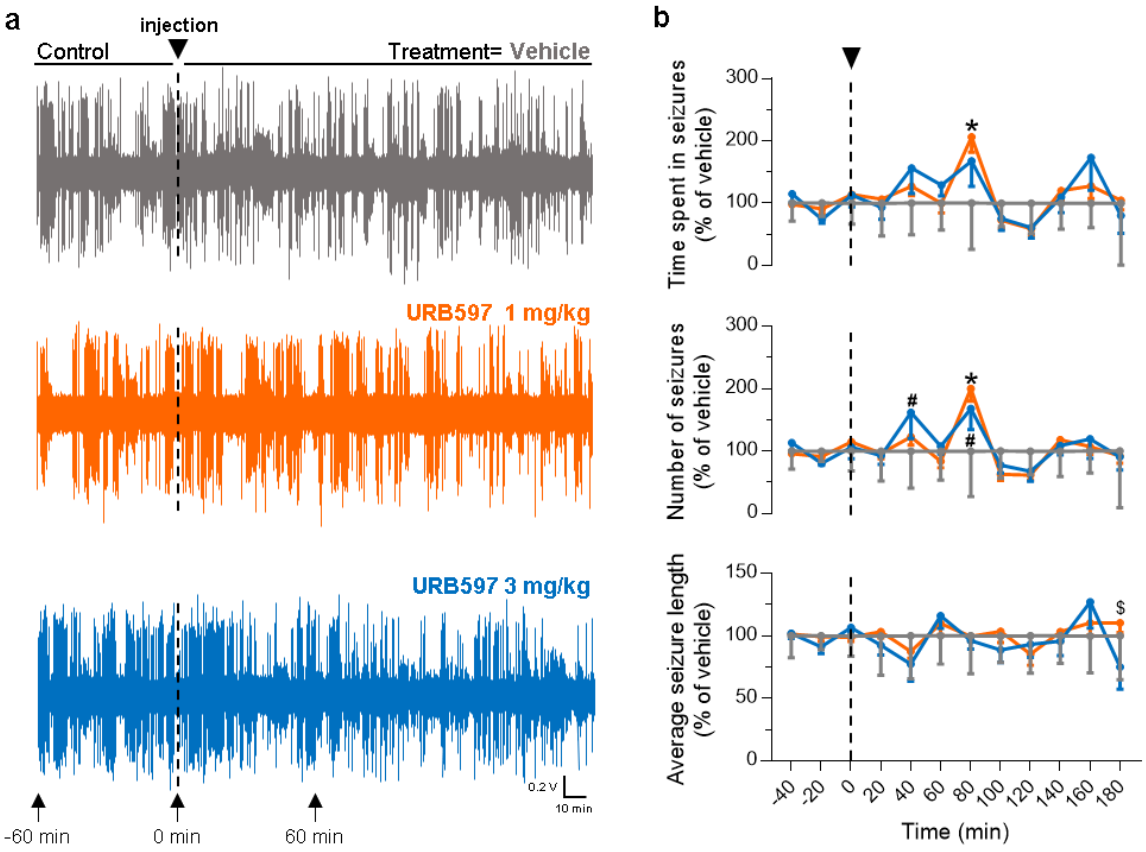

Figure 22. The block of the catabolism of the endocannabinoid anandamide transiently exacerbates SWDs in GAERS rats.

(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after acute i.p. injection (arrow/dashed line) of vehicle (grey) and FAAH inhibitor URB597 at 1 mg/kg (orange) or 3 mg/kg (blue). (**b**) Time course (mean ± SEM) of the time spent in seizures (top), number of seizures (middle) and length of a seizure (bottom) in vehicle (grey; n=8), URB at 1 mg/kg (orange; n=18) or 3 mg/kg (blue; n=8) treated GAERS before and after the injection time (dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. * - $p<0.05$, vehicle vs URB597 1 mg/kg. # - $p<0.05$, vehicle vs URB597 3 mg/kg, $ - $p<0.05$, URB 1 mg/kg vs URB597 3 mg/kg.

These results suggest that the FAAH inhibitor URB597, administrated i.p., did not affect absence seizures in GAERS rats, as the increase in SWD parameters were not consistent, since they were observed only in two time bins, which might have possibly occurred by chance.

The block of the catabolism of the endocannabinoid 2-AG transiently exacerbates SWDs and decreases the average seizure length in GAERS rats

GAERS rats, implanted with EEG electrodes, were recorded in the EEG recording system before and after acute i.p. injection of the MAGL inhibitor MJN110 in two different doses (1 mg/kg: n=10; 5 mg/kg: n=19) and the respective vehicle (n=18) (**Figure 23a**). MAGL enzyme is predominantly responsible for the degradation of the endocannabinoid 2-AG and MAGL inhibition, produced by MJN110, leads to an accumulation of 2-AG (Katona & Freund, 2012), which was used in this experiment to investigate the acute effect of an increased 2-AG tone on absence seizures in GAERS rats.

Two-way ANOVA RM with treatment x time as factors revealed no significant main effect of treatment MJN110 on time spent in seizures (treatment $F_{2,44}$= 0.2173; p= 0.8056). The time spent in seizures varied instead significantly over time ($F_{11,484}$= 2.229; p= 0.0122) and time-course of the seizures changed (treatment x time interaction $F_{22,484}$= 2.374; p= 0.0005) (**Figure 23b** *top*).

Similarly, two-way ANOVA RM showed a similar effect of MJN110 on number of seizures (**Figure 23b** *middle*) (treatment $F_{2,44}$= 1.853; p= 0.1687; time $F_{11,484}$= 2.699; p= 0.0022; interaction $F_{22,484}$= 2.896, p< 0.0001).

Conversely, two-way ANOVA RM revealed a significant main effect of MJN110 on average seizure length (**Figure 23b** *bottom*) (treatment $F_{2,44}$= 8.788; p= 0.0006; time $F_{11,484}$= 3.699; p< 0.0001; interaction $F_{22,484}$= 2.613; p= 0.0001).

Holm-Sidak's multiple comparison test showed that acute i.p. treatment with the higher does (5 mg/kg) of MAGL inhibitor MJN110 induced a late increase (140 min after drug injection) in the time spent in seizures that was visible during the last 40 min of recording (160 min time bin:

MJN110 5 mg/kg = 152 ± 23 %; vehicle vs MJN110 5 mg/kg, p= 0.0264; 180 min time bin: MJN110 5 mg/kg = 159 ± 24 %; vehicle vs MJN110 5 mg/kg, p= 0.0066) and in the number of seizures (160 min time bin: MJN110 5 mg/kg = 183 ± 26 %; vehicle vs MJN110 5 mg/kg, p= 0.0003; 180 min time bin: MJN110 5 mg/kg = 194 ± 32 %; vehicle vs MJN110 5 mg/kg, p< 0.0001) in comparison to the vehicle treated group (**Figure 23a, b** *top, middle*).

Moreover, higher dose of MJN110 (5 mg/kg) induced an increase in time spent in seizures that was significantly higher in comparison to the lower dose (1 mg/kg) during the last 20 minutes of recording (180 min time bin: MJN110 1 mg/kg= 86 ± 20 % vs MJN110 5 mg/kg = 159 ± 24 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p=0.0066) (**Figure 23b** *top*).

Also, higher dose induced an increase in the number of seizures in comparison to the lower dose during the first 20 minutes of the third hour after drug injection (140 min time bin: MJN110 1 mg/kg= 69 ± 12 % vs MJN110 5 mg/kg = 134 ± 24 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0277) and during the last 20 minutes of recording (l80 min: MJN110 1 mg/kg= 98 ± 19 % vs MJN110 5 mg/kg = 194 ± 32 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0002) (**Figure 23b** *middle*).

Higher dose of MJN110 (5 mg/kg) induced a decrease of the average seizure length in comparison to the vehicle group that started 20 minutes after the drug injection and was significant in all 20 min time bins until the end of the second hour of recording (40 min time bin: MJN110 5 mg/kg = 72 ± 6 %; vehicle vs MJN110 5 mg/kg, p= 0.0009; 60 min time bin: MJN110 5 mg/kg = 67 ± 5 %; vehicle vs MJN110 5 mg/kg, p= 0.0003; 80 min time bin: MJN110 5 mg/kg = 77 ± 8 %; vehicle vs MJN110 5 mg/kg, p= 0.01; 100 min time bin: MJN110 5 mg/kg = 67 ± 5 %; vehicle vs MJN110 5 mg/kg, p< 0.0001; 120 min time bin: MJN110 5 mg/kg = 73 ± 5 %; vehicle vs MJN110 5 mg/kg, p= 0.0017) and during 20 min of the third hour

after injection (160 min time bin: MJN110 5 mg/kg = 79 ± 7 %; vehicle vs MJN110 5 mg/kg, p= 0.0145) (**Figure 23b** *bottom*).

This decrease of average seizure length induced by the higher dose (5 mg/kg), was also significant in comparison to the lower dose (1 mg/kg) of MJN110, during the last 40 min of the first hour after drug injection (40 min time bin: MJN110 1 mg/kg= 93 ± 7 % vs MJN110 5 mg/kg = 72 ± 6 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0389; 60 min time bin: MJN110 1 mg/kg= 100 ± 10 % vs MJN110 5 mg/kg = 70 ± 5 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0021), during the first 40 minutes of the second hour after drug injection (80 min time bin: MJN110 1 mg/kg= 98 ± 7 % vs MJN110 5 mg/kg = 77 ± 8 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0434; 100 min time bin: MJN110 1 mg/kg= 89 ± 5 % vs MJN110 5 mg/kg = 67 ± 5 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0291) and during 20 min of the third hour after injection (160 min time bin: MJN110 1 mg/kg= 109 ± 11 % vs MJN110 5 mg/kg = 79 ± 7 %; MJN110 1 mg/kg vs MJN110 5 mg/kg, p= 0.0046) (**Figure 23b** *bottom*).

Moreover, the lower dose of MJN100 (1 mg/kg) induced a decrease in the average seizure length in comparison to the vehicle group during the last 20 minutes of the second hour after drug injection (120 min time bin: MJN110 1 mg/kg = 78 ± 8 %; vehicle vs MJN110 1 mg/kg, p= 0.0424) and during the last 20 minutes of the third hour after drug injection (180 min time bin: MJN110 1 mg/kg = 77 ± 11 %; vehicle vs MJN110 1 mg/kg, p= 0.0386) (**Figure 23b** *bottom*).

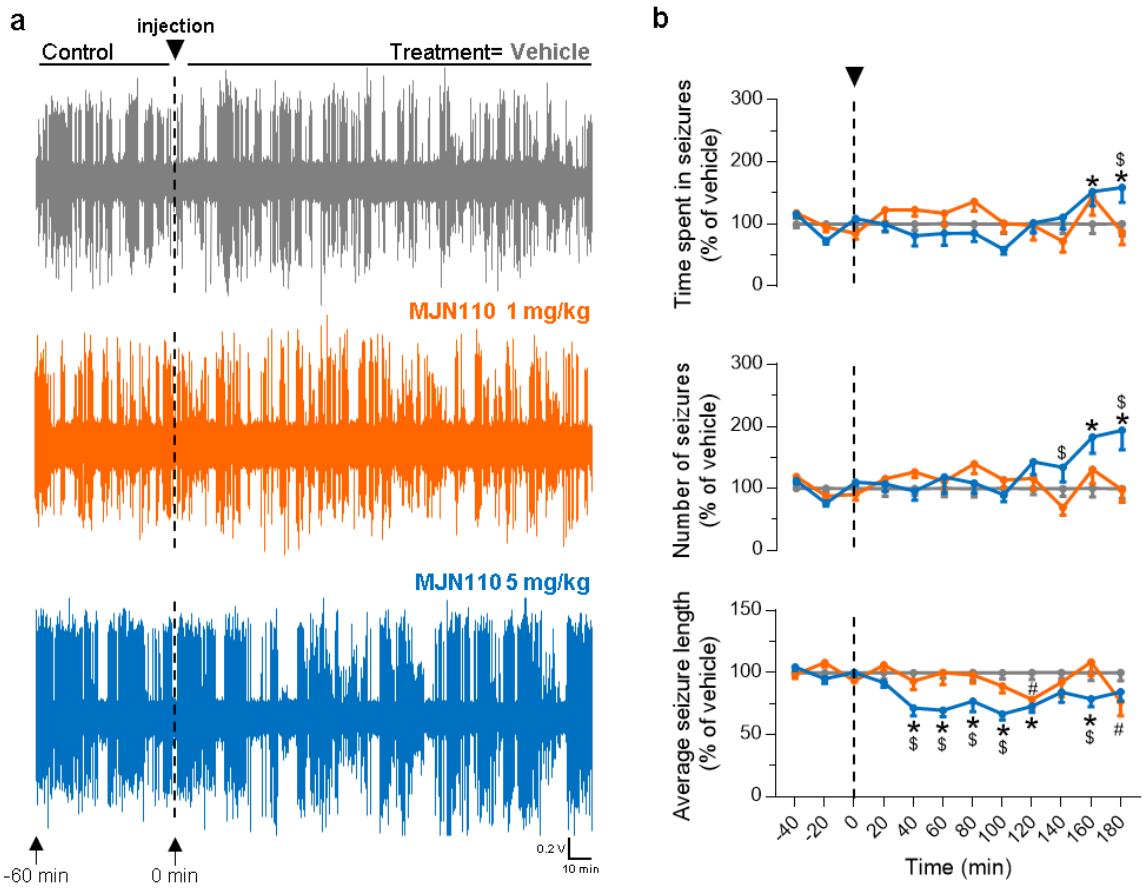

Figure 23. The block of the catabolism of the endocannabinoid 2-AG decreases the average seizure length and causes a late exacerbation of SWDs in GAERS rats.
(**a**) Example traces of SWDs recorded in the EEG of freely moving GAERS rats before and after acute i.p. injection (arrow/dashed line) of vehicle (grey) and MAGL inhibitor MJN110 at 1 mg/kg (orange) or 5 mg/kg (blue). (**b**) Time course (mean ± SEM) of the time spent in seizures (top), number of seizures (middle) and average length of a seizure (bottom) in vehicle (grey; n=18), MJN110 at 1 mg/kg (orange; n=10) or 5 mg/kg (blue; n=19) treated GAERS before and after the injection time (dashed line). All values are normalized to the control period (-40 to 0 min) and expressed as a percentage of their respective vehicle group. Two-way ANOVA RM (treatment x time interaction) was performed. * - $p<0.05$, vehicle vs MJN110 5 mg/kg. # - $p<0.05$, vehicle vs MJN110 1 mg/kg, $ - $p<0.05$, MJN110 1 mg/kg vs MJN110 5 mg/kg.

In conclusion, acute i.p. injection of MAGL inhibitor MJN 110 induced a decrease of average seizure length, which for the higher dose (5 mg/kg) lasted almost throughout the whole 3-hour EEG recording. Moreover, the higher dose induced a late exacerbation of absence seizures during the third hour after injection.

Discussion

GAERS are a good model of absence epilepsy, displaying SWDs, reactive to the anti-absence drugs, and showing affective behavioral comorbidities

As already mentioned in the introduction, ASs are accompanied by different neuropsychological comorbidities, and patients with AS have greater odds of developing affective behavioral disorders than the normal population (Gruenbaum et al., 2021).

Moreover, the same comorbidities have been shown in the GAERS animal models of absence epilepsy (Bouilleret et al., 2009; Jones et al., 2008; Marques-Carneiro et al., 2014; Powell et al., 2014). The first study investigating the differences in the affective behavior between GAERS and NEC, both male and female, was conducted by Jones and colleagues (Jones et al., 2008); they found that i) GAERS show a reduction in general locomotor activity in comparison to NEC; ii) GAERS show a more anxious behavior and iii) a more depressive-like behavior in comparison to NEC; moreover, iii) those changes were observed before and after the onset of seizures, indicating that ASs and these affective behavioral changes share a common cause.

Similarly, a study conducted on female GAERS and NEC rats, found that GAERS exhibited a more anxious behavior and had increased cortical, amygdala and ventricular volumes, and increased thickness of the somatosensory cortex in comparison to NEC (Bouilleret et al., 2009). Moreover, another study found that female GAERS displayed higher anxiety and depressive-like behavior in comparison to NEC; moreover, they showed that GAERS had an increased whole brain volume and increased somatosensory cortical width in comparison to NEC (Powell et al., 2014).

Finally, the first study that included Wistar rats as a second control was conducted by Marques-Carneiro and colleagues (Marques-Carneiro et al., 2014) as they investigated anxiety and locomotion in GAERS and found that: i) GAERS showed faster habituation to a novel environment; ii) GAERS displayed lower exploratory activity than NEC and Wistar rats; iii) NEC appeared less anxious than Wistars and GAERS, while iv) anxiety level of Wistar and GAERS was similar; moreover, volumetric analysis showed that v) NEC had a larger amygdala volume; while vi) GAERS had a larger thalamus volume in comparison to Wistar and NEC. The authors conclude that i) the increased amygdala volume in NEC is associated with their anxiety level; ii) inbreeding of GAERS did not affect the anxiety-related behavior, as their anxiety level is similar to Wistars; and iii) stress the importance of including Wistar as second control to avoid biased results as a consequence of inbreeding (Marques-Carneiro et al., 2014).

Further evidence for bidirectionality between AS and affective disorders, comes from studies on animal models, investigating the effect ETX, the first-line treatment for children with CAE, on both disorders (Dezsi et al., 2013; Russo et al., 2011; K. Y. Sarkisova et al., 2010).

Indeed, it has been shown that ETX has disease-modifying properties, on both ASs and depressive-like behavior in WAG-Rij animal model of absence epilepsy, as i) chronic oral administration of ETX (300 mg/kg/day), from postnatal day 21 onward to 5 months of age, almost completely suppressed the development of AS; moreover ii) ETX-treated WAG-Rij rats did not show depressive-like symptoms in comparison to untreated rats; iii) after discontinuation of ETX, AS emerged, however a reduction in SWD severity was observed for over a month after ETX discontinuation; but interestingly, iv) no effects of ETX on anxiety was found in WAG-Rij rats (K. Y. Sarkisova et al., 2010). Similarly, another study found that early long-term ETX treatment reduces both SWDs and depressive-like behavior in WAG/Rij rats, however, this was

not seen for other AEDs used in this study, namely zonisamide, carbamazepine, levetiracetam (Russo et al., 2011).

Furthermore, Dezsi and colleagues (Dezsi et al., 2013) investigated the effect of early chronic ETX treatment (ETX 300 mg/kg/day, in drinking water, from 3 to 22 weeks of age) on AS and behavioral comorbidities in GAERS animal of absence epilepsy, and they found that i) ETX suppressed AS and this effect was maintained for the 3-month after treatment cessation, indicating a disease-modifying effect of the ETX treatment; moreover ii) anxiety-like behavior was reduced by chronic ETX treatment in adult GAERS rats; while iii) ETX did not reduce anxiety-like behavior in GAERS rats at 7 weeks, prior to AS onset, which suggests that there is an indirect effect of ETX on the anxiety that is dependent on the presence of AS; moreover iv) ETX treatment was associated with increased expression of DNA methyltransferase enzyme (DNMT) messenger RNA (mRNA) in somatosensory cortex. More interesting results about the anxiety-related behavior in GAERS compared NEC were shown, i) at 7 weeks of age, before the onset of ASs, GAERS displayed a more anxiogenic behavior compared to NEC; however, ii) at 34 weeks of age, after ASs onset, these differences in the anxiety level between control GAERS and NEC, were not clear in all parameters (Dezsi et al., 2013).

We investigated the effect of ETX and VPA, the two most efficient AEDs to treat AS in children with CAE (Masur et al., 2013), firstly on SWDs in GAERS rats, and secondly on anxiety-like behavior of GAERS rats and two control strains, NEC and normal Wistar rats.

It has already been shown that ETX and VPA are able to suppress SWDs in GAERS rats (Depaulis et al., 2016; Marescaux et al., 1992). We showed that ETX (200 mg/kg) almost

completely blocked SWDs in GAERS rats during the whole 3 hour long EEG recording, which is line with previous studies showing ETX (100 mg/kg) mean efficacy exceeding 90% SWD suppression in GAERS rats (Marescaux et al., 1992). In contrast, we found that VPA (200 mg/kg) induced a 75-90 % decrease of SWD parameters, but only during the first 80 minutes after injection. Although it has been shown that VPA (200 mg/kg) mean efficacy exceeds 90% SWD suppression in GAERS rats (Marescaux et al., 1992), we found that ETX is more efficacious in suppressing SWDs.

Next, we investigated what is the effect of AEDs, ETX and VPA, on anxiety-like behavior in GAERS rats and two control strains, NEC and normal Wistar rats. Including normal Wistar rats is important for two main reasons: i) to understand if AEDs affect anxiety-related behavior in normal animals, which would help to explain their effect on comorbid anxiety in absence epilepsy, and the bidirectionality between them (Gruenbaum et al., 2021); ii) since both NEC and GAERS are inbred strains, and the presence of SWDs is polygenic, it is possible that the inbreeding affected other traits such as anxiety, so comparing them with the strain they originate from provides valuable data, taking into account that some Wistar rats also have SWDs (Marques-Carneiro et al., 2014).

We found multiple difference between the three strains, when comparing the vehicle groups. In comparison to normal Wistar rats, GAERS show: i) fewer Head Dips; ii) fewer Face Grooming episodes, shorter in duration; iii) fewer Edge Sniffs episodes, shorter in duration; iv) more Front Paw Licking episodes, longer in duration; v) longer Immobile Sniffing episodes. Moreover, there were multiple differences between NEC and Wistar rats, in comparison to normal Wistar rats, NEC show: i) fewer Head Dips; ii) fewer Face Grooming episodes, shorter in duration; iii) fewer Edge Sniff episodes, shorter in duration; iv) more Front Paw Licking episodes; v) longer

Immobile Sniffing episodes; iv) longer Rearing episodes; vi) more Front Paw Licking episodes, longer in duration.

Surprisingly, we did not find many differences between NEC and GAERS, except that GAERS did more Head Dips than NEC rats.

Based on differences of several behavioral components, such as more Head Dips and grooming activity, shorter Immobile Sniffing episodes, we can conclude that Wistar are least anxious between the three strains. These results are interesting as they imply that anxious behavior seen in NEC and GAERS, might be related to inbreeding. These findings are in contrast with the only other study that used NEC and Wistars as controls and showed that Wistar and GAERS have a similar anxiety level and are more anxious than NEC rats (Marques-Carneiro et al., 2014). Moreover, our results show that NEC and GAERS treated with vehicle exhibit a fairly similar behavior in HB, which is partly in line with the study conducted by Dezsi (Dezsi et al., 2013), that found no difference between NEC and GAERS in the number of inner entries, a parameter of anxiety-related behavior, in the open field test. However, this finding is not in line with other studies showing that GAERS are more anxious than NEC rats (Bouilleret et al., 2009; Jones et al., 2008; Powell et al., 2014); a possible explanation could be that quantitative analysis is not sufficient to reveal these differences in HB, and a more advanced analysis should be performed, such as analysis of temporal relationships between the behavioral events (Casarrubea et al., 2017). Moreover, it has to be noted that no other study described, investigated the anxiety level in HB, so it is difficult to directly compare these results.

Our results show that ETX and VPA changed the behavior of Wistar, NEC and GAERS, when tested in HB. In Wistar rats, ETX and VPA treatment induced many changes in the behavior in comparison to vehicle-treated rats, interestingly most changes were similar, ETX and VPA both

induced: i) fewer Head Dips; ii) fewer Face Grooming episodes, shorter in duration; iii) fewer Immobile Sniffing episodes; iv) fewer Climbing episodes, shorter in duration; v) fewer Edge sniffs, shorter in duration; vi) more Front Paw Licking episodes, longer in duration. Moreover, only ETX induced: i) longer Immobile Sniffing episodes; ii) fewer Walking episodes; while only VPA induced more Immobility episodes, longer in duration in comparison to vehicle-treated Wistar rats. These results clearly show that AEDs affect the behavior in normal Wistar rats; both ETX and VPA induce a decrease in the locomotion, seen by a decrease in Walking and an increase in Immobility, respectively. Moreover, Wistar treated rats were less likely to vertically explore the environment, as shown by a decrease in Climbing activity, and less likely to perform focused exploration, as seen by a decrease in hole exploration. The changes in grooming activity are not uniform, we observed a decrease in Face Grooming, increase in Front Paw Licking, while Body Grooming and Hind Paw Licking were unaffected by AEDs in Wistar rats.

These results, induced hypolocomotion and especially a decrease in Head Dip, imply that ETX and VPA induce an anxiogenic effect in Wistar rats.

In NEC rats, ETX and VPA treatment induced some similar changes: i) shorter Immobile Sniffing episodes; ii) longer Walking episodes; iii) shorter Rearing episodes; iv) fewer Front Paw Licking episodes in comparison to vehicle treated NEC. Moreover, ETX induced: i) more Walking episodes; ii) fewer Climbing episodes, while VPA induced i) more Head Dips; ii) longer Immobility episodes; iii) more Hind Paw Licking episodes in comparison to vehicle-treated NEC. These results show that in NEC, both ETX and VPA induced increase locomotion, seen by a decrease in behaviors with a fixed position, such as Immobile Sniffing and Rearing, and an increase in Walking in comparison to the vehicle group. Both AEDs induced a decrease in Front Paw Licking, in contrast, VPA also induced an increase in Hind Paw Licking, while

other grooming activities were unaffected. Interestingly, ETX did not induce any changes in the hole exploration, while VPA induced an increase in Head Dip. However, although VPA induced an increase in some exploratory behaviors in comparison to the vehicle, it also induced an increase in immobility duration, which was not seen by ETX.

These results in NEC rats, show that ETX does not induce changes in the anxiety level, which is in line with the results found by chronic ETX treatment in NEC rats (Dezsi et al., 2013), while VPA possibly decreases the anxiety level. However, it is necessary to further evaluate these findings with different behavioral tests.

In GAERS rats, both ETX and VPA induced longer Walking episodes in comparison to the vehicle; moreover, ETX induced: i) shorter Rearing episodes; ii) fewer Climbing episodes; iii) fewer Front Paw Licking episodes, shorter in duration, while VPA induces: i) more Rearing episodes; ii) shorter Immobile Sniffing episodes. These results show that in GAERS rats, both ETX and VPA induced an increase in locomotion, seen by the increase in Walking duration. Moreover, ETX induced a decrease in vertical exploratory behaviors, which implies an anxiolytic effect; while VPA induced an increase in the vertical exploratory behavior Rearing. Grooming activities were mostly unaffected, only ETX induced a decrease in Front Paw Licking. Surprisingly, AEDs did not induce any changes in hole exploration behaviors in our study.

Finally, these results imply a possible anxiolytic effect of ETX in GAERS rats, although it is not excluded that this effect might be a consequence of the AS reduction. A finding that supports the anxiolytic effect of ETX in GAERS rats is that there is not a reduction of Immobility induced by AEDs, which could be directly related to AS suppression.

On the other hand, VPA did not show a clear anxiolytic nor anxiogenic effect in GAERS rats, which is line with the findings of a study conducted by Citraro and colleagues, where they show that VPA has no effect on anxiety behavior in the WAG/Rij model of ASs.

In conclusion, our results show that not only chronic ETX treatment (Dezsi et al., 2013), but also acute systemic administration in adult rats, can influence similar anxiety-related behavior changes in GAERS and NEC; interestingly, ETX showed more effects on behavior in comparison to VPA. Moreover, these results are important as they show that both ETX and VPA, induce anxiety-like behaviors in normal Wistar rats.

Lamotrigine, the third-choice monotherapy in children with CAE (Masur et al., 2013), has been previously shown to not decrease AS in GAERS and WAG/Rij rats, when acutely administered (Dalby & Nielsen, 1997; C. M. van Rijn, Weyn Banningh, & Coenen, 1994). Huang and colleagues investigated the effect of LTG on Long-Evans rats that show spontaneous SWDs in comparison to Long-Evans rats with no SWDs (Huang et al., 2012), and similar effects were seen, i) LTG had no immediate effect on SWDs, but ii) decreased SWDs two weeks after the initiation of a 35-day long treatment, and iii) SWDs were decreased for a month after the end of LTG treatment; moreover, iv) chronic LTG treatment also ameliorated anxiety and depressive-like behavior in Long-Evans rats showing spontaneous SWDs in comparison to Long-Evans rats with no SWDs (Huang et al., 2012).These results imply that not only ETX (Dezsi et al., 2013), but also LTG have disease-modifying properties.

Interestingly, it has been shown that antidepressants may influence the epileptogenesis in WAG/Rij rats, since i) early long-term treatment with antidepressants fluoxetine and duloxetine, before the onset of AS, reduces the development of seizures; ii) fluoxetine was able to prevent

the development of comorbid depressive-like behavior; moreover, iii) duloxetine reduced AS, while fluoxetine increase AS in adult WAG/Rij rats (Rita Citraro et al., 2015).

Surprisingly, even environmental factors are important in both AS expression and comorbid anxiety in GAERS rats (Dezsi et al., 2016). In fact, it has been shown that early environmental enrichment i) delays the onset of AS, ii) reduces AS severity in adulthood; iii) reduces anxiety levels; moreover, iv) these beneficial effects are seen even if the enrichment is initiated in adulthood; furthermore, v) these effects are heritable into the next generation and finally, vi) GAERS exposed to enrichment had reduced expression of corticotrophin-releasing hormone (CRH) mRNA, the key stress hormone, that alters anxiety levels (Dezsi et al., 2016). In contrast, it has been shown that environmental enrichment worsens SWD severity in the WAG/Rij model of absence epilepsy (Schridde & van Luijtelaar, 2004). Interesting results considering the environmental and epigenetic factors in AS come from a study conducted by Sarkisova and colleagues (K. Y. Sarkisova et al., 2017), they found that rearing WAG/Rij rats by a foster mother with a high level of maternal care induces a decrease in SWD severity and a decrease in depressive-like behavior. Moreover, chronic ETX treatment was associated with increased expression of DNA methyltransferase enzyme (DNMT) messenger RNA (mRNA) in the somatosensory cortex (Dezsi et al., 2013) a key region for AS expression in animal models of absence epilepsy (Meeren et al., 2002; Polack et al., 2007), suggesting that the cellular mechanism of the disease-modifying effects of ETX might involve epigenetic modifications. These disease-modifying effects of environmental enrichment and ETX, indicates there might be a common cause in AS and anxiety occurrence and supports the premise of a bidirectional relationship of these conditions (Dezsi et al., 2016). However, to better evaluate the effect of the anxiolytic effect of ETX, chronic ETX should be initiated after AS initiation; and more

behavioral tests should be conducted, to get a more comprehensive understanding of these effects (Dezsi et al., 2013).

Moreover, inconsistent results have been found in changes of cerebral structures of animal models of absence epilepsy and their control strains, for example, the amygdala – a structure involved in anxiety, has been shown to be increased in GAERS in one study (Bouilleret et al., 2009), while in another study NEC had a larger amygdala volume (Marques-Carneiro et al., 2014). Furthermore, GAERS had been shown to have a larger thalamus volume in comparison to Wistar and NEC (Marques-Carneiro et al., 2014), increased cortical and ventricular volumes, and increased thickness of the somatosensory cortex in comparison to NEC (Bouilleret et al., 2009). Further experiments should be done to evaluate the changes in the cerebral structures of animal models of absence epilepsy and their control strain, and the significance of these changes on psychiatric comorbidities.

Furthermore, we evaluated whether there is a correlation between SWDs and anxiety-like behavior, by testing adult GAERS in HB, a very well-known test to study anxiety-related behavior in rodents (Brown & Nemes, 2008; Casarrubea et al., 2017), while simultaneously recording their EEG, for the first time in literature. While being tested in HB, 60 % (33 rats) of GAERS showed SWDs (HB-SWDs^{+}), while 40 % did not show SWDs (HB-SWDs^{-}).

When comparing the HB parameters between the two groups, we found that HB-SWDs^{+} rats were more immobile and were walking less than HB-SWDs^{-} rats – these finding clearly show that reduced locomotion in HB-SWDs^{+} rats, and possibly a more anxious behavior of HB-SWDs^{+} rats, although it is difficult to draw this conclusion, since it may be only a consequence of the

presence of SWDs. However, we found interesting results when considering the hole exploration behaviors, Head-Dip (HD) and Edge-Sniff (ES), since ES frequency did not vary between the two groups, meaning that there were no differences in the hole exploration, which could be related to the occurrence of seizures; while the HB-SWDs^{+} rats did fewer HD than HB-SWDs^{-} rats, a parameter associated with anxiety-like behavior, as it is more likely that a less anxious rodent will explore the novel environment by putting its head in a hole (Brown & Nemes, 2008; Casarrubea et al., 2017).

These findings suggest that anxiety-like behavior is aggravated by SWD severity in GAERS rats. Moreover, further analysis on HB-SWDs^{+} rats showed that anxiety-related features of HB, namely HD/ES Ratio and HD are negatively correlated to the total time spent seizure, while HD is also negatively correlated to the average seizure length. These findings suggest that anxiety-like behavior is aggravated by SWD severity in GAERS rats, which is in line with studies showing evidence of bidirectionality AS and anxiety, although further research is necessary to elucidate the causality between the two disorders (Gruenbaum et al., 2021)

Limitations to our study are i) acute administration of AEDs; ii) only one behavioral test used; iii) no inclusion of female rats; iv) only quantitative analysis of behavior. However, our data provide valuable addition to the available literature, as no other study investigated the effects of GAERS in HB; and furthermore, we evaluated the behavior of GAERS, while simultaneously recording EEG, which allowed us to directly compare the behavior to differences in SWD occurrences.

Activation of the cannabinoid system exacerbates SWDs in GAERS rats

We evaluated the cannabinoid control of absence epilepsy in the GAERS animal model, by systemic i.p. administration of cannabinoids; that is by administration of synthetic cannabinoids, WIN55,212-2 and AM 251, by administration of the phytocannabinoid CBD, and by enhancing the tone of endocannabinoids (AEA and 2-AG) indirectly via inhibition of endocannabinoid hydrolysis enzymes (FAAH and MAGL).

To date, there are no studies published in the literature that investigated the effect of synthetic cannabinoids on absence seizure in the GAERS animal model of absence epilepsy.

Our results show that acute administration of the CB1 receptor agonist WIN55,212-2 exacerbates SWDs in GAERS rats. More precisely, i) WIN55,212-2 (5 mg/kg) dramatically increased total time spent in seizures and seizure frequency (over 200 % from baseline), starting from the second hour after injection and lasting till the end of the recording (three hours postinjection).; ii) two lower doses of WIN55,212-2 (1 and 2 mg/kg), however, did not induce significant changes of SWDs in GAERS rats; moreover iii) WIN55,212-2 (5 mg/kg) induces a decreased of the average seizure length during the first hour after injection.

A similar study was conducted in the WAG/Rij animal models of absence epilepsy by van Rijn and colleagues (Clementina M. Van Rijn et al., 2010), and showed different results: they found that i) in the first 2 hours after injection, WIN55,212-2 (3, 6 and 12 mg/kg) decreased SWDs frequency; ii) the observed decrease in SWDs frequency was present also during the third hour after injection, but only in rats treated with the highest dose (12 mg/kg); iii) WIN55,212-2 induced an increase of the average seizure length after the third hour postinjection; and iv) WIN55,212-2 dose-dependently decreased the motor activity. A proposed explanation by van

Rijn and colleagues (Clementina M. Van Rijn et al., 2010) of this biphasic modulation induced by WIN55,212-2 – initial reduction of seizure frequency, followed by a late increase in average seizure length, is possible rapid desensitization of CB1 receptors localized on GABAergic terminals afferent to VB, which may occur in response to WIN55,212-2, thereby leaving GABA release unopposed 4–6 h following agonist exposure. Moreover, van Rijn and colleagues found downregulation of CB1 receptors in the RTN and VB of 8-month-old WAG/Rij rats, which show spontaneous absence seizures as compared with age-matched ACI (Augouti Copenhagen Irish) control rats (Clementina M. Van Rijn et al., 2010).

We found that treatment with CB1 receptor antagonist AM251 (2 mg/kg) alone, did not induce any significant changes of SWDs in GAERS rats. This is in line with the study conducted by van Rijn and colleagues, as AM251 alone did not induce any changes of seizure parameters in WAG/Rij rats (Clementina M. Van Rijn et al., 2010). Moreover, van Rijn and colleagues showed that AM251at the lower dose used, (6 mg/kg), was unable to block the lowering effects of WIN55,212-2 on SWD frequency, while at a higher dose used (12 mg/kg) it blocked those effects in the first 2 hours after injection; moreover, a combined injection of AM251(12 mg/kg) and WIN55,212 (6 mg/kg) increased the incidence the SWD frequency 4 and 5 hours after injection;

On the other side, our results show that pretreatment with AM251(2 mg/kg), 15 minutes before injection of WIN55,212-2 8 (5 mg/kg), blocked the WIN55,212-2 induced seizure aggravation – proving that the seizure aggravation was mediated through activation of the CB1 receptors in GAERS.

Moreover, acute administration of the CB1 receptor agonist WIN55,212-2 (5 mg/kg) induces a decrease of the average seizure length (mean duration) during the whole first hour after injection,

which was not blocked by pretreatment with AM251, suggesting that it was caused by some other mechanism, not involving CB1 receptors. This is in contrast with the mean duration elongation observed first by van Rijn and colleagues (Clementina M. Van Rijn et al., 2010) and more recently confirmed by the same group in WAG/Rij rats (M. F. J. Perescis, Flipsen, van Luijtelaar, & van Rijn, 2020).

In this latter study, Perescis and colleagues (M. F. J. Perescis et al., 2020), conducted a study to investigate the effect of a subchronic (2 weeks) treatment with WIN55,212-2 (6 mg/kg) in the WAG/Rij strain, and found that: i) in contrast to their previous results (Clementina M. Van Rijn et al., 2010), acute WIN55,212-2 (6 mg/kg) injection did not induce any changes in the SWD frequency; moreover ii) subchronic treatment did not induce any changes in the SWD frequency; iii) both acute and subchronic treatment induced an increase in the mean SWD duration; iv) also, both acutely and subchronically, WIN55,212-2 induced a decrease in the hazard rates in the seizures lasting 5-20 seconds, meaning that once an SWD reaches a duration of 5 s, it is less likely to be terminated in comparison to controls, resulting in a small number of very long SWDs; v) WIN55,212-2 (6 mg/kg, acutely or subchronically) did not induce any changes in the motor activity and finally vi) WIN55,212-2 did not produce tolerance, as all effects were the same for acute and subchronic treatment.

This study (M. F. J. Perescis et al., 2020), shows contrasting findings in comparison to their previous study (Clementina M. Van Rijn et al., 2010), since in the previous study a decrease in the seizure frequency was observed during 3 hours post-injection. The authors explain that probably the decrease of motor activity found in the previous study rather than the drug itself, caused the decrease in seizure frequency, however this explanation is not sufficient – as the same dose (6 mg/kg) used in this study (M. F. J. Perescis et al., 2020), induced a decrease in the

seizure frequency in the first two hours postinjection of the previous study (Clementina M. Van Rijn et al., 2010). An increase in the mean SWD duration in WAG/Rij rats induced by WIN55,212-2, imply that CB1 receptor activation alters the SWD stopping mechanisms, possibly through activation of CB1 receptors on the GABAergic neurons in the NRT (M. F. J. Perescis et al., 2020).

Another study conducted on the WAG/Rij strain, locally applying WIN55,212-2 bilaterally to different structures of the thalamocortical circuit, namely NRT, ventroposteromedial thalamic nucleus (VPM) and S1po, showed that i) locally applied (0.1, 0.3 and 1 μg/0.5 μl) in any of the structures, it reduced seizure frequency and total time spent in seizures, ii) intracerebroventricular (i.c.v.) injection reduced seizure frequency and total time spent in seizures only at the highest doses (1 and 2 μg/2 μl); moreover iii) injection of the CB1 receptor antagonist/inverse agonist, SR141716A (0.5, 1, 2.5 μg/0.5 μl) in the NRT or S1po did not affect SWDs, while the injection in the VPM induced a non-dose-dependent and significant increase in SWD number and duration, between 90 and 180 min after injection (R. Citraro, Russo, Ngomba, et al., 2013). To the best of our knowledge there are no similar studies conducted in the GAERS model of absence epilepsy.

Based on the available literature, we propose that the exacerbation of SWDs produced by WIN55,212-2 in GAERS rats is a consequence of an increase in GABAergic tonic current in the TC neurons. It has been previously shown that the highest expression of CB1 receptors in the brain is in the SNr, on striatal inhibitory terminals – that is in synapses coming from the inhibitory GABAergic neurons of the striatum to the SNr, where the activation of CB1 receptors induces a robust decrease of GABA release (Freund, Katona, & Piomelli, 2003; Mátyás et al., 2006; Sales-Carbonell et al., 2013; Wallmichrath & Szabo, 2002). Since GABAergic projections

of SNr to TC neuron are involved in the control of thalamocortical circuit during AS in GAERS rats (Paz et al., 2007), it is probable that the activation of CB1 receptors induced by WIN55,212-2 at the striatal inhibitory terminals, will inhibit the GABAergic neurons of the striatum, which will, in turn, cause a decrease of GABAergic transmission of SNr; this depolarization-induced suppression of inhibition caused by CB1R activation will activate SNr neurons and result in an increase of GABAergic transmission to TC neurons and an increased GABAergic tonic current in TC neurons, that will lead to the SWD increase (Cope et al., 2009). The experimental confirmation of this hypothesis is currently under investigation in our laboratory.

When comparing our results with published evidence in WAG/Rij rats, we observed a different scenario in GAERS rats as regarded the effect of cannabinoids on seizure length, with WIN55,212-2, but also by increasing the concentration of the endocannabinoid 2-AG, causing a clear CB1 receptor-independent shortening of the seizure length. Possible alterations in the SWD stopping mechanisms induced by WIN55,21-2, have been proposed by Perescis and colleagues (M. F. J. Perescis et al., 2020) in WAG/Rij rats, but for some reason, these are causing an opposite effect in GAERS rats – terminating the seizures earlier than vehicle-treated GAERS. This interesting finding is worthy to be further investigated.

Results from a recent study conducted by Roebuck and colleagues, showed the effect of phytocannabinoids THC and CBD on GAERS rats; they found that TCH, a partial agonist of CB receptors, when injected intraperitoneally dose-dependently (1–10 mg/kg) exacerbated SWDs, which is in line with our results showing SWD exacerbation induced by CB1 receptor agonist WIN55212-2 (Roebuck et al., 2020).

In addition, Roebuck and colleagues (Roebuck et al., 2020) investigated the effect of smoked cannabis on GAERS rats, they used two different cannabis formulations, one High THC-Low

CBD, the other Low THC- High CBD, and found that: i) increase in the total time spent in seizures induced by the High THC-Low CBD formulation, that was not blocked by pretreatment with the CB1 receptor antagonist SR141716A; ii) increase of average seizure length induced by the High THC-Low CBD formulation during the first hour, whereas pretreatment with the CB1 receptor antagonist SR141716A increased average duration during the whole recording (2 hours postinjection); iii) High THC-Low CBD formulation alone or with pretreatment with the CB1 receptor antagonist SR141716A showed no significant effects on the seizure frequency; iv) Low THC- High CBD did not induce any changes of the SWDs.

Since pretreatment with a CB1R antagonist did not prevent the high-THC cannabis smoke from increasing SWDs, it is suggested that the THC-mediated increase might be mediated by a non-CB1R mediated mechanism, possibly interacting directly with the T-type Ca2+ channel (Ross, Napier, & Connor, 2008); however the value of these findings is questionable, as this effect was produced by cannabis smoke, and other little researched cannabinoids found in cannabis formulations may cause this effect via unknown mechanisms and interactions.

Moreover, Roebuck and colleagues (Roebuck et al., 2020) investigated the effect of CBD on absence seizures in GAERS rats, when intraperitoneally injected and they found that CBD (10-100 mg/kg) dose-dependently modestly decreased SWDs; more precisely i) two higher doses used (30 and 100 mg/kg) decreased the total time spent in seizures by 50 % compared to baseline, while the lowest dose used (10 mg/kg) did not have any effects; ii) only the highest dose used (100 mg/kg) induced a decrease of the average seizure length; while iii) there was no effect of CBD (10, 30 and 100 mg/kg) on the seizure frequency; finally iv) altogether, the lowest dose used (30 mg/kg) did not induce any changes of the SWDs.

Our results show that acute administration of two lower doses of CBD used (50 and 100 mg/kg) induced an increase in the total time spent in seizures and the seizure frequency in the last 20 minutes of the second hour of recording; however, no such effect has been observed with the highest dose used (200 mg/kg). This exacerbation of SWDs is transient, lasting only for 20 minutes during a total of 3 hours of recording, possibly indicating an accidental finding, which is in line with our results of subchronic CBD (100 mg/kg; once per day/7 days) treatment, showing no effect on seizure frequency or total time spent in seizures.

Although in contrast with the findings from Roebuck and colleagues (Roebuck et al., 2020), which show a modest decrease of the total time spent in seizures induced by systemic injection of CBD, our results are in line with their findings of Low THC- High CBD smoked cannabis not inducing any changes of the SWDs in GAERS rats.

Moreover, our results agree with the findings of studies in children with Lennox-Gastaut syndrome and Dravet syndrome, who suffer from many different types of seizures, where CBD as an adjunctive treatment has shown effectiveness in reducing non-convulsive seizures in general, but not in reducing absence seizures (Devinsky et al., 2017; Thiele et al., 2018). So, it is not surprising that phase 2 clinical trials investigating the effects of CBD oral solution in the treatment of pediatric participants with treatment-resistant childhood absence seizures have been terminated early, due to a lack of a clinically meaningful reduction of seizure count (ClinicalTrials.gov #NCT03336242, ClinicalTrials.gov #NCT03355300).

Endogenous cannabinoids have been shown to play a protective role in different disorders associated with neuronal hyperexcitability (Mikheeva, Shubina, Matveeva, Pavlik, & Kitchigina, 2017; Naidoo et al., 2011). Moreover, targeting the cannabinoid system, by changing the endocannabinoid tone has shown promising anti-seizure effects in different acute experimental

models of seizures (Rosenberg, Patra, & Whalley, 2017). Moreover, endocannabinoids, especially AEA, are an interesting target, as it has been shown that inhibition of FAAH, the degradative enzyme of AEA is not inducing impairment of memory, which is usually associated with direct cannabinoid receptor activation (Colangeli, Pierucci, Benigno, Campiani, & Butini, 2017). In contrast, 2-AG hydrolysis blockade has been shown to alter learning and memory performance (Griebel et al., 2015).

In the literature, no studies have investigated the effects of endocannabinoids in the GAERS model of absence epilepsy. We analyzed how the enhancement of the endocannabinoid tone, by blocking the degrading enzymes, affects SWDs in GAERS rats. First, we tested the effect of an acute systemic injection of the FAAH inhibitor, URB597, on SWDs in GAERS rats. Inhibition of FAAH leads to accumulation of AEA and an increased AEA tone, and our results show that i) lower dose used (URB597 1 mg/kg) induced a transient increase in total time spent in seizures and in seizure frequency, for 20 minutes at the beginning of the second hour after injection; while ii) higher dose (URB597 3 mg/kg) produced no effect on the total time spent in seizures; however, ii) higher dose (URB597 3 mg/kg) induced an increase in the seizure frequency, for 40 minutes during the first 2 hours after injection. However, this exacerbation of SWDs is transient and most probably indicating an accidental finding, which suggests that the FAAH inhibitor URB597 has no effect on SWDs in GAERS rats.

Second, we tested the effect of an acute systemic injection of the MAGL inhibitor, MJN110, on SWDs in GAERS rats. Inhibition of MAGL leads to accumulation of 2-AG and an increased 2-AG tone, and our results show that i) lower dose used (1 mg/kg) did not induce any changes in time spent in seizures or seizure frequency; ii) higher dose used (5 mg/kg) induced an increase in the total time spent in seizures and seizure frequency during the last 40 minutes of a 3 hour long

EEG recording; moreover, iii) MJN110 administration induced a decrease in average seizure length. These results suggest that enhancement of the 2-AG tone reduce the seizure length in GAERS rats.

There are not many studies investigating the role of endocannabinoid in absence seizure. Some contrasting results on how AEA affects SWDs in WAG/Rij rats come from a study conducted by Citraro and colleagues in WAG/Rij animal models of absence epilepsy; they found that i) AEA (0.5, 1, 3, and 10 mg/2 ml) administered i.c.v. induced a dose-dependent decrease in the number and duration of SWDs that reached a peak at 90 min after administration and the effect was maintained until the end of the recording (4 h after infusion); moreover ii) pretreatment with SR141716 (CB1 receptor antagonist/ inverse agonist) completely blocked the seizure suppressing effects of AEA during the entire recording (R. Citraro, Russo, Scicchitano, et al., 2013). In addition, it has been shown that focal and bilateral injection of AEA, in the thalamocortical regions, namely the NRT, VPM, and S1p, reduces the seizure frequency and the total time spent in seizures, recorded in the EEG in the WAG/Rij animal model of absence epilepsy (R. Citraro, Russo, Ngomba, et al., 2013). It is not clear whether this effect is mediated through CB receptors, or possibly via T-type calcium channels, since it has been shown that can AEA can directly block T-type calcium channels (Chemin, Monteil, Perez-Reyes, Nargeot, & Lory, 2001; R. Citraro, Russo, Ngomba, et al., 2013). These results are not directly comparable with our findings, not only because they have been done in another animal model, but also because they used AEA directly, while we enhanced the tone of naturally produced endocannabinoids indirectly. However, further research should be done to elucidate these findings.

There are many limitations to our study, i) the effect of cannabinoids was tested in the EEG only in GAERS rats, while control strains NEC, and normal Wistar rats were not tested; ii) female GAERS were not used in these experiments; iii) the effect of endocannabinoids was not further evaluated by pretreatment with CB1 receptor antagonists; iv) in the light of the findings by provoking 2-AG enhancement, it would be informative to repeat the experiments by using a DGL inhibitor, to see the effect on SWDs in GAERS rats; v) EEG was only quantitatively analyzed.

The main advantage of our study in comparison to other studies investigating the effects of cannabinoids in the EEG of animal models of absence epilepsy is the presentation of results in 20 min time bins, which provides a more sensitive look to the time-dependent effect of the drugs used.

The complexity of the endocannabinoid signaling in epilepsy is demonstrated by the differences in the response between different models, caused by an interplay of many factors, such as i) different brain regions involved and different expression of CB receptors in the brain; ii) cannabinoids used in research show an effect on multiple other ligand targets, apart from CB receptors; iii) possible desensitization of a variety of ligand targets, producing variable results depending on the time duration, and localization of the treatment (Rosenberg et al., 2017).

Further experiments should be done to investigate the effects of cannabinoids on absence seizure in GAERS rats, it is especially important to investigate the role of cannabinoids by injecting them locally, in the structures of the thalamocortical circuit and to the SNr, a key point of AS modulation by basal ganglia. Moreover, it would be of great value to include control strains and female animals in future experiments.

Conclusion

In conclusion, our results confirm that GAERS is a good model of absence epilepsy, showing not only AS, but also comorbid anxiety. Moreover, we show that AEDs, ETX and VPA, change the behavior of normal Wistar rats towards a more anxious spectrum; nevertheless, more research should be done on these interesting findings of the influence of AEDs on affective behavior, to elucidate the exact mechanisms involved. Furthermore, we show that seizure severity is in correlation with the anxiety level, a finding that adds valuable information to the study of bidirectionality between AS and affective disorders.

In regards to cannabinoid control of absence seizures, we show that direct activation of the cannabinoid system by systemic administration of the CB1 receptor agonist, aggravates SWDs in GAERS. On the other hand, CBD, a very well-known and extensively studied phytocannabinoid, does not have an effect on seizures in GAERS rats, which is in line with findings in children with absence seizures. Enhancement of AEA tone, did not have an effect, while enhancement of 2-AG tone reduced seizure length in GAERS rats.

Our study adds important data to the scarce knowledge on the cannabinoid control of absence seizures in GAERS and provides a comprehensive understanding of the effects of systematically administered cannabinoids on absence seizures, while also providing a foundation for future research on complex cannabinoid signaling in the brain.

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