Università degli Studi di Verona

Department of Neurosciences,
Biomedicine, and Movement Sciences

Graduate School of Life and Health Science

doctoral program in

Neuroscience,
Psychological and Psychiatric Sciences,
and Movement Sciences

Cycle XXXVI, 2020

doctoral thesis

Brain excitability and synaptic plasticity in experimental epilepsy.

S.S.D. BIO/17

Coordinator: Prof. Michela Rimondini, PhD

Tutor: Prof. Giuseppe Bertini, MD, PhD

Doctoral Student: Dott. Valentina Salari

Table of Contents

Abstract

Epilepsy is a chronic neurological disorder characterized by repeated seizures, i.e. periods of paroxysmal brain activity commonly attributed to an imbalance between excitatory and inhibitory neuronal pathways. Understanding the cellular and molecular mechanisms underlying brain hyperexcitability is of central importance in epilepsy research.

Here, I report two separate studies based on experimental mouse models of epilepsy aimed at: 1) detecting and quantifying mechanisms of synaptic plasticity in an epileptic encephalopathy syndrome; and 2) describing the pattern of expression of an ATP receptor associated with epileptogenesis in a model of temporal lobe epilepsy, as well as understanding its contribution to the disorder.

Study 1. An inbred mouse strain, the A/J JAX, has been shown to present repeated spike-wave discharges (SWD) during slow-wave sleep, resembling an insidious, drug-resistant epileptic syndrome of childhood. We carried out, in both affected and control (A/J OLA) mice, in-vivo electrophysiological recordings and histological analyses of brain tissue, aimed at detecting and quantifying both excitatory and inhibitory synaptic contacts, with and without extended training in a motor learning task. Unlike OLA controls, all JAX mice examined at a young age were affected by spontaneously occurring SWDs and failed to improve in the performance of the task after training. In older JAX mice, SWDs were instead absent, in line with the progressive disappearance of discharges described over the years in human patients. Compared to the OLA group, JAX mice showed higher numbers of glutamatergic synapses in the cerebral cortex as well as a lower number of GABAergic contacts, a pattern that was modified by motor training, with differential effects on the two strains.

Study 2. Temporal lobe epilepsy is the most frequent type of epilepsy in adult patients and the most common cause of drug-resistant seizures. The P2X7 receptor (P2X7R) is increasingly recognized to contribute to pathological neuroinflammation and brain hyperexcitability and has been postulated as a treatment target for epilepsy. Its inhibition can, however, produce both pro- and anti-seizure effects. To understand the basis for these opposing actions, we generated mice lacking the P2rx7 gene in either microglia (P2rx7:Cx3cr1-Cre) or neurons (P2rx7:Thy-1-Cre). Mice lacking P2rx7 in microglia displayed less severe acute seizures and developed a milder form of epilepsy and a molecular profile characteristic of an

anti-inflammatory phenotype in microglia. In contrast, mice lacking P2X7R in neurons showed more severe evoked seizures and developed more frequent spontaneous seizures. Our results provide an explanation for the opposing actions of P2X7R in epilepsy and pave the way for investigating which neuronal subtypes are involved in this mechanism.

1. Introduction

1.1. Human epilepsy

Epilepsy is a widespread, chronic neurological disorder affecting over 50 million people worldwide (Beghi 2020). According to a recent International League Against Epilepsy (ILAE) definition, epilepsy is a "disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by [its] consequences" (Fisher et al. 2017). In turn, an epileptic seizure is a "transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher et al. 2017).

Importantly, the terms seizure and epilepsy are not equivalent. Indeed, up to 5% of the world's population may have a single seizure at some time in their lives, but a diagnosis of epilepsy is reserved to those who have at least two unprovoked recurring seizures at least 24 hours apart (Huff and Murr 2023; Fisher et al. 2005; Fisher et al. 2014).

1.1.1. Classification

Rather than a specific disease, the term epilepsy encompasses a variety of different diseases and conditions (Fisher et al. 2014), whose classification has proven challenging. Based on decades of research and clinical experience, in 2017 the ILAE drafted the most up-to-date operational classification of seizure types and epilepsies consisting of a multilevel classification (Scheffer et al. 2017, Figure 1).

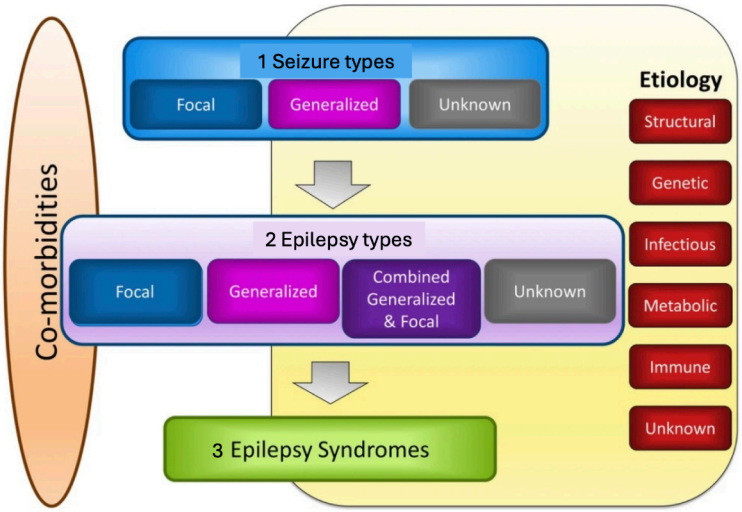

Figure 1. ILAE classification of epilepsies (adapted from Scheffer et al., 2017).

According to this new classification, the first step to classify epilepsy is to define the seizure type, assuming that the clinician already made a definite diagnosis of an epileptic seizure. The second step is to define the epilepsy type, assuming the patient is diagnosed with epilepsy based on the 2014 definition (Fisher et al. 2014). In this regard, a proper diagnosis of epilepsy may present challenges (Benbadis 2009) related to the presence of confounding conditions leading to erroneous interpretation of the symptoms, such as non-epileptic events mimicking epileptic seizures or real seizures caused by a non-neurological condition (Blume 2003). Finally, the third level consists of the diagnosis of an epilepsy syndrome.

Step 1: Epileptic seizures can be classified into two principal categories: focal and generalized, but some seizures cannot be included in these two main groups and are classified as having unknown onset (Table 1) (Fisher et al. 2017). In **focal** seizures, the abnormal electrical discharges start in a localized brain area. These discharges may remain localized or spread to the whole brain and become generalized *(secondary generalized seizures)*. Focal seizures can be classified as "aware" or "impaired-awareness seizures" according to the level of consciousness for the duration of the seizure. After considering the level of awareness for focal

Table 1. ILAE Classification of seizure types, expanded version (modified from Fisher *et al.*, 2017).

<table>
<tr><th colspan="2">Focal Onset</th><th>Generalized Onset</th><th>Unknown Onset</th></tr>
<tr><td>Aware</td><td>Impaired Awarness</td><td rowspan="4">Motor
Tonic-clonic
Tonic
Clonic
Myoclonic
Myoclonic tonic-clonic
Myoclonic-atonic
Atonic
Epileptic Spasm

Nonmotor
Typica
Atypical
Myoclonic
Eyelid Mioclonia</td><td rowspan="2">Motor
Tonic-clonic
Epileptic Spasm

Nonmotor
Behavior arrest</td></tr>
<tr><td colspan="2">Motor Onset
Automatism
Atonic
Clonic
Epileptic Spasm
Hyperkinetc
Myoclonic
Tonic

Nonmotor Onset
Autonomic
Behavior arrest
Cognitive
Emotional
Sensory</td></tr>
<tr><td colspan="2">Focal to bilateal tonic-clonic</td><td>Unclassified</td></tr>
</table>

seizures, the next step is to establish the onset as "motor" and "non-motor," including more detailed subdivisions, e.g., motor: automatism, atonic, clonic; non-motor: autonomic, cognitive, emotional. In **generalized** seizures, the abnormal discharges start from both sides of the brain simultaneously. In this classification, the step that defines the level of awareness is omitted since awareness is impaired in most generalized seizures. The distinction between "motor" and "non-motor (absence)" is used here as well.

Step 2: Epilepsy can be classified into four categories: generalized epilepsies, focal epilepsies, combined generalized and focal epilepsies, and unknown epilepsies. In generalized epilepsy, the patient has a typical generalized spike-wave activity on electroencephalography (EEG). Focal epilepsies involve unifocal and multifocal disorders or seizures involving one hemisphere. Combined generalized and focal epilepsies refer to those patients who have both generalized and focal seizures. The term "unknown" is used when the patient has epilepsy, but the clinician is not able to determine if the epilepsy is focal or generalized because of a lack of information. The epilepsy type may also be the final step of classification when the clinician is not able to make an epilepsy syndrome diagnosis.

Step 3: The epilepsy syndrome diagnosis summarizes a cluster of features incorporating seizure types, EEG, and imaging features that tend to occur together. It may also have distinctive comorbidities and associated etiologic, prognostic, and treatment implications (Figure 1) (Scheffer et al. 2017).

1.1.2. Etiology

Epilepsy can be caused by a variety of factors and conditions, which can be subdivided into six etiological categories.

Structural: Certain structural abnormalities in the brain, usually detectable with neuroimaging techniques, have a high risk of being associated with epilepsy. Such alterations may be due to strokes, trauma, tumors, infections, or congenital malformations such as cortical dysplasia (Ettinger 1994).

Genetic: This category encompasses conditions associated with a known or presumed genetic mutation, in which seizures are the main manifestations. The disorder may come from a single gene or a copy number variant with more severe effects. An example is Dravet syndrome, in which the majority of the patients have a mutation of the SCN1A gene (McTague et al. 2016), and the benign familial neona-

tal epilepsy syndrome, where most families carry a mutation of the potassium channel genes KCNQ2 or KCNQ3. It is important to emphasize that "genetic" does not necessarily mean inherited. Indeed, an ever-growing number of de-novo mutations are being identified in epilepsy.

Infectious: These epilepsies are caused by a known infection, in which seizures are the main symptom, or they can develop at a later time as a postinfectious consequence. Common infections that can lead to this kind of epilepsy include neurocysticercosis, human immunodeficiency virus (HIV), cerebral malaria, and cerebral toxoplasmosis (Vezzani et al. 2016).

Metabolic: Epilepsies resulting from an inherited metabolic disorder such as uremia, porphyria, and aminoacidopathies (Tumiene et al. 2022).

Immune: this group includes epilepsies directly linked to an auto-immune response, i.e. when there is evidence of autoimmune-mediated central nervous system (CNS) inflammation and patients are seropositive for neural-specific antibodies, such as anti-NMDA (N-methyl-d-aspartate) receptor encephalitis and anti-LGI1 encephalitis (Lancaster and Dalmau 2012).

Unknown: This category includes all the epilepsies whose causes are unknown.

The identification of a type of etiology may depend on the circumstances since a patient's epilepsy may be classified into more than one etiologic group. For example, epilepsy in a patient with tuberous sclerosis can be attributed to both a genetic and a structural etiology (Scheffer et al. 2017).

1.1.3. Epidemiology

Epilepsy does not know geographical, ethnic, or social confines. It occurs in both women and men and can begin at any age, but is most frequently diagnosed in childhood, adolescence, and advanced age. (Wirrell et al. 2011; Sen et al. 2020)

It has been estimated that the mean worldwide prevalence of active epilepsy (i.e. in the presence of recurring seizures or ongoing treatment) is approximately 8.2 per 1,000 of the general population. However, studies in developing countries suggest a prevalence in excess of 10 per 1,000.

In developed countries, the annual incidence of epilepsy is more or less 50 per 100,000 of the overall population. In developing countries, however, studies suggest a nearly double figure, i.e. around 100 per 100,000. One of the leading rea-

sons for the higher incidence of epilepsy in developing countries is the higher risk of experiencing conditions leading to permanent brain damage. These conditions include neurocysticercosis, meningitis, malaria, pre- and perinatal complications, and malnutrition (WHO | Epilepsy 2017). Furthermore, in developing countries, there is a more conspicuous number of patients who are refractory to treatment ("pharmacoresistant" epilepsy). Due to the above reasons and/or inadequate access to health care, the administration of appropriate antiseizure drugs (ASDs) can be delayed. In many cases, it may take several months or even years before an epileptic patient is eventually taken to consultation, by which time the treatment frequently turns out to be ineffective (Chakir et al. 2006).

1.1.4. Diagnostic evaluation

As stated above, the first step in diagnosing epilepsy is that the patient has at least two unprovoked seizures at least 24 hours apart (Fisher et al. 2014). Then, where possible, all three diagnostic levels described above should be sought.

A correct diagnosis of epilepsy is the result of a combination of different evaluations, including physical examination followed by electroencephalography (EEG) recording, blood tests, and structural and functional neuroimaging (such as computed tomography (CT) scan or magnetic resonance imaging (MRI) scan) (Bandopadhyay et al. 2021).

EEG is considered the gold standard method for diagnosis, allowing the monitoring of brain activity by non-invasive electrodes placed on the scalp. EEG evaluates the spontaneous electrical activity in the brain, measuring voltage fluctuations resulting from ionic currents (e.g., Na^+, K^+, Ca^{++}, and Cl^-). EEG signals result from the combined activity of a large number of cortical neurons in the vicinity of the electrode.

1.1.5. Status epilepticus

Status epilepticus (SE) is an extreme form of seizure that can cause irreversible brain damage and even death. While a "normal" seizure is self-limiting in time, SE is characterized by seizures of prolonged duration. According to a traditional definition, SE can be diagnosed when a seizure lasts more than 30 minutes. More recently, the threshold has been substantially reduced. In the case of generalized tonic-clonic convulsions, a seizure lasting more than 5 minutes is considered

a medical emergency requiring immediate pharmacological intervention; a duration of 30 minutes or more is likely to cause severe brain damage (Trinka et al. 2015).

1.1.6. Treatment

At present, there are no effective treatments for epilepsy. All currently available drugs are used to prevent or control seizures without altering the underlying disease. A good control of seizures with proper pharmacological treatment can be achieved in about 70% of patients (Fattorusso et al. 2021).

Antiseizure drugs (ASDs) act by reducing the tendency of the brain to be hyper-activated, both by increasing the inhibition or decreasing the excitation. Both the electrical activity (ion channels) and chemical neurotransmission can be targeted by drugs. For example, inhibiting voltage-gated sodium channels decreases neuronal excitation by avoiding depolarization, while inhibiting voltage-gated calcium channels prevents neurotransmitter release. Instead, drugs binding γ-aminobutyric acid (GABA) receptors can be used to enhance the inhibitory pathway (Rogawski and Löscher 2004).

Depending on the type of epilepsy, the administration of the proper drug is the first line of treatment. If seizures persist, other strategies may be employed, from dietary changes to surgical resection of lesioned brain tissue (Riva et al. 2021). In older epileptic patients, pharmacotherapy is more complex than in other age groups because of aging-related changes in pharmacokinetics and pharmacodynamics. Additionally, the elderly population often takes multiple drugs because of other comorbidities, which requires careful consideration to avoid possible toxic drug-drug interactions (Sen et al. 2020).

Despite the development of a significant number of ASDs in the last two decades, at least 30% of patients suffer from drug-resistant epilepsy (DRE). This means they remain refractory to common pharmacological treatments (Picot et al. 2008).

The ILAE defines DRE as “failure of adequate trials of two tolerated, appropriately chosen and used ASDs schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom” (Kwan et al. 2010). Individuals experiencing DRE face elevated risks of premature death, injuries, psychosocial dysfunction, and a diminished quality of life. Consequently, there is an urgent clinical demand for the development of more efficacious therapies. However, the diverse

forms of epilepsy, the varying types of seizures, and the intricate temporal patterns of refractoriness contribute to the complexity of this issue.

Two types of epilepsy frequently characterized by drug resistance are temporal lobe epilepsy (TLE) and epileptic encephalopathies (EE), both relevant for the present project and described here in some detail.

1.1.7. Temporal Lobe Epilepsy

TLE is the most common form of focal (partial) epilepsy and, as stated above, the most common cause of drug-resistant seizures. While antiseizure drugs can often control or at least reduce seizure occurrence, approximately one-third of patients remain resistant to pharmacological treatment (Löscher & Potschka, 2005; "WHO | Epilepsy," 2017). Surgical methods, such as anterior temporal lobectomy and amygdalo-hippocampectomy, reach control rates in 67-85% of patients, with low morbidity and mortality rates. Although the prognosis depends on several factors, surgical treatment can significantly improve the quality of life of these patients (Volcy, 2004).

The term TLE commonly refers to epilepsy presentations marked by focal seizures attributed to lesions within the temporal lobe or influenced by structures within the temporal lobe. There are two types of TLE. The primary and prevalent subtype is medial TLE, characterized by the involvement of the internal structures of the temporal lobe, while the second subtype, termed neocortical TLE, involves the outer cortical regions of the temporal lobe.

In clinical practice, TLE manifests seizures originating from the hippocampus, entorhinal cortex, or amygdala, and the diagnosis is based mainly on electroencephalography and magnetic resonance imaging results.

TLE is more difficult to recognize in children than in adults. Ictal symptoms in children are less stereotyped and less noticeable, and the neuropathological substrate is more heterogeneous than in adults (Fontana et al., 2006).

1.1.8. Epileptic encephalopathies

Epileptic encephalopathies (EEs) are a group of developmental syndromes in which repeated seizure activity is considered responsible for brain damage, leading to the slowing of cognitive function and the evolution of severe behavioral disorders. (Berg et al. 2010; Khan and Al Baradie 2012).

These disorders vary in etiology, age of onset, developmental outcome, EEG patterns, neuropsychological deficits, type of seizures, and prognosis. In spite of this, all of them may have a devastating impact on children's neurodevelopment (Kural and Ozer 2012) and are usually refractory to AED treatment (Donat 1992).

According to the most recent ILAE classification, as many as eight distinct conditions can be included in the EE spectrum. As this concept evolves, other syndromes may be incorporated (Table 2). All these pathologies are characterized by:

1. aggressive EEG paroxysmal activity;
2. multiform and often intractable seizures;
3. persistence of cognitive, behavioral, and neurological deficits;
4. in the most serious cases, early death (Scheffer et al. 2017).

Different types of EE preferentially affect specific age ranges, spanning early infancy to late childhood and early adolescence (Table 2). The diffuse epileptic activity through this expanded developmental period has a devastating impact on long-term cognitive and behavioral outcomes (Zuberi et al. 2022).

An EE condition characterized by diffuse spike-and-wave discharges (SWD, Figure 2) concentrated during slow-wave sleep (SWS), has been termed "encephalopathy related to electrical *status epilepticus* during slow sleep" (ESES) (Rubboli et al. 2023; Tassinari et al. 2012). The syndrome onset is between 2 and 12 years of age (with a peak around 4-5 years), and the abnormal EEG activity can be almost con-

Table 2. Classification of Epileptic Encephalopathies.

Neonatal period	Infancy	Childhood	Other severe epileptic encephalopathies
Early myoclonic encephalopathy (EME)	Epilepsy of infancy with migrating focal seizures	Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS) (including Landau-Kleffner syndrome)	Kozhevnikov-RasmUssen syndrome
Ohtahara syndrome	West syndrome		Fever-induced refractory epileptic encephalopathy
	Dravet syndrome	Lennox-Gastaut syndrome	
	Myoclonic encephalopathy in nonprogressive disorders		Hemiconvulsion-hemiplegia-epilepsy syndrome

tinuous (up to 85% of the total SWS time) and can persist for months or years, a period during which various neurological and neuropsychological deficits can develop. Although ESES syndromes are considered relatively uncommon, they are particularly insidious. Since SWDs are selectively concentrated during SWS and lack overt clinical signs, the electrical SE is subclinical. Thus, neurological disturbances are often misdiagnosed as autism spectrum disorders, and ESES cases are probably underestimated (Issa 2014). In addition, as already mentioned, ESES is a typically drug-resistant condition.

ESES syndromes affect a variety of cognitive and behavioral functions. A striking example is the subacute onset of aphasia, which is the hallmark of Landau-Kleffner syndrome (Nieuwenhuis and Nicolai 2006). Specifically, children between 2 and 8 years old, with previously normal language development, present "verbal auditory agnosia," a failure to give a semantic significance to sounds, thus causing the inability to understand spoken words. The agnosia is followed by progressive loss of articulation and reduction in the amount of speech.

More generally, all ESES conditions can lead to lower IQs, deterioration of language, temporo-spatial disorientation, behavioral changes, reduced attention span

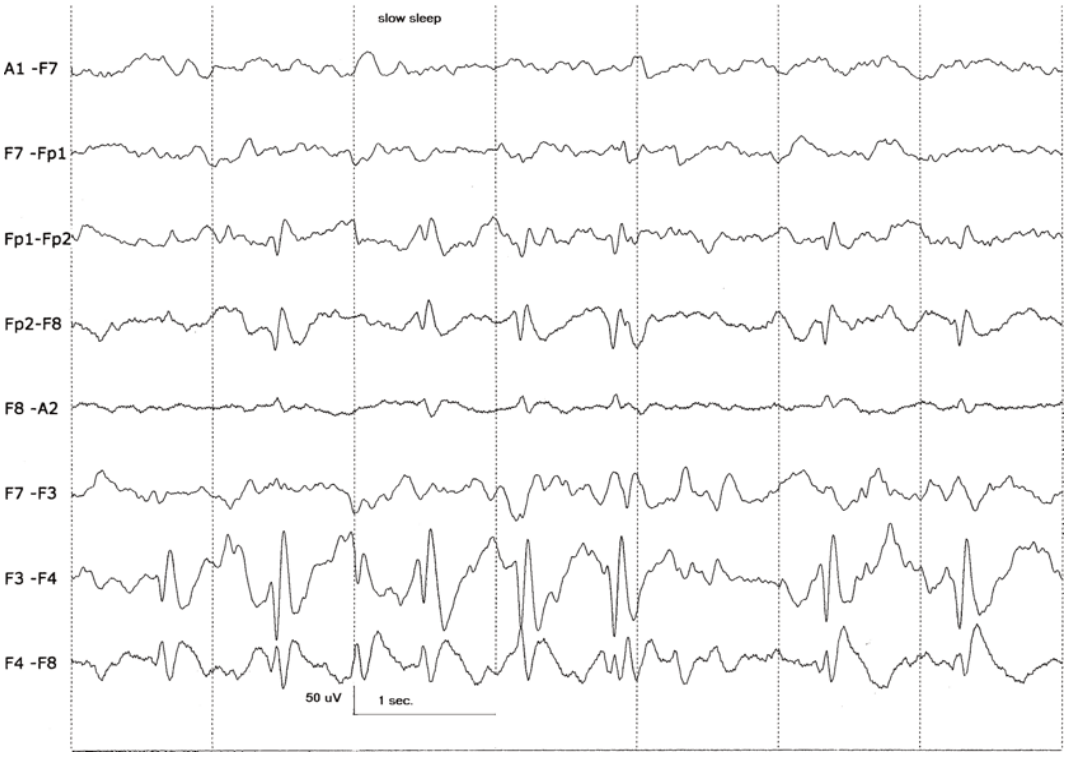

Figure 2. Typical example of sleep EEG recording with continuous spike-and-wave discharges (SWD) in the right frontal lobe. Modified from Caraballo et al., 2015.

and hyperactivity, aggressiveness, impaired social interaction, and communication (Tassinari et al. 2012). Motor impairments have also been reported, including dystonia, dyspraxia, and ataxia. While the electrical abnormalities tend to reduce over the years and eventually disappear, the neuropsychological consequences of ESES can persist and may result in permanent deficits.

1.2. Animal models of seizure and epilepsy

Epilepsy represents a significant disease burden worldwide, which underscores the need for more research into new approaches for the diagnosis, treatment, and prevention of epilepsy and its consequences. Experimental research carried out in human patients with epilepsy has obvious limitations (Engel et al. 1998). First and foremost, ethical considerations impose strict constraints on the procedures that can be applied, particularly those associated with the invasive techniques needed to pursue fundamental investigative questions. In addition, controlling clinical variables is inherently difficult, and control data may not be possible to obtain. Statistical analysis frequently requires large populations that cannot be obtained from most clinical practices. Finally, the cost of conducting research projects on patients can be prohibitive. Consequently, despite a tremendous increase in opportunities for non-invasive research on the human brain provided by modern neuroimaging, as well as access to direct investigations in the epilepsy surgery setting, animal models of epilepsy and epileptic seizures are and most likely will remain for the foreseeable future essential to epilepsy research.

In general, establishing and validating an epilepsy animal model requires one of two different approaches: the observation of clinical manifestations of epilepsy in animals carrying known or unknown genetic modifications, and inducing epilepsy in an otherwise normal animal. With the advantage of techniques such as CRISPR/Cas9 gene editing, known mutations can be introduced in murine embryonic stem cells. The mouse resulting from these mutated cells will develop spontaneous seizures.

Induced epilepsy consists of the electrical or chemical stimulation of the brain, precipitating seizure episodes. The electrical stimulation is characterized by the implantation of deep electrodes in the limbic regions or the topical stimulation of the corneas. Chemical stimulation instead involves the systemic or intracerebral injection of excitatory agents, leading to seizure onset (Figure 3) (Löscher 2011).

Incidentally, in the present project, we utilized a genetic and an induced animal model of epilepsy to simulate, respectively, ESES and TLE.

1.2.1. Induced mouse models

A distinction has to be made between seizure models and epilepsy models. The former do not necessarily result in chronic epilepsy, and they are useful to test new anti-epileptic drugs. Compounds often used to trigger acute seizures are pentylenetetrazol (PTZ), strychnine, N-methyl-D,L-aspartate, tetanus toxin, and penicillin (Kandratavicius et al. 2014).

The induction of chronic epilepsy in rodents can also be obtained in several ways. Over the past four decades, animal models mirroring features observed in patients with TLE have been established to comprehend its pathophysiology and, consequently, to develop novel pharmacological treatments. The primary chemoconvulsant agents employed to induce TLE in rodents are pilocarpine and kainic acid (Leite et al. 2002; Cavalheiro 1995).

The pilocarpine model of temporal lobe epilepsy: Pilocarpine is a muscarinic acetylcholine receptor agonist, and its intraperitoneal (i.p.) or intracerebral

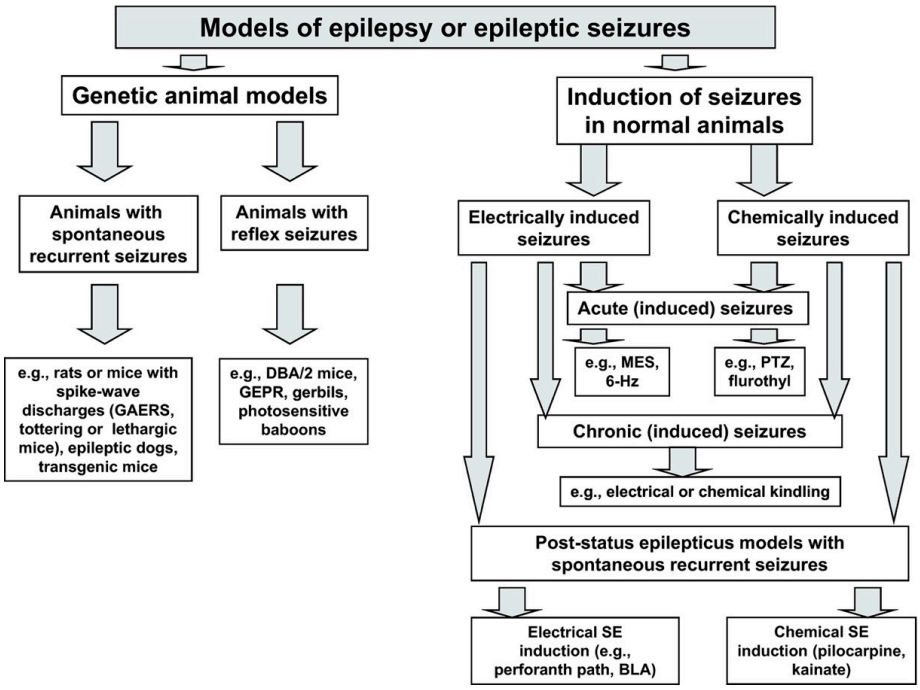

Figure 3. Schematic overview of models of epilepsy and epileptic seizures. Note that there are numerous models not shown in this figure, including chronic epilepsy models in which spontaneous recurrent seizures develop after traumatic brain injury, ischemic brain damage, or febrile seizures. Adapted from Loscher et al., 2011.

injection triggers a sequence of events, which eventually leads to structural brain damage and to the emergence of spontaneous recurrent seizures. These seizures closely resemble the characteristics of complex partial seizures in humans. Pilocarpine administration causes hippocampal lesions, but it also damages the neocortex (Cavalheiro 1995; Sharma et al. 2007; Wang et al. 2022).

The kainic acid model of temporal lobe epilepsy: Kainic acid (KA) is a cyclic analog of L-glutamate that specifically binds ionotropic KA receptors (KARs). When administered systemically or intracerebrally, it causes an excitotoxicity cascade characterized by neuronal hyperstimulation stemming from excessive depolarization (Sharma et al. 2007). Specifically, depolarization causes an excessive calcium influx, which leads to i) activation of phospholipase, endonucleases, and proteases and ii) mitochondrial dysfunction. In turn, this leads to increased reactive oxygen species (ROS) production, culminating in cell apoptosis (Beck et al. 2003; Rusina et al. 2021). Rodents injected with KA exhibit recurrent seizures with variable frequency. The KA model usually causes hippocampal-restricted injuries and is associated with hippocampal sclerosis. Specifically, KA provokes neuronal loss in the hippocampal hilus and CA1/CA3 regions and aberrant sprouting of granule cell axons (mossy fibers) into the inner molecular layer of the dentate gyrus (Raedt et al. 2009; Buckmaster ; Lévesque and Avoli 2013; Wang et al. 2022).

KA can be administered via different modalities. The most commonly used are systemic, intracerebral (intraventricular, intrahippocampal, supra-hippocampal, intra-amygdaloid), and intranasal. KA administration triggers SE, characterized by continuous seizure activity as described above. SE is usually followed by a latent period in which seizures are absent, culminating in the eventual onset of chronic epilepsy (Reddy and Kuruba 2013; Rusina et al. 2021).

1.2.2. Genetic mouse models

In both human epilepsy and animal models, mutations in ion channels are associated with one or multiple seizure activity patterns, with the majority exhibiting dominant Mendelian inheritance patterns (Steinlein and Noebels 2000). The identification of spontaneous, genetic epilepsies in mice has proven invaluable for modeling human epilepsies (Noebels and Sidman 1979; Seyfried and Glaser 1985). Moreover, with advancements in gene editing techniques, transgenic mice now exist to mimic specific human epileptic conditions (Yu et al. 2006; Zeng et al. 2008). Here, we focus on models of ESES.

A promising model of ESES is the mouse knockout for the Grin2a gene ($Grin2a^{-/-}$). This gene encodes for the α2 subunit of N-methyl-D-aspartate (NMDA) glutamatergic receptor (GluN2A), and it has been shown that microdeletions, nonsense, splice-site and missense mutations of GRIN2A gene are associated with human ESES, including Landau Kleffner's (Lesca et al. 2013; Strehlow et al. 2022; Carvill et al. 2013).

Another interesting model is the A/J mouse from Jackson Laboratories. The A/J inbred mouse is widley used in immunology applications, cardiovascular research and developmental biology. Several years ago, the A/J mouse bred at the Jacksons Laboratories (A/J JAX or simply JAX, for short) has been shown to present spontaneous spike-wave discharges, mainly concentrated during non-rapid eye movement (REM) sleep, similar to those observed in human patients with ESES (Strohl et al. 2007; Letts et al. 2014). The presence of an epileptic phenotype in JAX mice but not in mice purchased from other vendors suggests the occurrence of an unknown mutation within the A/J line at Jacksons Laboratories. Like "absence" events observed in prior studies with mice and rats, the spike-and-wave activity can be mitigated or eliminated by antiseizure medications. This suggests that the JAX strain exhibits an epilepsy phenotype, which may offer insights into the complex nature of human sleep-related epilepsy.

1.3. Hyperexcitability and synaptic dysfunction in epilepsy

The underlying mechanisms of epilepsy are complex and can involve various factors, including alterations in synaptic transmission (Casillas-Espinosa et al. 2012). Generally speaking, such alterations can lead to an imbalance between excitatory and inhibitory (E/I) neurotransmission, eventually culminating in abnormal synchronization of neuronal firing, which is the hallmark of epileptic seizures.

Two main theories, not mutually exclusive, have been proposed to explain the imbalance between excitatory and inhibitory neurotransmission:

Hyperexcitation: This theory suggests an abnormal increase in excitatory neurotransmission, leading to excessive neuronal activity and seizures. For example, an excessive release of glutamate, the primary excitatory neurotransmitter in the brain, may contribute to hyperexcitation (Niemeyer et al. 2022; Elmér 1997).

Lower Inhibition: Another view is that decreased inhibitory neurotransmission removes a necessary control over excitatory activity. GABA is the primary in-

hibitory neurotransmitter in the brain, and a deficiency in GABAergic inhibition might result in increased neuronal excitability (Sloviter 1987; Sloviter et al. 2012).

Overall changes in neurotransmission can take place in many different ways, including the formation and removal of synaptic contacts, as well as changes in their strength. Synaptic plasticity is crucial for normal brain function, and dysregulation of the underlying mechanisms can contribute to neurological disorders, including epilepsy.

1.3.1. Synaptic plasticity

Synaptic plasticity refers to the ability of synapses—the junctions between neurons—to change in strength and number over time, allowing the neural circuitry of the brain to adapt and reorganize. These modifications span temporal domains ranging from milliseconds to hours or days, and presumably even longer, and are fundamental for learning, memory, and various other cognitive processes (Citri and Malenka 2008)**.**

Short-term plasticity: All those forms of synaptic plasticity that last on the order of milliseconds to several minutes have been termed short-term synaptic plasticity and are thought to play important roles in short-term adaptations to sensory inputs, transient changes in behavioral states, and short-lasting forms of memory. Usually, these events are initiated by brief bursts of activity, leading to a transient accumulation of calcium within the presynaptic terminals. This increase in presynaptic calcium changes the probability of exocytosis of synaptic vesicles by directly influencing the release of neurotransmitters (Zucker and Regehr 2002).

Long-term plasticity: It is generally recognized that experiences can influence future behavior by inducing long-lasting modification of synaptic strength. This hypothesis implies that the storage of new information (i.e., the formation of memories) occurs when activity in a neural circuit leads to a persistent change in the pattern of synaptic weights. This idea originated in the late 1940s when Donald Hebb suggested that associative memories originated from synaptic modifications that enhance connections when there is a correlation between presynaptic activity and postsynaptic firing (Hebb 1949). This concept provided a cellular basis for phenomena like Pavlovian classical conditioning (Pavlov 2010). However, experimental evidence supporting the theory of activity-dependent synaptic changes was not available until the early 1970s when Bliss and colleagues demonstrated that repetitive stimulation of excitatory synapses in the hippocampus induced long-lasting po-

tentiation of synaptic strength (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973). This phenomenon was eventually termed long-term potentiation (LTP) and has been extensively studied over the last decades as a key mechanism for memory formation (Martin et al. 2000; Pastalkova et al. 2006).

Nevertheless, it has become evident that LTP represents just one variant of long-term synaptic plasticity in the mammalian brain. In this regard, many synapses capable of LTP can also decrease the strength of synaptic connections through an opposite phenomenon called long-term depression.

Recent research has identified additional forms of synaptic plasticity, including homeostatic plasticity and metaplasticity (Turrigiano and Nelson 2004; Abraham and Bear 1996). Homeostatic plasticity is a phenomenon known as "synaptic scaling" that involves global adjustments in synaptic strength across neurons in response to sustained changes in network activity. In contrast, metaplasticity refers to activity-dependent modifications in the synaptic ability to undergo long-term plasticity to modulate the threshold for subsequent synaptic modifications. Together, these findings highlight the complexity and diversity of synaptic plasticity mechanisms that contribute to the dynamic regulation of neural circuits, which is crucial for learning, memory, and normal brain functions.

Dynamic changes in the number of synaptic contacts: The above mechanisms refer to the ability of preexisting synapses to change in strength and structure over time. However, the number and distribution of synaptic contacts change continuously within the mature brain at an astonishing high rate (Attardo et al. 2015; Südhof 2021). As demonstrated in several studies, the dynamic turnover in the number of synapses aligns with the continued expression of proteins implicated in synapse formation throughout life (Saunders et al. 2018; Chen et al. 2020; Tabula Muris Consortium et al. 2018). We relied on the notion of rapid formation and removal of synapses in the design of Study 1.

1.3.2. Long-term synaptic plasticity in epilepsy

The process of remodeling brain circuits can occur in any life period in response to a variety of stimuli, including brain damage (Wall and Egger 1971). While plastic changes can compensate for brain damage, in some cases they may contribute to the development of chronic neurological disorders or neurodegenerative conditions (Ismail et al. 2017).

In a healthy brain, neuronal excitability and synaptic strength are homeostatically regulated to keep neuronal network activity within physiological boundaries. On the other hand, epileptogenesis is a complex and poorly understood process, in which neuroplastic changes are believed to predispose the brain to develop seizures (Pitkänen and Sutula 2002). A wide range of cellular and molecular changes have been hypothesized to contribute to neuronal loss, inflammation, compromised blood-brain barrier permeability, angiogenesis, neurogenesis, axonal sprouting, and synaptogenesis, eventually leading to the reorganization of neuronal networks (Patel et al. 2019; Bertini et al. 2013; Fabene et al. 2013).

Several studies have shown alterations in short- and long-term synaptic plasticity responses in the hippocampus and cerebral cortex following epileptic seizures (Reid and Stewart 1997; Zhou et al. 2007; Abegg et al. 2004), which may explain the cognitive deficits observed in patients with uncontrolled epilepsy. Therefore, epilepsy and neuroplasticity seem to be closely related, as the two processes can reciprocally affect each other. However, it is currently unclear whether it is a disturbance in physiological synaptic plasticity that leads to epilepsy or *vice versa*.

1.3.3. Role of sleep in normal brain plasticity

Despite the existing massive body of scientific knowledge and rapidly accumulating new evidence, sleep remains one of the most elusive brain functions. Sleep is extraordinarily preserved in phylogeny, and prolonged sleep deprivation has been shown to lead to severe illness and even death. Still, a convincing, unifying explanation for why we spend a substantial proportion of our lives in a state of behavioral inactivity and limited consciousness is not yet available.

Among other fields of investigation, a crucial role for sleep in synaptic plasticity has long been hypothesized, with a specific emphasis on the facilitating effect of sleep in consolidating recently acquired memories, both declarative and procedural. There is abundant evidence, for example, that retention and recall of previous experience are enhanced by an intervening period of undisturbed sleep, compared to a comparable length of time spent in wakefulness (Diekelmann and Born 2010).

Different mechanisms have been proposed as a neurobiological basis for sleep-dependent neural plasticity. A notable hypothesis posits that synaptogenesis is generally high during waking hours, with a tendency to form synapses in excess

and/or to strengthen existing ones. As a homeostatic control mechanism, synchronized sleep is accompanied by generalized synaptic "downscaling," leading to improved signal-to-noise ratio for salient, behaviorally relevant connections (Tononi and Cirelli 2003). On the other hand, there is evidence that normal sleep may promote synapse formation (Yang et al. 2014), so a complete picture of how different vigilance states contribute to brain plasticity has yet to be obtained.

In any case, disruption or deprivation of physiological sleep has been repeatedly shown, both in the clinical setting and in the laboratory, to cause transient cognitive impairments (Colavito et al. 2013), including reduced attention, reduced ability to recall information, and reduced improvements in procedural skills, which would be expected following a period of training. Such deficits are likely due to alterations in the neural plasticity mechanisms that normally occur during sleep. For example, mice trained in the Rotarod apparatus progressively develop the ability to hang on to a suspended, rotating rod and a period of such motor learning has been shown to promote dendritic spine formation and remodeling in the mouse cortex (Yang et al. 2009). In addition, it has been reported that several hours of sleep deprivation following a training session significantly interferes with the phenomenon (Yang et al. 2014).

1.4. Role of purinergic signaling in central nervous system

Purinergic receptors, or purinoceptors, are plasma membrane proteins located on the cell surfaces of nearly all mammalian tissues, including the central nervous system (CNS) (Tozaki-Saitoh, Tsuda, and Inoue 2011). Based on their endogenous ligands, they are classified into P1 and P2 categories (Geoffrey Burnstock 2008). P1 or adenosine receptors (ARs) are a family of G protein-coupled receptors (GPCRs) that include four subtypes: A1, A2A, A2B, and A3. P2 receptors are divided into two categories: the ligand-gated ion channel receptors P2X, with seven subtypes (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2X7), and the G protein-coupled metabotropic receptors P2Y, which have eight subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) (Geoff Burnstock 2018). Most of these receptors are expressed in the nervous system, including neurons and glial cells in central and peripheral nervous system (CNS and PNS), and they are generally considered to be involved in bi-directional neuronal–astroglial communication, exerting long-term effects on proliferation, differentiation, migration, and apoptosis (Neary and Zimmermann 2009). In the CNS, adenosine 5′-triphosphate (ATP),

known as an energy source for neurons and glial cells, also functions as an extracellular purinergic signaling molecule, regulating communication between brain cells (Geoffrey Burnstock 2006). The cytosolic ATP concentration remains high, typically ranging between 5 and 10 mM, while in the extracellular space, it is very low in the nanomolar (nM) range (Bhattacharya and Biber 2016). However, under pathological conditions and CNS insults such as trauma, ischemic stroke, epileptogenic seizures, cellular stress, neuroinflammation, and neurodegenerative disorders, there is a release of high concentrations of ATP into the extracellular region as a danger signal. This initiates a cascade of events that ultimately leads to neuronal damage (Roszek and Czarnecka 2015).

For all these reasons, in the last decades, purinergic signaling, via extracellularly released ATP, has emerged as a promising therapeutic approach in the field of neurological disorders. This is bolstered by evidence from both experimental models and clinical studies, with a particular focus on the ionotropic P2X7 receptor.

1.4.1. The P2X7 Receptor

The P2X7 receptor (P2X7R) is a ligand-gated ion channel belonging to the purinergic type 2 receptor family (P2) (Abbracchio et al. 2006). Among the P2 receptor family, the P2X7R is one of the most studied and, when activated by a high concentration of ATP, forms a channel for the passage of cations including K^+, Na^+, and Ca^{2+} (Jimenez-Mateos et al. 2019; Kopp et al. 2019). The lower affinity of the receptor for ATP when compared to the remaining P2XRs (Surprenant et al. 1996) suggests the receptor serves as a damage-activated initiator of inflammation. Indeed, a key consequence of P2X7R activation is the release of the pro-inflammatory cytokine interleukin-1β (IL-1 β) (Giuliani et al. 2017). Other effects of P2X7R activation have been reported, including promoting aberrant synaptic plasticity and neurogenesis, changes in blood-brain barrier (BBB) permeability, and cell death (Giuliani et al. 2017).

1.4.2. P2X7 Receptor and Epilepsy

The purinergic P2X7 receptor plays a crucial role in brain disorders. It has been postulated as a treatment target for epilepsy, where it has been shown to contribute to neuroinflammation and the generation of hyperexcitable neuronal networks (Engel et al. 2021; Engel 2023) (Figure 4). Several studies have reported the anticonvulsive effects of blocking or deleting the P2X7R during SE (Engel et al. 2012;

Jimenez-Pacheco et al. 2013; Nieoczym et al. 2017). Other reports, however, have detected either no effect of a P2X7R block on SE (Fischer et al. 2016; Doğan et al. 2020) or even an exacerbation of seizures (Kim and Kang 2011; Rozmer et al. 2017).

There has also been some inconsistency in the effects of pharmacological targeting of the P2X7R in experimental epilepsy, with studies reporting either reduced seizure duration (Amhaoul et al. 2016) or frequency (Jimenez-Pacheco et al. 2013). The cause of such variability in seizure outcomes may be the cell type-specific expression of the P2X7R and how these changes during brain hyperexcitability.

While there is widespread agreement for its presence on glial cells including microglia and oligodendrocytes, it remains debated whether P2X7Rs are expressed and functional on neurons (Teresa Miras-Portugal et al. 2017; Illes et al. 2017). Both cellular locations have been detected using immunohistochemical-based methods in experimental and human epilepsy (Engel et al. 2012; Jimenez-Pacheco et al. 2013; Doná et al. 2009; Beamer et al. 2022). It is uncertain which, if any, neuronal subtypes express P2X7Rs and their specific roles during seizures, and whether similar expression patterns are observed in human TLE.

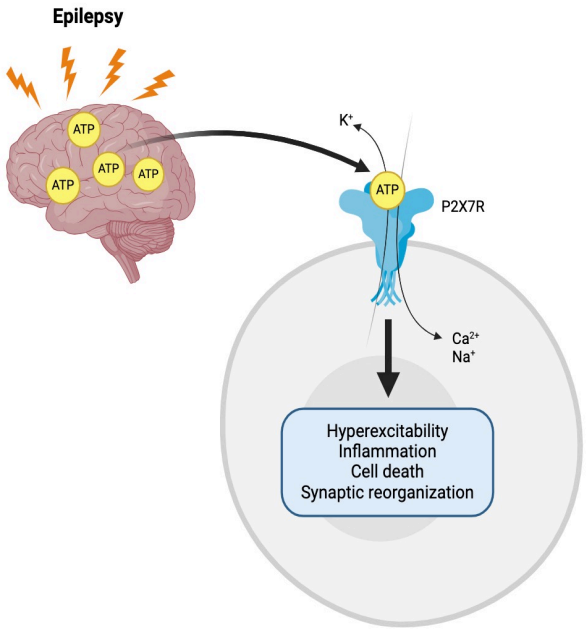

Figure 4. The ATP-P2X7 receptor axis as a major convergence pathway linking hyperexcitability in epilepsy. Seizures and epilepsy lead to high extracellular concentrations of ATP in the brain, activating P2X7Rs. P2X7R activation, in turn, promotes several pathological mechanisms such as inflammation, cell death, aberrant synaptic plasticity, and changes in the release and uptake of neurotransmitters, driving epileptogenesis. Created with BioRender.com

2. Aim of the project

The general goal of my doctoral research program was to gain insights into synaptic mechanisms in epilepsy. To achieve this goal, we set out to perform two distinct sets of experiments in rodent models of drug-resistant epilepsy. Resistance to pharmacological treatment poses significant challenges in the management of epilepsy and is a high priority in epilepsy research. Both epileptic encephalopathies (EE) and temporal lobe epilepsy (TLE) are characterized by drug resistance and a high impact on the patients' well-being.

In **Study 1,** we focused on the role of synaptic plasticity in a subclass of epilepsies, the epileptic encephalopathies with status epilepticus during sleep (ESESs), using a putative rodent model of the human condition, the A/J JAX mouse.

In **Study 2**, centered on the kainic acid model of temporal lobe epilepsy, we aimed to unveil the cell-specific role of the purinergic P2X7 receptor, thought to contribute to neuroinflammation and neuronal hyperexcitability, and postulated as a treatment target for epilepsy.

3. Study 1: Role of synaptic plasticity in the pathogenesis of neurological disorders in epileptic encephalopathies during slow-wave sleep

3.1. Rationale

The present experiment addresses the general question of whether the electrical status epilepticus that characterizes ESESs affects synaptic plasticity and, in turn, if alterations of plasticity may justify the deficits observed in human patients.

We planned to study such patterns by combining in-vivo electrophysiology, training in a procedural memory task, and ex-vivo histological examination of synapse density in target brain areas in a putative mouse model of ESESs (JAX).

3.2. Materials and Methods

3.2.1. Ethics statements

All the experiments were performed at the Department of Neuroscience, Biomedicine and Movement Science (section of Anatomy and Histology) of the University of Verona, Italy.

Animal care and experimental procedures were conducted in accordance with the European Union directive 2010/63/EU and all protocols were approved by the local ethical committee (Centro Interdipartimentale di Servizi per la Ricerca che utilizza Animali da Laboratorio – C.I.R.S.A.L., University of Verona) and the Italian Ministry of Health (Authorisation 239/2016-PR).

3.2.2. Animals

In this study, 4-week-old (w.o.) A/J mice, both males and females, were purchased from Jackson Laboratory (JAX; ♀=10, ♂=11). A similar cohort of A/J mice was acquired from a different vendor (Envigo, A/J OLA[1]; ♀=9, ♂=10). Upon arrival, animals were quarantined in the animal facilities for 2 weeks, with food and water *ad libitum*, under veterinarian care. Mice were then transferred to the labora-

[1] From the original source of the same mouse strain (Harlan Olac LTD.)

tory, in individual cages kept in a sound-attenuated room at a constant temperature (23 ± 1.0 °C) and humidity (60% ± 5%), for an additional 2-week period, during which the room was kept on a 12/12-hour inverted light/dark cycle. Zeitgeber time (ZT) 0, corresponding to lights-on time was set at 7:00 p.m. All mice used in this study entered the experimental protocol at 8 weeks of age, with a weight ranging between 25 and 30 g.

3.2.3. Experimental Design

All 21 JAX animals were used in this study as the **experimental group**, expected to show epileptic discharges. The 19 OLA mice, with presumably normal EEG, represented the **control group** (Figure 5).

The main experimental steps, described in detail below, were as follows:

1) All mice underwent surgery to implant chronic electrodes for long-term EEG and EMG recordings (see below).
2) Following post-surgical recovery, a baseline electrophysiological profile was recorded for one week.
3) During this time, baseline behavioral tests were conducted.
4) Next, OLA and JAX mice were further divided into two subgroups "**trained**" and "**untrained**".
5) Training consisted in repeated sessions of the Rotarod test over two days, in order to trigger motor learning, while "untrained" animals were kept undisturbed in their home cages.

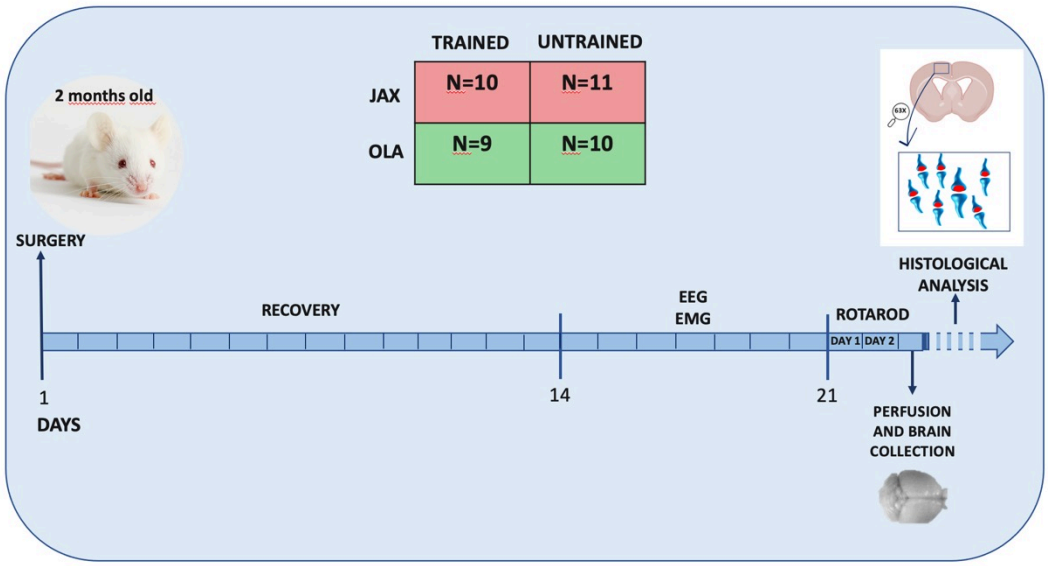

Figure 5. Experimental Design.

6) After training, animals were perfused and brain sections were processed for immunofluorescence studies at the confocal microscope.
7) Glutamatergic and GABAergic synaptic contacts were labeled and quantified in the motor cortex (MC), and in the somatosensory cortex (SSC).

Due to logistical and technical limitations (number of available amplifier channels, number of Rotarod lanes), the above experimental steps were conducted in batches of 6-8 mice each. JAX, OLA, trained, and untrained mice were balanced in each batch.

Two additional cohorts of mice were employed in this study: Six-month-old (m.o.) A/J OLA mice (n=10) were used in a pilot study to assess motor learning in the Rotarod apparatus and define the protocol for subsequent evaluations. Eight m.o. A/J mice (5 JAX and 5 OLA) were used to assess the presence of SWDs in older adult mice.

3.2.4. Chronic electrode surgical implant

In all A/J mice, surgery was performed under deep gas anesthesia (isoflurane: 4% for induction and 2% for maintenance) using an isoflurane vaporizer (Ugo Basile S.R.L (VA). The anesthetized mice were placed in a stereotaxic apparatus under a stereomicroscope frame (Stoelting Co., Wood Dale, IL). After exposing the animal's skull, 4 narrow craniotomies were drilled through the right frontal, right parietal, left parietal, and interparietal bones at commonly adopted (Jakubcakova et al. 2012) stereotaxic coordinates (Franklin and Paxinos 2013) (Figure 6). A stainless-steel screw was inserted into each craniotomy and served as epidural EEG electrode. Short stainless-steel wires were soldered to each screw head and to a plastic connector fixed to the skull with acrylic cement.

Two additional EMG recording electrodes, represented by stainless steel wires, were inserted into the left nuchal muscle and stitched in place. EEG EMG in freely behaving mice is a critical step to correlate behavior and physiology with sleep and wakefulness. In particular, the combination between EEG and EMG enables discrimination between REM sleep and non-REM sleep. At the end of the surgery, the animals were treated subcutaneously (sc) with an analgesic (carprofen, Rimadyl®, 1 mL/kg, 1:10) and a wide-spectrum antibiotic (enrofloxacin, Baytril®, 10 mL/kg, 1:50), and returned to their home cages. Analgesic and antibiotic administrations were repeated daily for the following 2 and 3 days, respectively.

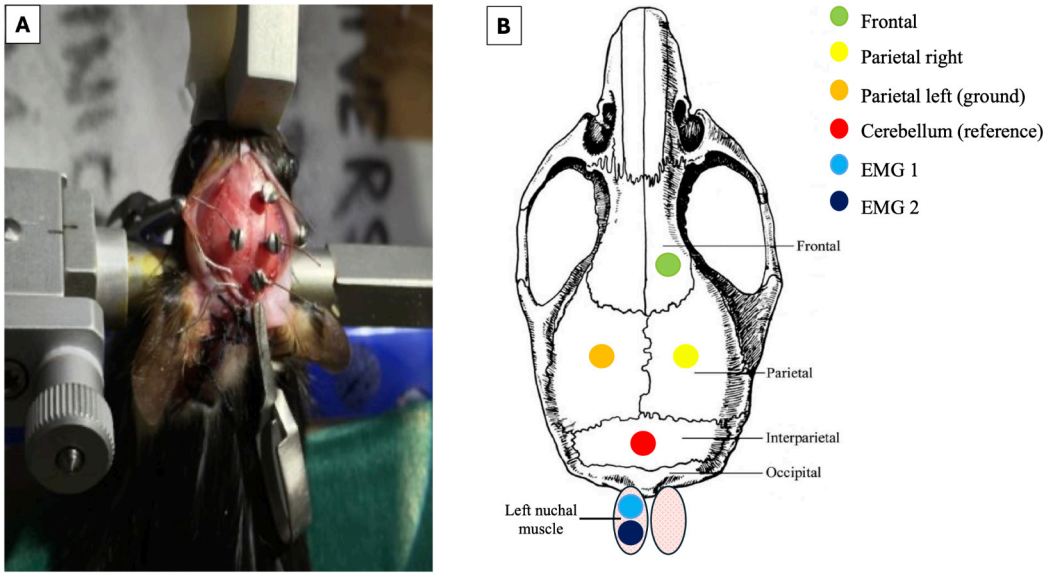

Figure 6. Chronic electrode surgical implant. A) Picture capturing an example electrode location on a mouse skull. B) EEG and EMG electrode locations: one frontal (1.7 mm anterior and 1.5 mm to the right of bregma), two parietal (2.5 mm posterior and 2.0 mm on either side of bregma), and one over the cerebellum (5.8 mm posterior to bregma, on the midline). A bipolar montage was used (frontal vs right parietal). The cerebellar electrode (red) was used as a reference, and the left parietal (orange) was used as ground. EMG electrodes were sutured in the left neck muscle, and a bipolar montage was used.

Mice were allowed to recover from surgery for 14 days. At the end of the recovery period, all animals appeared in good health and did not display overt behavioral anomalies.

3.2.5. Video-EEG and EMG recording

Two weeks after surgery, video-EEG-EMG recordings were carried out continuously for 1 week. Briefly, animals were housed in 30 x 30 x 55 (height) cm plexiglass cages (PhenoTyper® apparatus, Noldus®, Wageningen, The Netherlands), equipped with a video camera embedded in the top lid. Water and food were always available *ad libitum*. A custom-made cable was plugged into the head implant at one end and to the signal amplifier (Grass Comet-Plus, Grass Technologies) at the other (Figure 7). The cable was made weightless by a spring suspension, and animals were therefore free to move in the apparatus.

EEG and EMG signals were amplified, filtered (EEG: high-pass at 0.3 Hz; low-pass at 70 Hz; EMG: high-pass at 10 Hz; 50-Hz notch filtering for both sig-

nals), and digitized at 400 Hz. While data were collected during the entire week, the analyses reported here refer to the first (D1) and the seventh (D7) day of EEG/EMG recording.

3.2.6. Detection and analysis of epileptiform events

We define spike-wave discharges (SWDs, Figure 8) as a high-voltage (at least 1.5-fold increase over background signal), sharp discharge pattern of at least 4 cycles (Salmi et al. 2018; Salmi et al. 2019). SWDs were detected in the fronto-parietal EEG derivation with an overly permissive automated detection algorithm written in MATLAB® (Mathworks, Natick MA, USA) followed by visual inspection to remove false-positive events. The beginning and end of each SWD episode were marked (Figure 8) so that EEG segments limited to the duration of the discharge could be extracted. EMG traces and video recordings were checked alongside the EEG traces to assign each SWD to a specific vigilance state (wakefulness, non-REM, and REM sleep). Movement artifacts or electrical noises in the recordings were tagged and the corresponding epochs were excluded from subsequent analyses.

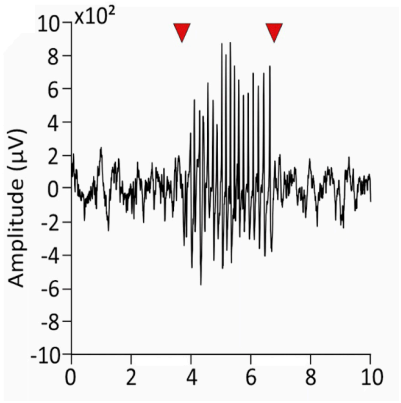

Figure 8. Typical example of a single spike and wave discharge

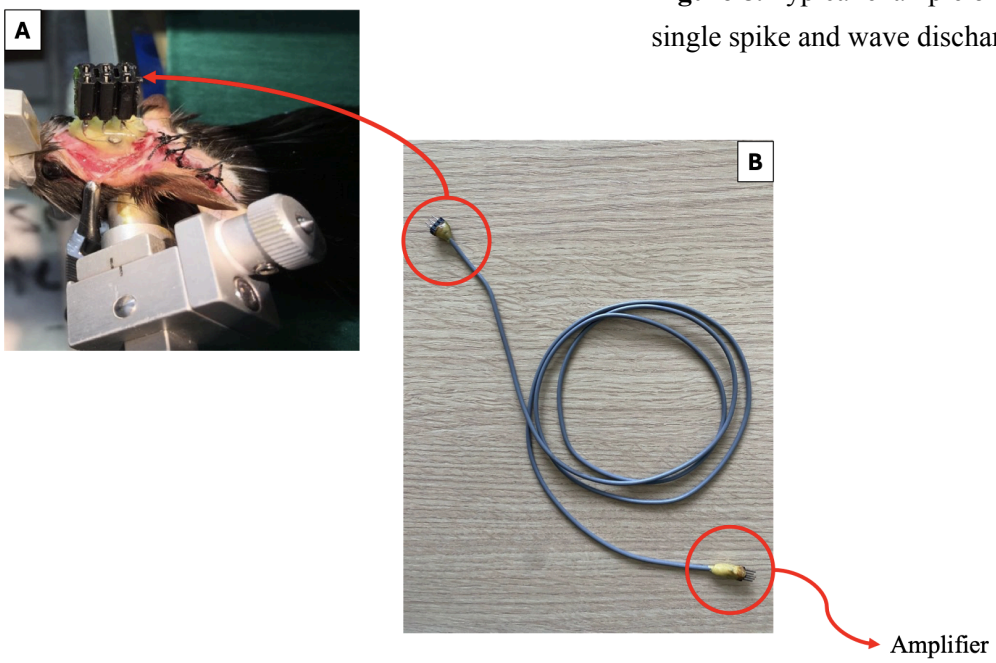

Figure 7. Recording set-up. A) Head implant with female connectors fixed on the skull; B) Custom-made cable connecting the head stage to the amplifier.

3.2.7. Rotarod tests

Fine-tuning the motor-learning protocol: The Rotarod apparatus (Ugo Basile s.r.l.) has long been used to evaluate motor coordination in rodents, especially in models of neurological disorders (Hamm et al. 1994). Briefly, the Rotarod consists of a horizontal rotating cylinder suspended at about 30 cm from the ground. The animal placed on the cylinder tends to spontaneously hang on to it by walking in the opposite direction of the rod's rotation. In our setup, the rod is divided in 5 parallel lanes, separated by large plastic discs (Figure 9), which allows to test 5 mice at once.

We adopted a modified version of the standard protocol (Yang et al. 2014) in order to emphasize the learning aspect, i.e. the progressive acquisition of a skilled behavior that takes place during training. Briefly, the standard protocol often consists of a single trial, where the rod rotates at increasing speeds, and the time spent by the animal before falling from the apparatus is taken as a measure of performance. In our "training" protocol, the test was instead administered multiple times a day, in order to assess performance improvements. All tests were administered in parallel to blocks of 5 animals.

To establish the optimal number of training days, sufficient to induce significant learning while minimizing stress, we conducted preliminary tests on a separate cohort of mice. Each mouse underwent training sessions for five consecutive days. Each session consisted of 40 repetitions of the test. Before each session, mice were weighed to account for any weight-related influences on the performance and were allowed a 30-minute habituation period in the Rotarod environment. Before the first actual trial, each mouse was placed for one minute on the suspended rod, at a speed of 5 rotations per minute (rpm). At such low speed, the test poses no real challenge, and is used to familiarize the animal with the apparatus.

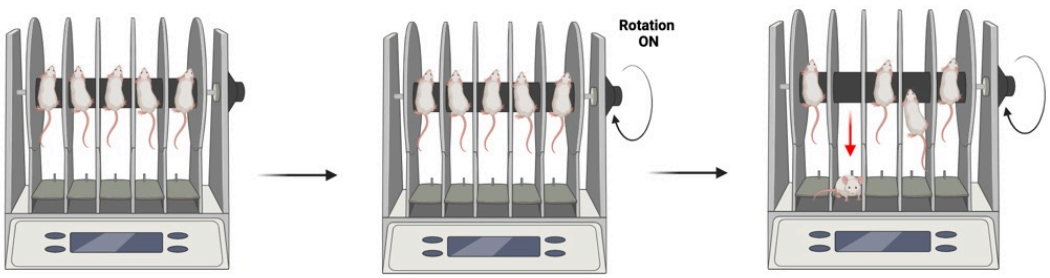

Figure 9. Schematic illustration of the rotarod setup and test principle. Created with BioRender.com.

Familiarization was immediately followed by the test trials, where the rod accelerated from 5 rpm to a maximum of 80 rpm over 135 seconds. The trial ended when the mouse fell off the Rotarod or after 135 seconds. After 10 consecutive trials, a 5-minute break was given to the mice to reduce fatigue and improve performance consistency.

Based on these preliminary tests (see Results section) we chose to induce and assess motor learning by administering two training sessions to the experimental cohorts.

Triggering and assessing motor skill learning: At the end of the video-EEG/EMG monitoring period, both JAX and OLA mice were unplugged from the recording setup and randomly assigned to one of two groups: "trained" and "untrained". The former entered the motor learning protocol established as described above, while mice in the latter group were left undisturbed in their home cage for the duration of the training sessions. All tests were conducted at the end of the "active" circadian phase (the lights-off period).

At the end of each training day, animals were returned to their home cages and left undisturbed for the 12 following hours, corresponding to a full lights-on phase.

3.2.8. Immunohistochemistry

About 12 hours after the end of the Rotarod protocol, all trained mice, together with the untrained group, were deeply anesthetized by intraperitoneal (i.p.) injection of 250 mg/kg tribromoethanol (Fluka) and transcardially perfused with ice-cold 0.01 M phosphate-buffered saline (PBS) pH 7.4, followed by 4% paraformaldehyde (PFA) in PBS.

Brains were removed from the skull and placed in 4% PFA at 4ºC for a 24 h post-fixation period, then transferred to 30% sucrose in PBS for 48 h for cryopreservation. Coronal sections, 20-µm-thick, were cut using the cryostat (Leica Biosystems, Wetzlar, Germany) and stored in PBS with sodium azide at 4ºC.

Eight sections that included both the motor and the somatosensory cortex were selected for each mouse, evenly spaced from approximately 2 mm anterior to -0.1 mm posterior to bregma. Sections were mounted on glass slides (4 sections each). Slides were immersed in an antigen unmasking solution (Vector Laboratories, Burlingame, USA) and processed in a microwave oven at 600 W (3 x 5 minutes).

After washes and a 1h pre-incubation step with blocking solution (2% of normal donkey serum and 0.3% triton in PBS), sections were incubated overnight with primary antibodies. For glutamatergic pre-synaptic endings, we used an anti-vesicular glutamate transporter type 2 (VGLUT2) antibody (guinea pig anti-mouse VGLUT2, 1:500, Synaptic Systems). For the GABAergic pre-synaptic terminals, we employed anti vesicular inhibitory amino acid transporter (VGAT) antibody (VGAT; guinea pig anti-mouse VGAT, 1:500, Synaptic Systems). After washing in PBS, sections were incubated for 90 minutes with fluorescent secondary antibodies (anti-guinea pig, Alexa Fluor 647-conjugated, 1:1000, InVitrogen Corporation) followed by a short incubation with DAPI (1:1000; Sigma-Aldrich, Italy) and cover-slipping with fluorescence mounting medium (Dako, Hamburg, Germany).

3.2.9. Confocal acquisitions

Images from the motor and somatosensory cortex were acquired at the confocal laser scanning microscope (Leica TCS-SP5, Leica Microsystems Mannheim, Germany) with 63X oil immersion objectives (HCX PL APO with 1.25 and 1.4 NA, respectively), 200 Hz acquisition rate, and a 3.5x digital zoom. The acquired "stacks" were 70.31 × 70.31 μm wide, and 5.035 μm thick. Acquired images had a 1024 × 1024 pixel resolution. In total, 16 image stacks were acquired per mouse, 8 for the GABAergic contacts (4 in the motor cortex and 4 in the somatosensory cortex) and 8 for the glutamatergic contacts (4 MC + 4 SSC).

3.2.10. Image processing

Images were deconvolved with the Huygen Professional software (18.10 version, Scientific Volume Imaging B.V.) using the CMLE (Classic Maximum Likelihood Estimation) algorithm. Deconvolved images were then quantitatively analyzed to detect and count the average number of GABAergic and glutamatergic presynaptic contacts using IMARIS (Oxford Instruments, version 9.6). Briefly, the algorithm searched for clusters of nearby voxels with fluorescent signal exceeding a threshold set by the operator to exclude background noise. In order to be identified as a putative presynaptic terminal, the cluster had to be at least 0.6 μm wide, when measured on the XY plane, or 1.2 μm along the Z axis.

3.2.11. Statistical analysis

Statistical analyses were carried out with Prism v.8 (GraphPad Software Inc., CA, USA). Data are reported as mean ± SEM values. An alpha level of $p < 0.05$ * was used to detect statistically significant differences.

Between-group comparisons were performed by two-way analysis of variance (ANOVA) followed by the Tuke's multiple comparison test. Pearson correlation analysis was used to assess the linear association between two continuous variables. The Pearson correlation coefficient (r) was employed as a quantitative measure of the strength and direction of the linear relationship between the variables.

3.3. Results

3.3.1. All young JAX mice displayed SWD during SWS.

Results from the continuous recordings confirm the early reports of spontaneous SWD episodes in young JAX mice (Figure 10). Notably, the disorder was consistently observed in all young JAX mice and in none of the OLA mice (Figure 10A). While the frequency of discharges was fairly variable between subjects (SD = 171.1) it was stable over time, in that no significant within-subject differences were observed when comparing the number of discharges detected during the first 24 hours (D1) vs the second 24-hour recording epoch (D7) (Figure 10B). Because of this, all subsequent analyses on electrical abnormalities were performed after concatenating data from D1 and D2 into a single 48 h recording window.

Aside from SWDs, the cortical activity of JAX mice was indistinguishable from that of OLA controls (Figure 10D).

On average, a higher frequency of discharges was accompanied by slightly longer episodes (Figure 10C). The correlation, however, was rather weak ($r = 0.428$ $P = 0.086$).

Interestingly, in contrast to what was observed in young JAX mice (2 m.o.), no discharges were detected in the older JAX cohort (8 m.o.) (Figure 10A). As expected, older OLA mice were also SWD-free.

A systematic inspection of the continuous video, EEG, and EMG recordings revealed an overall normal alternation of wakefulness and sleep, with no overt signs of sleep interruption following episodes of epileptiform activity. Furthermore, virtually all discharges occurred during periods of SWS.

3.3.2. Preliminary motor learning test

In the preliminary round of motor learning sessions, aimed at validating the test and establishing the protocol for subsequent experiments, all OLA mice in the cohort were able to correctly perform the Rotarod task in each of the 5 training sessions.

Performance in the Rotarod improved over time. On average, the rotation speed at which mice fell from the rod was 17.66 rpm at D1 and increased to 23.02 rpm by D5. The gains were statistically significant (one-way RM-ANOVA, F = 6.040, P = 0.0032) and mostly took place between D1 and D2 (Tukey's post hoc test, P = 0.0057), while the following daily sessions resulted in only modest average improvements (Figure 11A).

In the first training session, the variability of performance between animals was relatively low (SD: 2.067) but increased substantially at D2 and remained high until the end of the series (SD from D2 to D5: 3.862, 5.891, 4.370, 5.580). The likely explanation for this result is that 3 of the 10 subjects showed no learning at

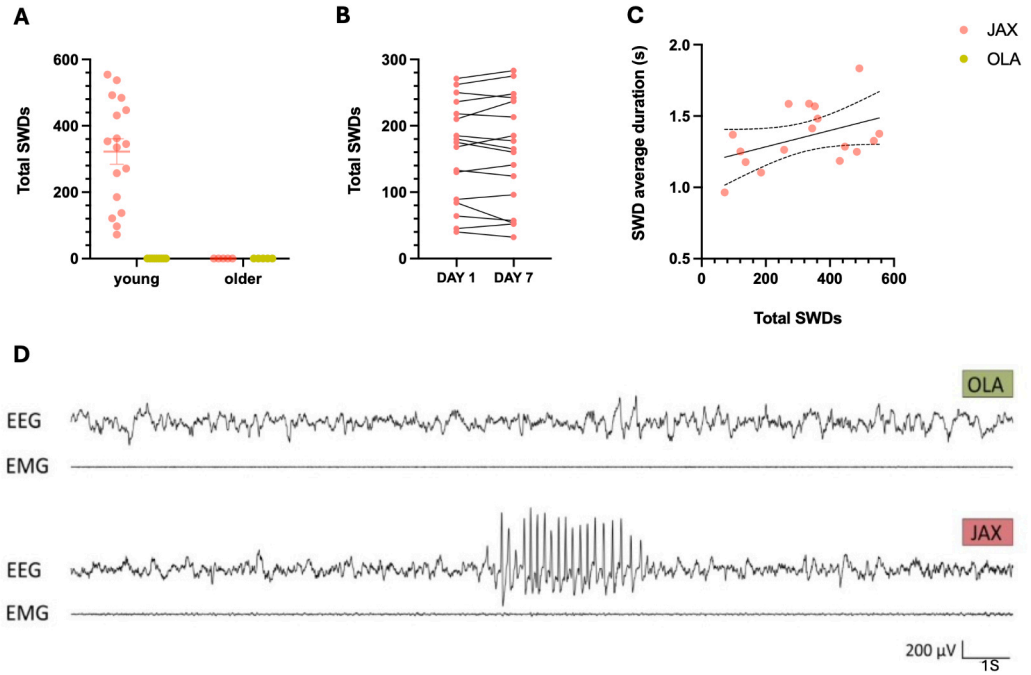

Figure 10. SWD activity in JAX mice. A) Mean number of SWD episodes/48 h. B) Mean number of SWD at recording days 1 and 7 in 24 h. C) Correlation of SWD mean duration and SWD number in the JAX group. Dashed line indicates the 95% confidential interval. D) Normal slow-wave sleep EEG/EMG sample epochs in a single OLA mouse and a single, 4-second-long SWD in a sample trace of a JAX mouse. Aside from SWD, the cortical activity of the JAX mouse is equal to that of OLA. C Pearson correlation analysis.

all, while the others raised their threshold rpm, thereby increasing the spread (Figure 11B).

To exclude the influence of body weight on performance, we performed a correlation test between these two variables, but no effect was found in this cohort of mice (r = -0.183, Figure 11C).

3.3.3. JAX mice show an impairment in motor learning

As stated in the Methods section, results of the preliminary motor learning tests were used to fine-tune the Rotarod protocol for the main experiment. Thus, given that most of the performance gains were observed between D1 and D2, we shortened the training routine to only 2 sessions, in order to reduce animal stress.

Similarly to what observed during the preliminary tests, all mice, both OLA and JAX, were able to successfully complete both Rotarod sessions.

Results in the OLA group were comparable to what already observed in the preliminary tests: on average, performance increased from 34.9 to 43.5 rpm, a 25% gain. On the other hand, JAX mice were unable to improve their performance from the first session (34.2 rpm) to the second (32.9 rpm) (Figure 12A, B). A 2-way, mixed-model ANOVA confirmed that the difference in training outcome between the 2 groups was statistically significant (strain × day interaction, $F(1, 16) = 9.57$, $P = 0.007$).

We explored the possibility of a link between severity of the EEG anomalies and learning proficiency at an individual level. Correlating performance gains with the number of discharges observed in each JAX mouse (r = -0.63, P = 0.09)

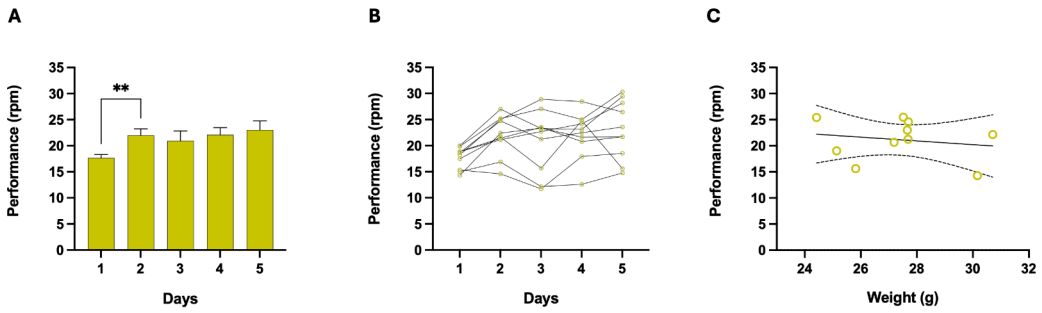

Figure 11. Motor skill learning in OLA mice (n=10) on modified rotarod test. A) Performance mean from day 1 to day 5 B) Individual performance from day 1 to day 5. C) Correlation between weight and performance. Dashed line indicates the 95% confidential interval. A One-way ANOVA with Tukey's multiple comparison test. C Pearson correlation analysis. Data are shown as mean ± SEM. **P < 0.01.

(Figure 12C). Finally, we repeated in these cohorts the control for the possible influence of body weight on performance, but found no correlation between the two variables, as shown in Figure 12D.

3.3.4. Confocal imaging of synaptic contacts in immunofluorescent brain sections

DAPI staining, applied to all sections, allowed the identification of neuronal nuclei (Figure 13A, B), which served as visual references to frame and focus on the regions of interest.

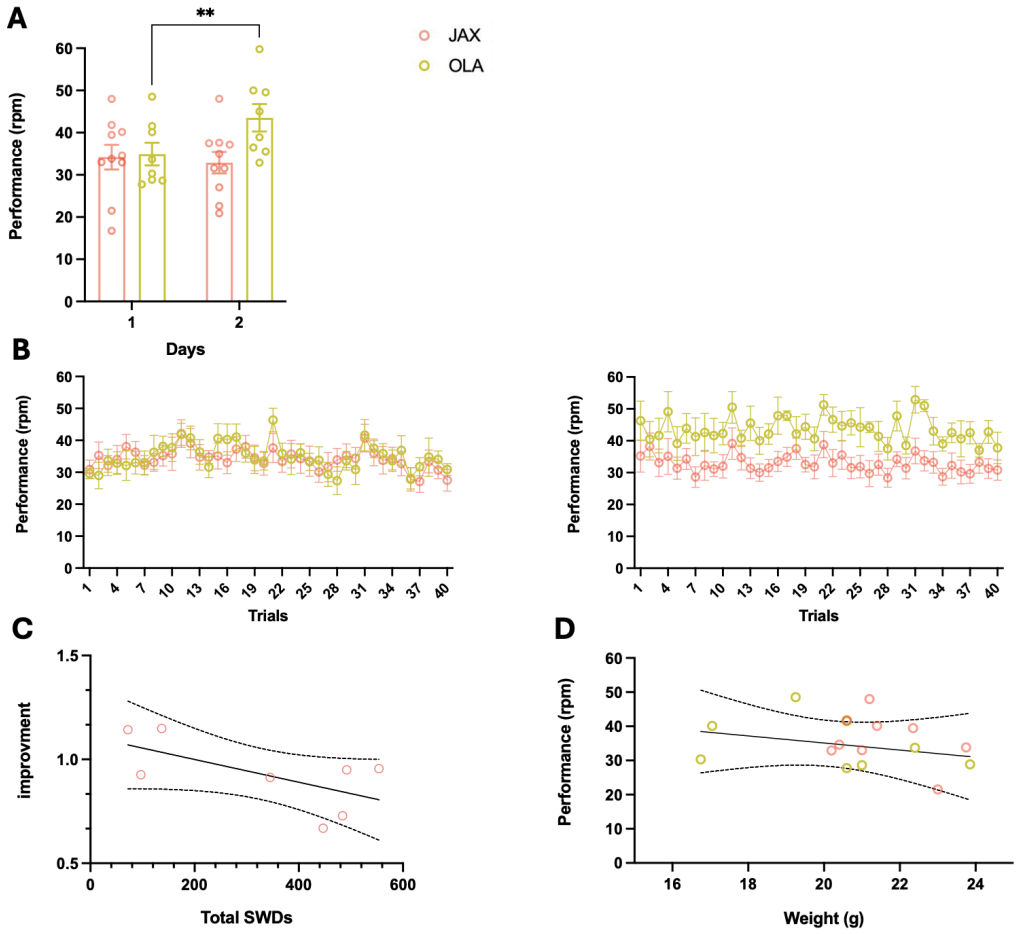

Figure 12. Motor skill learning between day one and day 2. A) Performance mean of day one and day two. B) Performance of each single trial on day one (right) and day two (left). C) Correlation between SWD number and performance improvement in JAX mice. D) Correlation between weight and performance. Dashed lines indicate the 95% confidential interval. A Two-way ANOVA with Tukey's multiple comparison test. C, D Pearson correlation analysis. Data are shown as mean ± SEM. **P < 0.01.

Immunofluorescent staining against both presynaptic markers, VGLUT2 and VGAT, provided clear microscopic images of glutamatergic and GABAergic puncta, respectively, in the cerebral cortex of both mouse strains (Figure 13).

A small number of brain sections displayed less-than-optimal staining and were therefore excluded from the analyses. In order to keep constant the total number of analyzed images per subject, we acquired extra stacks from nearby sections. Synaptic terminals, both glutamatergic and GABAergic, were detected in all studied animals, independent of strain and training.

The results of the quantitative analysis of synaptic terminals in the neocortex of A/J mice are presented here in terms of total number of putative contacts detected per image stack.

We observed a fairly complex pattern of results, with several statistically significant effects of training depending on mouse strain, revealed by 2-way ANOVAs computed for each cortical area and for each synaptic marker (full results shown in Table 3).

In order to provide a clearer description of the results, we first summarize the results for the untrained animals, and then we will add the data collected after

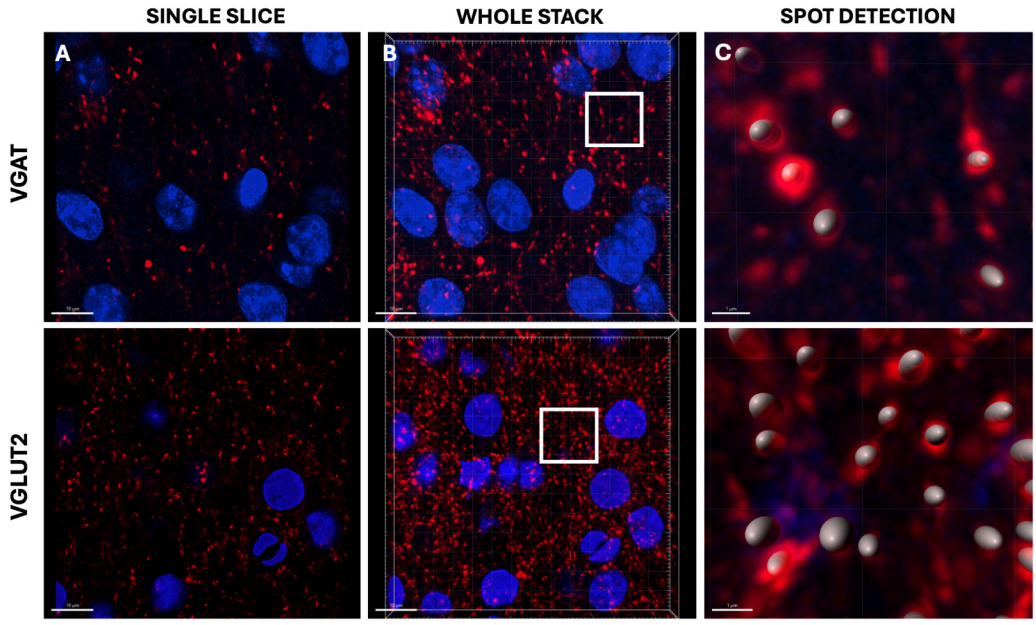

Figure 13. Representative VGAT and VGLUT2 staining (red) in the motor cortex. A) Single slice, i.e. focal plane. Nuclei are stained in blue with DAPI. B) Confocal stack in 3D view. White squares correspond to the details magnified in C. C) Examples of VGAT and VGLUT2 positive terminals as detected by Imaris software. 63X lens plus 3.5 digital zoom. Scale bar A,B 10 µm; C 1 µm.

training, to complete the picture. The P values reported in the following paragraphs refer to the results of Tukey's post-hoc pairwise comparisons.

3.3.5. Higher number of glutamatergic and fewer GABAergic synapses in untrained JAX mice compared OLA counterparts

In mice that did not experience the Rotarod test, two opposing patterns of synapse counts were observed between SWD-affected and control A/J mice, depending on synapse type.

In particular, JAX mice had a higher number of glutamatergic presynaptic terminals than OLA mice in both MC (798.3 ± 49.19 vs 637.1 ± 41.79, P = 0.047) and SSC (873,7 ± 78.6 vs 683.7 ± 23.66, P = 0.028; Figure 14A). In contrast, the number of GABAergic contacts was lower in untrained JAX mice compared to

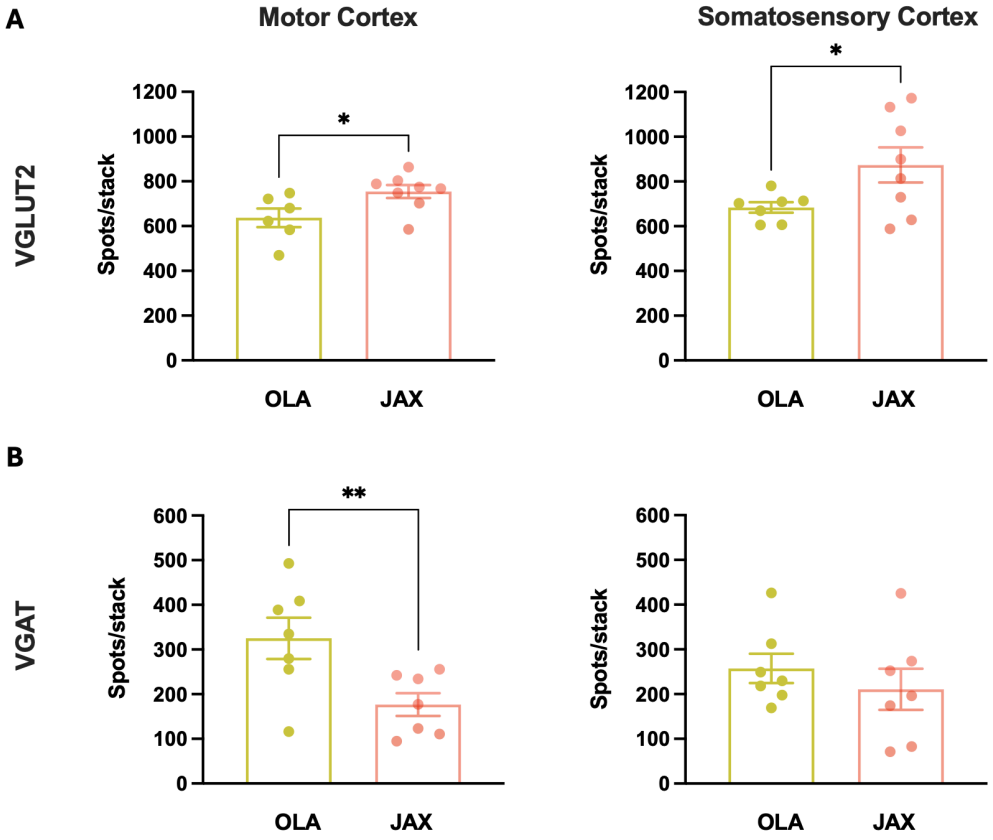

Figure 14. VGLUT2 and VGAT presynaptic terminals in the motor and somatosensory cortex in untrained mice. A) Average number of VGLUT2 spots in MC and SSC. B) Average number of VGAT spots in MC and SSC. The total number of spots per mouse is represented as the average of four different stacks from the same area of the cortex. The acquired stacks were 70.31 × 70.31 µm wide, and 5.035 µm thick. Two-way ANOVA with Tukey's multiple comparison test. Data are shown as mean ± SEM. *P < 0.05.

Table 3. Full 2-way ANOVA tables of the comparisons between synaptic terminal counts.

	Interaction	Training	Strain
MC VGLUT2	F (1, 26) = 11,99; P=0,0019	F (1, 26) = 0,2607; P=0,6140	F (1, 26) = 0,3654; P=0,5507
SSC VGLUT2	F (1, 27) = 8,581; P=0,0068	F (1, 27) = 9,841; P=0,0041	F (1, 27) = 1,872; P=0,1826
MC VGAT	F (1, 26) = 2,726; P=0,1108	F (1, 26) = 11,11; P=0,0026	F (1, 26) = 12,44; P=0,0016
SSC VGAT	F (1, 26) = 0,6066; P=0,4431	F (1, 26) = 32,07; P<0,0001	F (1, 26) = 0,7233; P=0,4028

OLA (MC: 176.07 ± 25.74 vs 325.2 ± 46.26, P = 0.0077; SSC: 210.7 ± 46.06 vs 257.4 ± 32.85, P = 0.68; Figure 14B).

3.3.6. Fewer glutamatergic synapses in trained JAX mice

In order to evaluate training-dependent synaptic plasticity, as well as the impact of SWD activity on it, we compared the above results with those obtained in both OLA and JAX mice after Rotarod training.

Analysis of glutamatergic synapses showed that OLA mice had a higher number of VGLUT2 presynaptic terminals in MC (637.1 ± 41.79 vs 754.1 ± 29.03, P = 0.209) after training, in line with the hypothesis that training promotes synaptogenesis in brain areas recruited during the prolonged execution of a task (Figure 15). In contrast, the number of VGLUT2 presynaptic terminals in JAX mice decreased significantly in both MC (754.1 ± 29.03 vs 640.8 ± 34.4, P = 0,033) and SSC (873,7 ± 78.6 vs 605.5 ± 20.65, P = 0,0009) after training (Figure 15).

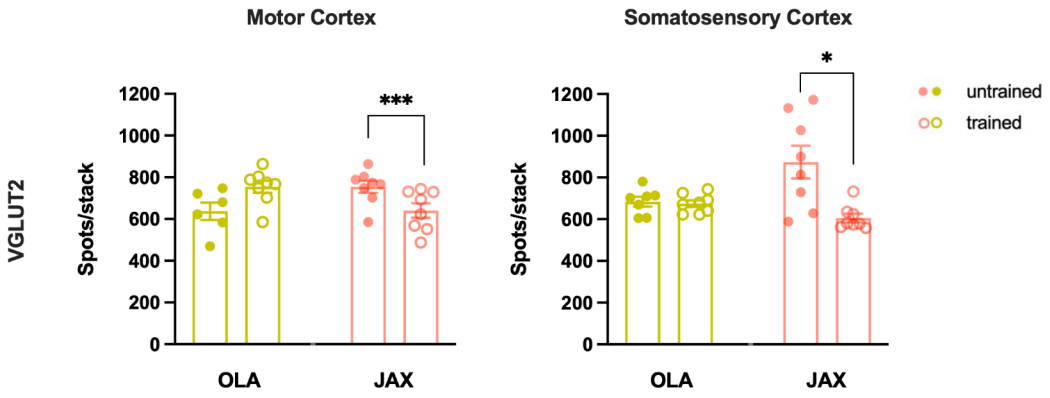

Figure 15. VGLUT2 presynaptic terminals in motor and somatosensory cortex in untrained versus trained mice. The total number of spots per mouse is represented as the average of four different stacks from the same area of the cortex. The acquired stacks were 70.31 × 70.31 µm wide, and 5.035 µm thick. Two-way ANOVA with Tukey's multiple comparison test. Data are shown as mean ± SEM. *P < 0.05; ***P < 0.001.

3.3.7. Increased GABAergic synapses in both OLA and JAX mice after training

The analysis of GABAergic synapses after the Rotarod sessions showed a general increase in the number of VGAT presynaptic terminals in OLA mice in both MC (325.2 ± 46.26 vs 373.4 ± 17.42, P = 0.63) and SSC (257.4 ± 32.85 vs 397.4 ± 12.19, P = 0.0097), suggesting a dynamic change in GABAergic synapses after training (Figure 16).

Finally, training was associated with an increase in the number of GABAergic synapses in JAX mice as well, both in MC (176.07 ± 25.74 vs 319.6 ± 21.5 P = 0.0082) and inSSC (210.7 ± 46.06 vs 395.4 ± 17.3 P = 0.0006) (Figure 16). This increase in GABA levels might result from an adaptive homeostatic mechanism acting to counterbalance excessive excitatory activity in these mice.

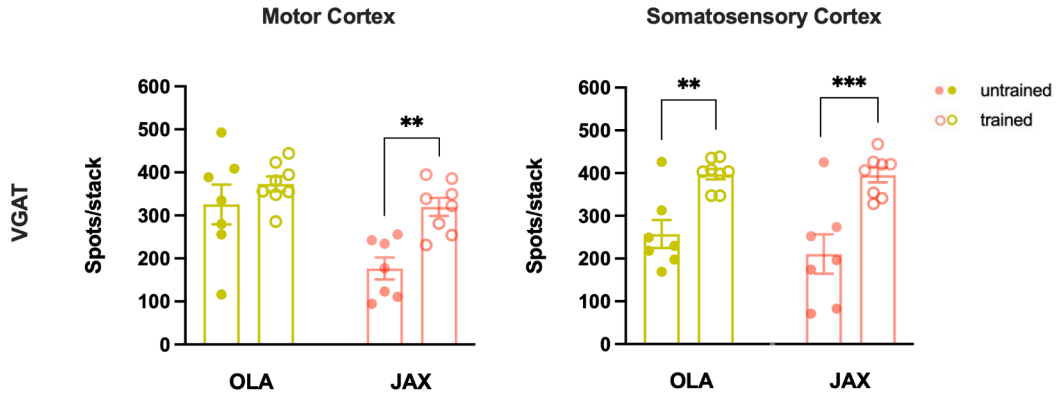

Figure 16. VGAT presynaptic terminals in the motor and somatosensory cortex in untrained versus trained mice. The total number of spots per mouse is represented as the average of four different stacks from the same area of the cortex. The acquired stacks were 70.31 × 70.31 µm wide, and 5.035 µm thick. Two-way ANOVA with Tukey's multiple comparison test. Data are shown as mean ± SEM. **P < 0.01; ***P < 0.001.

3.4. Discussion

By definition, epileptic encephalopathies are brain disorders caused by repeated seizures. Compared to most other epilepsies, when studying EEs, there is a partial shift of focus from what *causes* a seizure to how seizures themselves can damage the brain.

The ultimate goal of investigating animal models of EEs is to unravel the mechanisms by which the abnormal electrical activity leads to behavioral and cognitive disorders in human patients.

The core hypothesis of this study was that abnormal electrical activity may interfere with the synaptic plasticity mechanisms underlying normal brain function. Specifically, we investigated the association between: 1) repeated spike-wave discharges (SWD) during slow-wave sleep (SWS); 2) the ability to acquire a novel motor skill; and 3) the number of excitatory and inhibitory synapses detectable in the cerebral cortex with and without training.

We previously characterized the A/J JAX mouse as a putative model for the epileptic encephalopathy with *status epilepticus* during slow-wave sleep (ESES).

3.4.1. SWDs during SWS in JAX mice

Here, we confirmed that all young mice from this strain are affected by spontaneous SWDs, almost entirely occurring during phases of SWS, as originally reported (Strohl et al. 2007) and as previously observed in our laboratory (Del Gallo et al, in preparation). None of the OLA mice displayed the anomalous activity. Importantly, aside from the discharges, no other EEG or behavioral alterations could be observed between JAX and OLA mice.

The observed variability in the number and mean duration of SWD episodes between individual mice suggests individual differences in susceptibility or other contributing factors, as previously reported in patients with ESES (Sánchez Fernández et al. 2012; Patry et al. 1971; Van Hirtum-Das et al. 2006)

In spite of the variability in discharge frequency between subjects, however, the number of SWDs detected within each JAX mouse was remarkably consistent over the 7-day observation epoch. This result supports the view that each subject was characterized by a relatively stable clinical profile, at least at this stage (Sánchez Fernández et al. 2012; Patry et al. 1971; Van Hirtum-Das et al. 2006). It is important to note, however, that the overall frequency of epileptiform episodes in JAX mice was relatively modest, compared to the massive presence of discharges observed in many human patients. Ideally, to reproduce the human pathology, an animal model of ESES should show EEG patterns of similar severity, and to such aim there are at least two experimental strategies to pursue.

First, inducing in the JAX mouse a higher discharge frequency, for example by treating subjects with agents capable of lowering seizure thresholds. It is well known that the administration of (lipopolysaccharide) LPS significantly increases

seizure susceptibility, as reported for several other animal models of epilepsy (Eun et al. 2015; Sayyah et al. 2003). We are currently investigating the possibility that systemic administration of LPS in JAX mice increases the number of SWDs while, presumably, having no detectable effects in OLA mice.

A second possibility is to resort to a different animal model altogether. In this respect, a promising mouse strain is the Grin2a$^{-/-}$ mouse, a knockout for the Grin2a gene. It is known that microdeletions, nonsense, splice-site, and missense mutations of the GRIN2A gene are associated with human ESES (Carvill et al. 2013; Strehlow et al. 2022; Lesca et al. 2013). In a collaborative study on the Grin2a mouse, we reported a pattern of SWDs during SWS similar to that observed in JAX mice (Salmi et al. 2019). Overall, the number of discharges was significantly higher in the former, compared to the latter, making the Grin2a$^{-/-}$ a promising candidate for further investigations. On the other hand, this strain is costly and laborious to maintain.

The EEG recordings reported here were carried out in mice at 2-3 months of age, which in human terms correspond to young adults. The choice of age for our study could be considered less than ideal, since the pathological discharges in human ESES affect children and tend to reduce and eventually disappear in adulthood. Indeed, in a separate study, we carried out recordings during the 3rd postnatal week, with data collected acutely, with intracortical electrodes (Salmi et al. 2019). On the other hand, our focus was to carry out long-term epidural recordings in freely moving animals, which is not possible to accomplish in animals of young age, due to the size of the skull and of the brain itself. Nevertheless, epileptiform activity and behavioral phenotype at earlier developmental stages (P2-P30) in JAX mice remains an open question.

Another relevant finding was the absence of SWD episodes in 8-month-old JAX animals, an age roughly corresponding to full adulthood in humans. The result is in agreement with the typical evolution of the human pathology (Sánchez Fernández et al. 2012; Scholtes et al. 2005; Fernández et al. 2012) and it prompts a further question. At more advanced ages, would the pattern of behavioral and structural changes be different from what we observed at 2-3 months? Further studies are needed to address this issue.

3.4.2. Motor learning

In a translational study of ESES, it would be natural to assess, first and foremost, the animals' cognitive function, instead of motor learning, which is procedural in nature. Several different behavioral tests have long been available to measure different types of declarative memory and working memory. Indeed, in previous studies we tested JAX mice with the Morris water maze, the novel object recognition, and the fear conditioning tasks with often disappointing results, due to the propensity of these animals to shy away from the spontaneous behaviors expected to be elicited by the above tasks. This trait was confirmed in a series of tests carried out in the elevated "plus" maze, where JAX mice spent significantly more time in the protected arms of the apparatus compared to the exploratory mode of normal controls (Del Gallo et al, in preparation). Thus, for the present study, we opted for a design that might circumvent this behavioral limitation while triggering robust learning-related plasticity (Chen et al. 2019).

As stated above, the Rotarod task is commonly employed to assess motor function in animal models of neurological diseases (Hamm et al. 1994; Mizoguchi et al. 2002). We have adopted a modified protocol that emphasizes task repetition, which is assumed to promote motor learning and, subsequently, improved performance. More specifically, we carried out training sessions according to a previously published study (Yang et al. 2014) which demonstrated that Rotarod training promotes synapse formation in brain areas recruited during the execution of the task.

It is generally assumed that learning-related plastic changes in synaptic contacts are a function of the amount and effectiveness of the training. In this view, it was important to ensure that all animals would be subjected to the same number of training sessions, while striking a balance between a sufficiently long training and an excessive duration of the protocol, which might increase stress and possibly introduce confounds.

Following the preliminary Rotarod sessions and the decision to limit subsequent training to only two sessions, we reported significant overnight performance improvements in OLA but not in JAX mice, which indicates a differential response to the execution of the motor task.

The difference is in line with the hypothesis that the epileptiform activity in JAX mice may play a role in motor skill acquisition deficits. The finding is also consistent with studies indicating that children with ESES display impairment of

cognitive functions, including learning and memory (Rubboli et al. 2023; Caraballo et al. 2015; Tassinari et al. 2000).

It is reasonable to assume that, when a pathology is associated with a functional deficit, the severity of the symptom is proportional to the severity of the disease. This type of correlation has been reported in pediatric epilepsy, where previous research suggests that the severity of cognitive deficits may be related to the frequency and duration of epileptiform discharges (Ramantani et al. 2013; Hermann et al. 2008).

Indeed, we detected within the JAX cohort a modest inverse correlation between SWD frequency and improvements in the Rotarod. While in the present study the effect was not statistically significant, it would be interesting to revisit this question based on a larger sample size.

3.4.3. Spots

It is well established that, at the synaptic level, brain connectivity is highly dynamic. It can be influenced by a variety of internal and external factors, some of which have slow and long-lasting effects, while others can trigger synaptic plasticity quite rapidly (Laperchia et al. 2017). Either way, the initiation and maintenance of synaptic changes involve intricate cellular and molecular mechanisms that take place over a time window of a few hours (Caroni et al. 2012). This temporal dimension requires careful consideration in the experimental design; in a behavioral, electrophysiological, and histological protocol, the choice of timing for each step is always the result of a cost/benefit analysis. In this study, we chose to avoid interruptions of the light and dark phases, and intervene only in close proximity to a light switch: training took place at the very end of the active (lights-off) phase, the resting circadian phase (lights-on) was left undisturbed, in order to maximize the impact of sleep on synaptic plasticity, and animal sacrifice and perfusion took place at the beginning of the next active period.

The comparisons between mouse strains in the number of detected synaptic contacts offer a relatively complex picture, with opposing results depending on synaptic marker and training regimen.

First, we consider untrained animals as a reference baseline. JAX mice had a higher number of glutamatergic presynaptic terminals and a lower number of GABAergic contacts, compared to OLA untrained mice, in both examined cortical

areas. The excess glutamatergic (excitatory) and reduced GABAergic (inhibitory) transmission suggests the possibility of an overall increased neuronal excitability in the JAX mouse brain.

Imbalances between excitatory and inhibitory neurotransmission have been frequently reported in various forms of epilepsy (Bozzi et al. 2018; Avoli et al. 2016). In particular, the equilibrium between excitation and inhibition, often referred to as the excitation/inhibition (E/I) balance, tends to shift towards hyperexcitation, resulting in the uncontrolled paroxysmal activity that constitutes a seizure. The conditions responsible for such shift may include an increase in glutamatergic synaptic activity, such as seizure-induced sprouting or enhanced connectivity among excitatory pyramidal neurons, or changes in ion currents responsible for membrane depolarization, such as inward Na+ or Ca2+ flux (Raol et al. 2001; Barker-Haliski and White 2015). Conversely, an E/I imbalance can also occur when GABAergic inhibitory synaptic activity is reduced, e.g. when ion currents facilitating membrane hyperpolarization are strongly activated, for instance, through outward K+ or inward Cl− flux (Feng et al. 2022).

The finding of significant differences in the numbers of synaptic contacts between SWD-affected animals and normal mice, in baseline ("untrained") conditions, suggests a long-lasting state of the brain, not specifically related to a behavioral contingency. By training groups of animals, we instead aimed to investigate the impact of abnormal discharges on plastic changes triggered by a recent, novel experience requiring the rapid development of proprioceptive and motor control strategies.

The increase of VGLUT2 presynaptic terminals observed in the motor cortex of OLA mice after training is in line with the established notion that training-induced synaptogenesis and increased glutamatergic synapses are associated with learning and memory processes (Xu et al. 2009; Ulloa Severino et al. 2023).

The interpretation of the results in JAX mice is more challenging. In contrast to the increase detected in OLA mice, JAX mice showed a decrease in the number of VGLUT2 presynaptic terminals in both MC and SSC after training. What type of mechanism could be responsible for this effect? In designing the study, the training protocol was intended as a diagnostic tool, i.e. to help unveil behavioral and synaptic deficits in SWD-affected mice. However, what if training per se has the ability to affect the underlying epileptic pathology? It has been proposed that, aside from general psychophysical benefits, physical exercise may re-

duce seizure frequency (Pimentel et al. 2015). The effect has also been reported in an animal model of epilepsy, where long-term exercise was found to reduce seizure frequency (Peixinho-Pena et al. 2012).

The reduction of VGLUT2 terminals in JAX mice might hint at a beneficial effect of training accompanied by a moderation of excitatory activity in the cortex. Such a speculative hypothesis could be supported by a finding of reduced SDW activity in the hours following training sessions. To address this question, further experiments would be required, as additional EEG data collection in the present study would have been unfeasible.

The analysis of GABAergic synapses in both OLA and JAX mice revealed a general increase in the number of VGAT presynaptic terminals after training. In OLA mice, this result indicates that inhibitory neurotransmission may play a crucial role in the changes associated with learning or training, as previously demonstrated in different brain regions (Kida et al. 2016; Holmgren and Zilberter 2001; Komatsu and Yoshimura 2000). As mentioned above, it is known that excitatory synaptic transmission is enhanced by learning, and strengthening also inhibitory signaling restores the overall balance in the network. The increase in GABAergic contacts appeared to be stronger in JAX than in OLA mice, which could point to an adaptive response aimed at limiting overall brain excitability, not unlike the previously described training-dependent reduction in glutamatergic contacts.

Taken together, these results suggest that SWDs have a significant impact on synaptogenesis, especially in view of the fact that brain samples were analyzed at the end of a 12-hour rest period, during which physiological sleep has been shown to promote the formation of dendritic spines in the mouse motor cortex after Rotarod training (Yang et al. 2014). Moreover, the structural data are in alignment with the Rotarod test results, in which performance improvement was observed in OLA but not in JAX mice.

4. Study 2: Cell type-specific effects of the P2X7 receptor in epilepsy

4.1. Rationale

The P2X7 receptor (P2X7R) is increasingly recognized to contribute to neuroinflammation and brain hyperexcitability. Its inhibition can, however, produce both pro- and anti-seizure effects.

Therefore, the objective of the present study was to gain clarity on which cell types express P2X7Rs during epilepsy, what are their contributions to the disease and whether this can be used to develop new treatment strategies for better and more effective seizure control.

To understand the basis for these opposing actions, we generated mice lacking the P2rx7 gene in either microglia (P2rx7:Cx3cr1-Cre) or neurons (P2rx7:Thy-1-Cre) to test whether a cell type-specific loss of the P2X7R impacts the severity of seizures in both a mouse model of TLE and acute evoked seizures.

4.2. Materials and Methods

4.2.1. Ethic statement

All animal experiments were performed in accordance with the principles of the European Communities Council Directive (2010/63/EU). Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (RCSI) (REC 1322) and Health Products Regulatory Authority (HPRA) (AE19127/P038; AE19127/P013). The animals were treated according to European standards/regulations for animal experiments and all efforts were made to minimize animal suffering and reduce the numbers of animals under experiments.

4.2.2. Animals

Our study included 8-10 weeks old male C57/Bl6 OlaHsd mice, P2X7R knock-out (KO) ($P2rx7^{-/-}$) mice (6NTac; B6N-P2rx7tm1d(EUCOMM)Wtsi/Ieg) which lack exon 2 of the P2rx7 gene, tamoxifen-inducible Cre B6.129P2(Cg)-Cx3cr1tm2.1 (cre/ERT2) Litt/WganJ (Cx3cr) (JAX stock #021160) and Tg (Thy1-cre/ERT2,-EYFP) HGfng/PyngJ (Thy-1) (JAX stock #012708) lines, crossed to

mice where the murine exon 2 of the P2rx7 gene is flanked with loxP sites generated by the European Conditional Mouse Mutagenesis (EUCOMM) Program [P2rx7tm1a(EUCOMM)Wtsi] to obtain conditional mice with a P2X7R KO in microglia (P2X7$^{-/-}$-M) and neurons (P2X7$^{-/-}$-N). Animals were housed in a controlled biomedical facility on a 12-hour light/dark cycle at 22±1 ºC and humidity of 40-60% with food and water provided *ad libitum*.

4.2.3. Experimental Design

We first generated mice lacking the P2rx7 in either microglia or neurons. Using the conditional Cre-LoxP system, we targeted the P2X7R in microglia by crossing P2rx7$^{-/-}$ mice with the Cx3cr1-Cre line, generating offspring, P2rx7$^{-/-}$-M. We generated mice lacking the P2X7R in neurons by crossing P2rx7$^{-/-}$ mice with the Thy-1-Cre line, generating the offspring P2rx7$^{-/-}$-N (Sahasrabuddhe and Ghosh 2022; Young et al. 2008) (Figure 17**A**). Both lines used a tamoxifen-inducible Cre-LoxP system, thereby avoiding the effects of P2X7R deficiency during development and allowing analysis of the loss of the P2X7R initiated after SE to track effects on epilepsy development without interfering with the initial insult.

To test whether a cell type-specific loss of the P2X7R impacts the severity of seizures, mice lacking the P2X7R in microglia (*P2rx7*$^{-/-}$-M) and neurons (*P2rx7*$^{-/-}$-N), along with wt mice (*P2rx7*$^{+/+}$) were subjected to intra-amygdala microinjection of kainic acid (IAKA) (Figure 17**B**).

Then, to confirm the effects of cell type-specific deletion on seizures, we tested additional mice using the PTZ mouse model, a frequently used model to assess anti-or proconvulsant effects (Figure 17**C**).

To determine if disruption to P2X7R signaling in microglia or neurons affects the development of epilepsy, additional mice of each genotype were treated with tamoxifen beginning 24 h after IAKA-induced SE. Using this approach, the deletion of P2X7R occurred after SE, and the effect of its loss in either cell type was followed by continuous seizure monitoring as epilepsy developed (Figure 17**D**).

Finally, we wanted to explore how cell type-specific deletion of P2X7R contributes to brain hyperexcitability, analyzing gene expression in hippocampal samples from *P2rx7*$^{-/-}$-M compared to wt mice.

4.2.4. *Tamoxifen treatment*

Tamoxifen was used to induce the expression of Cre (Cx3cr1 and Thy-1) to delete the P2X7R in both microglia (P2X7$^{-/-}$-M) and neurons (P2X7$^{-/-}$-N). This treatment was applied for a period of 7 consecutive days by an intraperitoneal (i.p.) injection of tamoxifen once daily (40 mg/kg; prepared in 10% of 100% ethanol and 90% of

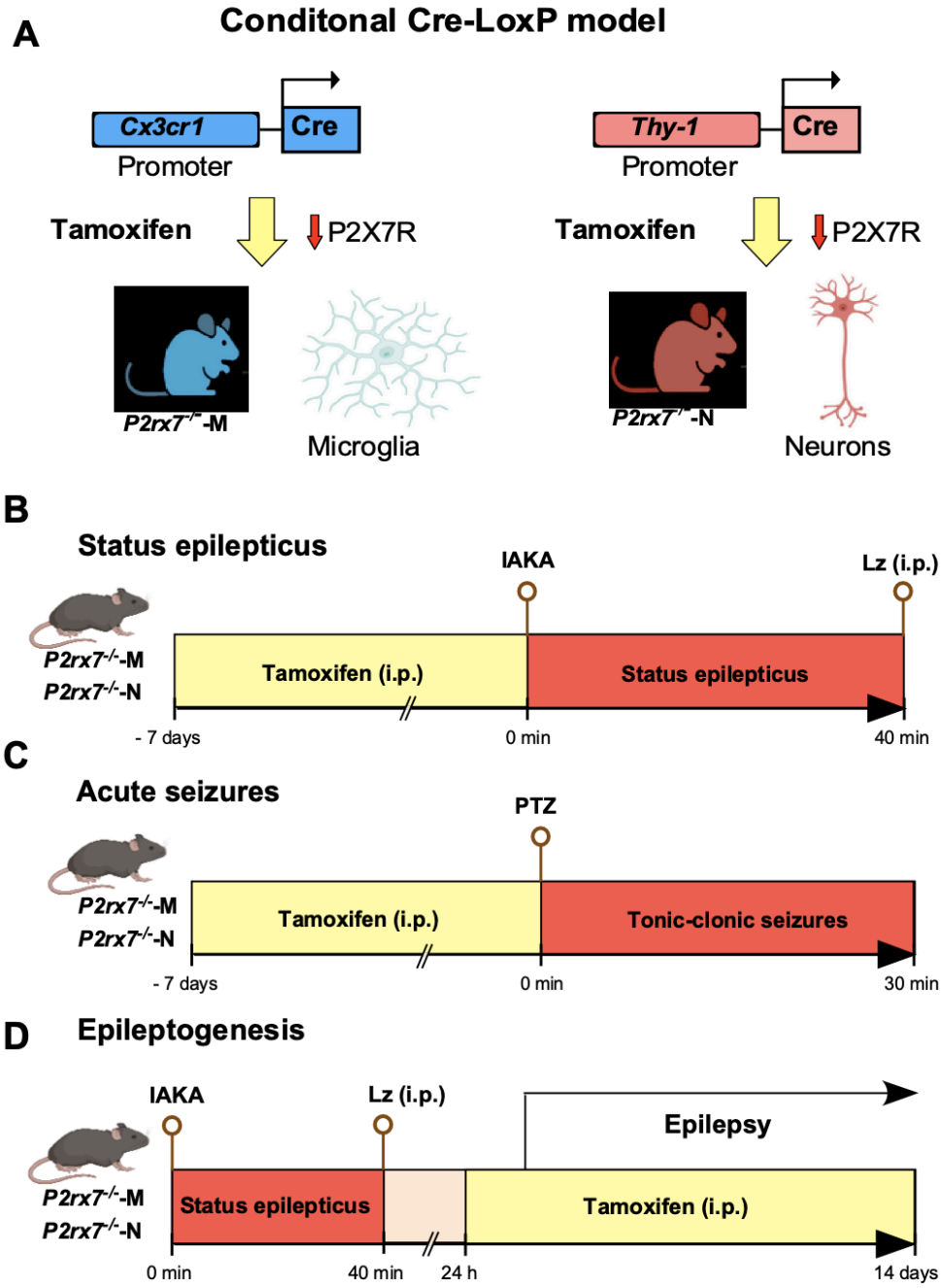

Figure 17. Experimental design. A) Cell type-specific approach. B) Experimental approach to study the impact of P2X7R knockdown on IAKA-induced SE. C) Experimental approach to study the impact of P2X7R knockdown on PTZ-induced seizures. D) Design to study the impact of P2X7R knockdown on epileptogenesis.

peanut oil; volume injection = 100 µl). For cell type-specific KO mice experiments, animals negative for Cre but homozygous for loxP were used as controls ($P2X7^{+/+}$). Tamoxifen was applied to mice expressing Cre and loxP ($P2X7^{-/-}$-M, $P2X7^{-/-}$-N), and loxP wt mice ($P2X7^{+/+}$).

4.2.5. Seizure models

Status epilepticus (SE) was induced in mice via IAKA or via intraperitoneal (i.p.) KA (Morgan et al. 2020). During stereotaxic procedures, mice were anesthetized using isoflurane (5% induction, 1-2% maintenance) and maintained normothermic by means of a feedback-controlled heat blanket (Harved Apparatus Ltd, Kent, UK). Once fully anesthetized, mice were placed in a stereotaxic frame and a midline scalp incision was performed to expose the skull. A guide cannula for IAKA injection (coordinates from Bregma; AP = -0.94 mm, L = -2.85 mm) and three cortical electrodes (IAKA and i.p. KA), one on top of each hippocampus and the reference electrode on top of the frontal cortex, were fixed in place with dental cement. For IAKA-induced SE, approximately 1 h post-surgery, SE was induced in awake, hand-restrained mice by a microinjection of 0.3 µg KA in 0.2 µl phosphate-buffered saline (PBS) (Sigma-Aldrich, Dublin, Ireland) into the right basolateral amygdala. All vehicle-injected control animals received 0.2 µl of PBS (pH = 7.4) solution. The anticonvulsant lorazepam (6 mg/kg) (Wyetch, Taplow, UK) was delivered intraperitoneal (i.p.) 40 min following IAKA or vehicle to curtail seizures and reduce morbidity and mortality. For i.p. KA-induced SE, mice were injected with 10 mg/kg KA. Electroencephalogram (EEG) was recorded using a Xltek recording system (Optima Medical Ltd, Guildford, UK) starting 10 min before administration of IAKA or i.p. KA. For the induction of generalized tonic-clonic seizures, mice were equipped with three cortical EEG electrodes as described above and treated i.p. with 60 mg/kg Pentylenetetrazol (PTZ). Mice were observed for 30 minutes post-PTZ administration.

4.2.6. Analysis of seizure severity and epilepsy

Behavioral seizures during IAKA and i.p. KA-induced SE were scored according to a modified Racine Scale as reported previously (Jimenez-Mateos et al. 2012). Score 1, immobility and freezing; score 2, forelimb and or tail extension, rigid posture; score 3, repetitive movements, head bobbing; score 4, rearing and falling; score 5, continuous rearing and falling; score 6, severe tonic–clonic

seizures. The highest score attained during each 5-minute period was recorded by an observer blinded to treatment. In the PTZ model, behavioral seizures were classified as: Score 0, Normal behavior; score 0.5, abnormal behavior; score 1, isolated myoclonic jerk; score 2, atypical clonic seizure; score 3, bilateral forelimb clonus; score 3.5, bilateral forelimb clonus with body twist; score 4, tonic-clonic seizure with suppressed tonic phase; score 5, fully developed tonic-clonic seizure; score 6, tonic-clonic seizure followed by death. To analyze EEG frequency and amplitude signal (power spectral density and EEG spectrogram of the EEG data), EEG data were uploaded into Labchart7 software (AD instruments Ltd, Oxford, UK) and analyzed as before (Morgan et al. 2020). EEG total power (μV^2) is a function of EEG amplitude over time and was analyzed by integrating frequency bands from 0 - 100 Hz and the amplitude domain filtered from 0 - 50 mV (Beamer et al. 2022). Epilepsy analysis was carried out via continuous video monitoring. Animals were housed in pairs (mice were distinguished by different headset colors) in clear Perspex cages. Webcam-style cameras connected to laptop computers were placed in front of each cage in a room with safe lights for night-time recordings. Images were captured using VirtualDub 1.9.11 (SourceForge.net) with a sampling rate of 10 frames per second and data transfer rate of 140 kb s-1. Videos were reviewed by an observer unaware of experimental treatment.

4.2.7. RNA extraction and qPCR

RNA extraction was performed using the Trizol method (Alves et al. 2019). The quantity and quality of RNA were measured using a nanodrop spectrometer (Thermo Scientific, Rockford, IL, U.S.A). Samples with a 260/280 ratio between 1.8 - 2.0 were considered acceptable. 500 ng of total RNA was used to produce complementary DNA (cDNA) by reverse transcription using SuperScript III reverse transcriptase enzyme (Invitrogen, CA, U.S.A) primed with 50 pmol of random hexamers (Sigma, Dublin, Ireland). Quantitative real-time polymerase chain reaction (qPCR) was performed using the QuantiTech SYBR Green kit (Qiagen Ltd, Hilden, Germany) and the LightCycler 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany). Each reaction tube contained 2 µl cDNA sample, 10 µl SyBR green Quantitect Reagent (Qiagen Ltd, Hilden, Germany), 1.25 µM primer pair (Sigma, Dublin, Ireland) and RNAse free water (Invitrogen, CA, U.S.A) to a final volume of 20 µl. Using LightCycler 1.5 software, data were analyzed and normalized to the expression of β-actin. Primers used (Sigma, Dublin, Ireland) are listed in Table 4.

Table 4. Primer sequences used for qPCRs (Sigma-Aldrich).

Primer	Forward sequence	Reverse sequence
P2rx7	ACTGGCAGGTGTGTGTTCCATA	TTGGCAAGATGTTTCTCGTG
β-Actin	GGTTGGCCTTAGGGTTCAGG	GGGTGTGATGGTGGGAATGG
gfap	AGAAAACCGCATCACCATTC	TCACATCACCACGTCCTTGT
P2ry12	ATCACAGAGGGCTTTGGGAA	ATGGGAAGAGAACCTGGGTG
Iba-1	TGGAGGGGATCAACAAGCAA	ACCCCAAGTTTCTCCAGCAT
CD206	AAGGAAACCATGGACAACGC	ACTTTGCTCCCATCCATCCA
ARG1	GCTGGGAAGGAAGAAAAGGC	TGCCGTGTTCACAGTACTCT
CD86	GCACGTCTAAGCAAGGTCAC	CATATGCCACACACCATCCG
IL-6	GCCAGAGTCCTTCAGAGAGA	ATGGTCTTGGTCCTTAGCCA

4.2.8. Western blotting

Western blot analysis was performed as described (Jimenez-Pacheco et al. 2013). Lysis buffer (100 mM NaCl, 50 mM NaF, 1% Tx-100, 5 mM EDTA pH 8.0, 20 mM HEPES pH 7.4) containing phosphatase and protease inhibitors were used to homogenize brain tissue and extract proteins, which was quantified using a Tecan plate reader at 560 nm. 30-50 µg of protein per sample was loaded onto an acrylamide gel and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were transferred to a nitrocellulose membrane (GE Health Care) and immunoblotted with primary antibodies (Table 5). Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5000; Sigma-Aldrich, Dublin, Ireland). Protein bands were visualized using Fujifilm LAS-4000 system with chemiluminescence (Imombilon western HRP substrate, Merck Millipore, Cork, Ireland) and analyzed using Alpha-EaseFC4.0 software. Spot dense option was used to evaluate the optical density of each protein band. Protein quantity was normalized to the loading control β-Actin (1:1000; anti-mouse; Sigma-Aldrich, Wicklow, Ireland) or GAPDH (1:1000; anti-rabbit; Cell-signaling, Massachusetts, USA).

4.2.9. Immunofluorescence

Mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) and brains were removed. Following 24 h of post-fixation in 4% PFA at 4ºC, brains were transferred to PBS and immersed into 4% agarose. 50 µm sagittal sections were cut using the VT1000S vibratome (Leica Biosystems, Wetzlar, Germany), and sections were stored at -20ºC in glycol. Tissue sections were incubated with 0.1% triton/PBS, followed by 1 M glycine and with 1% BSA-PBS. Sections were then incubated with primary antibodies overnight (Table 5). Af-

Table 5. Antibodies used for Western blotting and Immunostaining. *Abbreviations*: WB, Western blot; IF, Immunofluorescence.

Gene name	Host	Supplier	WB dilution	Immunostaining dilution
β-Actin	Mouse monoclonal	Sigma (A2228)	1:1000	-
CD206	Goat	R&D systems (AF2535-SP)	-	1:40 (IF)
GFAP	Mouse	Sigma (G3893)	1:1000	1:400 (IF)
Iba-1	Rabbit Goat	Wako(019-19741) Abcam (ab5076)	-	1:400 (IF) 1:400 (IF)
CD16/32	Rat	BD bioscience (553141)	-	1:100 (IF)
P2X7R	Rabbit	Alomone labs (APR-004) Synaptic system (177003)	1:200	-
P2X7 nanobody (7E2-rbIgG)	Rabbit	Nolte Lab	-	1:400 (IF)

ter washing in PBS, tissue was incubated with fluorescent secondary antibodies, AlexaFluor 568 (Cat #: A-11011) or 488 (Cat #: A11008) (1/400 prepared in 1% BSA-PBS; anti-rabbit-IgG; BioSciences, Dublin, Ireland), followed by a short incubation with DAPI (1:500; Sigma-Aldrich, Dublin, Ireland). FluorSave™ (Millipore, Dublin, Ireland) was used to mount tissue. Confocal images were taken with a Zeiss 710 LSM NLO confocal microscope equipped with four laser lines (405, 488, 561, and 653 nm) using a 40X immersion oil objective and ZEN 2010B SP1 software. For immunofluorescence using P2X7R nanobodies {Kaczmarek-Hajek, 2018 #16}, tissue sections were washed with PBS, then washed in 0.1% Triton-X100 for 7 min and blocked with 0.05% saponin/3% BSA/15 mM NH4Cl/PBS (blocking buffer) for 20 min. Primary antibodies against P2X7R (1:200; anti-rabbit nanobody; Nolte group, Hamburg, Germany) and GFP (1:400; anti-chicken, Invitrogen, Waltham, Massachusetts, USA) were incubated overnight at 4°C in blocking buffer without saponin. After 3 washes with PBS, sections were incubated for 2 h at room temperature (RT) with secondary antibodies, AlexaFluor-488 and AlexaFluor-647 (1:400; Life Technologies, Eugene, Oregon, USA), washed 3 times with PBS, shortly stained with DAPI (1 mg/L, Carl Roth, Karlsruhe, Germany), washed with water and mounted (PermaFluor, Thermo Fisher, Dreieich, Germany) for con-

focal microscopy on a Zeiss LSM880 equipped with four laser lines (405, 488, 561 and 633 nm) using a 40X immersion oil objective and ZEN 2.3 SP1 FP1 (black) software.

4.2.10. 3D morphological analysis of microglia

To analyze morphological changes of microglia, we performed an immunofluorescence staining, as described in the previous section. Microglia cells were identified via Iba-1 (1:400; anti-goat; Abcam, Cambridge, UK). Z-stacks of ≈ 450 slices (~15 μm thickness) were taken with a Zeiss 710 LSM NLO confocal microscope using a 40x immersion oil objective and ZEN 2010B SP1 software. Z-stacks were taken from each hippocampal subfield (i.e. CA1, CA3 and DG), amounting to 3 images per slice, N = 3 per group (Control) and N = 3 per group (SE) for both wt and P2X7 KO mice in microglia. Images were subsequently rendered in 3D using FluoRender Version 2.25.0. Three cells from each subfield were randomly selected by a reviewer blind to groups and isolated from the z-stack. Once isolated, the background signal was removed using the 'threshold slider'. Cell process length was then measured on the same software, using the multipoint measurement tool, beginning from the border of the soma (located using DAPI) to the most extremity of the cell process. Only primary processes were analyzed, meaning processes that extended directly from the soma, as opposed to secondary or tertiary processes branching of the primary cell process. The average process length was calculated as the mean length of all primary processes extending from the cell body of each individual cell.

4.2.11. Fluoro-Jade B staining

Coronal sections 12 μm thick at the medial level of the hippocampus (Bregma AP = -1.94 mm) were cut on a cryostat. Tissue was fixed in formalin, rehydrated in ethanol, and then transferred to a 0.006% potassium permanganate solution followed by incubation with 0.001% FjB (Chemicon Europe Ltd, Chandlers Ford, UK). Sections were mounted in DPX mounting solution. Then, using an epifluorescence microscope, cells including all hippocampal subfields (dentate gyrus (DG), CA1, and CA3 regions) were counted by a person unaware of treatment under a 40x lens in two adjacent sections, and the average determined for each animal.

4.2.12. Cytokine measurement in brain tissue

IL-1β, TNF-α and IL-10 levels were measured using the DuoSet ELISA kits from R&D Systems following the manufacturer's instructions (mouse IL-1β/IL-1F2, Cat #: DY401-05, mouse TNF-α, catalog #DY410-05, mouse IL-10 catalog #DY417-05). First, detection antibody was incubated overnight in a 96-well ELISA plate at RT. Then, 100 µl of samples (50 ng) and standard curve (IL-1β: from 15.6 - 1000 pg/ml; TNF-α and IL-10: 31.2–2000 pg/ml) were added to wells and incubated for 2 h at RT, followed by incubation with 100 µl of streptavidin-HRP complex. A color reaction, caused by the addition of a substrate solution (100 µL) and terminated via stopping solution (50 µL), was quantified at 450 and 570 nm using a microplate reader. Cytokine concentration was obtained following the manufacturer's recommendations; 570 nm values were subtracted from the 450 nm values. The log10 of the standard curve values were plotted, and a line of best fit was generated. The amount of cytokines was extrapolated using standard curve and average of calculated triplicate. Cytokine concentration was then normalized to milligrams of total protein concentration in tissue. Data is presented as n-fold of control samples.

4.2.13. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 and STATVIEW software (SAS Institute, Cary, NC, U.S.A). Data are presented as means ± standard error of the mean (SEM). One-way ANOVA parametric statistics with *post hoc* Fisher's protected least significant difference test was used to determine statistical differences between three or more groups. Unpaired Student's t-test was used for a two-group comparison. Two-way ANOVA was used for repeated measures between groups where a series of measurements have been taken from the same mouse at different time-points. Significance was accepted at * $P < 0.05$.

4.3. Results

4.3.1. Efficacy of tamoxifen treatment

Analysis confirmed that P2rx7 messenger RNA and protein levels were reduced by ~20-40% post-tamoxifen treatment in the hippocampus of P2rx7$^{-/-}$-M and P2rx7$^{-/-}$-N mice under baseline conditions and following SE (8 and 24 h) (Figure 18A, B). Co-immunostaining using a highly specific P2X7R nanobody (Kacz-

marek-Hajek et al. 2018) and the microglia marker Iba-1 confirmed the absence of P2X7R on microglia in P2rx7$^{-/-}$-M mice post-tamoxifen treatment (Figure 18C). P2X7R deficiency in microglia post-tamoxifen was further confirmed via fluorescent activated cell sorting (FACS) followed by qPCR, showing no P2rx7 mRNA amplification in microglia (CD11^{+}/CD45^{-}) or macrophages (CD11^{-}/CD45^{+}) (Figure 18D).

4.3.2. Cell type-specific effects of the P2X7R on acute seizures (IAKA model)

Compared to wt mice, P2rx7$^{-/-}$-M mice experienced reduced seizure severity as measured by surface EEG recordings during the 40 min following IAKA until the administration of the anticonvulsant lorazepam (Figure 19A, B). In contrast, mice lacking the P2X7R in neurons (P2rx7$^{-/-}$-N), developed more severe seizures during IAKA-induced SE compared to wt animals (Figure 19A, B).

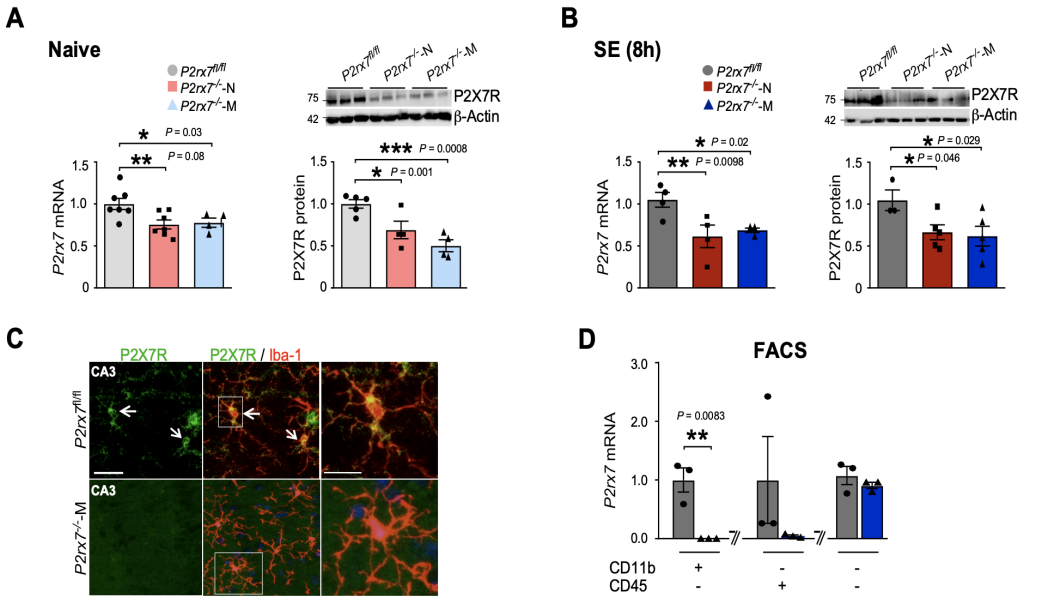

Figure 18. Analysis post-tamoxifen treatment. A) Hippocampal P2X7R mRNA and protein levels post-tamoxifen treatment (mRNA: n = 7 (wt), 7 (P2rx7-/--N), 4 (P2rx7-/--M); protein: n = 5 (wt), 4 (P2rx7-/--N), 4 (P2rx7-/--M)) in naïve conditions and (B) 8 h post-IAKA (mRNA: n = 4 (wt), 4 (P2rx7-/--N), 4 (P2rx7-/--M)); protein: n = 3 (wt), 5 (P2rx7-/--N), 5 (P2rx7-/--M)). (C) Co-localization (arrows) of P2X7R (green) with Iba-1 (red) in the hippocampus post-tamoxifen treatment. D) P2X7R mRNA levels in FACS-separated hippocampal microglia and macrophages (n = 3/group). A, B One-way ANOVA followed by Fischer's multiple-comparison test. D Unpaired Student's t-test. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001

Analysis of brain samples 72 h post-SE revealed that P2rx7$^{-/-}$-N mice lacking neuronal P2X7R displayed increased neurodegeneration and astrocytosis compared to wt mice. In contrast, mice lacking the P2X7R in microglia displayed similar levels of seizure-induced neuronal death and GFAP-positive cells in the hippocampus. No difference was observed in the number of Iba-1-positive cells across genotypes (Figure 20 A-C).

4.3.3. Cell type-specific effects of the P2X7R on acute seizures (PTZ model)

Consistent with the earlier findings in the IAKA model, P2rx7$^{-/-}$-N mice developed more severe behavioral seizures when compared to wt mice and P2rx7$^{-/-}$-M mice, whereas P2rx7$^{-/-}$-M mice displayed a delay to the first myoclonic tonic-clonic seizure (Figure 21A-C). Moreover, all P2rx7$^{-/-}$-M survived the first 30 min post-PTZ injection compared to only ~70% of wt mice and approximately 50% of P2rx7$^{-/-}$-N (Figure 21D). These findings suggest the cell type-specific effects of

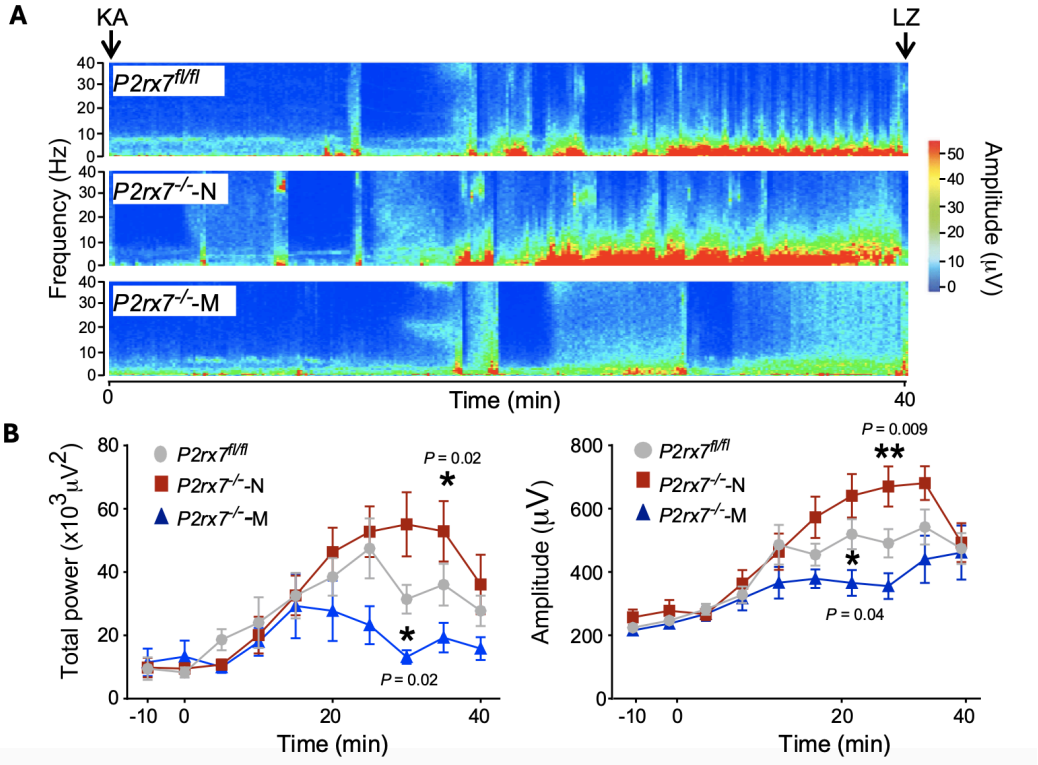

Figure 19. Cell type-specific effects of the P2X7R after IAKA injection A) Representative EEG heat maps during IAKA-induced SE. B) EEG total power and amplitude from IAKA until lorazepam (n = 18 (P2rx7+/+), 14 (P2rx7-/--N), 13 (P2rx7-/--M). Two-way ANOVA with Tukey's multiple comparisons test. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01

deleting the P2X7 receptor from neurons or microglia are independent of the method of seizure induction.

4.3.4. *Cell type-specific effects of the P2X7R on epilepsy development*

Deletion of the P2X7R in microglia reduced the number of spontaneous seizures in mice during a 14-day recording period, starting at 3 days post-SE. In contrast, the loss of P2X7R from neurons resulted in a slightly more severe epilepsy phenotype when compared to wt mice (Figure 22).

Together, these data demonstrate a cell-type-specific contribution of the P2X7R to the generation of seizures and epilepsy. Neuronally-expressed P2X7R may confer modest anti-seizure effects in this context, whereas the P2X7R in microglia enhances brain hyperexcitability.

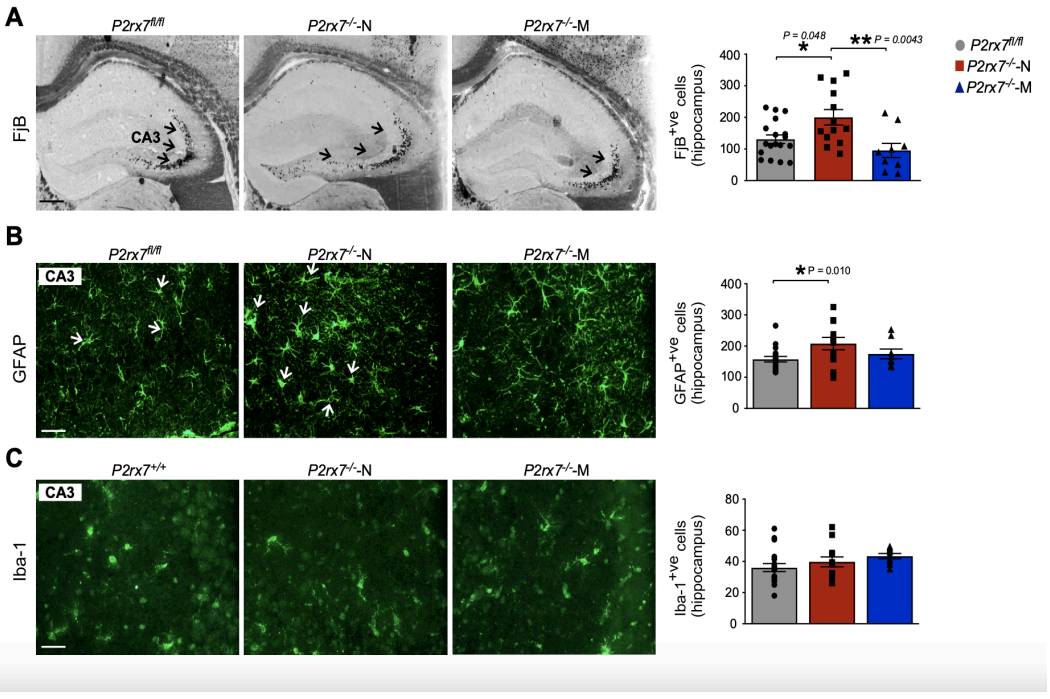

Figure 20. Lack of P2X7R in neurons increases neurodegeneration and astrogliosis following IAKA-induced SE. A) Representative FjB images (5x lens) and corresponding graph showing more FjB-positive cells in the ipsilateral hippocampus of P2X7-/--N mice when compared to wt or P2X7-/--M 24 h post-IAKA-induced SE. Scale bar = 500mm. B) Photomicrograph (40x lens) showing a significant increase in the number of GFAP-positive cells in the ipsilateral hippocampus of P2X7-/--N mice when compared to wt mice 24 h following IAKA-induced SE. Scale bar = 50mm. C) Photomicrograph (40x lens) showing no difference in the number of Iba-1 positive-cells in the ipsilateral hippocampus between

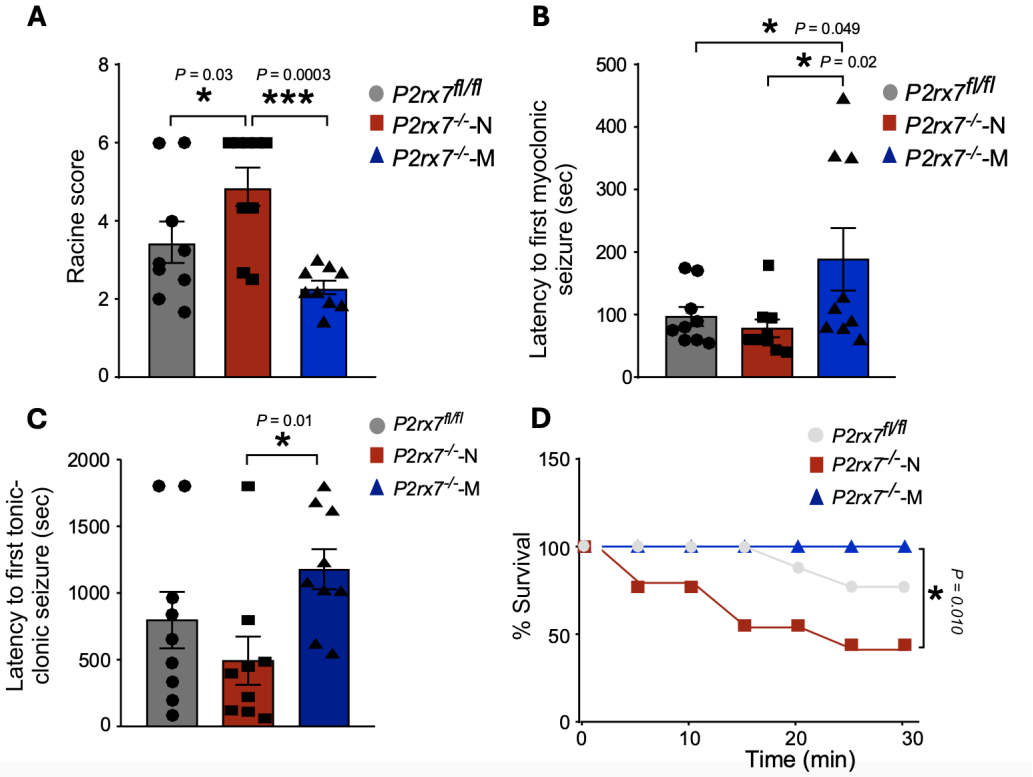

Figure 21. Cell type-specific effects of the P2X7R after PTZ injection A) Behavior seizures of tamoxifen-treated mice post-PTZ (n = 9/group). B) Threshold to first myoclonic and C) tonic-clonic seizure in tamoxifen-treated mice post-PTZ (n = 9/group). D) Survival analysis of tamoxifen-treated mice 30 min post-PTZ (n = 9/group). A, B, C One-way ANOVA with Fischer's multiple-comparison test. D Log-rank (Mantel-Cox) Test. Data are shown as mean ± SEM. *P < 0.05, *P < 0.01, ***P < 0.001.

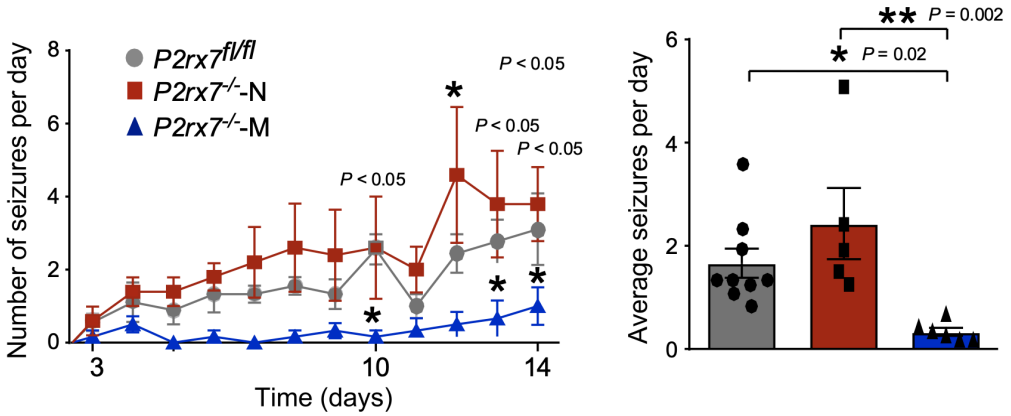

Figure 22. Daily seizure counts in tamoxifen-treated mice post-IAKA. (n = 9 (P2rx7+/+), 5 (P2rx7-/--N), 6 (P2rx7-/--M). Two-way ANOVA with Tukey's multiple comparisons test. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01.

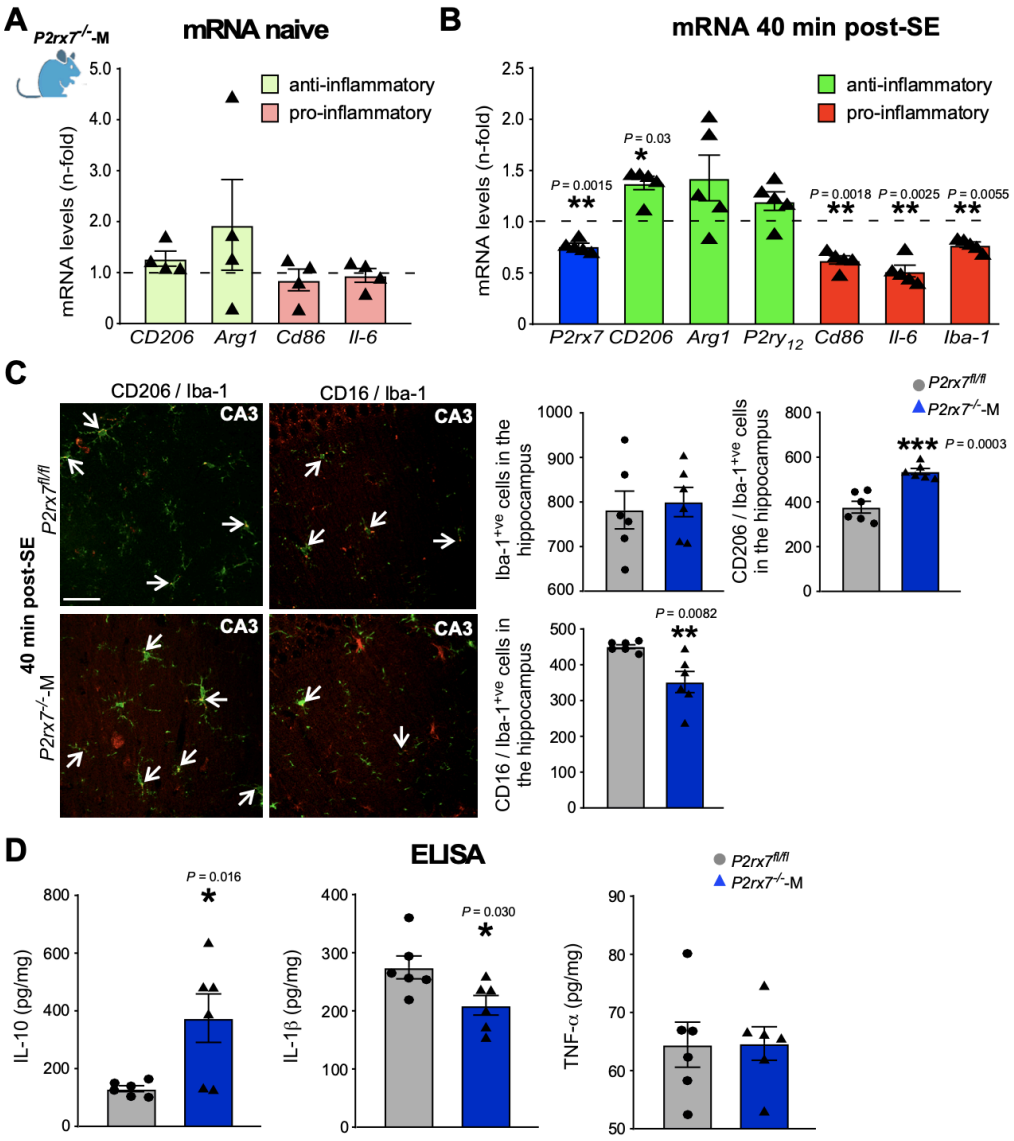

Figure 23. P2X7R deficiency in microglia promotes an anti-inflammatory microglia phenotype post-SE. A) Hippocampal mRNA levels of the anti-inflammatory markers Cd206 and Arg1 and pro-inflammatory markers Cd86 or Il-6 of tamoxifen-treated mice (n = 4/group). B) Hippocampal (ipsilateral) mRNA levels of the anti-inflammatory marker Cd206, and pro-inflammatory markers Cd86, Il-6 and Iba-1 in tamoxifen-treated P2rx7+/+ and P2rx7-/--M mice 40 min post-IAKA (n = 5/group). C) Degree of co-localization of CD206 (red) or CD16 (red) with microglia marker Iba-1 (green) in the ipsilateral hippocampus of tamoxifen-treated P2X7+/+ and P2rx7-/--M mice 40 min post-IAKA. White arrows indicate co-localization (n = 6/group). D) IL-10, IL-1β and TNF-α levels in the ipsilateral hippocampus of tamoxifen-treated P2rx7+/+ and P2rx7-/--M mice 40 min post-IAKA (n = 6/group). B, One-way ANOVA with Fischer's multiple-comparison test. C (right graphs), D, Unpaired Student's t-test. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

4.3.5. *P2X7R deficiency on microglia promotes an anti-inflammatory microglia phenotype following SE*

Analysis of gene expression in hippocampal samples from P2rx7$^{-/-}$-M compared to wt mice revealed no baseline differences in microglia activation markers. In contrast, there was enhanced expression of anti-inflammatory genes (Mrc1 (Cd206), Arg1, p2ry12) and lower expression of pro-inflammatory genes (Cd86, Il-6, Iba-1) in P2rx7$^{-/-}$-M mice when compared to IAKA-injected wt mice (Figure 23A,B). This shows that the P2X7R on microglia contributes to pro-inflammatory responses to SE. No changes in microglial genes were observed when comparing gene profiles from Cre mice with wt mice, confirming these changes are due to deletion of P2X7Rs on microglia and not a non-specific effect of the expression of Cre (Figure 24). To extend these findings, we performed double staining for the microglia marker Iba-1 and the anti-inflammatory microglia marker CD206. This showed an elevated number of CD206-positive cells in the hippocampus of P2rx7$^{-/-}$-M mice post-IAKA (Figure 23**C**). In contrast, there was a reduced number of CD16-positive cells, a pro-inflammatory marker, in the hippocampus of P2rx7$^{-/-}$-M mice after SE.

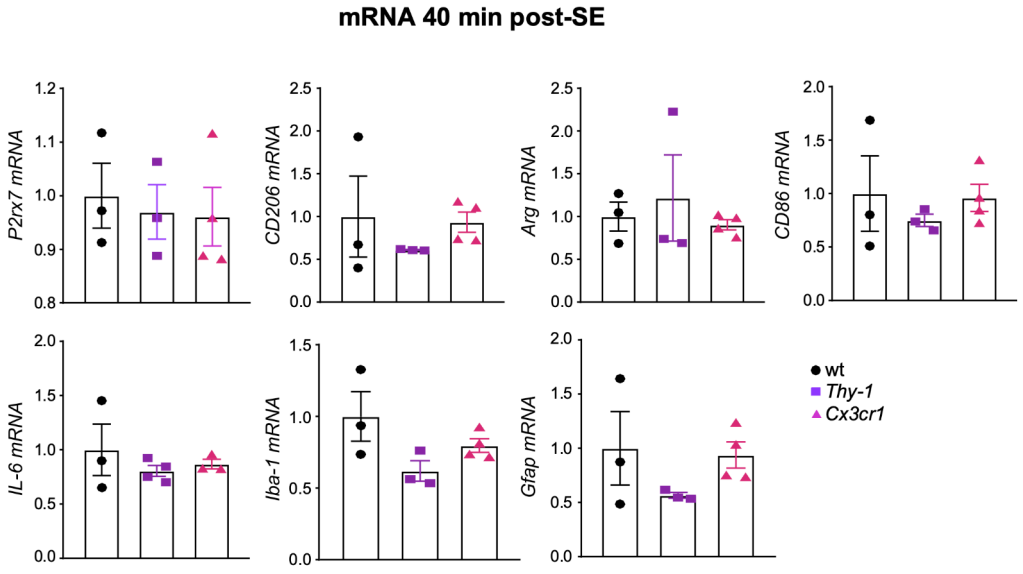

Figure 24. No impact of Cre on microglial gene expression. Graphs showing no difference in mRNA levels of P2rx7, anti-inflammatory markers CD206, Arg, pro-inflammatory markers CD86, IL-6 and Iba-1, and astrocyte marker Gfap between Thy-1, Cx3cr1 and wt mice pre-treated with tamoxifen mice during 40 min of SE (n = 3 (wt) 3 (Thy-1) and 4 (Cx3cr1). One-way ANOVA followed by Fischer's multiple-comparison test. Data are shown as mean ± SEM.

While no obvious changes in microglia morphology between genotypes could be observed post-SE, microglia in P2rx7$^{-/-}$-M mice showed slightly longer primary processes, suggesting a more anti-inflammatory phenotype (Figure 25) (Leyh et al. 2021). ELISA analysis of hippocampal tissue obtained 40 min post-IAKA showed higher levels of the anti-inflammatory cytokine IL-10 and lower levels of the pro-inflammatory cytokine IL-1β in P2rx7$^{-/-}$-M mice when compared to wt mice. No change in TNF-α levels was observed between genotypes (Figure 23D).

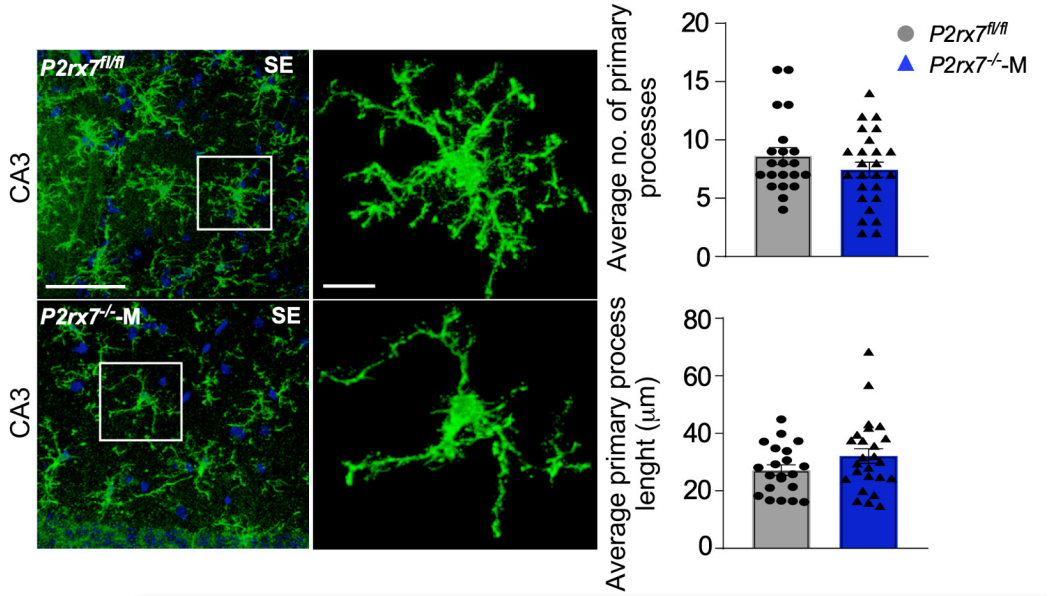

Figure 25. No obvious change in microglial morphology after SE in mice with a microglial P2X7R knock-out. Photomicrographs of IBA1+ve cells from the hippocampus and isolated IBA1 +ve cells (outlined by white square) taken 40 mins post status epilepticus induction in both mice lacking P2X7R expression in microglia and controls. Graphs showing average number of 1° processes and average 1° process length (µm) of isolated microglia. From controls (n = 21 cells from 3 mice,) and mice lacking microglia P2X7R (n = 25 cells from 3 mice). Unpaired Student's t-test. Data are shown as mean ± SEM. Scale bar = 50 µm

4.4. Discussion

Here, we break down the cell type-specific functions of the P2X7R and show divergent effects in regulating brain hyperexcitability in epilepsy. We show that loss of the P2X7R from microglia has anti-convulsive and anti-epileptogenic effects whereas its deletion from neurons increases excitability and leads to a more severe epileptic phenotype. Together, the findings may explain previous conflicting findings on the seizure-modifying actions of the P2X7R and indicate P2X7R-based

precision and gene therapy approaches to enhance inhibitory neuron function to treat epilepsies.

Although it is well-established that the P2X7R is an important initiator of neuroinflammation, and neuroinflammation is a well-known contributor to brain hyperexcitability (Vezzani et al. 2019), there have been conflicting reports on the efficacy of targeting the receptor during seizures and epilepsy (Beamer et al. 2021). This is most likely, at least in part, due to the cell types that express P2X7R and how its expression profile changes during disease progression. The complexity of how P2X7Rs impact seizures and epilepsy may derive from the bi-directional effects of microglia on inflammation and brain excitability, with both proconvulsant and anti-convulsant functions being reported (Kinoshita and Koyama 2021), and the potentially counter-acting effects of any presence of neurons which could lead to increased or decreased network excitability according to whether the effect is to boost inhibitory or excitatory drive. Therefore, the objective of the present study was to gain clarity on which cell types express P2X7Rs during epilepsy, what are their contributions to the disease, and whether this can be used to develop new treatment strategies for better and more effective seizure control.

A key finding of the present study was that microglial P2X7R drives inflammatory signaling and contributes to hyperexcitability. This is based on the finding that mice lacking the receptor in microglia show less severe seizures during IAKA-induced SE and following PTZ treatment and develop a milder epileptic phenotype. These results align with inflammation contributing to brain hyperexcitability and drugs blocking neuroinflammation increasing the seizure threshold (Vezzani et al. 2019). The P2X7R has been described as a key driver of inflammation, contributing to the activation of the NLRP3 inflammasome and the proliferation and activation of microglia (Campagno and Mitchell 2021; Monif et al. 2009; Pelegrin 2021). Previous studies have shown P2X7R antagonism to reduce IL-1β levels in the hippocampus post-SE (Engel et al. 2012) and to reduce microgliosis and astrogliosis during epilepsy (Jimenez-Pacheco et al. 2016). More recently, it has been shown that increased P2X7R activation post-SE leads to a pro-inflammatory phenotype in microglia (Beamer et al. 2022), and, conversely, that blocking of the P2X7R leads to an anti-inflammatory microglial phenotype following seizures in a mouse model of neonatal hypoxia induced-seizures (Smith et al. 2023). Of note, our study shows a strong up-regulation of the cytokine IL-10 and reduction of IL-1β in the hippocampus of mice with a microglia P2X7R deletion. The pro-con-

vulsant function of IL-1β is well-established (Maroso et al. 2010) and IL-10 is a known anti-epileptic cytokine. Interestingly, recent data show that IL-10 enhances GABAergic signaling which is antagonized by IL-1β (Maroso et al. 2010). IL-10 has also been shown to reduce IL-1β levels (Maroso et al. 2010). While our results suggest that the proconvulsant effects of P2X7R on microglia are mediated via altering inflammatory signaling, we cannot exclude other effects contributing to seizures and epilepsy. The P2X7R has been shown to regulate numerous pathways in microglia (e.g., clearance of extracellular and intracellular debris) (Campagno and Mitchell 2021), which contribute to seizures and epilepsy (Kinoshita and Koyama 2021). Of note, while microglia had previously been ascribed a mainly pro-convulsant role, data also suggests a protective function of microglia with the depletion of microglia leading to a more severe seizure phenotype (Badimon et al. 2020). This suggests that targeting specific pathways (e.g., P2X7R) is most likely more effective than blocking microglia function as a whole.

It is important to remember that while representing an invaluable tool to examine cell-type-specific contributions of target genes to diseases, both *Cx3cr1* and *Thy-1* have been shown to target additional cell types such as macrophages and T-cells (Bradley et al. 2009; Zhao et al. 2019). While we have shown an almost complete knock-down of P2X7R in microglia (*Cx3cr1*) and neurons (*Thy-1*), we can, therefore, not rule out that other cell types that show high P2X7R expression (*e.g.*, oligodendrocytes (Kaczmarek-Hajek et al. 2018)), contribute to the observed phenotypes and a possible contribution of these cells should be evaluated in future studies. For our study, we have used male mice; possible gender effects on the described P2X7R function need to be addressed. It has been previously shown, however, that both male and female mice with an altered P2X7R expression respond similarly to IAKA (Beamer et al. 2022).

5. Conclusion

While distinct in methodology and scope, the two studies reported here reinforce the importance of a multidisciplinary approach in the field of translational research in animal models of disease, with data obtained from behavioral assessments, electrophysiological recordings, molecular and pharmacological assays, and quantitative histological analyses.

In conclusion, in the first study, we demonstrated that the spike-wave epileptic discharges observed in JAX mice present important similarities with clinical ESES, including a strong association with non-REM sleep and their disappearance in adulthood. We also demonstrated that the electrical status epilepticus that characterizes ESES syndromes affects both learning and synaptic plasticity in JAX mice. The observed changes suggest an imbalance in the excitation/inhibition ratio, possibly contributing to learning deficits and emphasizing the impact of SWD on synaptic function.

In the second study, we demonstrated for the first time a cell type-specific contribution of the P2X7R to seizures and epilepsy. We showed that loss of the P2X7R from microglia has anti-convulsive and anti-epileptogenic effects, whereas its deletion from neurons increases excitability and leads to a more severe epileptic phenotype. These findings suggest that P2X7R in neurons may act as an innate defense mechanism against pathological brain hyperexcitability.

6. References

Abbracchio, Maria P., Geoffrey Burnstock, Jean-Marie Boeynaems, Eric A. Barnard, José L. Boyer, Charles Kennedy, Gillian E. Knight, et al. 2006. "International Union of Pharmacology LVIII: Update on the P2Y G Protein-Coupled Nucleotide Receptors: From Molecular Mechanisms and Pathophysiology to Therapy." Pharmacological Reviews 58 (3): 281–341.

Abegg, Mathias H., Natasa Savic, Markus U. Ehrengruber, R. Anne McKinney, and Beat H. Gähwiler. 2004. "Epileptiform Activity in Rat Hippocampus Strengthens Excitatory Synapses." *The Journal of Physiology* 554 (Pt 2): 439–48.

Abraham, W. C., and M. F. Bear. 1996. "Metaplasticity: The Plasticity of Synaptic Plasticity." *Trends in Neurosciences* 19 (4): 126–30.

Alves, Mariana, Jonathon Smith, and Tobias Engel. 2019. "Differential Expression of the Metabotropic P2Y Receptor Family in the Cortex Following Status Epilepticus and Neuroprotection via P2Y1 Antagonism in Mice." *Frontiers in Pharmacology* 10: 1558.

Amhaoul, Halima, Idrish Ali, Marco Mola, Annemie Van Eetveldt, Krystyna Szewczyk, Stephan Missault, Kenny Bielen, et al. 2016. "P2X7 Receptor Antagonism Reduces the Severity of Spontaneous Seizures in a Chronic Model of Temporal Lobe Epilepsy." *Neuropharmacology* 105 (June): 175–85.

Attardo, Alessio, James E. Fitzgerald, and Mark J. Schnitzer. 2015. "Impermanence of Dendritic Spines in Live Adult CA1 Hippocampus." *Nature* 523 (7562): 592–96.

Avoli, Massimo, Marco de Curtis, Vadym Gnatkovsky, Jean Gotman, Rüdiger Köhling, Maxime Lévesque, Frédéric Manseau, Zahra Shiri, and Sylvain Williams. 2016. "Specific Imbalance of Excitatory/inhibitory Signaling Establishes Seizure Onset Pattern in Temporal Lobe Epilepsy." *Journal of Neurophysiology* 115 (6): 3229–37.

Badimon, Ana, Hayley J. Strasburger, Pinar Ayata, Xinhong Chen, Aditya Nair, Ako Ikegami, Philip Hwang, et al. 2020. "Negative Feedback Control of Neuronal Activity by Microglia." *Nature* 586 (7829): 417–23.

Bandopadhyay, Ritam, Tanveer Singh, Mohammed M. Ghoneim, Sultan Alshehri, Efthalia Angelopoulou, Yam Nath Paudel, Christina Piperi, et al. 2021. "Recent Developments in Diagnosis of Epilepsy: Scope of MicroRNA and Technological Advancements." *Biology* 10 (11). https://doi.org/10.3390/biology10111097.

Barker-Haliski, Melissa, and H. Steve White. 2015. "Glutamatergic Mechanisms Associated with Seizures and Epilepsy." *Cold Spring Harbor Perspectives in Medicine* 5 (8): a022863.

Beamer, Edward, Manvitha Kuchukulla, Detlev Boison, and Tobias Engel. 2021. "ATP and Adenosine-Two Players in the Control of Seizures and Epilepsy Development." *Progress in Neurobiology* 204 (September): 102105.

Beamer, Edward, James Morgan, Mariana Alves, Aida Menéndez Méndez, Gareth Morris, Béla Zimmer, Giorgia Conte, et al. 2022. "Increased Expression of the ATP-Gated P2X7 Receptor Reduces Responsiveness to Anti-Convulsants during Status Epilepticus in Mice." *British Journal of Pharmacology* 179 (12): 2986–3006.

Beck, Joe, Brett Lenart, Douglas B. Kintner, and Dandan Sun. 2003. "Na-K-Cl Cotransporter Contributes to Glutamate-Mediated Excitotoxicity." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 23 (12): 5061–68.

Beghi, Ettore. 2020. "The Epidemiology of Epilepsy." *Neuroepidemiology* 54 (2): 185–91.

Benbadis, S. 2009. "The Differential Diagnosis of Epilepsy: A Critical Review." *Epilepsy & Behavior: E&B* 15 (1): 15–21.

Berg, Anne T., Samuel F. Berkovic, Martin J. Brodie, Jeffrey Buchhalter, J. Helen Cross, Walter van Emde Boas, Jerome Engel, et al. 2010. "Revised Terminology and Concepts for Organization of Seizures and Epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005-2009." *Epilepsia* 51 (4): 676–85.

Bertini, Giuseppe, Placido Bramanti, Gabriela Constantin, Michele Pellitteri, Beatrice Mihaela Radu, Mihai Radu, and Paolo Francesco Fabene. 2013. "New Players in the Neurovascular Unit: Insights from Experimental and Clinical Epilepsy." *Neurochemistry International* 63 (7): 652–59.

Bhattacharya, Anindya, and Knut Biber. 2016. "The Microglial ATP-Gated Ion Channel P2X7 as a CNS Drug Target." Glia 64 (10): 1772–87.

Bliss, T. V., and A. R. Gardner-Medwin. 1973. "Long-Lasting Potentiation of Synaptic Transmission in the Dentate Area of the Unanaestetized Rabbit Following Stimulation of the Perforant Path." *The Journal of Physiology* 232 (2): 357–74.

Bliss, T. V., and T. Lomo. 1973. "Long-Lasting Potentiation of Synaptic Transmission in the Dentate Area of the Anaesthetized Rabbit Following Stimulation of the Perforant Path." *The Journal of Physiology* 232 (2): 331–56.

Blume, Warren T. 2003. "Diagnosis and Management of Epilepsy." *CMAJ: Canadian Medical Association Journal = Journal de l'Association Medicale Canadienne* 168 (4): 441–48.

Bozzi, Yuri, Giovanni Provenzano, and Simona Casarosa. 2018. "Neurobiological Bases of Autism-Epilepsy Comorbidity: A Focus on Excitation/inhibition Imbalance." *The European Journal of Neuroscience* 47 (6): 534–48.

Bradley, John E., Gustavo Ramirez, and James S. Hagood. 2009. “Roles and Regulation of Thy-1, a Context-Dependent Modulator of Cell Phenotype.” *BioFactors* 35 (3): 258–65.

Buckmaster, Paul S. n.d. “Mossy Fiber Sprouting in the Dentate Gyrus.” In *Jasper’s Basic Mechanisms of the Epilepsies*, edited by Jeffrey L. Noebels, Massimo Avoli, Michael A. Rogawski, Richard W. Olsen, and Antonio V. Delgado-Escueta. Bethesda (MD): National Center for Biotechnology Information (US).

Burnstock, Geoff. 2018. “Editor’s Note.” Purinergic Signalling 14 (3): 213–213.

Burnstock, Geoffrey. 2006. “Historical Review: ATP as a Neurotransmitter.” Trends in Pharmacological Sciences 27 (3): 166–76. 2008. “Purinergic Signalling and Disorders of the Central Nervous System.” Nature Reviews. Drug Discovery 7 (7): 575–90.

Campagno, Keith E., and Claire H. Mitchell. 2021. “The P2X7 Receptor in Microglial Cells Modulates the Endolysosomal Axis, Autophagy, and Phagocytosis.” *Frontiers in Cellular Neuroscience* 15 (March): 645244.

Caraballo, Roberto Horacio, Sebastian Fortini, Santiago Flesler, María Constanza Pasteris, Luciana Caramuta, and Ernesto Portuondo. 2015. “Encephalopathy with Status Epilepticus during Sleep: Unusual EEG Patterns.” *Seizure: The Journal of the British Epilepsy Association* 25 (February): 117–25.

Caroni, Pico, Flavio Donato, and Dominique Muller. 2012. “Structural Plasticity upon Learning: Regulation and Functions.” *Nature Reviews. Neuroscience* 13 (7): 478–90.

Carvill, Gemma L., Brigid M. Regan, Simone C. Yendle, Brian J. O’Roak, Natalia Lozovaya, Nadine Bruneau, Nail Burnashev, et al. 2013. “GRIN2A Mutations Cause Epilepsy-Aphasia Spectrum Disorders.” *Nature Genetics* 45 (9): 1073–76.

Casillas-Espinosa, Pablo M., Kim L. Powell, and Terence J. O’Brien. 2012. “Regulators of Synaptic Transmission: Roles in the Pathogenesis and Treatment of Epilepsy.” *Epilepsia* 53 Suppl 9 (December): 41–58.

Cavalheiro, E. A. 1995. “The Pilocarpine Model of Epilepsy.” *Italian Journal of Neurological Sciences* 16 (1-2): 33–37.

Chakir, A., P. F. Fabene, R. Ouazzani, and M. Bentivoglio. 2006. “Drug Resistance and Hippocampal Damage after Delayed Treatment of Pilocarpine-Induced Epilepsy in the Rat.” *Brain Research Bulletin* 71 (1-3): 127–38.

Chen, Kai, Yuhan Zheng, Ji-An Wei, Huan Ouyang, Xiaodan Huang, Feilong Zhang, Cora Sau Wan Lai, Chaoran Ren, Kwok-Fai So, and Li Zhang. 2019. “Exercise Training Improves Motor Skill Learning via Selective Activation of mTOR.” *Science Advances* 5 (7): eaaw1888.

Chen, Michelle B., Xian Jiang, Stephen R. Quake, and Thomas C. Südhof. 2020. "Persistent Transcriptional Programmes Are Associated with Remote Memory." *Nature* 587 (7834): 437–42.

Citri, Ami, and Robert C. Malenka. 2008. "Synaptic Plasticity: Multiple Forms, Functions, and Mechanisms." *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology* 33 (1): 18–41.

Colavito, Valeria, Paolo F. Fabene, Gigliola Grassi-Zucconi, Fabien Pifferi, Yves Lamberty, Marina Bentivoglio, and Giuseppe Bertini. 2013. "Experimental Sleep Deprivation as a Tool to Test Memory Deficits in Rodents." *Frontiers in Systems Neuroscience* 7 (December): 106.

Diekelmann, Susanne, and Jan Born. 2010. "The Memory Function of Sleep." *Nature Reviews. Neuroscience* 11 (2): 114–26.

Doğan, Elif, Hatice Aygün, Gökhan Arslan, Emil Rzayev, Bahattin Avcı, Mustafa Ayyıldız, and Erdal Ağar. 2020. "The Role of NMDA Receptors in the Effect of Purinergic P2X7 Receptor on Spontaneous Seizure Activity in WAG/Rij Rats With Genetic Absence Epilepsy." *Frontiers in Neuroscience* 14 (May): 414.

Doná, Flavia, Henning Ulrich, Daniele Suzete Persike, Isaltino Marcelo Conceição, João Paulo Blini, Esper Abrão Cavalheiro, and Maria José Silva Fernandes. 2009. "Alteration of Purinergic P2X4 and P2X7 Receptor Expression in Rats with Temporal-Lobe Epilepsy Induced by Pilocarpine." *Epilepsy Research* 83 (2-3): 157–67.

Donat, Jane F. 1992. "Topical Review Article: The Age-Dependent Epileptic Encephalopathies." *Journal of Child Neurology* 7 (1): 7–21.

Elmér, Eskil. 1997. *Mechanisms of Hyperexcitability in the Kindling Model of Epilepsy*. Section of Restorative Neurology, Department of Clinical Neuroscience, University of Lund.

Engel, Jerome, Timothy A. Pedley, and Jean Aicardi. 1998. *Epilepsy: A Comprehensive Textbook*. Vol. 3. Lippincott-Raven.

Engel, Tobias. 2023. "The P2X7 Receptor as a Mechanistic Biomarker for Epilepsy." *International Journal of Molecular Sciences* 24 (6). https://doi.org/10.3390/ijms24065410.

Engel, Tobias, Rosa Gomez-Villafuertes, Katsuhiro Tanaka, Guillaume Mesuret, Amaya Sanz-Rodriguez, Paula Garcia-Huerta, M. Teresa Miras-Portugal, David C. Henshall, and Miguel Diaz-Hernandez. 2012. "Seizure Suppression and Neuroprotection by Targeting the Purinergic P2X7 Receptor during Status Epilepticus in Mice." *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 26 (4): 1616–28.

Engel, Tobias, Jonathon Smith, and Mariana Alves. 2021. “Targeting Neuroinflammation via Purinergic P2 Receptors for Disease Modification in Drug-Refractory Epilepsy.” *Journal of Inflammation Research* 14 (July): 3367–92.

Ettinger, A. B. 1994. “Structural Causes of Epilepsy. Tumors, Cysts, Stroke, and Vascular Malformations.” *Neurologic Clinics* 12 (1): 41–56.

Eun, Baik-Lin, Jayne Abraham, Lauren Mlsna, Min Jung Kim, and Sookyong Koh. 2015. “Lipopolysaccharide Potentiates Hyperthermia-Induced Seizures.” *Brain and Behavior* 5 (8): e00348.

Fabene, Paolo F., Carlo Laudanna, and Gabriela Constantin. 2013. “Leukocyte Trafficking Mechanisms in Epilepsy.” *Molecular Immunology* 55 (1): 100–104.

Fattorusso, Antonella, Sara Matricardi, Elisabetta Mencaroni, Giovanni Battista Dell’Isola, Giuseppe Di Cara, Pasquale Striano, and Alberto Verrotti. 2021. “The Pharmacoresistant Epilepsy: An Overview on Existant and New Emerging Therapies.” *Frontiers in Neurology* 12 (June): 674483.

Feng, Yan, Zi-Han Wei, Chao Liu, Guo-Yan Li, Xiao-Zhi Qiao, Ya-Jing Gan, Chu-Chu Zhang, and Yan-Chun Deng. 2022. “Genetic Variations in GABA Metabolism and Epilepsy.” *Seizure: The Journal of the British Epilepsy Association* 101 (October): 22–29.

Fernández, Iván Sánchez, Jurriaan M. Peters, Stavros Hadjiloizou, Sanjay P. Prabhu, Marcin Zarowski, Karen M. Stannard, Masanori Takeoka, Alexander Rotenberg, Sanjeev V. Kothare, and Tobias Loddenkemper. 2012. “Clinical Staging and Electroencephalographic Evolution of Continuous Spikes and Waves during Sleep.” *Epilepsia* 53 (7): 1185–95.

Fischer, Wolfgang, Heike Franke, Ute Krügel, Heiko Müller, Klaus Dinkel, Brian Lord, Michael A. Letavic, David C. Henshall, and Tobias Engel. 2016. “Critical Evaluation of P2X7 Receptor Antagonists in Selected Seizure Models.” *PloS One* 11 (6): e0156468.

Fisher, Robert S., Carlos Acevedo, Alexis Arzimanoglou, Alicia Bogacz, J. Helen Cross, Christian E. Elger, Jerome Engel Jr, et al. 2014. “ILAE Official Report: A Practical Clinical Definition of Epilepsy.” *Epilepsia* 55 (4): 475–82.

Fisher, Robert S., J. Helen Cross, Jacqueline A. French, Norimichi Higurashi, Edouard Hirsch, Floor E. Jansen, Lieven Lagae, et al. 2017. “Operational Classification of Seizure Types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology.” *Epilepsia* 58 (4): 522–30.

Fisher, Robert S., Walter van Emde Boas, Warren Blume, Christian Elger, Pierre Genton, Phillip Lee, and Jerome Engel Jr. 2005. “Epileptic Seizures and Epilepsy: Definitions

Proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE)." *Epilepsia* 46 (4): 470–72.

Fontana, Elena, Francesca Negrini, Stefano Francione, Roberto Mai, Elisa Osanni, Elisa Menna, Francesca Offredi, Francesca Darra, and Bernardo Dalla Bernardina. 2006. "Temporal Lobe Epilepsy in Children: Electroclinical Study of 77 Cases." *Epilepsia* 47 Suppl 5: 26–30.

Franklin, Keith B. J., and George Paxinos. 2013. *Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates*. Amsterdam: Academic Press, an imprint of Elsevier.

Giuliani, Anna Lisa, Alba C. Sarti, Simonetta Falzoni, and Francesco Di Virgilio. 2017. "The P2X7 Receptor-Interleukin-1 Liaison." *Frontiers in Pharmacology* 8 (March): 123.

Hamm, R. J., B. R. Pike, D. M. O'Dell, B. G. Lyeth, and L. W. Jenkins. 1994. "The Rotarod Test: An Evaluation of Its Effectiveness in Assessing Motor Deficits Following Traumatic Brain Injury." *Journal of Neurotrauma* 11 (2): 187–96.

Hebb, D. O. 1949. "The Organization of Behavior; a Neuropsychological Theory." *Https://psycnet.apa.org › Recordhttps://psycnet.apa.org › Record* 335. https://psycnet.apa.org/fulltext/1950-02200-000.pdf.

Hermann, Bruce P., Jana E. Jones, Raj Sheth, Monica Koehn, Tara Becker, Jason Fine, Chase A. Allen, and Michael Seidenberg. 2008. "Growing up with Epilepsy: A Two-Year Investigation of Cognitive Development in Children with New Onset Epilepsy." *Epilepsia* 49 (11): 1847–58.

Holmgren, Carl D., and Yuri Zilberter. 2001. "Coincident Spiking Activity Induces Long-Term Changes in Inhibition of Neocortical Pyramidal Cells." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 21 (20): 8270–77.

Illes, Peter, Tahir Muhammad Khan, and Patrizia Rubini. 2017. "Neuronal P2X7 Receptors Revisited: Do They Really Exist?" *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 37 (30): 7049–62.

Ismail, Fatima Yousif, Ali Fatemi, and Michael V. Johnston. 2017. "Cerebral Plasticity: Windows of Opportunity in the Developing Brain." *European Journal of Paediatric Neurology: EJPN: Official Journal of the European Paediatric Neurology Society* 21 (1): 23–48.

Issa, Naoum P. 2014. "Neurobiology of Continuous Spike-Wave in Slow-Wave Sleep and Landau-Kleffner Syndromes." *Pediatric Neurology* 51 (3): 287–96.

Jakubcakova, Vladimira, Cornelia Flachskamm, Rainer Landgraf, and Mayumi Kimura. 2012. “Sleep Phenotyping in a Mouse Model of Extreme Trait Anxiety.” *PloS One* 7 (7): e40625.

Jimenez-Mateos, Eva M., Tobias Engel, Paula Merino-Serrais, Ross C. McKiernan, Katsuhiro Tanaka, Genshin Mouri, Takanori Sano, et al. 2012. “Silencing microRNA-134 Produces Neuroprotective and Prolonged Seizure-Suppressive Effects.” *Nature Medicine* 18 (7): 1087–94.

Jimenez-Mateos, Eva M., Jonathon Smith, Annette Nicke, and Tobias Engel. 2019. “Regulation of P2X7 Receptor Expression and Function in the Brain.” *Brain Research Bulletin* 151 (September): 153–63.

Jimenez-Pacheco, Alba, Miguel Diaz-Hernandez, Marina Arribas-Blázquez, Amaya Sanz-Rodriguez, Luis A. Olivos-Oré, Antonio R. Artalejo, Mariana Alves, et al. 2016. “Transient P2X7 Receptor Antagonism Produces Lasting Reductions in Spontaneous Seizures and Gliosis in Experimental Temporal Lobe Epilepsy.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 36 (22): 5920–32.

Jimenez-Pacheco, Alba, Guillaume Mesuret, Amaya Sanz-Rodriguez, Katsuhiro Tanaka, Claire Mooney, Ronan Conroy, Maria Teresa Miras-Portugal, Miguel Diaz-Hernandez, David C. Henshall, and Tobias Engel. 2013. “Increased Neocortical Expression of the P2X7 Receptor after Status Epilepticus and Anticonvulsant Effect of P2X7 Receptor Antagonist A-438079.” *Epilepsia* 54 (9): 1551–61.

Kaczmarek-Hajek, Karina, Jiong Zhang, Robin Kopp, Antje Grosche, Björn Rissiek, Anika Saul, Santina Bruzzone, et al. 2018. “Re-Evaluation of Neuronal P2X7 Expression Using Novel Mouse Models and a P2X7-Specific Nanobody.” *eLife* 7 (August). https://doi.org/10.7554/eLife.36217.

Kandratavicius, Ludmyla, Priscila Alves Balista, Cleiton Lopes-Aguiar, Rafael Naime Ruggiero, Eduardo Henrique Umeoka, Norberto Garcia-Cairasco, Lezio Soares Bueno-Junior, and Joao Pereira Leite. 2014. “Animal Models of Epilepsy: Use and Limitations.” *Neuropsychiatric Disease and Treatment* 10 (September): 1693–1705.

Khan, Sonia, and Raidah Al Baradie. 2012. “Epileptic Encephalopathies: An Overview.” *Epilepsy Research and Treatment* 2012 (November): 403592.

Kida, Hiroyuki, Yasumasa Tsuda, Nana Ito, Yui Yamamoto, Yuji Owada, Yoshinori Kamiya, and Dai Mitsushima. 2016. “Motor Training Promotes Both Synaptic and Intrinsic Plasticity of Layer II/III Pyramidal Neurons in the Primary Motor Cortex.” *Cerebral Cortex* 26 (8): 3494–3507.

Kim, Ji-Eun, and Tae-Cheon Kang. 2011. “The P2X7 Receptor-Pannexin-1 Complex Decreases Muscarinic Acetylcholine Receptor-Mediated Seizure Susceptibility in Mice.” *The Journal of Clinical Investigation* 121 (5): 2037–47.

Kinoshita, Shinichi, and Ryuta Koyama. 2021. “Pro- and Anti-Epileptic Roles of Microglia.” *Neural Regeneration Research* 16 (7): 1369–71.

Komatsu, Y., and Y. Yoshimura. 2000. “Activity-Dependent Maintenance of Long-Term Potentiation at Visual Cortical Inhibitory Synapses.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 20 (20): 7539–46.

Kopp, Robin, Anna Krautloher, Antonio Ramírez-Fernández, and Annette Nicke. 2019. “P2X7 Interactions and Signaling - Making Head or Tail of It.” *Frontiers in Molecular Neuroscience* 12 (August): 183.

Kural, Zekiye, and Ali Fahir Ozer. 2012. “Epileptic Encephalopathies in Adults and Childhood.” *Epilepsy Research and Treatment* 2012 (September): 205131.

Kwan, Patrick, Alexis Arzimanoglou, Anne T. Berg, Martin J. Brodie, W. Allen Hauser, Gary Mathern, Solomon L. Moshé, Emilio Perucca, Samuel Wiebe, and Jacqueline French. 2010. “Definition of Drug Resistant Epilepsy: Consensus Proposal by the Ad Hoc Task Force of the ILAE Commission on Therapeutic Strategies.” *Epilepsia* 51 (6): 1069–77.

Lancaster, Eric, and Josep Dalmau. 2012. “Neuronal Autoantigens--Pathogenesis, Associated Disorders and Antibody Testing.” *Nature Reviews. Neurology* 8 (7): 380–90.

Laperchia, Claudia, Roberta Imperatore, Idris A. Azeez, Federico Del Gallo, Giuseppe Bertini, Gigliola Grassi-Zucconi, Luigia Cristino, and Marina Bentivoglio. 2017. “The Excitatory/inhibitory Input to Orexin/hypocretin Neuron Soma Undergoes Day/night Reorganization.” *Brain Structure & Function* 222 (8): 3847–59.

Leite, J. P., N. Garcia-Cairasco, and E. A. Cavalheiro. 2002. “New Insights from the Use of Pilocarpine and Kainate Models.” *Epilepsy Research* 50 (1-2): 93–103.

Lesca, Gaetan, Gabrielle Rudolf, Nadine Bruneau, Natalia Lozovaya, Audrey Labalme, Nadia Boutry-Kryza, Manal Salmi, et al. 2013. “GRIN2A Mutations in Acquired Epileptic Aphasia and Related Childhood Focal Epilepsies and Encephalopathies with Speech and Language Dysfunction.” *Nature Genetics* 45 (9): 1061–66.

Letts, V. A., B. J. Beyer, and W. N. Frankel. 2014. “Hidden in Plain Sight: Spike-Wave Discharges in Mouse Inbred Strains.” *Genes, Brain, and Behavior* 13 (6): 519–26.

Lévesque, Maxime, and Massimo Avoli. 2013. “The Kainic Acid Model of Temporal Lobe Epilepsy.” *Neuroscience and Biobehavioral Reviews* 37 (10 Pt 2): 2887–99.

Leyh, Judith, Sabine Paeschke, Bianca Mages, Dominik Michalski, Marcin Nowicki, Ingo Bechmann, and Karsten Winter. 2021. “Classification of Microglial Morphological Phenotypes Using Machine Learning.” *Frontiers in Cellular Neuroscience* 15 (June): 701673.

Löscher, Wolfgang. 2011. “Critical Review of Current Animal Models of Seizures and Epilepsy Used in the Discovery and Development of New Antiepileptic Drugs.” *Seizure: The Journal of the British Epilepsy Association* 20 (5): 359–68.

Löscher, Wolfgang, and Heidrun Potschka. 2005. “Drug Resistance in Brain Diseases and the Role of Drug Efflux Transporters.” *Nature Reviews. Neuroscience* 6 (8): 591–602.

Maroso, Mattia, Silvia Balosso, Teresa Ravizza, Jaron Liu, Eleonora Aronica, Anand M. Iyer, Carlo Rossetti, et al. 2010. “Toll-like Receptor 4 and High-Mobility Group Box-1 Are Involved in Ictogenesis and Can Be Targeted to Reduce Seizures.” *Nature Medicine* 16 (4): 413–19.

Martin, S. J., P. D. Grimwood, and R. G. Morris. 2000. “Synaptic Plasticity and Memory: An Evaluation of the Hypothesis.” *Annual Review of Neuroscience* 23: 649–711.

McTague, Amy, Katherine B. Howell, J. Helen Cross, Manju A. Kurian, and Ingrid E. Scheffer. 2016. “The Genetic Landscape of the Epileptic Encephalopathies of Infancy and Childhood.” *Lancet Neurology* 15 (3): 304–16.

Mizoguchi, Kazushige, Mitsutoshi Yuzurihara, Atsushi Ishige, Hiroshi Sasaki, and Takeshi Tabira. 2002. “Chronic Stress Impairs Rotarod Performance in Rats: Implications for Depressive State.” *Pharmacology, Biochemistry, and Behavior* 71 (1): 79–84.

Monif, Mastura, Christopher A. Reid, Kim L. Powell, Megan L. Smart, and David A. Williams. 2009. “The P2X7 Receptor Drives Microglial Activation and Proliferation: A Trophic Role for P2X7R Pore.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 29 (12): 3781–91.

Morgan, James, Mariana Alves, Giorgia Conte, Aida Menéndez-Méndez, Laura de Diego-Garcia, Gioacchino de Leo, Edward Beamer, Jonathon Smith, Annette Nicke, and Tobias Engel. 2020. “Characterization of the Expression of the ATP-Gated P2X7 Receptor Following Status Epilepticus and during Epilepsy Using a P2X7-EGFP Reporter Mouse.” *Neuroscience Bulletin* 36 (11): 1242–58.

Neary, Joseph T., and Herbert Zimmermann. 2009. “Trophic Functions of Nucleotides in the Central Nervous System.” Trends in Neurosciences 32 (4): 189–98.

Niemeyer, James E., Poornima Gadamsetty, Chanwoo Chun, Sherika Sylvester, Jacob P. Lucas, Hongtao Ma, Theodore H. Schwartz, and Emre R. F. Aksay. 2022. “Seizures Initiate in Zones of Relative Hyperexcitation in a Zebrafish Epilepsy Model.” *Brain: A Journal of Neurology* 145 (7): 2347–60.

Nieoczym, Dorota, Katarzyna Socała, and Piotr Wlaź. 2017. "Evaluation of the Anticonvulsant Effect of Brilliant Blue G, a Selective P2X7 Receptor Antagonist, in the Iv PTZ-, Maximal Electroshock-, and 6 Hz-Induced Seizure Tests in Mice." *Neurochemical Research* 42 (11): 3114–24.

Nieuwenhuis, Lotte, and Joost Nicolai. 2006. "The Pathophysiological Mechanisms of Cognitive and Behavioral Disturbances in Children with Landau-Kleffner Syndrome or Epilepsy with Continuous Spike-and-Waves during Slow-Wave Sleep." *Seizure: The Journal of the British Epilepsy Association* 15 (4): 249–58.

Noebels, Jeffrey L., and Richard L. Sidman. 1979. "Inherited Epilepsy: Spike-Wave and Focal Motor Seizures in the Mutant Mouse Tottering." *Science* 204 (4399): 1334–36.

Pastalkova, Eva, Peter Serrano, Deana Pinkhasova, Emma Wallace, André Antonio Fenton, and Todd Charlton Sacktor. 2006. "Storage of Spatial Information by the Maintenance Mechanism of LTP." *Science* 313 (5790): 1141–44.

Patel, Dipan C., Bhanu P. Tewari, Lata Chaunsali, and Harald Sontheimer. 2019. "Neuron–glia Interactions in the Pathophysiology of Epilepsy." *Nature Reviews. Neuroscience* 20 (5): 282–97.

Patry, G., S. Lyagoubi, and C. A. Tassinari. 1971. "Subclinical 'Electrical Status Epilepticus' Induced by Sleep in Children. A Clinical and Electroencephalographic Study of Six Cases." *Archives of Neurology* 24 (3): 242–52.

Pavlov, P. Ivan. 2010. "Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex." *Annals of Neurosciences* 17 (3): 136–41.

Peixinho-Pena, Luiz Fernando, Jansen Fernandes, Alexandre Aparecido de Almeida, Fabiano Guimarães Novaes Gomes, Ricardo Cassilhas, Daniel Paulino Venancio, Marco Tulio de Mello, Fulvio Alexandre Scorza, Esper Abrão Cavalheiro, and Ricardo Mario Arida. 2012. "A Strength Exercise Program in Rats with Epilepsy Is Protective against Seizures." *Epilepsy & Behavior: E&B* 25 (3): 323–28.

Pelegrin, Pablo. 2021. "P2X7 Receptor and the NLRP3 Inflammasome: Partners in Crime." *Biochemical Pharmacology* 187 (May): 114385.

Picot, Marie-Christine, Michel Baldy-Moulinier, Jean-Pierre Daurès, Pierre Dujols, and Arielle Crespel. 2008. "The Prevalence of Epilepsy and Pharmacoresistant Epilepsy in Adults: A Population-Based Study in a Western European Country." *Epilepsia* 49 (7): 1230–38.

Pimentel, José, Raquel Tojal, and Joana Morgado. 2015. "Epilepsy and Physical Exercise." *Seizure: The Journal of the British Epilepsy Association* 25 (February): 87–94.

Pitkänen, Asla, and Thomas P. Sutula. 2002. “Is Epilepsy a Progressive Disorder? Prospects for New Therapeutic Approaches in Temporal-Lobe Epilepsy.” *Lancet Neurology* 1 (3): 173–81.

Raedt, R., A. Van Dycke, D. Van Melkebeke, T. De Smedt, P. Claeys, T. Wyckhuys, K. Vonck, W. Wadman, and P. Boon. 2009. “Seizures in the Intrahippocampal Kainic Acid Epilepsy Model: Characterization Using Long-Term Video-EEG Monitoring in the Rat.” *Acta Neurologica Scandinavica* 119 (5): 293–303.

Ramantani, Georgia, Navah Ester Kadish, Karl Strobl, Armin Brandt, Angeliki Stathi, Hans Mayer, Susanne Schubert-Bast, et al. 2013. “Seizure and Cognitive Outcomes of Epilepsy Surgery in Infancy and Early Childhood.” *European Journal of Paediatric Neurology: EJPN: Official Journal of the European Paediatric Neurology Society* 17 (5): 498–506.

Raol, Y. H., D. R. Lynch, and A. R. Brooks-Kayal. 2001. “Role of Excitatory Amino Acids in Developmental Epilepsies.” *Mental Retardation and Developmental Disabilities Research Reviews* 7 (4): 254–60.

Reddy, Doodipala Samba, and Ramkumar Kuruba. 2013. “Experimental Models of Status Epilepticus and Neuronal Injury for Evaluation of Therapeutic Interventions.” *International Journal of Molecular Sciences* 14 (9): 18284–318.

Reid, I. C., and C. A. Stewart. 1997. “Seizures, Memory and Synaptic Plasticity.” *Seizure: The Journal of the British Epilepsy Association* 6 (5): 351–59.

Riva, Antonella, Alice Golda, Ganna Balagura, Elisabetta Amadori, Maria Stella Vari, Gianluca Piccolo, Michele Iacomino, et al. 2021. “New Trends and Most Promising Therapeutic Strategies for Epilepsy Treatment.” *Frontiers in Neurology* 12 (December): 753753.

Rogawski, Michael A., and Wolfgang Löscher. 2004. “The Neurobiology of Antiepileptic Drugs.” *Nature Reviews. Neuroscience* 5 (7): 553–64.

Roszek, Katarzyna, and Joanna Czarnecka. 2015. “Is Ecto-Nucleoside Triphosphate Diphosphohydrolase (NTPDase)-Based Therapy of Central Nervous System Disorders Possible?” Mini Reviews in Medicinal Chemistry 15 (1): 5–20.

Rozmer, Katalin, Po Gao, Michelle G. L. Araújo, Muhammad Tahir Khan, Juan Liu, Weifang Rong, Yong Tang, et al. 2017. “Pilocarpine-Induced Status Epilepticus Increases the Sensitivity of P2X7 and P2Y1 Receptors to Nucleotides at Neural Progenitor Cells of the Juvenile Rodent Hippocampus.” *Cerebral Cortex* 27 (7): 3568–85.

Rubboli, Guido, Elena Gardella, Gaetano Cantalupo, and Carlo Alberto Tassinari. 2023. “Encephalopathy Related to Status Epilepticus during Slow Sleep (ESES).

Pathophysiological Insights and Nosological Considerations." *Epilepsy & Behavior: E&B* 140 (March): 109105.

Rusina, Evgeniia, Christophe Bernard, and Adam Williamson. 2021. "The Kainic Acid Models of Temporal Lobe Epilepsy." *eNeuro* 8 (2). https://doi.org/10.1523/ENEURO.0337-20.2021.

Sahasrabuddhe, Vinaya, and Hiyaa Singhee Ghosh. 2022. "Cx3Cr1-Cre Induction Leads to Microglial Activation and IFN-1 Signaling Caused by DNA Damage in Early Postnatal Brain." *Cell Reports* 38 (3): 110252.

Salmi, Manal, Radu Bolbos, Sylvian Bauer, Marat Minlebaev, Nail Burnashev, and Pierre Szepetowski. 2018. "Transient Microstructural Brain Anomalies and Epileptiform Discharges in Mice Defective for Epilepsy and Language-Related NMDA Receptor Subunit Gene Grin2a." *Epilepsia* 59 (10): 1919–30.

Salmi, Manal, Federico Del Gallo, Marat Minlebaev, Andrey Zakharov, Vanessa Pauly, Pauline Perron, Alexandre Pons-Bennaceur, et al. 2019. "Impaired Vocal Communication, Sleep-Related Discharges, and Transient Alteration of Slow-Wave Sleep in Developing Mice Lacking the GluN2A Subunit of N-Methyl-D-Aspartate Receptors." *Epilepsia* 60 (7): 1424–37.

Sánchez Fernández, Iván, Tobias Loddenkemper, Jurriaan M. Peters, and Sanjeev V. Kothare. 2012. "Electrical Status Epilepticus in Sleep: Clinical Presentation and Pathophysiology." *Pediatric Neurology* 47 (6): 390–410.

Saunders, Arpiar, Evan Z. Macosko, Alec Wysoker, Melissa Goldman, Fenna M. Krienen, Heather de Rivera, Elizabeth Bien, et al. 2018. "Molecular Diversity and Specializations among the Cells of the Adult Mouse Brain." *Cell* 174 (4): 1015–30.e16.

Sayyah, M., M. Javad-Pour, and M. Ghazi-Khansari. 2003. "The Bacterial Endotoxin Lipopolysaccharide Enhances Seizure Susceptibility in Mice: Involvement of Proinflammatory Factors: Nitric Oxide and Prostaglandins." *Neuroscience* 122 (4): 1073–80.

Scheffer, Ingrid E., Samuel Berkovic, Giuseppe Capovilla, Mary B. Connolly, Jacqueline French, Laura Guilhoto, Edouard Hirsch, et al. 2017. "ILAE Classification of the Epilepsies: Position Paper of the ILAE Commission for Classification and Terminology." *Epilepsia* 58 (4): 512–21.

Scholtes, F. B. J., M. P. H. Hendriks, and W. O. Renier. 2005. "Cognitive Deterioration and Electrical Status Epilepticus during Slow Sleep." *Epilepsy & Behavior: E&B* 6 (2): 167–73.

Sen, Arjune, Nathalie Jette, Masud Husain, and Josemir W. Sander. 2020. "Epilepsy in Older People." *The Lancet* 395 (10225): 735–48.

Seyfried, T. N., and G. H. Glaser. 1985. “A Review of Mouse Mutants as Genetic Models of Epilepsy.” *Epilepsia* 26 (2): 143–50.

Sharma, Alok K., Rachel Y. Reams, William H. Jordan, Margaret A. Miller, H. Leon Thacker, and Paul W. Snyder. 2007. “Mesial Temporal Lobe Epilepsy: Pathogenesis, Induced Rodent Models and Lesions.” *Toxicologic Pathology* 35 (7): 984–99.

Sloviter, Robert S., Argyle V. Bumanglag, Robert Schwarcz, and Michael Frotscher. 2012. “Abnormal Dentate Gyrus Network Circuitry in Temporal Lobe Epilepsy.” In *Jasper's Basic Mechanisms of the Epilepsies*, edited by Jeffrey L. Noebels, Massimo Avoli, Michael A. Rogawski, Richard W. Olsen, and Antonio V. Delgado-Escueta. Bethesda (MD): National Center for Biotechnology Information (US).

Sloviter, R. S. 1987. “Decreased Hippocampal Inhibition and a Selective Loss of Interneurons in Experimental Epilepsy.” *Science* 235 (4784): 73–76.

Smith, Jonathon, Aida Menéndez Méndez, Mariana Alves, Alberto Parras, Giorgia Conte, Anindya Bhattacharya, Marc Ceusters, et al. 2023. “The P2X7 Receptor Contributes to Seizures and Inflammation-Driven Long-Lasting Brain Hyperexcitability Following Hypoxia in Neonatal Mice.” *British Journal of Pharmacology* 180 (13): 1710–29.

Steinlein, O. K., and J. L. Noebels. 2000. “Ion Channels and Epilepsy in Man and Mouse.” *Current Opinion in Genetics & Development* 10 (3): 286–91.

Stephen Huff, J., and Najib Murr. 2023. *Seizure*. StatPearls Publishing.

Strehlow, Vincent, Claudine Rieubland, Sabina Gallati, Sukhan Kim, Scott J. Myers, Vincent Peterson, Amy J. Ramsey, Daniel D. Teuscher, Stephen F. Traynelis, and Johannes R. Lemke. 2022. “Compound-Heterozygous GRIN2A Null Variants Associated with Severe Developmental and Epileptic Encephalopathy.” *Epilepsia* 63 (10): e132–37.

Strohl, Kingman P., Laura Gallaugher, Audrey Lynn, Lee Friedman, Annie Hill, Jonathan B. Singer, Eric S. Lander, and Joseph Nadeau. 2007. “Sleep-Related Epilepsy in the A/J Mouse.” *Sleep* 30 (2): 169–76.

Südhof, Thomas C. 2021. “The Cell Biology of Synapse Formation.” *The Journal of Cell Biology* 220 (7). https://doi.org/10.1083/jcb.202103052.

Surprenant, A., F. Rassendren, E. Kawashima, R. A. North, and G. Buell. 1996. “The Cytolytic P_{2Z} Receptor for Extracellular ATP Identified as a P_{2X} Receptor ($P2X_7$).” *Science* 272 (5262): 735–38.

Tabula Muris Consortium, Overall coordination, Logistical coordination, Organ collection and processing, Library preparation and sequencing, Computational data analysis, Cell type annotation, Writing group, Supplemental text writing group, and Principal

investigators. 2018. “Single-Cell Transcriptomics of 20 Mouse Organs Creates a Tabula Muris.” *Nature* 562 (7727): 367–72.

Tassinari, C. A., G. Rubboli, L. Volpi, S. Meletti, G. d’Orsi, M. Franca, A. R. Sabetta, et al. 2000. “Encephalopathy with Electrical Status Epilepticus during Slow Sleep or ESES Syndrome Including the Acquired Aphasia.” *Clinical Neurophysiology: Official Journal of the International Federation of Clinical Neurophysiology* 111 Suppl 2 (September): S94–102.

Tassinari, C., G. Cantalupo, B. Bernardina, F. Darra, M. Bureau, C. Cirelli, G. Tononi, and G. Rubboli. 2012. “Encephalopathy Related to Status Epilepticus during Slow Sleep (ESES) Including Landau-Kleffner Syndrome.” *Syndromes in Infancy …*, 255–76.

Teresa Miras-Portugal, M., Álvaro Sebastián-Serrano, Laura de Diego García, and Miguel Díaz-Hernández. 2017. “Neuronal P2X7 Receptor: Involvement in Neuronal Physiology and Pathology.” *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 37 (30): 7063–72.

Tononi, Giulio, and Chiara Cirelli. 2003. “Sleep and Synaptic Homeostasis: A Hypothesis.” *Brain Research Bulletin* 62 (2): 143–50.

Tozaki-Saitoh, Hidetoshi, Makoto Tsuda, and Kazuhide Inoue. 2011. “Role of Purinergic Receptors in CNS Function and Neuroprotection.” Advances in Pharmacology 61: 495–528.

Trinka, Eugen, Hannah Cock, Dale Hesdorffer, Andrea O. Rossetti, Ingrid E. Scheffer, Shlomo Shinnar, Simon Shorvon, and Daniel H. Lowenstein. 2015. “A Definition and Classification of Status Epilepticus--Report of the ILAE Task Force on Classification of Status Epilepticus.” *Epilepsia* 56 (10): 1515–23.

Tumiene, Birute, Carlos R. Ferreira, and Clara D. M. van Karnebeek. 2022. “2022 Overview of Metabolic Epilepsies.” *Genes* 13 (3). https://doi.org/10.3390/genes13030508.

Turrigiano, Gina G., and Sacha B. Nelson. 2004. “Homeostatic Plasticity in the Developing Nervous System.” *Nature Reviews. Neuroscience* 5 (2): 97–107.

Ulloa Severino, Francesco Paolo, Oluwadamilola O. Lawal, Kristina Sakers, Shiyi Wang, Namsoo Kim, Alexander David Friedman, Sarah Anne Johnson, et al. 2023. “Training-Induced Circuit-Specific Excitatory Synaptogenesis in Mice Is Required for Effort Control.” *Nature Communications* 14 (1): 5522.

Van Hirtum-Das, Michele, Eliot A. Licht, Susan Koh, Joyce Y. Wu, W. Donald Shields, and Raman Sankar. 2006. “Children with ESES: Variability in the Syndrome.” *Epilepsy Research* 70 Suppl 1 (August): S248–58.

Vezzani, Annamaria, Silvia Balosso, and Teresa Ravizza. 2019. “Neuroinflammatory Pathways as Treatment Targets and Biomarkers in Epilepsy.” *Nature Reviews. Neurology* 15 (8): 459–72.

Vezzani, Annamaria, Robert S. Fujinami, H. Steve White, Pierre-Marie Preux, Ingmar Blümcke, Josemir W. Sander, and Wolfgang Löscher. 2016. “Infections, Inflammation and Epilepsy.” *Acta Neuropathologica* 131 (2): 211–34.

Volcy, M. Gómez. 2004. “Mesial Temporal Lobe Epilepsy: Its Physiopathology, Clinical Characteristics, Treatment and Prognosis.” *Revista de Neurologia* 38 (7): 663–67.

Wall, P. D., and M. D. Egger. 1971. “Formation of New Connexions in Adult Rat Brains after Partial Deafferentation.” *Nature* 232 (5312): 542–45.

Wang, Yilin, Penghu Wei, Feng Yan, Yumin Luo, and Guoguang Zhao. 2022. “Animal Models of Epilepsy: A Phenotype-Oriented Review.” *Aging and Disease* 13 (1): 215–31.

“WHO | Epilepsy.” 2017. World Health Organization. February 10, 2017. http://www.who.int/mediacentre/factsheets/fs999/en/.

Wirrell, Elaine C., Brandon R. Grossardt, Lily C. L. Wong-Kisiel, and Katherine C. Nickels. 2011. “Incidence and Classification of New-Onset Epilepsy and Epilepsy Syndromes in Children in Olmsted County, Minnesota from 1980 to 2004: A Population-Based Study.” *Epilepsy Research* 95 (1-2): 110–18.

Xu, Tonghui, Xinzhu Yu, Andrew J. Perlik, Willie F. Tobin, Jonathan A. Zweig, Kelly Tennant, Theresa Jones, and Yi Zuo. 2009. “Rapid Formation and Selective Stabilization of Synapses for Enduring Motor Memories.” *Nature* 462 (7275): 915–19.

Yang, Guang, Cora Sau Wan Lai, Joseph Cichon, Lei Ma, Wei Li, and Wen-Biao Gan. 2014. “Sleep Promotes Branch-Specific Formation of Dendritic Spines after Learning.” *Science* 344 (6188): 1173–78.

Yang, Guang, Feng Pan, and Wen-Biao Gan. 2009. “Stably Maintained Dendritic Spines Are Associated with Lifelong Memories.” *Nature* 462 (7275): 920–24.

Young, Paul, Li Qiu, Dongqing Wang, Shengli Zhao, James Gross, and Guoping Feng. 2008. “Single-Neuron Labeling with Inducible Cre-Mediated Knockout in Transgenic Mice.” *Nature Neuroscience* 11 (6): 721–28.

Yu, Frank H., Massimo Mantegazza, Ruth E. Westenbroek, Carol A. Robbins, Franck Kalume, Kimberly A. Burton, William J. Spain, G. Stanley McKnight, Todd Scheuer, and William A. Catterall. 2006. “Reduced Sodium Current in GABAergic Interneurons in a Mouse Model of Severe Myoclonic Epilepsy in Infancy.” *Nature Neuroscience* 9 (9): 1142–49.

Zeng, Ling-Hui, Lin Xu, David H. Gutmann, and Michael Wong. 2008. “Rapamycin Prevents Epilepsy in a Mouse Model of Tuberous Sclerosis Complex.” *Annals of Neurology* 63 (4): 444–53.

Zhao, Xiao-Feng, Mahabub Maraj Alam, Yuan Liao, Tingting Huang, Ramkumar Mathur, Xinjun Zhu, and Yunfei Huang. 2019. “Targeting Microglia Using Cx3cr1-Cre Lines: Revisiting the Specificity.” *eNeuro* 6 (4). https://doi.org/10.1523/ENEURO.0114-19.2019.

Zhou, Jun-Li, Tatiana N. Shatskikh, Xianzeng Liu, and Gregory L. Holmes. 2007. “Impaired Single Cell Firing and Long-Term Potentiation Parallels Memory Impairment Following Recurrent Seizures.” *The European Journal of Neuroscience* 25 (12): 3667–77.

Zuberi, Sameer M., Elaine Wirrell, Elissa Yozawitz, Jo M. Wilmshurst, Nicola Specchio, Kate Riney, Ronit Pressler, et al. 2022. “ILAE Classification and Definition of Epilepsy Syndromes with Onset in Neonates and Infants: Position Statement by the ILAE Task Force on Nosology and Definitions.” *Epilepsia* 63 (6): 1349–97.

Zucker, Robert S., and Wade G. Regehr. 2002. “Short-Term Synaptic Plasticity.” *Annual Review of Physiology* 64: 355–405.

7. List of abbreviations

ASDs Antiseizure Drugs

ATP Adenosine Triphosphate

BBB Blood-Brain Barrier

CA1/3 Cornu Ammonis1/3

CD Cluster of Differentiation

CNS Central Nervous System

CT Computed Tomography

DG Dentate Gyrus

EEG Electroencephalography

EEs Epileptic Encephalopathies

EMG Electromyography

ESES Encephalopathies with Status Epilepticus during low Sleep

GABA γ- aminobutyric acid

GFAP Glial fibrillary acidic protein

IAKA Intra-amygdala kainic acid

IL Interleukin

ILAE International League Against Epilepsy

KA Kainic Acid

KAR Kainic Acid receptors

KO Knock-out

LTD Long-Term Depression

LTP Long-Term Potentiation

MC Motor Cortex

MRI Magnetic Resonance Imaging

NMDA N-methyl-D-aspartate

PBS Phosphate-buffered saline

PFA Paraformaldehyde

PTZ Pentylenetetrazol

REM Rapid Eye Movement

RPM Rotations Per Minute

SE Status Epilepticus

SSC Somatosensory Cortex

SWD Spike-and-Wave Discharge

SWS Slow-Wave Sleep

TLE Temporal Lobe Epilepsy

TNF-α Tumor necrosis Factor-α

VGAT Vesicular GABA Transporter

VGLUT2 Vesicular Glutamate Transporter type 2

ZT Zeitgeber time