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Exploring the potential of trilostane as antiepileptogenic and disease-modifying agent able to modulate neurosteroids' levels in the hippocampus and neocortex of kainic acid-treated rats

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Abbreviations

17α-OHP	17α-Hydroxyprogesterone
3α-HSD	3α-Hydroxysteroid Dehydrogenase
3β-HSD	3β-Hydroxysteroid Dehydrogenase/Isomerase System
11β-HSD	11β-hydroxysteroid dehydrogenase
5-HT	Serotonin
5α-DHP	5α-Dihydroprogesterone
ALLO	Allopregnanolone
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid
ASMs	Antiseizure Medications
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BLA	Basolateral Amygdala
CCL2	Chemokine (C-C Motif) Ligand 2
Ca^{2+}	Calcium Ion
Can	Calcineurin
CB	Calbindin
CNS	Central Nervous System
CR	Calretinin
CSF	Cerebrospinal Fluid
DG	Dentate Gyrus
DHDOC	5α-dihydrodeoxycorticosterone
DHEA	Dehydroepiandrosterone

DHEAS	Dehydroepiandrosterone Sulfate
DS	Dravet Syndrome
EcoG	Electrocorticographic
EEG	Electroencephalogram
FST	Forced Swimming Test
GABA	γ-Aminobutyric Acid
GFAP	Glial Fibrillary Acidic Protein
GLUT1	Glucose Transporter Member 1
GluR	Glutamate Receptor
GAD65	Glutamic Acid Decarboxylase 65
GlyRs	Glycine Receptors
GNX	Ganaxolone
HMGB1	High-Mobility Group Box 1
HAC	Hyperadrenocorticism
IL-1β/17	Interleukin 17/1β
ILAE	International League Against Epilepsy
IPSPs	Inhibitory Postsynaptic Potentials
K+	Potassium Ion
KA	Kainic Acid
KARs	Kainic Acid Receptors
LPS	Lipopolysaccharide
LMol	Lacunosum Moleculare Layer
MCP-1	Monocyte Chemoattractant Protein-1

MFs	Mossy Fibers
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
mTLE	Mesial Temporal Lobe Epilepsy
MTS	Mesial Temporal Sclerosis
Na+	Sodium Ion
NSCE	Non-Convulsive Se
NMDA	N-Methyl-D-Aspartate
nAChRs	Nicotinic Acetylcholine Receptors
PGB	Pregabalin
PGE2	Prostaglandin E2
PNS	Peripheral Nervous System
P450scc	Cytochrome P450 Cholesterol Side Chain Cleavage
PREG	Pregnanolone
PREGS	Pregnenolone Sulfate
PROG	Progesterone
ROS	Reactive Oxygen Species
SCN9A	Sodium Voltage-Gated Channel Alpha Subunit 9
SRSs	Spontaneous Recurrent Seizures
SSRIs	Selective Serotonin Reuptake Inhibitors
TGF-β	Transforming Growth Factor-β

THDOC	Tetrahydrodeoxycorticosterone
TLE	Temporal Lobe Epilepsy
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor-α
TSC	Tuberous Sclerosis Complex
I.V.	Intravenous
S.C.	Subcutaneous

Abstract (English Version)

Epileptogenesis and the progression of epilepsy present complex challenges in understanding and managing seizures. Two separate studies were conducted to investigate the role of neurosteroids, particularly allopregnanolone (ALLO), in both the initiation of epileptogenesis and the modulation of epileptic seizures in the kainic acid (KA) model of temporal lobe epilepsy.

In the first study, we aimed to understand the impact of neurosteroid manipulation on the development of epilepsy. Rats were subjected to status epilepticus (SE) through intraperitoneal administration of KA (15 mg/kg), and subsequently received trilostane, a 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD) inhibitor known to increase brain levels of neurosteroids. Trilostane (50 mg/kg) was administered subcutaneously once daily for up to six consecutive days, starting 10 minutes after KA injection. Trilostane did not alter the onset or duration of SE. However, rats receiving six daily trilostane injections displayed a remarkable delay in the onset of the first spontaneous electrocorticographic (ECoG) seizure and subsequent tonic-clonic spontaneous recurrent seizures (SRSs) compared to the vehicle-treated group. Conversely, rats treated with only the first trilostane injection during SE did not differ from vehicle-treated rats in developing the SRSs. Notably, trilostane did not affect neuronal cell densities or hippocampal damage but decreased activated microglia morphology in the subiculum. ALLO and other neurosteroid levels significantly increased in the hippocampus and neocortex of rats treated for six days, returning to baseline after a week of trilostane washout.

In the second study, we explored the effects of trilostane on epileptic rats to determine its influence on seizure progression. Trilostane treatment (50 mg/kg/day for one week) was initiated 64 days after KA administration. Rats receiving trilostane displayed increased levels of various neurosteroids, including ALLO, in both the neocortex and hippocampus. Importantly, the trilostane-treated group did not experience an increase in seizure occurrence, in contrast to the vehicle-treated group, indicating a disease-modifying effect of enhanced neurosteroid availability in epileptic rats.

These findings collectively highlight the potential therapeutic implications of neurosteroid modulation in both the initiation of epileptogenesis and the management of epilepsy. Increasing ALLO levels through trilostane treatment may not only delay epileptogenesis but also mitigate the

progression in severity of recurrent seizures, shedding light on a promising avenue for improving epilepsy management.

Key words: Allopregnanolone; trilostane; epileptogenesis; neurosteroids; Spontaneous recurrent seizures; temporal lobe epilepsy

Abstract (Italian version)

Titolo in italiano: Validazione del trilostano come farmaco antiepilettico che agisce aumentando i livelli di molteplici neurosteroidi nell'ippocampo e nella neocorteccia di ratti trattati con acido kainico

L'epilettogenesi ed il naturale peggioramento dell'epilessia rappresentano vere e proprie sfide per una completa comprensione ed efficace gestione delle crisi epilettiche. Ho effettuato due studi indipendenti per caratterizzare il ruolo dei neurosteroidi, in particolare dell'allopregnanolone (ALLO), nell'influenzare l'epilettogenesi oppure nel modulare le crisi epilettiche di ratti già epilettici nel modello di epilessia del lobo temporale indotto dall'acido kainico.

Nel primo studio, il nostro obiettivo era quello di comprendere l'impatto della manipolazione dei neurosteroidi nello sviluppo dell'epilessia dopo una lesione iniziale. I ratti sono stati trattati con la somministrazione intraperitoneale di acido kainico (15 mg/kg) per indurre uno stato epilettico (SE) e, successivamente, hanno ricevuto l'iniezione di trilostano, un inibitore dell'enzima 3β-idrossisteroide deidrogenasi/Δ^{5-4} isomerasi noto per provocare l'aumento dei livelli di neurosteroidi nel cervello. Il trattamento con trilostano (50 mg/kg) è stato ripetuto per via sottocutanea una volta al giorno per un massimo di sei giorni consecutivi, cominciando 10 minuti dopo l'iniezione di acido kainico. Il trilostano non ha modificato né l'insorgenza né la durata di SE. Tuttavia, i ratti trattati con un ciclo completo di trilostano hanno presentato un notevole ritardo nell'insorgenza della prima crisi epilettica spontanea registrata all'elettrocorticogramma, nonché delle crisi ricorrenti spontanee tonico-cloniche (SRS), quando confrontati al gruppo di ratti epilettici trattati con il solvente del trilostano. Al contrario, i ratti trattati con una sola iniezione di trilostano durante il SE non hanno mostrato differenze rispetto ai ratti trattati con il solvente. Poi, è interessante notare che il trilostano non ha modificato le densità cellulari neuronali né le lesioni all'ippocampo determinate dall'acido kainico e dallo SE, ma ha ridotto la morfologia di tipo attivato della microglia nel subicolo. I livelli di ALLO e di altri neurosteroidi sono aumentati significativamente nell'ippocampo e nella neocorteccia dei ratti trattati per sei giorni con trilostano, tornando ai valori di base dopo una settimana dall'interruzione del trattamento.

Nel secondo studio, abbiamo esplorato gli effetti del trilostano su ratti epilettici per determinarne l'influenza del trattamento farmacologico sulla progressione delle crisi epilettiche. Il trattamento

con trilostane (50 mg/kg/giorno per una settimana) è stato iniziato 64 giorni dopo la somministrazione dell'acido kainico. I ratti trattati con trilostano hanno presentato un aumento dei livelli di vari neurosteroidi, tra cui l'ALLO, sia nella neocorteccia sia nell'ippocampo. Il gruppo trattato con trilostano non ha presentato un aumento del numero di crisi epilettiche con convulsioni, a differenza del gruppo trattato con il solvente che ha significativamente aumentato il loro numero, indicando un effetto in grado di modificare l'andamento della malattia da parte del trilostano, grazie all'aumento della disponibilità dei neurosteroidi nei ratti epilettici.

Questi risultati sottolineano complessivamente le potenziali implicazioni terapeutiche della modulazione dei neurosteroidi sia nell'epilettogenesi sia nella gestione dell'epilessia. L'aumento dei livelli di ALLO ottenuto con il trattamento con trilostane potrebbe non solo ritardare l'epilettogenesi ma anche attenuare la gravità delle crisi ricorrenti, gettando luce su una promettente strategia per migliorare la gestione dell'epilessia.

Parole chiave: Allopregnanolone; trilostano; epilettogenesi; neurosteroidi; crisi ricorrenti spontanee; epilessia del lobo temporale

Chapter 1

1. Epilepsy: General introduction

Epilepsy, as defined by the criteria established by the International League Against Epilepsy (ILAE), represents a complex neurological disorder characterized by the occurrence of a minimum of two unprovoked seizures, each separated by a minimum interval of 24 hours. Alternatively, individuals may receive an epilepsy diagnosis in the presence of a solitary unprovoked seizure, provided there exists a substantial probability exceeding 60% of experiencing a subsequent seizure within the ensuing decade. An additional diagnostic pathway entails the identification of a specific epilepsy syndrome [1]. It is of profound significance to acknowledge that epilepsy boasts a profound historical lineage, intertwined with cultural beliefs in malevolent forces and shrouded in enigmatic narratives. Furthermore, within contemporary society, epilepsy continues to be afflicted by pervasive social stigmatization [2]. The amalgamation of its extensive historical underpinnings and enduring societal implications confers upon epilepsy a truly distinctive status within the realm of neurological disorders.

Epilepsy, a subject of profound historical significance, can be traced back to antiquity. Its earliest documented account is found in a 4000-year-old Akkadian tablet uncovered in Mesopotamia. The tablet provides a vivid depiction of individuals experiencing seizures characterized by a turned neck, tense limbs, wide-open eyes, and frothing at the mouth, all while devoid of consciousness [2]. This historical artifact underscores the ancient world's recognition and documentation of epilepsy's clinical manifestations. Advancing nearly a millennium, the Late Babylonians meticulously chronicled epilepsy in a diagnostic manual known as Sakikku. This manual delves into the detailed description and classification of various seizure types based on their distinct presentations. Significantly, it demonstrates a nuanced understanding of prognostics, as evidenced by descriptions of diverse outcomes associated with different seizure types. The text also introduces specific terminology related to epilepsy, such as miqtu (fall), hayyatu (fit), and sibtu (seizure), shedding light on the ancient world's sophisticated comprehension of this neurological condition [2, 3]. Importantly, it is worth noting that during this era, prevailing beliefs attributed these seizures to malevolent spirits infiltrating the body, resulting in therapeutic interventions rooted in spiritual practices [3]. Ancient texts from various civilizations abound with references to epilepsy, with Greek

medical documents holding particular significance in shaping our contemporary comprehension of this neurological disorder. The term "epilepsy" itself finds its etymological roots in the Greek verb "epilambanein" (επιλαμβανεΐνη), signifying to seize, take hold of, or attack. Epilepsy's historical presence transcends geographical boundaries, encompassing ancient Egypt as well. One notable artifact in this regard is the Edwin Smith Surgical Papyrus, dating back to approximately 1700 BC. Within this ancient document, multiple instances of epilepsy are meticulously documented, with a specific case of particular significance. It narrates the experience of an individual afflicted with a "gaping wound in his head." Notably, when this cranial injury was palpated, it induced pronounced convulsive responses, causing the individual to "shudder exceedingly" [4]. This observation, distinct from the Mesopotamian belief attributing seizures to spiritual and divine influences, provides empirical support for the hypothesis that seizures may be elicited by disruptions within the cortical regions of the brain.

Beyond Egypt, evidence of epilepsy is discernible in historical Chinese texts dating from approximately 770-221 B.C. One notable work is The Yellow Emperor's Classic of Internal Medicine, also known as Huang Di Nei Ching, authored by a consortium of physicians. This seminal text offers detailed descriptions of generalized seizures. Notably, in 610 A.D., Cao Yuan Fang made significant strides in classifying and categorizing epilepsy. Traditional principles rooted in Yin Yang Wu Xing principles were employed in the treatment of epilepsy, encompassing therapeutic modalities such as herbal remedies, massage, and acupuncture [5]. These historical accounts serve as a compelling testament to the global recognition of epilepsy throughout ancient civilizations, offering multifaceted insights into both the understanding and therapeutic approaches to this neurological condition. Epilepsy's pathophysiological understanding was predominantly steeped in spiritual explanations until approximately the 5th century BC when the School of Hippocrates in Greece postulated a more neurologically oriented hypothesis. Hippocrates, a pivotal figure in this shift, contended that epilepsy, called the "Sacred Disease," possessed no inherent divine nature beyond other ailments. The designation as "sacred" stemmed from its unique and enigmatic clinical manifestations. He further posited that epilepsy, like other diseases, could be susceptible to treatment, albeit with a caveat that once it reached a chronic stage, curability became elusive [4]. Moreover, Hippocrates made pioneering contributions to the understanding of post-traumatic epilepsy. His keen observations of head injuries revealed a consistent pattern of contralateral convulsions to the site of head trauma. Essentially, Hippocrates stood among the earliest

proponents of attributing epilepsy to neurological factors, emphasizing its hereditary nature over contagion. He delineated the clinical presentation, highlighting unilateral motor symptoms accompanied by an aura, which served as a valuable forewarning enabling individuals to withdraw from public spaces before experiencing convulsions. He astutely recognized that society's misconceptions and reactions to epilepsy stemmed from a deep-seated, unfounded apprehension rooted in the supernatural realm [4].

The Hippocratic concept that epilepsy primarily represents a disorder of the brain began to gain substantial recognition in Europe, commencing in the 17th century and extending through subsequent centuries [3]. Samuel Tissot (1728-1797), a prominent Swiss physician, played a pivotal role in advancing this understanding. His significant work, "Traité de l'épilepsie," published in 1770 [6], was followed by a comprehensive four-volume treatise titled "Traite des Nerfs et du leurs Maladies" a decade later, which solidified his stature as a key figure in the Enlightenment-era medical community. William Cullen (1710-1790), a Scottish physician, made notable contributions by elucidating that seizures could manifest in specific parts of the body without necessarily resulting in a loss of consciousness [2, 7]. At the onset of the 19th century, a significant intellectual resurgence occurred within the realm of epileptology, driven by the contributions of prominent French physicians affiliated with the French medical school. Distinguished figures such as Maisonneuve (1745–1826) [8], Calmeil (1798–1895) [4], and Jean-Etienne Dominique Esquirol (1772–1840) made substantial advancements in the field. Maisonneuve's work assumed particular prominence as he underscored the imperative of hospitalizing individuals afflicted with epilepsy. His scholarly endeavors further entailed the refinement of epilepsy classification into idiopathic and sympathetic categories, as well as the elucidation of the concept of the "sensitive aura" associated with sympathetic epilepsy. Esquirol, an influential figure of his era, distinguished between petit and grand mal seizures. In collaboration with his students, Bouchet and Cazauvieilh, he embarked on a comprehensive exploration of the complex interplay between insanity and epilepsy. This multifaceted investigation spanned clinical observations and postmortem examinations, yielding invaluable insights into the intricate connections between these conditions [2]. Collectively, the scholarly contributions of these esteemed French physicians significantly enriched the scientific discourse surrounding epilepsy during this pivotal period in its history.

In 1849, Dr. Robert Bentley Todd introduced a groundbreaking hypothesis suggesting that the brain operates through electrical forces and posited that "electrical discharges" within the brain could

underlie seizures [3, 9]. Subsequently, he empirically substantiated his hypothesis by employing Michael Faraday's magnetoelectric rotation machine on rabbits [10]. John Hughlings Jackson (1835-1911) significantly advanced the scientific foundation of epileptology and conducted pioneering research on the localization of brain lesions that could trigger seizures. His influential work, "Study of Convulsion," encapsulated his comprehensive scientific findings. In the latter half of the 19th century, the field of medicine underwent a significant transformation with a focus on unraveling the pathophysiological intricacies of epilepsy and the precise topographic localization of epileptic seizures. Eminent physicians of their time, including Théodore Herpin (1799–1865) in 1852 and 1867, Louis Jean François Delasiauve (1804–1893) in 1854, John Russell Reynolds (1828–1896) in 1861, and Sir William Richard Gowers (1845–1915) in 1881, contributed seminal works that advanced the understanding of epileptogenesis, etiology, and the taxonomy of epilepsy. Notably, pivotal insights into the origin of epilepsy within the brain emerged through the experimental endeavors of physiologist Fritsch (1838–1927) and psychiatrist Hitzig (1838–1907). Their groundbreaking work, presented in the paper titled "On the Electric Excitability of the Cerebrum," featured experiments wherein seizures were elicited through electric stimulation of the cerebral cortex in dogs [11]. However, John Hughling Jackson (1835–1911) laid the enduring scientific foundation for epileptology. He significantly advanced the scientific foundation of epileptology and conducted pioneering research on the localization of brain lesions that could trigger seizures [12, 13]. Jackson conducted extensive research, delving into the pathological and anatomical underpinnings of epilepsy. His magnum opus, the "Study of Convulsions," represented the culmination of his studies and introduced a comprehensive definition of epilepsy as "occasional, sudden, excessive, rapid, and local discharges of grey matter" in 1873 [13].

At the dawn of the 20th century, Santiago Ramón y Cajal (1852–1934), a distinguished Spanish pathologist, histologist, and neuroscientist, ushered in a new era of understanding by making profound contributions to the microscopic structure of the brain and the nervous system. His pioneering work included the first descriptions of neuronal structure and synapses, marking a watershed moment in the history of neurology. These breakthroughs were the culmination of his efforts, commencing in 1887 with the employment of Golgi staining techniques in nervous system research, culminating in his Nobel Prize recognition in 1906 [14]. In 1907, Gowers authored the renowned publication "The Borderlands of Epilepsy," which not only delved into epilepsy but also addressed faints, vagal and vasovagal attacks, migraine, vertigo, and certain sleep-related

symptoms, notably narcolepsy [15]. The early 20th century witnessed significant milestones in the identification of neurotransmitters, with Dale (1875–1968) pinpointing acetylcholine in 1914, a discovery later confirmed by Loewi (1873–1961) in 1921, initially referred to as "Vagusstoff" due to its release by the vagus nerve [16-19]. William Lenox revolutionized the field by disproving the long-held belief in a vascular etiology for epilepsy. He demonstrated that there were no fluctuations in cerebral blood flow during seizures. Furthermore, he unveiled the presence of abnormal electrical changes preceding and intensifying during seizures, proposing this as the new etiology for epilepsy [20] The 1940s were marked by crucial discoveries in the realm of psychomotor epilepsy. Klüver (1897–1979), a German-American psychologist, and Bucy (1904–1992), an American neuropathologist, made significant strides by associating behavioral changes in monkeys with temporal lobe lesions, thereby introducing the Klüver-Bucy syndrome [21]. In 1941, Jasper (1906–1999) and Kershmann confirmed the temporal lobe as the origin of psychomotor seizures [22]. Concurrently, Moruzzi (1910–1986) and Magoun (1907–1991) unveiled the reticular formation in the brain, elucidating its role in sustaining alert wakefulness and facilitating sensory perception, higher cognitive functions, voluntary motor activities, and behaviors [23-25]. Hans Berger introduced the human electroencephalogram (EEG), enabling him to confirm that convulsions indeed stemmed from abnormal electrical activity within the brain [3]. Dawson, in 1947, recorded responses from the human scalp in response to somatosensory stimuli, pioneering the field of somatosensory evoked potentials [26]. In 1949, Roberts (1920-) and Frankel discovered γ-aminobutyric acid (GABA) [27]. The subsequent decades witnessed a proliferation of advancements in neuroscience and synapse physiology, spearheaded by luminaries such as Eccles (1903–1997), Kandel (1929-), Spencer (1931–1977), Speckmann (1939-), Purpura, Meldrum, and others [12, 28-40] [24–38]. In 1969, James Kiffin Penry (1929–1996) made seminal contributions through publications such as the "Basic Mechanisms of the Epilepsies" series, followed by works on antiepileptic drugs, neurosurgical management of epilepsy, complex partial seizures, their treatment, and antiepileptic drugs' mechanisms of action, all of which significantly advanced the field. Gastaut's endeavors led to the organization of a meeting in Marseilles in 1969, attended by 120 members of the ILAE. During this meeting, a preliminary classification of epilepsies was presented to a commission on the terminology of epilepsy. The General Assembly of the ILAE subsequently endorsed the first publication of the clinical and electroencephalographic classification of epileptic seizures [41, 42]. Dreifuss (1926–1997) made pioneering strides in video-

monitoring of absence seizures and contributed to the classification of various epileptic conditions [43]. Prince et al. conducted seminal studies examining cellular phenomena during epileptic events within the human cortex [44-46]. Meldrum et al. challenged the prevailing hypothesis connecting brain damage from seizures to hypoxia, demonstrating that excessive excitatory activity, rather than hypoxia, was responsible for brain cellular loss [47-49]. The ensuing decades witnessed a comprehensive exploration of brain damage in epilepsy, including mossy fiber sprouting and synaptic reorganization [50-53].

Epilepsy, as defined by the criteria established by the ILAE, represents a complex neurological disorder characterized by the occurrence of a minimum of two unprovoked seizures, each separated by a minimum interval of 24 hours accompanied by consequential cognitive, psychological, and social ramifications [1]. This foundational criterion is encapsulated succinctly in the oft-cited clinical maxim: "A single seizure does not epilepsy make" [54]. Notably, epilepsy exhibits a prevalence rate ranging from 0.5% to 1.0% within the global population, positioning it as the fifth most prevalent neurological disorder following stroke, migraine, dementia, and meningitis [55, 56]. An epileptic seizure represents a transient behavioral alteration that may encompass objective manifestations or subjective sensations, including loss of awareness, muscular stiffening, jerking movements, sensations rising from the abdomen to the chest, or sensory phenomena such as the smell of burnt rubber or déjà vu. These phenomena arise from abnormal, excessive, or synchronized neuronal activity within the brain. Seizure onset can be categorized into three primary types: focal, where aberrant neuronal activity initiates in one or more localized brain regions or hemispheres; generalized, where abnormal neuronal activity commences with widespread distribution across both hemispheres; or of unknown onset when available clinical and laboratory data fail to definitively determine whether the onset is focal or generalized. The determination of onset type relies on achieving a confidence level exceeding 80%, as ascertained through a comprehensive assessment of clinical features, electroencephalography, and neuroimaging findings [57]. Although the etiology of epilepsy remains elusive in many cases, seizures can manifest because of virtually any insult that disrupts normal brain function. In general, the causes of epilepsy encompass a range of factors, including structural causes such as stroke and brain tumors, genetic factors like sodium voltage-gated channel alpha subunit 1A (SCN1A)-related epilepsies, infectious causes involving bacterial or viral brain infections, metabolic factors like solute carrier family 2, facilitated glucose transporter member 1 (GLUT1) deficiency, immune-related factors such as

multiple sclerosis (MS) and autoimmune encephalitis, and unknown etiologies [58]. As of now, over 500 genes associated with epilepsy have been identified [59]. Gender-based disparities in incidence and prevalence were not evident in a systematic review [60]. Nevertheless, certain investigations have indicated a higher prevalence among males. This could be attributed to potential under-reporting by females, particularly in areas where a female diagnosed with epilepsy might face marginalization or be deemed ineligible for marriage if her condition were disclosed [61].

The treatment of epilepsy aims to achieve seizure freedom while minimizing adverse effects. This involves considering various factors, including the properties of the drugs and the patient's characteristics [62, 63]. Drug-related factors affecting the choice of medications include the efficacy of antiseizure medications (ASMs) for different seizure types, tolerability, safety, therapeutic index, pharmacokinetics, drug-drug interactions, approved indications, contraindications, available formulations, cost, and reimbursability. Patient-related factors that come into play include the type of seizures or epilepsy, age, gender, comorbidities, comedications, and risk factors for adverse effects.

The general approach to epilepsy treatment typically begins with monotherapy, which is often effective and better tolerated compared to using multiple drugs. If seizures persist despite monotherapy, a re-evaluation of the diagnosis and drug selection is necessary. In some cases, patients may achieve seizure remission with alternative monotherapy. Polytherapy (using multiple ASMs) is generally considered after two or three monotherapy attempts have failed to control seizures. However, it's essential to note that only a relatively small number of patients unresponsive to two appropriately chosen ASMs will achieve seizure freedom with other treatments [62].

1.1 The challenge of drug-resistant epilepsy

Pharmacoresistance in epilepsy was defined in 2010 by the ILAE as the failure to achieve sustained seizure freedom after adequate trials of two tolerated and appropriately prescribed AED schedules, either as monotherapies or in combination [64]. This condition affects approximately 20-30% of all patients with epilepsy [65] and presents significant challenges in epilepsy management due to its association with an increased risk of death, disabling psychosocial consequences [66], and uncontrolled seizures. It is important to note that not all types of epilepsy have the same proportion of pharmacoresistance, with certain syndromes, such as epileptic encephalopathies, having a worse response to treatment than others, like juvenile myoclonic epilepsy.

Uncontrolled seizures and drug toxicity, often related to polytherapy and high AED doses, can lead to learning disability, intellectual impairment, psychiatric comorbidities, reduced autonomy, decreased quality of life, and an elevated risk of seizure-related sudden death [67]. While drug resistance is often identified early in the disease, it can also develop after several years and may follow a prolonged seizure-free period. Specific etiologies and high seizure frequency are predictors of poor treatment response, and pharmacoresistance is frequently observed in patients with epileptic encephalopathies, documented cortical dysplasia, and hippocampal sclerosis [67, 68]. The persistence of seizures results in a higher usage of polytherapy, leading to increased side effects, a significant reduction in quality of life, greater direct medical costs, and increased mortality.

Despite the introduction of several newer ASMs over the last three decades for managing drug-resistant epilepsy, their overall impact on long-term outcomes in these patients remains modest [69]. Clinical trial data suggest that approximately 5-10% of patients with refractory epilepsy may achieve seizure freedom with newer ASMs [70, 71]. It is crucial to consider that AED use in clinical trials may differ from clinical practice, and many epilepsy syndromes have not been investigated in well-designed randomized trials [70, 71]. While epilepsy surgery offers the best chance of achieving seizure control in patients with pharmacoresistant epilepsy, eligibility criteria for surgery, including focal epilepsy with a clearly defined epileptogenic zone in a non-eloquent part of the brain, limit the number of suitable candidates. Ongoing research aims to elucidate the molecular basis of drug resistance and facilitate the discovery of more effective ASMs, focusing on mechanism-based approaches targeting anti-epileptogenic factors, multi-drug transporters reversal/inhibitors, drug target alterations, and drugs addressing neuroinflammatory pathways.

2. Classification of seizures and epilepsies

The early classification of epilepsies holds significant implications for clinical consultations and plays a pivotal role in advancing both basic and clinical epilepsy research, as well as shaping the development of new treatments. This classification serves as a fundamental framework for understanding not only the specific type of seizure experienced by the patient but also the likelihood of other seizure types, the underlying causes of these seizures, and the prognosis. Moreover, it extends its influence to risk assessments for comorbid conditions such as learning and intellectual disabilities, psychiatric attributes like autism spectrum disorder, and the potential for

mortality, notably sudden unexpected death in epilepsy. Importantly, this classification often serves as a guiding principle in the selection of appropriate anti-seizure therapies [58]. In 2017, the ILAE took a significant step in refining the classification of seizures and epilepsies [57, 72]. The revised seizure classification, building upon the 1981 framework, has been enhanced to offer a more practical approach [73, 74]. Similarly, the ILAE classification of epilepsies initially ratified in 1989, has undergone updates to reflect advancements in understanding the mechanisms underpinning epilepsies and evolving diagnostic and management approaches over time [58]. The novel classification of epilepsies is structured across three distinct levels (Figure 1). The initial level pertains to the classification of seizure types, encompassing focal onset, generalized onset, and unknown onset, assuming the clinician has already established a diagnosis based on the 2017 ILAE seizure classification [57]. The second level focuses on the diagnosis of epilepsy types, including focal epilepsy, generalized epilepsy, combined generalized and focal epilepsy, and an unknown epilepsy group. The third level delves into the diagnosis of epilepsy syndromes, allowing for comprehensive syndromic diagnoses [58]. Both the classification of seizure types and epilepsy types incorporate insights garnered from electroencephalography and neuroimaging studies, in addition to other investigations exploring the underlying etiology of epilepsy. Significantly, the new classification of epilepsies incorporates etiological considerations at each diagnostic level, emphasizing the pivotal role of etiology in guiding treatment decisions [58].

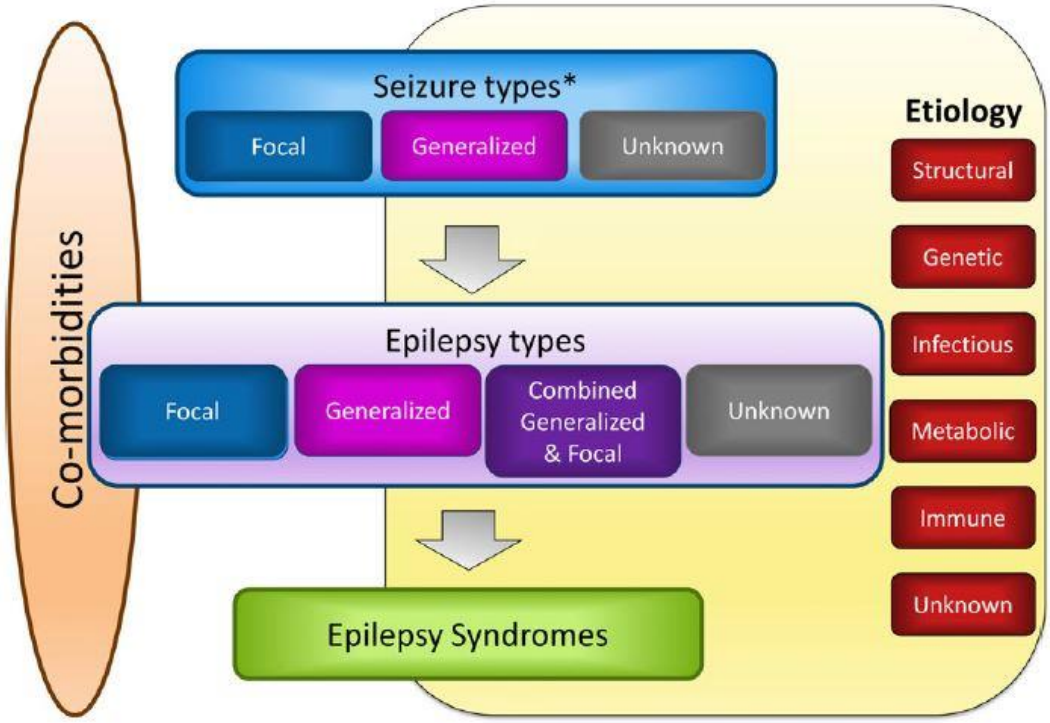

Figure 1. 2017 ILAE classification of epilepsies. Modified from Scheffer et at. (2017) [58]. *Denotes onset of seizure.

2.1. Seizure type

The classification of seizures encompasses three main types: focal, generalized, and unknown onset, as illustrated in Figure 2 and Figure 3 of the 2017 seizure classification. These two representations essentially depict the same classification system, with the basic version collapsing subcategories for a simplified view, while the expanded version offers insights that are more detailed. Seizure classification begins by determining whether the initial manifestations of the seizure are focal or generalized. When the onset is evident and localized, it falls under the category of focal seizures. Conversely, if the onset is unclear or missed, the seizure is classified as having an unknown onset. When a patient's EEG shows epileptiform activity or abnormalities that are indicative of epilepsy on brain imaging, it suggests an increased likelihood of experiencing recurrent seizures [57, 72, 75].

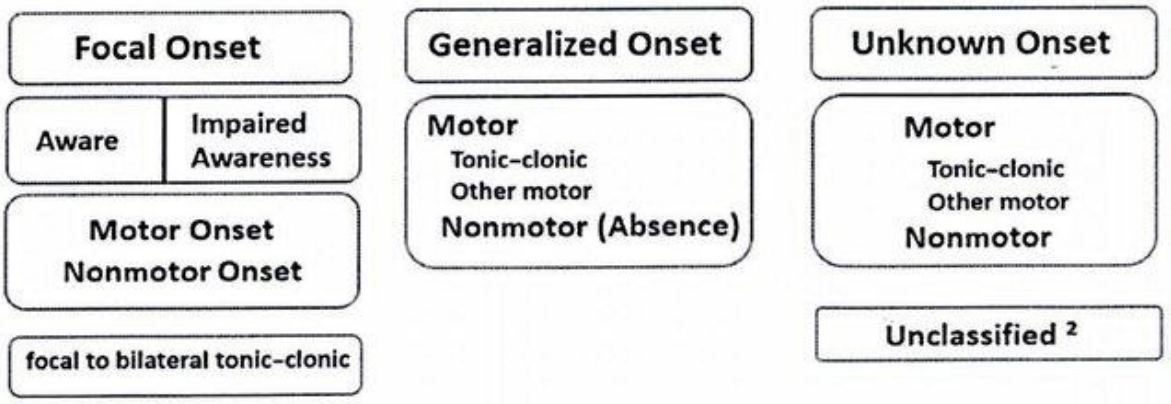

Figure 2. The basic ILAE 2017 operational classification of seizure types. The basic seizure classification is intended for use by practitioners not specialized in epilepsy.

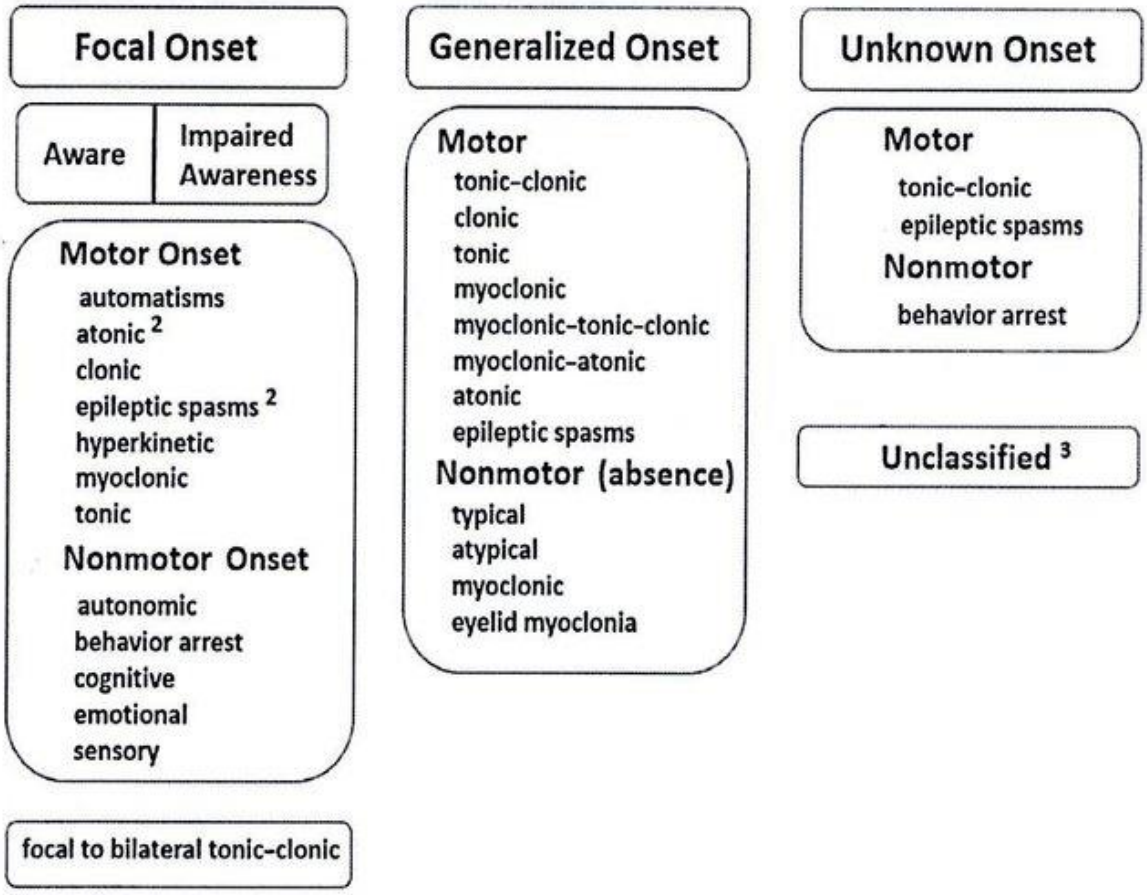

Figure 3. The expanded ILAE 2017 operational classification of seizure types. The expanded seizure classification is intended for use by clinicians with expertise in the diagnosis and treatment of epilepsy. This classification has a structure similar to that of the basic classification, but the motor and non-motor categories are further divided according to features that might be present during seizures, such as automatisms and myoclonus.

Focal seizures can further incorporate the level of awareness as an optional factor. Awareness becomes crucial, as it distinguishes between focal aware seizures, corresponding to the former term "simple partial seizures," and focal impaired awareness seizures, aligning with the previous concept of "complex partial seizures." Impaired awareness during any part of a seizure categorizes it as a focal impaired awareness seizure. Additionally, focal seizures are sub-classified based on the presence of motor or non-motor signs and symptoms at the onset. If both motor and non-motor signs are evident, motor signs generally take precedence unless non-motor symptoms, such as sensory experiences, dominate. The types of focal motor and non-motor seizures are described in the following table 1 and table 2 respectively. Focal awareness or impaired awareness seizures may also be further characterized by specifying the first prominent sign or symptom that occurs at the seizure's onset. For example, a focal impaired awareness seizure with automatism features would be labeled as such. Seizures are often classified based on the earliest prominent motor or non-motor characteristic, except for instances where the seizure is characterized by a continuous feature throughout its duration, such as focal behavior arrest, which categorizes it as having impaired awareness. The classification of an individual seizure can be as simple as "focal onset" or "generalized onset" with no additional elaboration. Alternatively, it can be more specific, incorporating descriptors like "focal sensory seizure," "focal motor seizure," "focal tonic seizure," "focal automatism seizure," and so forth. Additional qualifiers are encouraged, and their usage may depend on the expertise and objectives of the person classifying the seizure.

Table 1. Types and glossary of focal motor seizures [57, 58, 72, 75, 76].

Automatisms	Coordinated and repeated motor activity usually occur when cognition is impaired and is followed by amnesia afterward. This often resembles a voluntary movement and may be an inappropriate continuation of preictal motor activity.
Atonic	A sudden loss or diminution of muscle tone that occurs suddenly without apparent preceding myoclonic or tonic event lasting approx. 1-2 sec, involving head, trunk, jaw, or limb musculature and possible only on one side of the body.
Clonic	Rhythmica jerking, either symmetric or asymmetric, that is regularly repetitive, and involve the same muscle groups.
Epileptic Spasms	Sudden und controlled and often painful flexion, extension, or mixed extension–flexion of predominantly proximal and truncal muscles. Limited forms may occur including grimacing, head nodding, or subtle eye movements. Epileptic spasms may frequently occur in clusters and can be focal, generalized, or of unknown onset. Infantile spasms are the best-known form but can also occur at all ages.
Hyperkinetic	Exaggerated, and often uncontrolled muscle activities such as agitated kicking, Trashing, and peddling during the seizure.
Myoclonic	Similar to clonic seizures but sudden and brief (<100 msec) involuntary single or multiple contraction(s) of muscles(s) or muscle groups of a variable topography (axial, proximal limb, distal) and is less regularly repetitive and less sustained than clonus is.
Tonic	Sustained increase in muscle contraction that lasts for a few seconds or minutes and presents clinically as stiffening of a limb or the neck.

Types of *focal non-motor* seizures are structured in table 2.

Table 2. Types and glossary of focal non-motor seizures [57, 58, 72, 75, 76].

Autonomic	A distinct alteration of autonomic nervous system function involving cardiovascular, pupillary, gastrointestinal, sudomotor, vasomotor, and thermoregulatory functions and presenting symptoms such as rising sensation in the stomach, hot and cold feelings, a strange taste or smell.
Behavior arrest	Arrest of activities, freezing, immobilization, and unresponsiveness for the entired duration of the seizure.
Cognitive	Impaired language or other cognitive domains or positive features such as déjà vu, ha hallucinations, illusions, or perceptual distortions.
Emotional	Begins with an emotion such as fear, panic, anxiety, spontaneous joy or euphoria, laughing and crying.
Sensory	A perceptual abnormal experience including visual, olfactory, auditory, and gustatory sensations, which are not caused by stimuli of the environment.

Generalized seizures are subdivided into motor and non-motor (absence) seizures. These subcategories align with the 1981 classification, with the addition of seizure types like myoclonic–atonic seizures, myoclonic–tonic–clonic seizures, myoclonic absence, and absence seizures with eyelid myoclonia. Generalized seizures can occasionally manifest asymmetrical features, making them challenging to distinguish from focal-onset seizures. It is important to note that the term "absence" in "absence seizures" refers to more than just a vacant stare; it signifies a distinct seizure type with activity arrest, different from other seizure types with similar appearances. Generalized seizures are caused by abnormal neuronal activity that starts in a widespread distribution over both hemispheres at the same time. Some types of motor seizures have already been defined under focal motor seizures. Additional types of generalized motor seizures are specified below in table 3.

Table 3. Types and glossary of generalized motor seizures [57, 58, 72, 75, 76].

Tonic-clonic	A sequence consisting of a tonic followed by a clonic phase.
Myoclonic-tonic-clonic	One or a few jerks of limbs bilaterally. Characterized by arms jerking, tonic stiffening, and, clonic rhythmical jerking. Common in juvenile myoclonic epilepsy.
Myoclonic-atonic	A generalized seizure type with a myoclonic jerk leading to an atonic motor component. This type was previously called myoclonic–astatic. Often seen in patients with the Doose syndrome.

Further, types of non-motor seizures have already been presented in table 2. Additional types of generalized non-motor (absence) seizures are listed in subsequent table 4.

Table 4. Types and glossary of generalized non-motor (absence) seizures [57, 58, 72, 75, 76].

Absence, typical	A sudden onset, interruption of ongoing activities, a blank stare, possibly a brief upward deviation of the eyes. Usually, the patient will be unresponsive when spoken to. Duration is a few seconds to half a minute with very rapid recovery. EEG would show generalized epileptiform discharges during the event.
Absence, atypical	Change in a tone that are more pronounced than in typical absence or the onset and are often associated with slow, irregular, generalized spike-wave activity.
Eyelid myoclonia	Jerking of the eyelids at frequencies of at least 3 per second, commonly with upward eye deviation, usually lasting <10sec with possible loss of awareness.

For seizures with unknown onset, the 2017 classification allows for the addition of a limited number of qualifiers to provide a more comprehensive characterization of the seizure. These may include descriptors like motor, non-motor, tonic–clonic, epileptic spasms, and behavior arrest. Seizures of unknown onset can later be reclassified as either focal or generalized onset, but any associated behaviors from the previous unclassified phase still apply. The term "unknown onset" serves as a temporary placeholder reflecting a lack of knowledge about the seizure's origin rather than an inherent feature of the seizure itself.

The 2017 ILAE classification of seizures greatly reduces unclassifiable cases. Flexible combinations of awareness level and motor/non-motor features provide detailed seizure descriptions. Several unclassified cases, however, are still likely to arise, and this is mostly due to our lack of understanding of epilepsy. To date, the ILAE 2017 classification of seizures demonstrates a steady transition from the 1981 classification, with acceptable consistency and improvements. Finally, seizures with patterns that do not match the criteria of other categories and seizures with inadequate information to allow classification fall under the umbrella term unclassified [57, 72].

2.2. Classification and aetiology of epilepsy

Epilepsy is not a singular disorder but rather a spectrum of distinct conditions with diverse underlying causes, leading to various classifications. The latest ILAE classification system subdivides epilepsy based on three key aspects: seizure location, EEG pattern, and etiology, which refers to the original cause of abnormal brain activity. Seizure localization can be categorized as focal, generalized, focal & generalized, or unknown. Focal seizures originate from synchronized neuronal activity in specific brain regions, and their manifestations depend on the precise areas of the brain involved. Focal seizures can generalize when abnormal activity spreads from the focal point to the entire cortex, resulting in a loss of consciousness, vocalization, and tonic-clonic movements. Notably, focal seizures are often preceded by an aura, which can help pinpoint the brain region where the seizure initiates [77].

Focal Epilepsy: Characterized by focal epileptiform discharges, it includes unifocal and multifocal disorders, often involving one hemisphere. Focal epilepsy comprises focal aware seizures, focal impaired awareness seizures, focal motor seizures, focal non-motor seizures, and focal to the bilateral tonic–clonic seizures. Diagnosis primarily relies on clinical assessment with EEG support [58].

Generalized Epilepsy: Typically marked by generalized spike-wave activity on EEG, patients may experience absence, myoclonic, atonic, tonic, and tonic–clonic seizures. Diagnosis is mainly clinical, supplemented by the presence of typical interictal EEG discharges [58].

Combined Generalized and Focal Epilepsy: This category involves individuals with both generalized and focal seizures. Conditions like Dravet syndrome (DS) and Lennox-Gastaut syndrome may exhibit both seizure types. While the interictal EEG may display both generalized spike-wave and focal epileptiform discharges, it's not mandatory for diagnosis, which is primarily clinical with EEG support [58].

Unknown Epilepsy: Individuals with unknown epilepsy lack a specific diagnosis due to insufficient available information [58].

2.3. Epilepsy syndrome

Epilepsy syndrome, at the third level of classification, refers to a constellation of factors that consistently co-occur, encompassing seizure types, EEG patterns, and imaging characteristics. These syndromes often exhibit age-dependent features, such as age at onset and remission (when applicable), seizure triggers, diurnal variations, prognosis, and associated comorbidities, including intellectual and mental impairments [78]. Notably, epilepsy syndromes like childhood absence epilepsy, West syndrome, and DS are recognized examples [79]. However, it is essential to note that the ILAE has not formally established a comprehensive classification of epilepsy syndromes, and in most cases, diagnosis at the epilepsy type level remains the ultimate goal of classification. Furthermore, idiopathic generalized epilepsies, a subset of epilepsy syndromes, encompass childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy, and generalized tonic–clonic seizures alone. These can alternatively be termed genetic generalized epilepsies to reflect a genetic etiology when clinically relevant [58]. Additionally, certain self-limited focal epilepsies, typically originating in childhood, include benign epilepsy with centrotemporal spikes among others [58].

3. Etiology

Epilepsy is a complex neurological disorder with a diverse range of etiologies that significantly impact its clinical course and prognosis. Approximately 50% of epilepsy cases lack a discernible

cause, highlighting the intricate nature of this condition. The ILAE's classification of epilepsy etiology encompasses genetic, structural, metabolic, immune, infectious, and/or unknown causes [58].

Structural Etiology: This category encompasses abnormalities observable through structural neuroimaging. To attribute seizures to a structural etiology, clinical evaluation and imaging findings must strongly support the conclusion that the structural abnormality is the likely cause of the seizures. It is worth noting that employing advanced magnetic resonance imaging (MRI) techniques is often necessary to detect even subtle structural lesions. Structural etiologies can be genetic, such as various cortical developmental anomalies, or acquired, including instances like stroke, trauma, and infection. Notably, this association is frequently observed in conditions like mesial temporal lobe seizures with hippocampal sclerosis [79-81].

Genetic Etiology: Genetic factors play a substantial role in epilepsy, leading to seizures as a primary symptom. Genetic etiology can be established based on various criteria, including a family history of autosomal dominant disorders, clinical research findings within populations exhibiting the same syndrome (e.g., childhood absence epilepsy or juvenile myoclonic epilepsy), or molecular evidence linking a specific gene or copy number variation to a significant effect. Several genes, such as Potassium Voltage-Gated Channel Subfamily Q Member 2, SCN9A, and Chloride voltage-gated channel 2, have been associated with epilepsy [82, 83].

Infectious Etiology: Infections represent one of the most prevalent causes of epilepsy worldwide. This category refers to epilepsy that results directly from infections, with seizures serving as a central symptom. Examples include neurocysticercosis, tuberculosis, HIV, cerebral malaria, subacute sclerosing panencephalitis, cerebral toxoplasmosis, as well as congenital infections like Zika virus and cytomegalovirus [58].

Metabolic Etiology: Metabolic disorders can lead to epilepsy. These disorders encompass conditions such as GLUT1 deficiency, adenylosuccinate lyase deficiency, and serine biosynthesis defects [58, 59, 84]. It is important to note that the majority of metabolic epilepsies are attributed to genetic defects [85].

Immune Etiology: Autoimmune disorders can underlie the development of new-onset epilepsy. Various immune-related epilepsies have been described, often associated with conditions like MS and autoimmune encephalitis. Specific antibodies, both intracellular (glutamic acid decarboxylase 65 (GAD65), antineuronal nuclear antibody type1, Monoclonal antibodies) and cell surface (voltage-gated potassium channel complex, N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-

methyl-4-isoxazole propionic acid (AMPA), $GABA_B$, glutamate receptor 5 (GluR5)), have been linked to autoimmune encephalitis and epilepsy [58, 86, 87].

Unknown Etiology: Some epilepsy cases defy identification of a specific cause, and this is particularly prevalent in resource-constrained regions. The ability to evaluate potential causes depends on factors such as the patient, healthcare infrastructure, and geographic location. Unknown etiologies tend to be more common in less developed countries compared to wealthier nations [58].

These causes are not mutually exclusive, and epilepsy can often result from a combination of two or more etiologies. Historically, epilepsies have been categorized into monogenic, polygenic, and acquired forms [88]. Monogenic epilepsies stem from defects in the structure and function of a single protein and can be either inherited or the result of de novo mutations in a single gene. In contrast, polygenic epilepsy involves multiple genes that collectively lower the seizure threshold. Additionally, epilepsy can occur as a consequence of acquired disorders such as cerebral tumors, traumatic brain injuries, strokes, neurodegenerative diseases, infections, or autoimmune diseases [58].

4. Temporal Lobe Epilepsy

Among focal epilepsies, temporal lobe epilepsy (TLE) takes precedence as the most prevalent, accounting for approximately 60% of all cases within this category and therefore meriting the most comprehensive scrutiny. Hippocampal onset accounts for at least 80% of all temporal lobe seizures [89].

The main features of TLE are (i) epileptic foci in the limbic system; (ii) an “initial precipitating injury”; (iii) the so-called “latent period”; and (iv) the presence of hippocampal sclerosis leading to reorganization of neuronal networks [90]. There are two main types of TLE: the mesial, limbic form, which is by far the most common, and the lateral, neocortical form [91]. It is difficult to distinguish the two forms of TLE clinically, but patients with the neocortical form usually have auditory hallucinations before seizures. So, TLE can originate from either neocortical regions, specifically the lateral temporal lobe, or archicortical areas within the hippocampal formation of the mesial temporal lobe. The hippocampal formation, composed of the hippocampus and para-hippocampal structures, represents one of the most frequently impacted brain regions in epilepsy and is often a

prime target for surgical intervention [92]. Hallmarks of mesial temporal lobe epilepsy (mTLE) include typical histopathological features such as hippocampal sclerosis and hyperactivation of glial cells, encompassing astrocytes and glia. This hyperactivity likely results from the release of pro-inflammatory molecules like Adenosine triphosphate (ATP) and cytokines [92]. The initial signs of a seizure vary depending on the affected temporal lobe, the seizure network's location and function, and the seizure propagation pattern (Table 5) [93-96].

Table 5. Possible seizure patterns that may be seen in patients with TLE.

Seizure type	**Possible symptoms**
Seizure with autonomic symptoms	Rising discomfort from the stomach (epigastric aura), pupillary changes, nausea, vomiting (emetic seizures), flushing. goosebumps, palpitations, altered bowel function, cold, warmth
Seizure with motor elements	Behavioral arrest, automatisms, strange behavior
Seizure with sensory symptoms	Unpleasant smell (uncinate seizures), peculiar taste in the mouth, auditory or visual disturbances (illusions à la Alice in Wonderland syndrome or hallucinations)
Seizure with emotional symptoms	Anxiety, despondency, despair, laughter (gelastic seizures), crying (dacrystic seizures), intense happiness, ecstasy (Dostoevsky seizures)
Seizure with cognitive symptoms	Déja vu, jamais vu, forced thinking, memory disturbance, dysphasia, dyspraxia, neglect

The regions most profoundly affected within the hippocampal subfield are the Cornu Ammonis 1 (CA1) and CA3 regions, while the dentate gyrus (DG) primarily remains intact. However, the DG exhibits a characteristic widening of the cell granule layer, often referred to as "dispersion," stemming from abnormal newborn neuron formation with altered physiological characteristics. Notably, the selective elimination of adult neurogenesis in the hilus and DG through a genetic approach in animals injected with pilocarpine resulted in a prolonged reduction in seizure frequency and cognitive enhancement [97]. Some hypotheses posit that the DG might serve as a major site of seizure onset. This apparent incongruity could be attributed to the dual nature of granule cells, serving both excitatory and inhibitory roles. Despite uncertainty regarding the underlying mechanisms, granule cells express several inhibitory traits. Alongside their prominent excitatory profile, granule cells have been shown to release GABA and express the GABA-

synthesizing enzyme (GAD67). Moreover, granule cells express zinc and dynorphin, neuropeptides with complex modulatory functions. Interestingly, during epilepsy and heightened excitation, messenger ribonucleic acid (mRNA) levels of GAD67, GAD65, and GABA itself increase, suggesting a potential shift towards inhibitory activity during this phase. This phenomenon may represent an adaptive response to mossy fiber sprouting and the loss of lateral inhibition due to cellular death within the hippocampal subfield. These changes, coupled with the pathophysiological excitatory shift of GABA, may help elucidate why the DG constitutes a highly epileptogenic zone, despite granule cells' hyperpolarized membrane potential [98, 99].

The etiology of TLE can be diverse, encompassing genetic factors, tumorous growths, vascular pathologies, immune dysregulation, and brain injuries. One of the most prevalent associations in individuals with TLE is a history of febrile seizures or convulsions during childhood [100]. Temporal lobe seizures often commence with an experiential aura, followed by a phase of impaired awareness characterized by potential automatisms, such as manual fidgeting. Postictally, individuals may experience confusion and, in cases where the seizure originates in the dominant temporal lobe, temporary speech difficulties lasting several minutes. Generalization to bilateral tonic-clonic seizures is a rarity in TLE, with factors like sleep deprivation and stress heightening the risk of seizures [101]. Additionally, epilepsy can arise from various causes, including low-grade gliomas, arteriovenous malformations, cortical malformations, autoimmune or viral encephalitis, and hippocampal sclerosis [102]. Historically, dating back to 1825, autopsies had revealed hippocampal atrophy and calcification in individuals with epilepsy. In 1880, Sommer presented the earliest microscopic documentation of hippocampal changes in a 25-year-old man who experienced frequent focal seizures. During these seizures, the man believed he could fly after hearing divine messages, leading him to jump from a roof. Although he survived the fall, he succumbed to infection several years later [103]. Histologically, specific subregions of the hippocampus display neuronal loss, particularly among pyramidal cells (Figure 4). This phenomenon is accompanied by astrocyte proliferation, forming the basis for scarring and sclerosis. Additionally, other neurons and glial cells undergo alterations, with some patients experiencing sclerosis extending to nearby structures like the amygdala, entorhinal cortex, and parahippocampal gyrus. These morphological changes are believed to underlie the development of epileptic cell networks through dysfunctional synaptic reorganization or modifications in the internal properties of neurons and glial cells [104]. The etiology of hippocampal sclerosis has been a subject of vigorous debate over the years. In

children, it has been demonstrated that prolonged febrile seizures increase the risk of subsequent hippocampal sclerosis and epilepsy [102]. Genetic susceptibility likely plays a role in this context, with identified associations between mutations in the SCN1A gene, prolonged febrile seizures, and hippocampal sclerosis [105]. It is now widely acknowledged that hippocampal sclerosis can both trigger and result from epileptic seizures.

Patients with mTLE often exhibit a reduced hippocampal volume when visualized via MRI, sometimes accompanied by lesions such as tumors or brain malformations. The ictal scalp-EEG pattern depends on the underlying pathology and the location of seizure onset within the temporal lobe (lateral neocortex vs. mesial temporal onset) [106]. Scalp EEG, while non-invasive, is susceptible to artifacts stemming from patient mastication and muscle twitches and may not detect abnormal activity generated in the mesial temporal lobe until it has spread to the lateral neocortex. For enhanced spatial and temporal resolution, which is occasionally indispensable before surgical resection, intracranial EEG and sphenoidal electrodes (implanted beneath the zygomatic bone) are typically employed in conjunction with MRI scans. This approach aids in identifying the low-amplitude, high-frequency (>20Hz) trace characteristic of the initial seizure phase [92].

Medical treatments for TLE, especially in cases with severe hippocampal sclerosis, frequently yield limited success. The conventional treatment strategy involves monotherapy followed by combination therapy with two or more ASMs. Importantly, a higher rate of AED failures correlates with a reduced likelihood of effectively controlling seizures with subsequent drugs. Therefore, the criterion for defining drug resistance often hinges on the failure of two or more ASMs at adequate doses. While approximately 60-70% of patients achieve seizure freedom with AED therapy, the response to treatment hinges on the epilepsy's underlying cause [107].

Notably, drugs like carbamazepine, valproate, and clobazam exhibit higher retention rates in mTLE. Nevertheless, even the most effective drug, carbamazepine, yields relatively low one-year seizure-free rates (approximately 11%). Furthermore, adverse reactions are common, with symptoms encompassing dizziness, anxiety, memory impairment, nausea, ataxia, and vision disturbances [108]. Hippocampal sclerosis stands out as a predominant cause of drug-resistant epilepsy, with successful management achieved in only 25-40% of patients using ASMs [109]. In contrast, surgical resection in cases of hippocampal sclerosis can yield remarkable success, as more than 70% of patients become seizure-free. Hence, there is a growing consensus to encourage the early consideration of surgical resection as a viable treatment option for individuals grappling with

hippocampal sclerosis. Unfortunately, surgical resection remains a feasible choice for fewer than 10% of all individuals afflicted with drug-resistant epilepsies [108, 110].

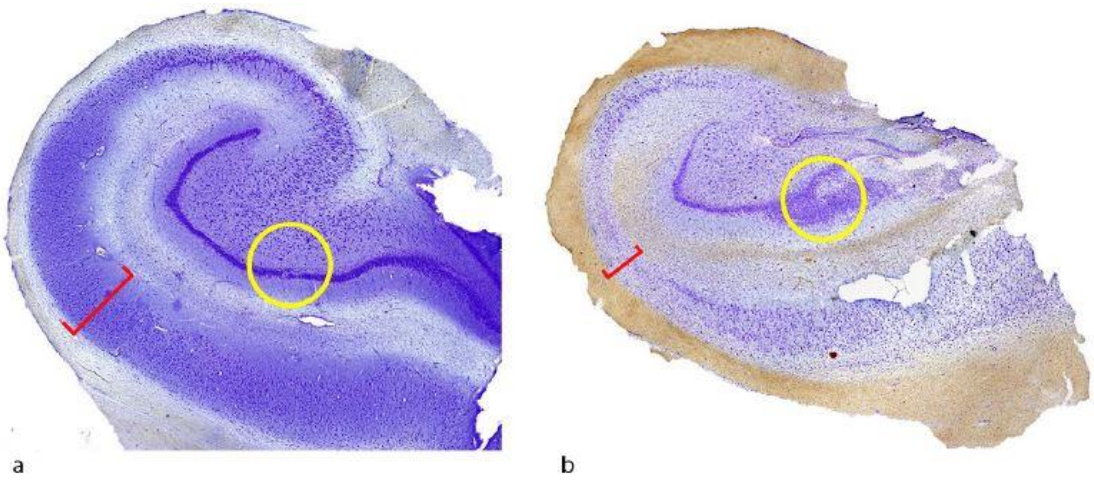

Figure 4. Cross-section of the hippocampus with Nissl staining of neurons. a) Normal hippocampus and b) sclerotic hippocampus. Significant narrowing of the pyramidal cell layer can be seen in specific areas of the sclerotic hippocampus due to cell death (red bracket). In addition, granule cell dispersion can be seen in another area, likely reflecting a migration defect (yellow circle).

4.1. The hippocampus and its circuits

The hippocampus, an elongated structure (Figure 5), resides within the temporal lobe, situated along the medial aspect of this region. It boasts extensive connections with various brain areas. Longitudinally, the hippocampal formation can be divided into three primary subsections: the CA1, CA3 (also referred to as the hippocampus proper), and the DG. Two main sources of hippocampal inputs are layer II and III of the entorhinal cortex, which send projections to the hippocampus via the perforant path fibers. One pathway involves projections from layer II to the DG (which then projects to CA3, and CA3 projects to CA1, forming the "trisynaptic circuit"). An alternative pathway originates in layer III, projecting primarily to CA1 and the subiculum [111].

Within the hippocampus, the DG projects to CA3 through mossy fibers (MFs); similarly, the axons from CA3 pyramidal cells, in addition to forming many recurrent excitatory connections, synapse onto CA1 neurons, passing through the small CA2 area, known as the "Schaffer collaterals." CA1 is classically recognized as the primary output region of the hippocampus and is connected to the entorhinal cortex and subiculum. The entorhinal cortex, in turn, projects to the prefrontal cortex and other parts of the neocortex [112].

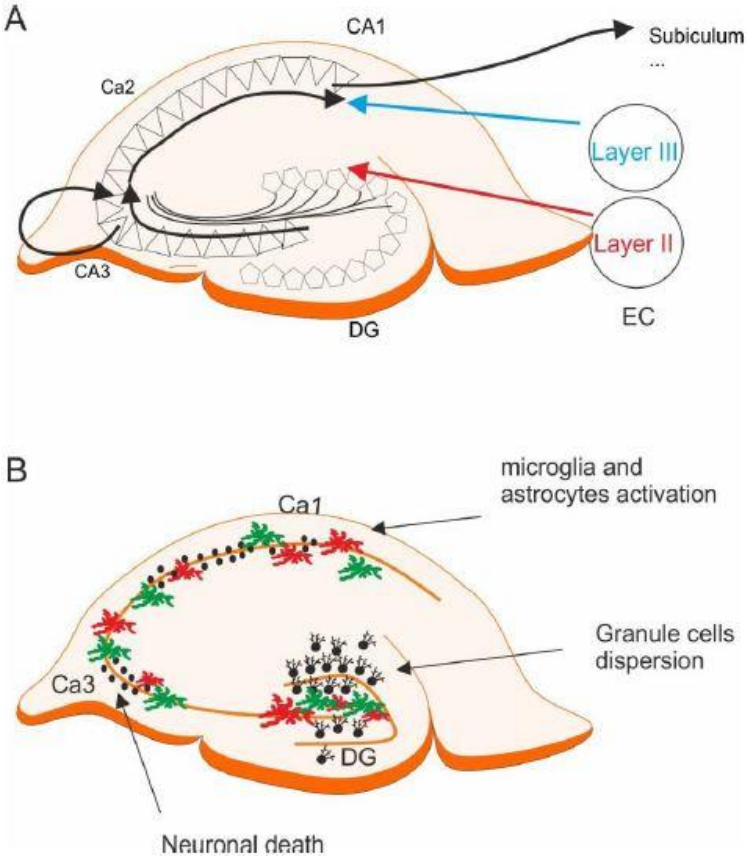

Figure 5. Schematic representation of hippocampal circuits and hippocampal sclerosis. A) Simplified hippocampal circuits. **B)** Typical consequences of an epileptic insult in the hippocampus;

Although there are some inter-species differences, the overall structure and principal connections of the hippocampus are well conserved between rodents, primates, and humans [111]. The hippocampus is organized into a five-layer structure – alveus, stratum moleculare, stratum pyramidale, stratum radiatum, and stratum oriens-lacunosum. Stratum pyramidale consists of densely packed pyramidal neurons, interspersed with fewer GABAergic inhibitory neurons strategically positioned to control excitation. Rhythmic activity of interneurons suppresses pyramidal neuronal firing, synchronizing them and giving rise to oscillations at different frequency bands. Distinct frequencies serve different biological functions. For instance, gamma oscillations (30-130 Hz) have been linked to memory formation, one of the hippocampus's primary functions.

Initially, memory is consolidated and reinforced in these circuits and subsequently stored for long-term retrieval in other parts of the cortex [113]. Human studies have shown that damage or removal of one or both hippocampi significantly impairs the ability to store new event-related memories [114]. Dorsal and ventral parts of the hippocampus are attributed to different types of memories, with the ventral part believed to be involved in emotional memory and the dorsal part more associated with spatial memory [115].

The hippocampus also exhibits other types of frequencies, such as theta oscillations (4-8 Hz), involved in the initial phase of learning, sleep phases, and exploratory behavior [113]. Additionally, the hippocampus contains place cells, whose activity correlates with an animal's speed and location. These cells fire bursts of action potentials when the animal passes through specific locations [112, 115]. Other frequencies detected in the hippocampus are fast ripples (140-200 Hz), believed to encode the reinforcement of previously acquired information after an initial theta phase and responsible for transferring information to higher cortical structures [115].

The hippocampus displays both structural and functional plasticity in an activity-dependent manner. This activity can be physiological, including hormones, stress, and learning, or pathological, such as trauma, stroke, or epilepsy. Experimental induction of mechanisms similar to those underlying memory formation and synaptic plasticity is achieved through paradigms like long-term potentiation (LTP) in slices. This protocol involves repeated and long-term stimulations that potentiate neuronal activity. As a result, spines increase in number and enlarge in shape [116]. The opening and recruitment of NMDA receptors at synapses are believed to represent fundamental mechanisms for inducing LTP. Pharmacological prevention of LTP using blocking agents of NMDA receptors significantly reduces LTP induction. Similar results are obtained when AMPA receptor mobility is prevented with specific antibodies against AMPA subunits [114, 117].

In recent years, it has been discovered that the hippocampus, particularly the DG, is among the adult niches capable of producing new-born neurons. The function of these neurons is still a topic of debate, with proposed roles in learning and neuroendocrine activity. Adult neurogenesis has also been observed following damage from seizures and may be directly involved in seizure generation [116, 118]. The DG to CA3 connection represents the second synapse in the classic view of the trisynaptic circuit. The DG has a distinctive shape, forming an acute angle that smooths from the dorsal to the ventral part of the hippocampus. Comprising the DG are granule cells, named for their characteristic small granular shape, densely packed together to create the central granule cell

layers. These granule cell dendrites project into the molecular layer, where they receive inputs from the medial and lateral perforant path fibers, interneurons, and other afferent fibers.

Below the granule cells lies the polymorphic layer, which houses the mossy cells. Mossy cells form the commissural fibers and receive inputs from MFs. MFs are large unmyelinated axons originating from granule cells. They release glutamate from large boutons at multiple release sites, exhibiting significant facilitation when the frequency of stimulation increases [119]. MFs have multiple sites of termination in the stratum lucidum within the CA3 area, rich in dendrites from excitatory neurons and, to a lesser extent, inhibitory neurons [120].

The DG is believed to serve as a filter and regulator of the excitation and information arriving from afferent fibers, such as those from the entorhinal cortex. In fact, granule cells are not inherently very active cells and maintain a relatively negative resting membrane potential [121]. Damage to this filtering function can result in the loss of its gating function [119]. Notably, glutamate is not the only neurotransmitter released at MF synapses. GABA, opioids, zinc, and other peptides are also released at these synapses and are believed to play essential neuromodulatory roles [122, 123]. Presynaptic GABA, NMDA, and kainate receptors have been described on MF boutons and are thought to regulate the potentially substantial excitatory input from MF [121].

5. Status epilepticus

Status epilepticus (SE) represents a critical neurological emergency characterized by prolonged seizure activity, often accompanied by a loss of consciousness. The outcome of SE depends on etiology, age, symptomatology, and duration of SE, and patients benefit from carefully chosen but rapidly administered efficacious anti-seizure medication and appropriate management of SE [124-132]. Over time, the definition of SE has evolved to encompass a more nuanced understanding of its clinical and pathophysiological aspects. This comprehensive definition now includes two pivotal time points, t1 and t2, which are instrumental in grasping the complexities of SE [133]. T1 signifies the critical moment when intervention should be promptly initiated to halt a seizure. It is notably set at 5 minutes for convulsive SE based on compelling evidence demonstrating that seizures persisting beyond this threshold are more likely to become prolonged and challenging to terminate [134, 135]. This revised definition underscores the importance of early identification and timely intervention as essential components of managing SE. T2, conversely, marks the time when

protracted seizure activity may lead to enduring consequences, including neuronal damage, injury, and alterations within neuronal networks. For convulsive SE, T2 continues to be defined as 30 minutes [133]. These time points, thoughtfully proposed by ILAE Task Force, provide invaluable guidance for the management and treatment of SE.

SE manifests in various forms, each characterized by distinct clinical features, including convulsive SE, epilepsia partialis continua, and nonconvulsive SE [136]. The ILAE classification makes a distinction between SE without prominent motor phenomena, often referred to as nonconvulsive SE, and SE characterized by prominent motor phenomena. This includes conditions such as bilateral tonic-clonic SE, which is also known as convulsive SE, as well as focal, myoclonic, tonic, and hyperkinetic SE (Figure 6 and Table 6). In convulsive SE, there are repetitive tonic-clonic movements, followed by a postictal state. In the case of epilepsia partialis continua, focal neurological deficits, such as aphasia and motor dysfunction, occur due to partial seizures, but there is no altered mental status. Continuous or fluctuating mental status changes are observed in nonconvulsive SE [137]. This diversity underscores the multifaceted nature of SE and highlights the importance of tailoring treatment strategies to its specific manifestations. Refractory SE refers to continuous seizure activity that remains uncontrolled despite the administration of first-line and second-line ASMs [138]. Super-refractory SE, on the other hand, is characterized by SE that does not respond to third-line agents [139] . An alternative definition suggests that super-refractory SE is present when SE persists for 24 hours or more after the administration of anesthesia [140].

Table 6. The Operational Defintion of Time t1 and Time t2

Type of status epilepticus	**Time t1**	**Time t2**
	Seizure activity does not stop spontaneously with a high probability, therefore, time t1 is the time at which emergency treatment of status epilepticus should be started	Seizure activity may cause long-term sequelae, therefore, time t2 is the time at which treatment should be successful to prevent long-term consequences
Bilateral tonic-clonic status epilepticus	5 minutes	30 minutes
Focal status epileptics with and 10 minutes without impairment of consciousness, absences	10 minutes	60 minutes

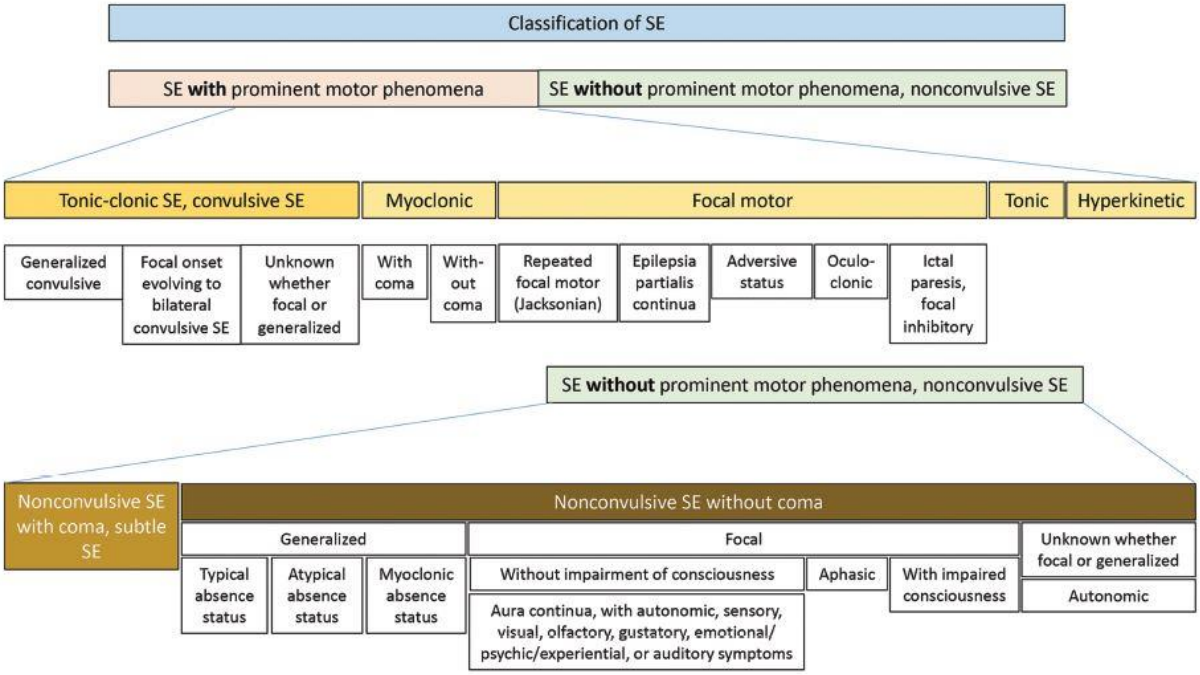

Figure 6. The classification of status epilepticus by the International League Against Epilepsy. Data from Trinka E, et al, Epilepsia.

Furthermore, SE is not an uncommon condition, with an incidence rate ranging from 10 to 41 cases per 100,000 individuals annually. Intriguingly, a significant proportion of individuals experiencing SE have no prior history of epilepsy, with approximately half of all SE episodes occurring in this population [136, 141]. The consequences of SE can be severe, with a mortality rate ranging from 10% to 20%. However, it is worth noting that advancements in treatment regimens have led to a notable reduction in mortality rates in the 21st century. Survivors of SE may face cognitive and neurological deficits, further emphasizing the critical nature of SE management [136, 141]. Therefore, the evolving definition of SE, with the incorporation of time points t1 and t2, serves as a vital framework for understanding and addressing this neurological emergency. SE encompasses a spectrum of clinical presentations, necessitating tailored treatment approaches. Its relatively high incidence rate and potential for severe consequences underscore the urgency of early identification and intervention in SE cases.

5.1. Causes

The etiological factors contributing to SE are diverse and have been extensively studied [142, 143]. Generally, these causes are categorized into three main groups: "cryptogenic/unknown," where no

clear cause is identified, "remote symptomatic," linked to previous insults like stroke or head trauma, and "acute symptomatic," arising from acute neurological conditions (such as stroke or central nervous system (CNS) infection) or systemic issues (like electrolyte disturbances or hypoxia), as well as progressive neurological disorders. It's important to note that febrile SE, while technically falling under the acute symptomatic category, is often classified separately due to its distinct clinical features and different prognosis compared to other forms of acute symptomatic SE.

The causes of SE exhibit variations based on age groups. Among adults, common identifiable causes of SE encompass trauma, tumors, vascular diseases, alcohol withdrawal, and noncompliance with antiseizure medications [144-146]. Conversely, in the pediatric population, the majority of cases are attributed to unknown causes or remote symptomatic factors. In acute pediatric cases, fever and infection are the most frequent identifiable causes [142, 147]. Febrile SE accounts for more than 25% of all pediatric SE cases, with over two-thirds occurring in children during their second year of life. It is defined as an episode of SE that meets the criteria for a febrile seizure, involving a seizure linked to a febrile illness in a child without acute CNS infection, trauma, or electrolyte disturbances, and who has no prior history of afebrile seizures. This subgroup is particularly intriguing for study due to its epidemiological association with hippocampal sclerosis and TLE [148] .

Previous research has indicated a genetic predisposition to prolonged seizures, with DS being a classic example. This syndrome can result from a mutation in the SCN1A gene, which encodes the alpha subunit of voltage-gated sodium channels. Children with DS often experience a fever-associated episode of SE before the age of one. Moreover, even in children without defined gene mutations, there is clear evidence of a familial predisposition to both febrile seizures and SE, including febrile SE [141, 149].

5.2. Pathophysiology

The pathophysiology of SE is intricate and multifaceted, involving various rapid cellular changes, neuronal injury, genetic and epigenetic factors, and alterations in neurotransmitter receptors. At the cellular level, SE initiates a cascade of rapid changes. Within milliseconds to seconds of seizure onset, key events include protein phosphorylation, neurotransmitter release, and ion channel modulation. In particular, clinical and experimental evidence suggests that changes in the localization and subunit composition of $GABA_A$ receptors are implicated in the pathophysiology of

SE [150]. Over the past two decades, extensive research has unveiled a sequence of maladaptive transformations that underlie the progression from an isolated seizure to the development of SE, contributing to its self-sustaining nature (Figure 7). Within the initial milliseconds to seconds following the onset of a seizure, crucial events such as neurotransmitter release, ion channel dynamics, and protein phosphorylation collectively lay the groundwork for a potential prolongation of the seizure [151]. Subsequent to these molecular events, there are alterations in receptor trafficking, including a decline in inhibitory GABAA β2/β3 and γ2 receptor subunits mediated by endocytosis [152, 153], accompanied by an elevation in excitatory NMDA receptors [48, 154]. These receptors, distributed throughout the brain, predominantly consist of the γ2 subunit and play a crucial role in responding to benzodiazepines, which are the primary agents for terminating seizures [155]. The internalization of these receptors results in a reduction in neuronal postsynaptic inhibition, leading to a gradual decrease in sensitivity to benzodiazepines. Intriguingly, extrasynaptic GABAA receptors, characterized by the presence of δ and/or α4 subunits, remain unaffected by this phenomenon [156]. These extrasynaptic receptors are primarily located in regions such as the hippocampus, thalamus, amygdala, hypothalamus, and cerebellum. The inclusion of the δ subunit in these receptors renders them impervious to various benzodiazepines, implying that they are subject to different modes of regulation [157].

It is noteworthy that the modulation of GABAA receptors is believed to play a significant role in the increasing resistance to benzodiazepines, a phenomenon that becomes more pronounced with the duration of SE [158, 159]. Further detrimental changes that transpire over the following minutes to hours involve shifts in excitatory and inhibitory neuropeptide expression, thereby maintaining a state of heightened excitability [160, 161]. The neurosteroids, namely ALLO, pregnanolone, and tetrahydrodeoxycorticosterone (THDOC), are recognized modulators of extrasynaptic GABAA receptors. Within the CNS, progesterone (PROG) undergoes active conversion to 5α-dihydroprogesterone (5α-DHP), and subsequently, with the aid of the enzyme 3α-hydroxysteroid oxidoreductase, to ALLO. Remarkably, ALLO serves as a potent positive allosteric modulator of GABAergic transmission by augmenting the opening of the chloride ion (Cl^-) channel in the GABAA receptor, thereby extending inhibitory postsynaptic currents [162, 163]. It is important to note that this neurosteroid exerts its influence not only on extrasynaptic GABAA receptors but also on synaptic ones, consequently enhancing both phasic (synaptic) and tonic (extrasynaptic) inhibition [164-166]. However, extrasynaptic GABAA receptors, characterized by the presence of the δ

subunit, exhibit greater sensitivity to ALLO modulation [167]. Similarly, THDOC also demonstrates increased potency for δ-containing extrasynaptic receptors [168]. Notably, the δ GABAA receptors expressed extrasynaptically in regions such as the DG play a pivotal role in mediating tonic inhibition, network shunting, and reducing susceptibility to seizures. Natural and synthetic neurosteroids, including ALLO and related pregnane analogs, maximize tonic inhibition within the hippocampus and offer effective protection against various limbic seizures and SE. Due to its distinctive activity across GABAA receptors and heightened sensitivity to extrasynaptic receptors, ALLO has been proposed as a potential treatment for benzodiazepine-resistant SE [169].

Comprehensive analysis of genetic and epigenetic alterations in the days and weeks following SE has uncovered changes in the expression of numerous genes, exhibiting both increases and decreases, which may contribute to the epileptogenic process [170, 171]. Additionally, epigenetic modifications, including widespread changes in DNA methylation within hippocampal cells, have been observed in a murine model of SE [172]. Furthermore, disruptions in the regulation of microRNA, responsible for post-transcriptional gene expression control, are believed to be implicated in epileptogenesis and the resulting neuronal damage induced by SE [173]. Prolonged seizure activity during SE escalates the risk of systemic complications, neurological injury, and mortality. Neuronal injury is a recognized consequence, as demonstrated in animal models, with injuries occurring in regions such as the neocortex, thalami, and hippocampi. Notably, even non-convulsive SE (NCSE) can lead to neuronal injury, challenging the perception that only convulsive seizures are harmful. In humans, serum neuron-specific enolase levels increase following both convulsive and NCSE, indicating neuronal injury. Potential mechanisms behind this injury encompass excitotoxicity, mitochondrial dysfunction, necrosis, and apoptosis [174, 175]. Neuroimaging findings offer insight into SE pathophysiology, reflecting the associated brain changes. Computed tomography may reveal cortical edema, sulcal effacement, loss of gray-white matter differentiation, reduced attenuation, and enhancement. MRI can exhibit T2 hyperintensity, restricted diffusion, and apparent diffusion coefficient alterations, resembling stroke-like changes in regions including the cortex, basal ganglia, thalami, hippocampi, and corpus callosum. Leptomeningeal enhancement and crossed cerebellar diaschisis may also be observed. Some findings resolve with time, but persistent hippocampal sclerosis and focal atrophy suggest lasting neuronal injury [174].

Animal models have significantly contributed to understanding SE's pathophysiology, primarily through convulsive SE induction using chemoconvulsants or electrical stimulation. However, models of NCSE are limited. The exact reasons behind some seizures transitioning into SE while others resolve spontaneously remain unclear. Failure of seizure termination in SE can result from failed inhibition or persistent excitation. Specifically, GABAA receptor-mediated inhibition plays a crucial role in SE. The reduced inhibition in SE correlates with modifications in GABAA receptor populations. These changes may involve receptor trafficking and post-translational modifications. Alterations in surface expression of AMPA and NMDA receptors have also been documented in SE [151-153, 160, 161, 175].

Furthermore, chloride transporters and channels, such as A-type potassium (K^+) and Hyperpolarization-activated cyclic nucleotide-gated channels, contribute to altering neuronal excitability during SE. Glutamate, acting through NMDA and AMPA receptors, plays a significant role in SE development and maintenance. A reduction in surface expression of the GluA2 subunit of AMPA receptors occurs during SE, exacerbating the reduction in inhibition. Additionally, chloride transporters can lead to chloride loading, resulting in a depolarizing response to GABA, contributing to hyperexcitability and epileptiform discharges [48, 170, 172]. In summary, the pathophysiology of SE is characterized by a complex interplay of rapid cellular changes, neuronal injury, genetic and epigenetic factors, and alterations in neurotransmitter receptors. These multifaceted mechanisms provide valuable insights into the understanding of SE, and they emphasize the critical need for effective treatments and interventions in this neurological emergency.

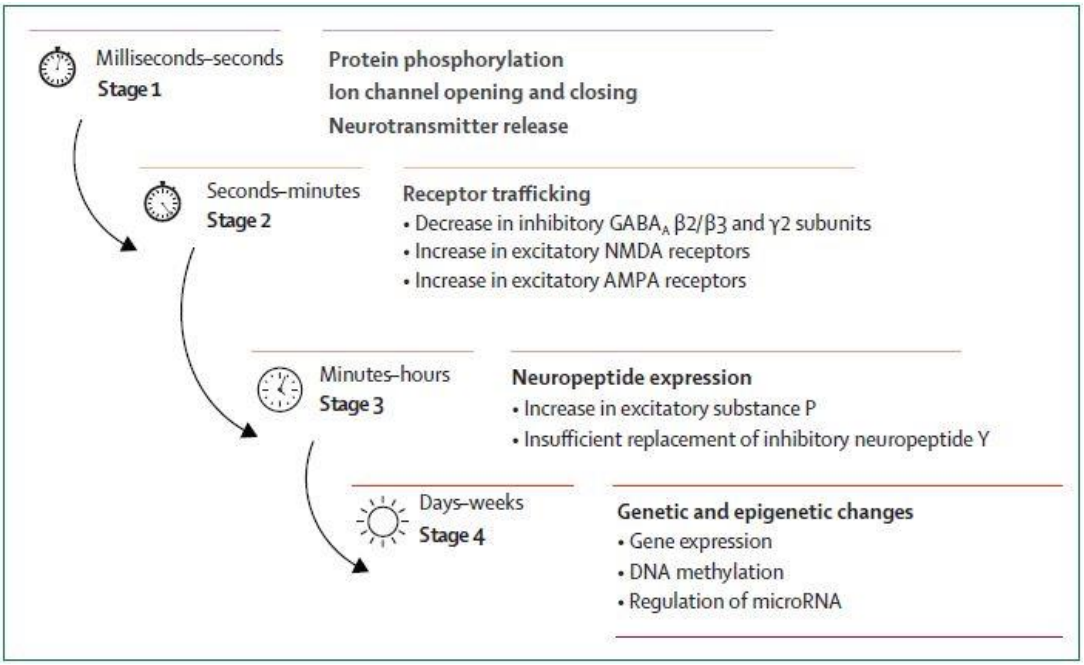

Figure 7: Cascade of selected mechanisms involved in the transition of a single seizure to status epilepticus

6. Animal models of mesial temporal lobe epilepsy

In light of the limited accessibility to human brain samples from surgical resections, researchers have long sought to replicate TLE in animal models. These models aim to faithfully replicate the electroencephalographic, behavioral, and neuropathological characteristics characteristic of this epileptic disorder, a pursuit that has spanned approximately four decades [176]. Classic models have been established through the utilization of chemoconvulsants, either administered locally or systemically, or the application of electrical stimuli, either subthreshold or suprathreshold, primarily in rodent subjects. Notably, two widely used chemoconvulsants are kainic acid (KA) and pilocarpine. These procedures induce an initial brain injury, leading to SE, followed by a latent period and the recurrence of spontaneous seizures originating from the temporal lobe. These models have found widespread utility in scientific investigations due to their remarkable similarity with the human disease. KA, an agonist of kainate receptors and AMPA receptors, can induce TLE-like pathology when administered via local stereotaxic injection or intraperitoneally. This leads to marked neuronal depolarization, hyperactivation of hippocampal circuitry, neurotoxicity, cell death in CA1 and CA3 areas, and mossy fiber sprouting. Furthermore, KA injection emulates a chronic acquired model of epilepsy, characterized by a latent interictal period preceding the establishment of TLE.

However, it is worth noting that this model exhibits high variability in the number and type of seizures developed by animals and is associated with a relatively high mortality rate [177].

Another prevalent chemoconvulsant, the M1 muscarinic agonist pilocarpine, triggers SE in the limbic system by promoting excessive glutamate release and recurrent activation of NMDA receptors [90]. Pilocarpine can be also used together with lithium, which activates the inflammatory response and damages the blood brain barrier. As with the KA model, the pilocarpine model exhibits variability and mortality rates. Despite the insights gained from chemical models, they bear the limitation that acute effects observed may not be direct consequences of epilepsy but rather side effects of the chemoconvulsant [178]. Therefore, electrical stimulation models have been instrumental in elucidating the molecular and pathophysiological mechanisms of TLE. Electrical stimulation is typically applied to ventral hippocampal or peri-hippocampal areas (such as the perforant path) or the amygdala. These models can be categorized into kindling models or post-epilepticus models. The kindling model involves a two-step electrical stimulation process, where the first day determines an after discharge threshold and habituates the rat to the procedure. Subsequently, on the second day, repeated sub-threshold electrical stimulation is administered until the animal reaches Racine score 5 several times. While this model replicates some aspects of TLE pathophysiology, spontaneous seizures do not consistently occur, and the extent of damage is relatively limited [179]. The perforant path model represents another prominent model of TLE. It consists of a one-step procedure involving repeated electrical stimulations over 2-3 hours, resulting in self-sustained status epilepticus. Mechanistically, this model involves hyper-activation of granule cells, which leads to damage in principal neurons across different hippocampal regions (CA3 and CA1), glial swelling, and neuronal loss of hilar interneurons. This, in turn, affects feedback inhibition and is associated with marked memory decline in behavioral tests. The PP stimulation model proves particularly useful for testing anti-epileptic drugs [180, 181].

Lastly, transgenic mice and rats with de novo or targeted genetic mutations exhibit varying susceptibility to seizure generation. For instance, certain rat strains are sensitive to audiogenic seizures, such as those induced by ethanol withdrawal. Additionally, stargazin mice experience seizures triggered by strong acoustic stimuli. In these cases, the initiation of seizures may not necessarily originate in the hippocampus but still involves this region in the generation of seizures [182].

6.1. The kainic acid (KA) model

KA was one of the first compounds used to model TLE in rodents [183]. The KA animal model offers a comprehensive platform for investigating the pathophysiology of SE and the subsequent development of epilepsy in rodents. It closely mirrors key aspects of the human condition, including neuronal degeneration and the progression from acute to chronic seizures, making it an invaluable resource for epilepsy research. This model is employed by administering KA through various routes, including systemic delivery via intraperitoneal, intravenous (i.v), and subcutaneous (s.c.) injections, as well as focal administration into specific brain regions such as the ventricle, amygdala, and hippocampus. It is noteworthy that focal KA administration typically results in more localized brain lesions compared to systemic injection, and is associated with lower mortality [184]. KA, a cyclic analog of L-glutamate and an agonist of the ionotropic KA receptors (KARs), was first shown by Nadler et al. (1978) that hippocampal pyramidal neurons are highly sensitive to damage induced by KA [185]. However, the foundation of using KA as an epilepsy model was established by Ben-Ari and colleagues [186, 187]. Their groundbreaking work involved unilateral intra-amygdaloid injections of KA in un-anaesthetized non-paralyzed rats. This procedure led to the development of focal seizures that progressively evolved into SE as the dosage increased. Histological examinations revealed significant neuronal degeneration and gliosis, predominantly in the CA3 region of the hippocampus, closely resembling pathological features observed in some epilepsy patients. This model not only replicates the acute phase of SE but also demonstrates the induction of spontaneous seizures in the days following the initial SE. This feature makes it a valuable tool for studying the transition from acute SE to chronic epilepsy, mirroring the clinical course observed in humans [188].

6.1.1. KA receptors (KARs)

Extensive biomolecular research has provided insights into the localization of KARs in the mammalian brain [189-193]. KARs, belonging to the ionotropic GluR family along with AMPA and NMDA receptors, are distributed across various brain regions, including the entorhinal cortex, cerebellum, amygdala, basal ganglia, and notably, the hippocampus [193]. KARs exhibit diverse functionality as both presynaptic and postsynaptic receptors. Presynaptic KARs act bidirectionally, displaying excitatory action through ionotropic activity and inhibition via a "non-canonical" metabotropic signaling pathway [194]. Postsynaptic KARs contribute to excitatory

neurotransmission [195] and modulate GABAergic neurotransmission, influencing both presynaptic and postsynaptic processes [196].

Five known subunits make up the KAR family: GluR5 (GluK1), GluR6 (GluK2), GluR7 (GluK3), KA1 (GluK4), and KA2 (GluK5). These subunits exhibit varying expression patterns in the hippocampus, with GluK4 predominantly found in the CA3 hippocampal field [197, 198]. GluK5 subunits are expressed in both CA1 and CA3 regions, as well as in other brain areas like the cortex and striatum [199]. Due to their high affinity for KA, GluK4 and GluK5 subunits may contribute to excitotoxic damage patterns and neuronal cell death in the CA3 region [200]. GluK1 subunits are primarily located in the CA3 field of the hippocampus, while GluK2 subunits are highly expressed in both CA1 and CA3 regions [193]. Despite their lower affinity for KA, GluK1 knock-out mice exhibit increased susceptibility to KA-induced epileptogenic effects, whereas GluK2 ablation prevents epileptiform discharges [201]. Overexpression of GluK2 leads to seizures and hyperexcitability [202]. GluR7 subunits, with the lowest affinity to glutamate, are downregulated by long-term KA-induced seizures [203]. KAR subunits' distinct roles in acute seizure induction and chronic epilepsy development underscore their complexity in epileptogenesis. GluK1 subunits may contribute to acute seizures, while GluK2 and GluK5 subunits, present in newly formed KARs within the mossy fiber network, likely play a key role in chronic epilepsy development [204, 205]. Increased GluK4 expression has been observed in patients with refractory TLE, and GluK4 knock-out mice exhibit neuroprotection in the CA3 hippocampal area following KA administration [206].

Exploring which KAR subtypes in specific cell types and brain regions primarily trigger epileptogenesis remains an intriguing avenue for future research. Recent advancements in photoswitchable regulators of ligand-gated channels offer the potential for precise control and investigation of specific KARs in a cell type and region-dependent manner [207].

6.1.2. Mechanism of action

KA induces excitotoxicity, a process in which neurons suffer severe damage, ultimately leading to cell death due to overstimulation by excitatory neurotransmitters, particularly glutamate [208]. This process involves a complex cascade of molecular interactions resulting in osmotic imbalance, excessive depolarization, and, ultimately, the rupture of postsynaptic membranes [209].

A central mechanism in KA-induced excitotoxicity involves the intracellular accumulation of calcium ions (Ca^{2+}) following the excessive activation of glutamate receptors. Elevated levels of Ca^{2+} can

have a profound impact on cellular organelles such as mitochondria and the endoplasmic reticulum [210]. Eliminating intracellular Ca^{2+} or preventing its influx into mitochondria can reduce cellular sensitivity to apoptotic stimuli [211]. Sodium (Na^{+}) and chloride ions (Cl^{-}) also play roles in this process, and their removal from the extracellular space can halt neurodegeneration. Furthermore, extracellular potassium ions are involved in KA-induced excitotoxicity [212]. Oxidative stress is another central player in cell death during excitotoxic damage. The excessive glutamate release initiates the formation of reactive oxygen species (ROS), leading to mitochondrial dysfunction and molecular damage [213, 214]. KA injection results in high levels of ROS [215, 216], a phenomenon also observed in brain tissue from TLE patients [217].

While oxidative stress is often considered the primary mechanism of cell death in TLE, some authors argue that apoptotic cell death also contributes to the brain damage induced by SE or may even predominate. SE-induced gene changes can lead to apoptotic neuronal death, possibly linked to the excitotoxic component of damage. Oxidative stress in mitochondrial membranes can activate apoptotic-inducing factor, which translocates into the nuclei and initiates DNA fragmentation [218]. Therefore, it is plausible that excessive glutamate release triggered by KA activation leads to excitotoxic cellular damage, activating apoptotic factors and resulting in both apoptotic and excitotoxic cell death. However, this aspect is still a subject of debate.

The precise mechanisms underlying KA-induced SE remain incompletely understood and may vary among different species, strains, and experimental conditions. Various factors, including SE induction methods (e.g., pilocarpine, traumatic brain injury, KA), species, strain, gender, and age, can lead to different phenotypic outcomes. The state of brain circuits at the moment of SE induction is likely a crucial determinant of the subsequent phenotype. While full comprehension of these mechanisms remains a challenge, it is essential for developing epilepsy models with specific desired traits. Significant research efforts are required to achieve this level of understanding. Currently, it is suggested that the focus should be on studying the phenotypic traits, as they may be of greater importance than the specific mechanisms of epileptogenesis [219].

6.1.3. Neuropathological changes

Systemic administration of KA leads to extensive neuronal damage primarily in the hippocampal and parahippocampal structures. Within 48 hours of KA injection, especially in animals experiencing robust convulsions during SE, there is a notable loss of pyramidal cells in several hippocampal

regions, including CA1, CA3, and CA4 [220-226], but parvalbumin (PV)-positiveinterneurons are also highly sensitive to KA, since they degenerate in the CA1 region, the entorhinal cortex and the subiculum [227, 228]. In animals surviving beyond the initial 48-hour period post-status epilepticus, additional patterns of neuronal damage become evident. These include neuron loss in the hilus and cell layer dispersion in the DG, mossy fiber sprouting, and shrinkage of nerve cells in various brain regions such as the piriform and entorhinal cortices, olfactory bulb, substantia nigra, thalamus, and mesencephalon [220, 229-231]. Interestingly, in the stratum oriens-alveus of CA1, KA-induced damage to interneurons can occur independently of pyramidal cell damage, particularly in young animals [232, 233]. Moreover, in the amygdala, KA leads to a significant reduction in the density of GABAergic interneurons, with somatostatin-expressing interneurons being primarily affected [234, 235]. Systemic injections of KA also lead to bilateral neuronal loss and affect extra-temporal regions. Within a 24-hour period after SE, neurodegeneration occurs in layer III of the entorhinal cortex, proximal subiculum, claustrum, thalamus, caudate putamen, and the cerebral cortex [223]. Animals that survive beyond 48 hours after SE exhibit bilateral gliosis, brain edema, or nerve cell shrinkage in the piriform and entorhinal cortices, olfactory bulb, substantia nigra, thalamus, and mesencephalon. Furthermore, cell layer dispersion is observed in the DG [220]. It is worth noting that these neuropathological changes are more prominent in animals that experience robust convulsions during SE, indicating that damage in extra-hippocampal regions may be linked to the propagation of epileptiform activity.

It is important to note that the extent and specific patterns of KA-induced neuronal damage may vary depending on factors such as the strain of animals used in experiments [221]. This strain-dependent variability underscores the importance of considering genetic and physiological differences in research involving KA-induced neuronal damage.

6.1.4. Response to anti-epileptic drugs

To establish valid animal models of TLE, it is essential that the initial SE induced by chemoconvulsants is followed by the development of spontaneous seizures, which are often resistant to pharmacological treatment. Some research has focused on the effects of anti-epileptic drugs in animals treated with KA and pilocarpine [236].

In the KA model, certain anti-epileptic drugs have demonstrated effectiveness when administered after chemoconvulsant exposure. For example, a single i.p administration of topiramate or

carbamazepine has been shown to reduce the frequency of spontaneous seizures, although the anti-ictogenic effect is short-lived [237, 238]. Conversely, daily oral administration of carbamazepine appears to be more effective, providing a long-lasting effect that may even completely prevent seizure occurrence [238, 239]. However, it remains unclear whether non-convulsive seizures are also blocked by these treatments, as no studies have been conducted on animals treated with KA, implanted with depth electrodes, and administered anti-epileptic drugs for multiple consecutive days. Additionally, in cases where treatment was discontinued, convulsive seizures tended to reoccur, suggesting that carbamazepine may primarily have an anti-convulsive effect.

6.2. Pilocarpine model

This isomorphic model, with its striking similarity to the human disease, has been widely adopted in research laboratories since its introduction a quarter of a century ago [240]. The histopathological findings in the pilocarpine model and its utility in evaluating the effectiveness of ASMs have been the subject of recent reviews [230, 241]. However, it is worth noting that systemic pilocarpine injection-induced SE leads to significantly more extensive brain damage than what is typically observed in human mTLE. This increased damage seems to be dose- and duration-dependent [242]. Injection of pilocarpine is most commonly administered systemically and is associated with several distinct phases: I. Induction of acute SE, characterized by tonic–clonic generalized seizures. II. A seizure-free interval known as the latent period, which precedes the emergence of spontaneous recurrent seizures (SRSs), marking the onset of the chronic epileptic phase [188, 243]. III. Development of neuropathological features, including mossy fiber sprouting, interneuron loss, and granule cell dispersion in the DG, mirroring the pathophysiological changes seen in patients with TLE. IV. A diminished response to ASMs akin to what is observed in individuals with TLE [244].

6.2.1. Pilocarpine and its receptors

Pilocarpine hydrochloride is a cholinergic agonist that is mainly known to be a hygroscopic, odorless, white crystal or powder that is soluble in water and alcohol. Pilocarpine's potential to induce SE appears to be contingent on the activation of the M1 muscarinic receptor subtype. This notion is substantiated by findings in M1 receptor knockout mice, which do not experience seizures when exposed to pilocarpine [245]. Other cholinomimetics, such as carbachol and oxotremorine,

also have the ability to induce seizures and provoke brain damage when administered either systemically or directly into the brain [240, 246]. Importantly, pilocarpine-induced SE can be blocked by the systemic administration of the muscarinic antagonist atropine [247]. However, once seizures are initiated, atropine loses its effectiveness [247]. Experiments conducted on cultured hippocampal neurons have revealed that pilocarpine, acting through muscarinic receptors, disrupts the balance between excitatory and inhibitory neurotransmission, ultimately leading to the generation of SE [248]. Moreover, in vivo microdialysis studies have shown that pilocarpine results in increased glutamate levels in the hippocampus following the onset of seizures [249]. A growing body of evidence suggests that, after initiation by M1 receptors, the maintenance of seizures involves the activation of NMDA receptors [249, 250].

6.2.2. Systemically injected pilocarpine

IP injection of pilocarpine induces SE followed by brain damage and the development of SRSs [90, 188]. The behavioral manifestations of pilocarpine administration are dose-dependent, progressing from immobility to gustatory and olfactory automatisms, and eventually culminating in convulsive seizures [90, 240, 251]. The extent of neuropathological changes is influenced by the duration and dose of SE, resulting in neuronal necrosis in various brain regions. Brain damage can range from the piriform cortex and anterior olfactory nuclei with lower doses (100 mg/kg) to more extensive damage in the amygdala, cortical and basal nuclei, as well as limbic-motor seizures with higher doses (200 mg/kg). The most significant neuropathological changes occur with an injection of 400 mg/kg [90].

After pilocarpine-induced SE, a silent or quiescent phase follows before the appearance of SRSs, lasting from a few days to several weeks in rodents [252]. During this phase, animals exhibit normal behavior [253, 254], but various pathophysiological changes linked to epileptogenesis occur, including mossy fiber sprouting, interneuron loss, synaptic circuit rewiring, glial cell activation, and ectopic cell proliferation. The duration of the preceding convulsive SE influences the development of interictal and ictal activity, and structural alterations in interictal discharges occur after the emergence of SRSs [255].

SRSs in pilocarpine-treated rats can be classified as partial seizures (1–3) and focal to bilateral tonic-clonic seizures (4–6) [256]. Partial seizures appear approximately seven days after SE, evolving into generalized seizures over subsequent days [256]. In animals with SRSs, damage is observed in the

lateral thalamic nucleus, substantia nigra, and dentate hilus [257]. Cell loss is reported in the subiculum [258, 259], amygdala [240], and layer III of the medial entorhinal cortex [260]. These areas also exhibit signs of atrophy, dendritic sprouting, disrupted laminar organization, reduced neuronal density, and reactive gliosis [261, 262]. Network reorganization is likely a consequence of neuronal loss and SE-induced sprouting [90, 263].

Local administration of pilocarpine, whether delivered intracerebroventricularly or directly into the hippocampus, has been utilized in studies examining seizure-induced alterations in amino acid levels and the effectiveness of certain anti-epileptic agents [264-268]. However, it is important to note that none of these studies conducted a comprehensive analysis of the model encompassing behavior, electrophysiology, and morphology. In contrast, a more recent study by Furtado et al. [269] demonstrated that intrahippocampal pilocarpine injection (2.4 mg/ml; injected volume 1.0 ml) induces SE with minimal mortality. This model also exhibited features such as Timm-positive mossy fiber sprouting and SRSs, with a comparable seizure frequency to that observed in systemically injected animals [269].

6.2.3. Response to antiseizure medications

ASMs for the treatment of SE have primarily been evaluated using the pilocarpine model. In pretreatment studies, diazepam, administered at doses of 5 or 10 mg/kg, has shown efficacy in preventing the development of behavioral and EEG abnormalities induced by pilocarpine. It also mitigates subsequent neuropathological changes in both lithium-pretreated and high-dose pilocarpine-treated rats [270, 271] and mice [251]. An extensive investigation of the effects of various classes of drugs on seizure onset was conducted by Morrisett et al. [271]. This study revealed that phenobarbital (32.5 mg/kg), carbamazepine (100 mg/kg), and paraldehyde (0.3 mg/kg) could effectively prevent SE when administered 15 minutes prior to pilocarpine administration. Phenytoin (200 mg/kg) prevented SE in two out of three rats and extended the latency to seizure initiation in the remaining rat. Sodium valproate (300 mg/kg) increased the latency to seizure onset but did not prevent SE [271].

In post-treatments studies, Morrisett et al. [271] conducted studies showing that diazepam (20 mg/kg), phenobarbital (32.5 mg/kg), phenytoin (100 mg/kg), valproate (300 mg/kg), and carbamazepine (100 mg/kg) were ineffective in halting SE in lithium-pilocarpine-treated rats. In

contrast, paraldehyde (0.3 mg/kg, intramuscular) exhibited efficacy, a finding corroborated by subsequent research [272].

Furthermore, Jones et al. [159] suggested that diazepam becomes ineffective when administered 15 minutes after the initiation of SE, possibly due to the development of pharmacoresistance. However, they found that co-administering diazepam (10 mg/kg) with pentobarbital (30 mg/kg; [273]) or phenytoin (60 mg/kg; [274]) could terminate both motor and electrographic seizures in pilocarpine-treated rats. Nonetheless, in animals with SE durations longer than 1 hour, recovery periods increased, and these animals eventually developed SRSs and morphological changes characteristic of the chronic model [273]. Interestingly, Goffin et al. [256] reported that diazepam (20 mg/kg) administered after 2 hours of SE was capable of terminating electrographic seizures within 3-4 hours.

It appears that that diazepam appears to be less effective in terminating motor seizures compared to other drugs, such as paraldehyde, or combinations of diazepam with barbiturates. Nonetheless, a single diazepam injection (20 mg/kg, i.p.) at different time intervals following SE onset led to significant differences in mortality and SRSs, with outcomes dependent on the duration of SE. In cases where diazepam was not administered, SE spontaneously remitted within a few hours after pilocarpine treatment [273].

After the administration of pilocarpine, epileptic rats were either treated by levetiracetam [275] or phenobarbital) [276]. levetiracetam was administered continuously via subcutaneously implanted osmotic minipumps in female Wistar rats [277], and it was reported that 38% of tested rats were responders, with complete or almost complete seizure control, another 38% were non-responders, while the remaining 24% of rats could not clearly be included in either group [275]. In the study with phenobarbital, the 50% of Sprague-Dawley female epileptic rats were non-responders, while another 50% of the animals displayed decreased seizure frequency and severity [276]. Although responders and non-responders did not differ in drug seizure frequency, drug plasma levels, or hippocampal neurodegeneration, behavioral differences were observed in anxiety models [278].

6.2.4. Limitations

Pilocarpine-induced seizures are highly persistent and prolonged, leading to extensive neuropathological damage that exceeds the damage observed in human mesial temporal lobe sclerosis. Compared to the KA model, the pilocarpine model exhibits higher mortality rates, with

approximately 30–40% of treated animals not surviving SE [90]. Both KA and pilocarpine are associated with neuronal damage, initially affecting the hippocampus and subsequently spreading to extrahippocampal and extra-temporal networks. However, the main difference between these two models is the intensity and timing of neuronal lesions. Systemic pilocarpine injections lead to significant and rapid neuronal loss in as little as 3 hours after SE, while KA-induced damage in the same regions takes around 8 hours to become evident [230]. These distinctions impact studies concerning the development of neuropathological changes following SE and during the latent period, and may also explain the higher mortality rate associated with pilocarpine.

The pilocarpine or Lithium-pilocarpine model has become widely adopted, especially during periods of limited availability of KA, which had previously been the preferred choice for establishing models of temporal lobe seizures with SE, resulting in spontaneous seizures and brain damage. The use of high pilocarpine doses for acute seizure induction is further complicated by the necessity for peripheral cholinergic antagonists. However, the cost-effectiveness of pilocarpine, in contrast to KA, provides a significant advantage in experimental research [279].

7. Epileptogenesis

In a mechanistic context, epileptogenesis is the process through which a previously normal brain network becomes functionally altered, leading to increased seizure susceptibility and an enhanced probability of generating SRSs [280]. Traditionally, epileptogenesis was associated with the "latent period," the time interval between the epileptogenic insult and the first clinical seizure. However, numerous studies have shown that the frequency and severity of SRSs continue to increase after the first spontaneous seizure [281-285], indicating that epileptogenesis is an ongoing and prolonged process. Various forms of molecular and cellular plasticity, leading to the occurrence of the first unprovoked seizure, persist beyond the initial seizure(s) and contribute to the progression of epilepsy [286-289]. Clinical studies have also suggested that human TLE, in particular, is progressive [290, 291]. With these new insights, ILAE Working Group revised the terminology related to disease modification, including epileptogenesis, with significant implications for experimental epilepsy research, treatment development, and biomarker identification [292, 293].

According to the updated terminology, epileptogenesis now refers to the development and extension of tissue capable of generating SRSs, leading to (1) the development of an epileptic

condition or (2) the progression of epilepsy after it is established. Importantly, the term "epileptogenesis" no longer pertains solely to the time between the epileptogenic insult and the diagnosis of epilepsy (Figure 8A); it now encompasses the mechanisms of progression that can continue even after the diagnosis of epilepsy (Figure 8B). Epileptogenesis often accompanies comorbidities arising from overlapping networks [294] and/or the effects of SRSs. Thus, disease or syndrome modification consists of two components: antiepileptogenesis and comorbidity modification. antiepileptogenesis refers to a process that counteracts the effects of epileptogenesis, which includes prevention, seizure modification, and cure. Prevention can be either complete, aborting the development of epilepsy, or partial, delaying the development or reducing its severity. antiepileptogenesis may also prevent or reduce the progression of epilepsy after its establishment. Cure implies a complete and permanent reversal of epilepsy, with no seizures occurring after treatment withdrawal. Antiepileptogenic treatment can be administered either before or after epilepsy onset, and it aims to prevent or delay the development of epilepsy. This differs from insult modification, where treatment before epilepsy onset modifies the insult itself. Regardless of the timing of treatment, if SRSs persist, they may become less frequent, shorter, milder, or more responsive to pharmacotherapy, and progression may be reduced. When administered after the diagnosis of epilepsy, such a treatment is clearly antiepileptogenic rather than insult modifying. Comorbidity-modifying treatment alleviates or reverses the symptomatic development or progression of epilepsy-related comorbidities, such as anxiety, depression, somatomotor impairment, or cognitive decline [292, 293]. Both antiepileptogenic and comorbidity-modifying treatments can also alleviate or reverse associated pathology.

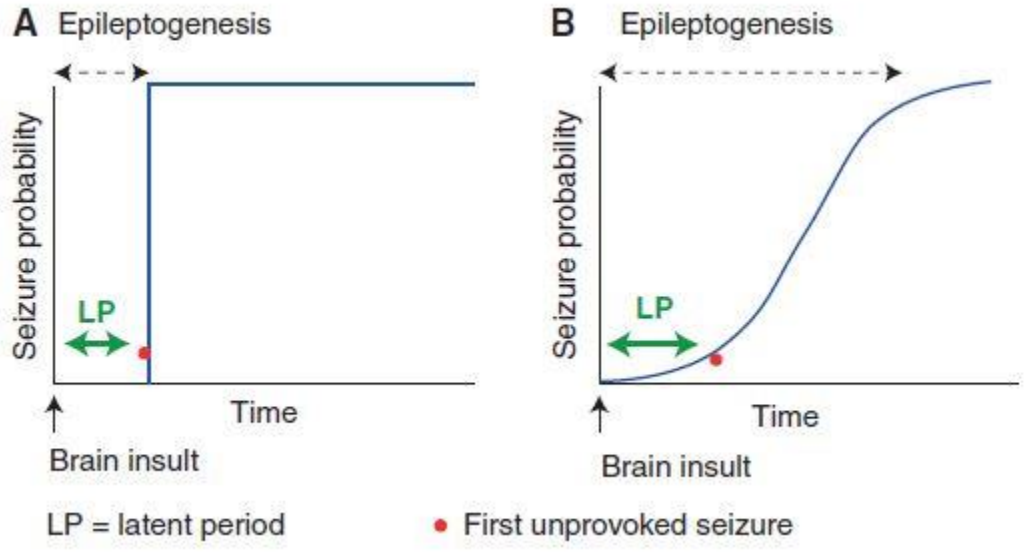

Figure 8. Definitions of epileptogenesis. (A) In the traditional definition, epileptogenesis referred to the latent period, which represented the time interval between the initial provoking insult and the onset of the first unprovoked clinical seizure. According to this view, the development of acquired epilepsy was considered a step function of time. (B) In a more recent perspective influenced by various experimental and clinical findings, the concept of epileptogenesis has evolved. It still encompasses the latent period, defined as the time between the precipitating injury and the first clinical seizure. However, this updated understanding recognizes that subconvulsive seizures may occur before the first clinical seizure, and that seizure frequency and severity progressively increase over time. These observations suggest that epileptogenesis can be an ongoing process, extending indefinitely [284, 285].

7.1. Neuroinflammation in epileptogenesis

Inflammatory processes are not confined to the chronic epileptic brain; they also become upregulated following an epileptogenic injury, and often persist during the latent phase preceding SRSs. This observation has led to the hypothesis that brain inflammation, beyond its established role in ictogenesis, may also contribute to the development of the epileptogenic process. Research regarding the involvement of brain inflammation in epileptogenesis is still in its early stages, but there is pharmacological evidence to support this notion [295]. In both animal and human studies, it has become increasingly evident that neuroinflammation plays a significant role in epilepsy and its associated comorbidities [296, 297]. Neuroinflammation, stemming from factors like brain damage or systemic inflammation, disrupts cellular communication within the CNS, affecting both neurons and glial cells. This imbalance in signaling molecules and increased inflammatory substances leads

to neuronal hyperexcitability, rendering the brain more susceptible to seizures and the processes of ictogenesis and epileptogenesis [296, 298]. Various inflammatory mediators have been under investigation in the context of epileptogenesis. Toll-like receptors (TLRs), part of the innate immune system, have been recognized as pivotal players in neuroinflammation [296]. Mounting evidence suggests that TLR4 activation can provoke seizures. High-mobility group box 1 (HMGB1), a DNA-binding protein, acts as a danger signal by engaging TLR4 and the receptor for advanced glycation end-products. TLR4 is the primary receptor for HMGB1, and its activation, mediated by HMGB1, leads to seizures through $Ca2^{+}$ influx resulting from the phosphorylation of the NR2B subunit of the NMDA receptor. HMGB1 is also capable of compromising the blood-brain barrier (BBB) [296, 299-301].

Under normal physiological conditions, HMGB1 is present at low levels in the brain. However, in pathological and inflammatory states, damaged cells can release HMGB1 as a damage-associated molecular pattern [302]. It has been demonstrated that HMGB1 secretion can occur in response to an inflammatory environment, hypoxia, or seizures and is triggered by an increase in ATP in the brain [301]. Studies by Maroso et al. have revealed elevated expression of HMGB1 and TLR4 in hippocampal samples from patients with drug-resistant TLE compared to controls. Moreover, their research demonstrated that blocking the HMGB1-TLR4 axis using specific antagonists reduced the number of seizures induced by KA in mice [303]. In a different vein, Hosseinzadeh and colleagues found that pretreatment of rat models of TLE with TLR4 and TLR2 ligands reduced the severity of seizures [304]. Additionally, other TLRs, including TLR3 and TLR7, have been implicated in neuroinflammation during seizures [305, 306].

Apart from TLRs, the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling pathway is a critical player in the progression of neuroinflammation and the pathogenesis of epilepsy. Activation of the mammalian target of rapamycin (mTOR) pathway influences synaptic plasticity, alters the expression of ionic channels, and results in neural hyperexcitability and increased susceptibility to seizures [307, 308]. Furthermore, the mTOR signaling pathway can modulate neuroinflammation by impacting immune cells and altering the secretion of pro-inflammatory cytokines (PICs) within the CNS. While this represents one of the potential mechanisms through which mTOR contributes to epilepsy, further research is needed to fully elucidate the relationship between mTOR and neuroinflammation [307, 308]. Genetic disorders that lead to epilepsy often act by dysregulating the mTOR pathway. The tuberous sclerosis complex

(TSC), resulting from mutations in TSC1 or TSC2 genes encoding hamartin and tuberin proteins, respectively, can hyperactivate mTOR and lead to intractable seizures. Moreover, other genetic disorders such as fragile X syndrome, megalencephaly, polyhydramnios, and neurofibromatosis type 1 are associated with the development of epilepsy through disruption of the mTOR pathway. It has also been demonstrated that acquired epilepsy can result in hyperactivation of the mTOR pathway. Importantly, even in cases of TSC, not all individuals develop epilepsy, suggesting that additional factors contributing to epileptogenesis exist beyond genetic predisposition [307-309].

Due to the breakdown of the BBB and the release of chemoattractant molecules, peripheral innate and adaptive immune cells infiltrate the CNS. Subsequently, they secrete inflammatory factors, including PICs like interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), Interferon gamma, and IL-17 in the CNS. These studies have demonstrated the migration of various immune cell types, such as monocytes, granulocytes, T cells, and B cells, into the CNS. Recent research has also shown that peripheral dendritic cells may enter the brain during prolonged seizures, and they can contribute to maintaining the inflammatory state in the CNS by releasing inflammatory mediators. Additionally, PICs released into the peripheral blood and albumin can enter the brain through the compromised BBB [298, 301, 310-313]. Inflammation in the CNS and brain injuries, including those that induce epilepsy, trigger the release of ROS and reactive nitrogen species. Once induced, oxidative stress increases the brain's susceptibility to seizures and exacerbates the inflammatory state by stimulating the release of PICs, transforming growth factor-β (TGF-β), and prostaglandin E2 (PGE2) [314]. Furthermore, TGF-β and PGE2 can stimulate astrocytes and influence glutamate release, ultimately leading to neural hyperexcitability [298]. PICs play a crucial role in various neuroinflammatory mechanisms, serving as both triggers and consequences of epilepsy. PICs heighten the hyperexcitable state of the brain and increase its susceptibility to seizures. Conversely, seizures exacerbate the inflammatory state of the CNS, creating a cyclical relationship between neuroinflammation and epilepsy, ultimately perpetuating the brain's susceptibility to seizures [315, 316].

7.2. Biomarkers for epileptogenesis and antiepileptogenesis

At the molecular and cellular levels, epileptogenesis resulting from a structural cause involves a series of molecular and cellular changes set in motion by an initial brain-damaging insult. These

changes ultimately culminate in the occurrence of unprovoked seizures. These cellular alterations encompass neurodegeneration, neurogenesis, axonal sprouting, damage to axons and myelin, dendritic remodeling, various forms of gliosis, the invasion of inflammatory cells, BBB impairment, angiogenesis, modifications in extracellular matrix composition, the possible accumulation of materials like iron and Ca^{2+}, and the acquisition of channelopathies [100, 289]. This complex interplay of pathologies creates a "cellular and molecular ecosystem" that leads to seizures and can be influenced by treatments. The epileptogenic tissue milieu produces and releases molecules that can serve as biomarkers, detectable in various epilepsy contexts, including therapy trials, through methods such as blood or cerebrospinal fluid (CSF) analysis, examination of brain tissue (e.g., cortical or hippocampal tissue), or indirectly via imaging or electrophysiology. It is crucial to note that at each stage of the epileptogenic process and biomarker discovery, genetic factors, microbiota, and environmental exposures (exposome) can all play modulatory roles.

At the network level, epileptogenesis involves cellular loss and neurogenesis, leading to axonal sprouting and synaptic reorganization. These changes not only affect local circuitry and broader excitability but also predispose the brain to increased synchronization of abnormal neuronal discharges, such as burst firing. The subsequent development of a localized epileptogenic region and a distributed epileptogenic network can be assessed through the recording of specific electrophysiological patterns [317, 318] (e.g., interictal spikes, high-frequency oscillations, or sleep spindles), as well as through structural and functional MRI. Emerging technologies like optogenetics and designer receptors exclusively activated by designer drugs (DREADDs) offer further avenues for investigation.

Given the intricacy of the epileptogenic process, it is expected that a single biomarker alone may not be sufficient to robustly measure the functions required for designing a cost-effective clinical trial of an antiepileptogenic agent. Instead, a profile of biomarkers will likely be needed to enhance sensitivity and specificity in this context.

7.3. Role of glial cells in epileptogenesis

Growing evidence suggests that a breakdown in glial protective mechanisms can act as a precursor to the transformation of seizures (ictal transformation) and the subsequent generalization of a focal

discharge [319]. Glial cells, while often overlooked, play multifaceted roles in their dynamic interactions with neighboring neuronal populations:

1. Synaptic neurotransmission regulation: Glial cells actively contribute to synaptic neurotransmission. They express glial glutamate receptors and facilitate the uptake of excitatory neurotransmitters, such as glutamate, through specialized transporters like glutamate transporter-1 [320].

2. Electrical communication through gap junctions: Gap junctions form intercellular channels that link mature glial cells, allowing the spread of voltage changes over relatively long distances, thanks to the movement of ions like $Ca2^{+}$ and K^{+} [321]. Moreover, ATP, a critical signaling molecule, can diffuse between cells, enabling intricate neuron-glial interactions [322].

3. Glutamate release by glia: Glial cells possess the capability to release glutamate, an excitatory neurotransmitter. This raises the intriguing possibility that glial cells might contribute to neuronal hyperexcitability through glutamate release [323-326].

4. Active Potassium (K^{+}) Uptake: Glial cells play a central role in removing excessive potassium ions from the extracellular space, particularly after neuronal activation has led to a substantial increase in extracellular K^{+} concentrations. This active K^{+} uptake helps in restoring the balanced ionic environment necessary for proper neuronal function.

The regulation of the neuronal microenvironment is a complex process, involving the interplay of excitatory and inhibitory potentials. In the case of pyramidal neurons, excitatory potentials hinge on depolarizing ion conductances, which are primarily regulated by the voltage-dependent activation/inactivation properties of Na^{+} and Ca^{2+} channels. These depolarizing potentials are terminated through the activation of intrinsic K^{+} conductances, alongside the release of inhibitory neurotransmitters by interneurons, which generate inhibitory postsynaptic potentials (IPSPs). Elevated extracellular K^{+} has a direct depolarizing effect on neurons and their terminals, making them more excitable. Remarkably, minor increases in K^{+} can simulate synaptic changes usually associated with the direct stimulation of presynaptic fibers, a phenomenon known as long-term potentiation [327].

Glial cells could potentially play pivotal roles in the expression of epileptogenesis. Their contributions may differ among various types of focal epilepsies, including conditions like hippocampal sclerosis, cortical dysplasia, and epilepsies induced by factors like tumors or vascular malformations. These roles might involve increased connectivity between glial cells through gap

junctions, dysfunction in their K^+ buffering capacity, and impaired clearance of glutamate. Further extensive investigations are necessary to unravel the intricacies of these interactions and their specific implications in the context of epileptogenesis [328].

We obtained evidence that a significant increase of Iba1 associated with the microglia-activated morphology was found in both the CA3 lacunosum moleculare layer (LMol) ($p < .001$) and subiculum ($p < .001$) of KA-treated rats when compared to healthy controls ($p < .001$). Nevertheless, the microglia-activated morphology in the Sub was significantly attenuated in rats treated with multiple injections of trilostane ($p = .004$), in respect to vehicle-treated rats. However, the single or repeated administration of trilostane in rats did not significantly affect the damage in the examined brain regions (CA3 LMol and Subiculum) [329].

7.4. $GABA_A$ receptors and epileptogenesis

In the CNS, mature $GABA_A$ receptors are crucial for fast synaptic inhibition. When these receptors are activated, they open chloride channels, resulting in hyperpolarization of the neuronal membrane potential. In the intricate neural networks of the brain, inhibitory connections play an equally important role as excitatory synapses. In cortical structures, the mediation of inhibitory neurotransmission primarily involves the release of the GABA by different classes of interneurons onto specific post-synaptic structures. This means that inhibitory inputs are strategically located at axodendritic, axosomatic, and axoaxonic segments, contributing to the intricate balance of neuronal activity [330].

While most excitatory neurotransmission aims to establish connections between relatively distant brain regions, the majority of interneuronal networks are intricately woven into the local circuitry. When examining the morphological aspects of cells in neocortical and hippocampal structures, it becomes evident that the principal cells exhibit relatively repetitive appearances, whereas GABA-ergic interneurons display a diverse range of cellular morphologies and structures [330].

GABA, in addition to its role in modulating inhibition, can also act as a trophic factor. During early prenatal brain development, GABA may even function as an excitatory neurotransmitter, primarily due to the more negative resting membrane potential during this period compared to the average $GABA_A$ reversal potential. This difference in membrane potential leads to the depolarization of the

cell, subsequently activating voltage-gated calcium channels and increasing intracellular Ca^{2+} levels in immature neurons [331-336].

The depolarizing effects of GABA receptor activation have been observed in immature cells from various brain regions, such as the neocortex and hippocampus. This depolarizing activity is crucial in the activation of NMDA receptors by providing the necessary depolarization to alleviate the Mg^{2+} block of the NMDA channel. The presence of this synergistic effect during early and late postnatal periods may serve as a synaptic substrate for the neuronal hyperexcitability observed in early life and potentially contribute to the onset of epileptogenesis [331-333, 335]. Age-dependent predisposition to epilepsy is a well-documented phenomenon, often associated with pediatric neurological conditions that involve seizures. These early-life seizures may influence or determine the appearance of epileptic disorders later in life. The maturational changes in neurotransmitter function and channel activity should be taken into consideration when identifying the cellular mechanisms that initiate seizures. A growing body of evidence suggests that impaired GABA function can lead to seizures and may be implicated in various types of epilepsy. Altered $GABA_A$ receptor function, for instance, could contribute to both inherited and acquired epilepsies [337, 338].

Studies conducted on tissues resected from patients with mesial temporal lobe and neocortical epilepsies have revealed reductions in $GABA_A$ receptors. However, these findings are not entirely consistent with other studies of human hippocampi resected from patients with TLE. These later studies have shown that GABA neurons are relatively preserved, and their axons sprout into the supragranular layer, resulting in aberrant innervation of the fascia dentata. Moreover, the densities of postsynaptic $GABA_A$ receptor proteins are increased throughout the extent of the fascia dentata molecular layer when compared to normal controls [339-343].

This intricate interplay between excitatory and inhibitory neurotransmission and the role of GABA and $GABA_A$ receptors in different brain regions highlights the complexity of neural circuitry in the context of epilepsy and provides valuable insights into potential mechanisms underlying seizure initiation and epileptogenesis. Further research is needed to fully understand these complex processes and their contributions to epilepsy.

8. $GABA_A$ receptor function in the epileptic brain

In the early 1980s, it was widely assumed that the failure of $GABA_A$ receptor-mediated inhibition was a critical prerequisite for the generation of epileptic discharges, a view that was supported by the marked decrease in interneurons observed in some experimental models of epilepsy [344, 345]. However, subsequent studies, which employed histological and electrophysiological techniques to investigate the function of GABAergic inhibition in both humans and animal models, have challenged this notion. In this section, we will provide a concise overview of the evidence related to changes in inhibition and inhibitory cells in patients with TLE and in various animal models of epilepsy. Additionally, we will examine experimental findings that shed light on the role played by $GABA_A$ receptor-mediated conductances in the synchronization of neuronal networks, presumably contributing to the facilitation of ictogenesis in the epileptic brain.

8.1. GABAA receptor-mediated inhibition in TLE

TLE is a partial epilepsy disorder characterized by seizure discharges that involve various brain regions, including the hippocampus proper, extrahippocampal structures such as the entorhinal and perirhinal cortices, the amygdala, and the temporal neocortex. TLE patients often exhibit a distinct pattern of brain damage known as Ammon's horn sclerosis, or mesial temporal sclerosis (MTS). This pathological condition is typically characterized by neuronal loss in specific regions, including the hippocampal CA1/CA3 subfields, the dentate hilus, layer III of the medial entorhinal cortex, and the amygdala [346, 347]. Similar histopathological changes have been observed in laboratory animals subjected to various experimental procedures. These models involve the injection of convulsant drugs like pilocarpine or KA or repetitive electrical stimulation of limbic pathways [90, 188, 348, 349]. These procedures induce an initial SE, followed by a chronic condition of recurrent limbic seizures that typically emerges 1 to 2 weeks later.

Interestingly, a comparable latent period, during which patients remain seizure-free, can also be observed in individuals with TLE. This latency may be associated with an initial insult experienced during early childhood, such as birth trauma, complicated febrile convulsions, brain injury, or meningitis. In these cases, partial seizures may develop during adolescence or early adulthood. This suggests a complex interplay between early insults, latent periods, and the eventual onset of TLE.

In patients with TLE and in animal models designed to mimic this disorder, inhibitory mechanisms are notably altered [350-357]. However, these alterations are intricate and cannot be succinctly summarized as a uniform reduction in the number of GABAergic cells accompanied by a collapse of inhibition. For instance, electrophysiological studies performed pre-surgically using intracranial electrodes or extracellular unit recordings in TLE patients have identified periods of robust firing depression, believed to correspond to IPSPs [358, 359]. Furthermore, histochemical investigations have shown that certain subtypes of interneurons are preserved in both human and experimental animal epileptic tissue [339, 360, 361]. However, it's important to note that some other subtypes of interneurons exhibit a clear reduction in number, particularly in specific limbic structures [258, 362]. These findings underscore the complex and multifaceted nature of inhibitory alterations in TLE.

In addition to variations in the number of interneurons (GABAergic inhibitory cells) characterizing epileptic tissue, research suggests that diminished network inhibition in epileptic regions can be attributed to a decrease in the excitatory input to inhibitory interneurons. This mechanism has been observed in the hippocampus and entorhinal cortex layers II/III [363-368]. Studies conducted in rats treated with pilocarpine, a chemoconvulsant, have shown that pharmacologically isolated IPSPs mediated by GABAA receptors exhibit more positive reversal potentials in neurons recorded in vitro from epileptic subregions such as the subiculum, perirhinal cortex, and amygdala [258, 369, 370]. These findings have often correlated with decreased levels of mRNA expression and immunoreactivity of the neuron-specific cotransporter Potassium Chloride Cotransporter 2, which plays a crucial role in regulating intracellular chloride concentration. This suggests that an imbalance in intracellular chloride levels is associated with epileptogenesis [371]. Furthermore, research indicates that GABAergic inhibition is more labile in human epileptogenic tissue than in non-epileptogenic tissue, and this functional discrepancy appears to arise from an inherent deficiency in the endogenous phosphorylation of $GABA_A$ receptors [372].

The relationship between $GABA_A$ receptors and epileptic disorders becomes even more intricate when examining changes in inhibition within the kindling model of TLE. Notably, in the DG of the hippocampus, inhibition appears to be enhanced rather than diminished following kindling [373]. Moreover, research by Buhl et al. [374] has revealed that in this particular region of the hippocampus, the excitatory input to inhibitory interneurons is increased, concomitant with a reduction in presynaptic autoinhibition of GABA release in brain slices obtained from kindled rats.

Interestingly, these experiments indicate that the heightened inhibition observed in epileptic tissue is mitigated by the release of Zn^{2+} from mossy fiber terminals of granule cells that sprout abnormally during seizures. This suggests that Zn^{2+} has a modulating effect on inhibition in the context of epilepsy. Additionally, there is evidence of selective changes in receptor subunit expression in animal models of TLE [375-378]. These findings emphasize the intricate nature of $GABA_A$ receptor involvement in epileptic disorders.

8.2. Loss of interneurons in TLE

Numerous studies have consistently reported a loss of GABAergic interneurons in both experimental models [350, 352, 365, 379-381] and human cases [382-385] of TLE. This loss of inhibitory interneurons would theoretically result in a decreased number of inhibitory synapses on postsynaptic cells.

Ultrastructural investigations have shown that the number of GABAergic terminals targeting the cell bodies (perisomatic region) of CA1 pyramidal cells is not altered in animal models of TLE, both before and after the development of SRSs [386, 387]. Interestingly, in one of these models using KA, a loss of perisomatic terminals marked with PV was observed, while the number of these terminals around the initial segment of CA1 pyramidal cells remained unchanged [380]. These findings suggest a transient deafferentation of pyramidal cell somata by GABAergic terminals, followed by a re-establishment of synaptic connections, indicating a reactive synaptogenesis. This transient loss of inhibitory terminals may explain the temporary loss of paired pulse inhibition [388].Notably, axonal sprouting of GABAergic neurons has been observed in a different preparation [389], and in the hippocampus, this could occur in parallel with the well-documented recovery of excitatory terminals through axonal sprouting in TLE [382, 390-392]. It is important to note that the impact on GABAergic terminals along the dendrites of principal cells has not been quantified. Nevertheless, a significant reorganization is expected along dendrites due to the identified loss of somatostatin-containing interneurons in TLE [393]. In the CA1 area, most somatostatinergic interneurons are situated in the stratum oriens, and they constitute the most prevalent population of GABAergic interneurons [394, 395]. These interneurons form extensive axonal arbors that create symmetric synapses primarily on the distal dendrites of pyramidal cells [396], precisely where perforant path afferents from the lacunosum-moleculare synapse. The loss of somatostatinergic interneurons could have important functional consequences because somatostatin exhibits anticonvulsant

properties [397, 398], and its loss results in disinhibition of pyramidal cells [399-401]. These interneurons are strongly activated by excitatory inputs, receive robust excitation from pyramidal cells, and many of them fire spontaneously, providing robust and precise inhibitory control at the site of perforant path afferences. A reduction in their activity could lead to direct excitation of CA1 pyramidal cells by the temporoammonic pathway, resulting in disinhibition [401].

The loss of GABAergic interneurons logically leads to the disinhibition of principal cells. This hypothesis is supported by the decrease in paired-pulse inhibition, a measure thought to be related to the "strength" of inhibition, reported in vivo [388, 402]. However, as mentioned earlier, paired-pulse inhibition recovers 7 to 8 days after the initial lesion-induced SE, indicating that the surviving inhibitory pathways can still function. This raises questions about the fate of the remaining GABAergic interneurons in TLE [388].

9. Inflammation and glial cells in the epileptic brain

There is a growing body of evidence supporting a correlation between inflammation and epilepsy. Activated microglia, reactive astrocytes, local expression of PICs, BBB leakage, and peripheral immune cell infiltration have all been observed in human TLE as well as in animal models [403]. Consequently, inflammatory mechanisms are believed to play a central role in the initiation and maintenance of seizures, including those starting in the acute phase during SE induction. Despite marked gliosis and inflammation being key features of hippocampal sclerosis in TLE, the potential involvement of glial cells, particularly microglia, in promoting epileptogenesis has been inadequately explored. Given that the role of astrocytes in epileptogenesis has been recently reviewed [404-406], this discussion will concentrate on microglia. Microglia, the immune cells of the CNS, are characterized by ramified processes, actively surveying their environment. Upon CNS injury, microglia swiftly undergo activation, traditionally viewed as detrimental to neuronal survival. However, recent insights suggest that microglia exhibit neuroprotective properties in various CNS pathologies, contributing not only to inflammation but also to tissue remodeling, repair, and neurogenesis [407]. In the context of epilepsy, morphologically-activated microglia are promptly observed after SE and remain activated for several days thereafter (Figure 9), implying a significant role in the disease's development and maintenance. Microglia activation correlates with the expression of PICs such as IL-1β, IL-6, and TNF-α, believed to contribute to neuronal cell death

following SE [403, 408]. Cytokines like IL-1b and TNFa have demonstrated the ability to increase neuronal excitability in brain slices, suggesting their involvement in epileptic activity development [403, 408]. Additionally, elevated levels of chemokines, including chemokine (C-C motif) ligand 2 (CCL2), CCL3, and CCL4, along with chemokine receptors like chemokine (C-X-C motif) receptor 4, are observed in the hippocampus of both human epileptic patients and pilocarpine-treated animals. The chemokine system is presumed to be crucial in recruiting peripheral inflammatory cells [403, 408]. Minocycline has been shown to reduce microglia activation and subsequent seizure susceptibility in young animals [409]. Overall, the data indicate a pro-inflammatory phenotype of microglia preceding neuronal injury and cell death, leading to their general classification as playing a pro-epileptogenic role.

Recent publications have presented evidence suggesting a beneficial role of microglia in pathological brain conditions. Specifically, under demyelinating conditions, microglia produce and secrete anti-inflammatory cytokines and neurotrophic factors, such as IL-10, TGF-β, and brain-derived neurotrophic factor [410]. Microglia have also demonstrated neuroprotective effects in Alzheimer's disease and ischemia, promoting axonal regeneration and providing instructive signals for neurogenesis [411]. Studies have shown that microglia play a crucial role in neuronal protection against excitotoxicity [412]. Additionally, recent findings indicate that microglia express major histocompatibility complex in the absence of co-stimulatory signals, along with membrane markers of immature dendritic cells, suggesting their primary function is to inhibit lymphocytic inflammation and promote tolerance [413]. Evidence supporting a beneficial role of microglia in epilepsy exists. For instance, the induction of TLE in IL-6 knockout mice results in decreased microglia activation. However, this decrease is accompanied by an increase in oxidative stress, neuronal cell death, and the severity of seizures, suggesting that inhibiting microglia activation might worsen epilepsy development [414]. Moreover, specific depletion of hippocampal microglia did not alter acute seizure sensitivity compared to normal animals, indicating that microglia are not responsible for disease development. In the same study, preconditioning with lipopolysaccharide (LPS) before acute seizure induction led to increased seizure activity and higher mortality in the absence of microglia, suggesting that activated microglia may have a protective function during SE [415].

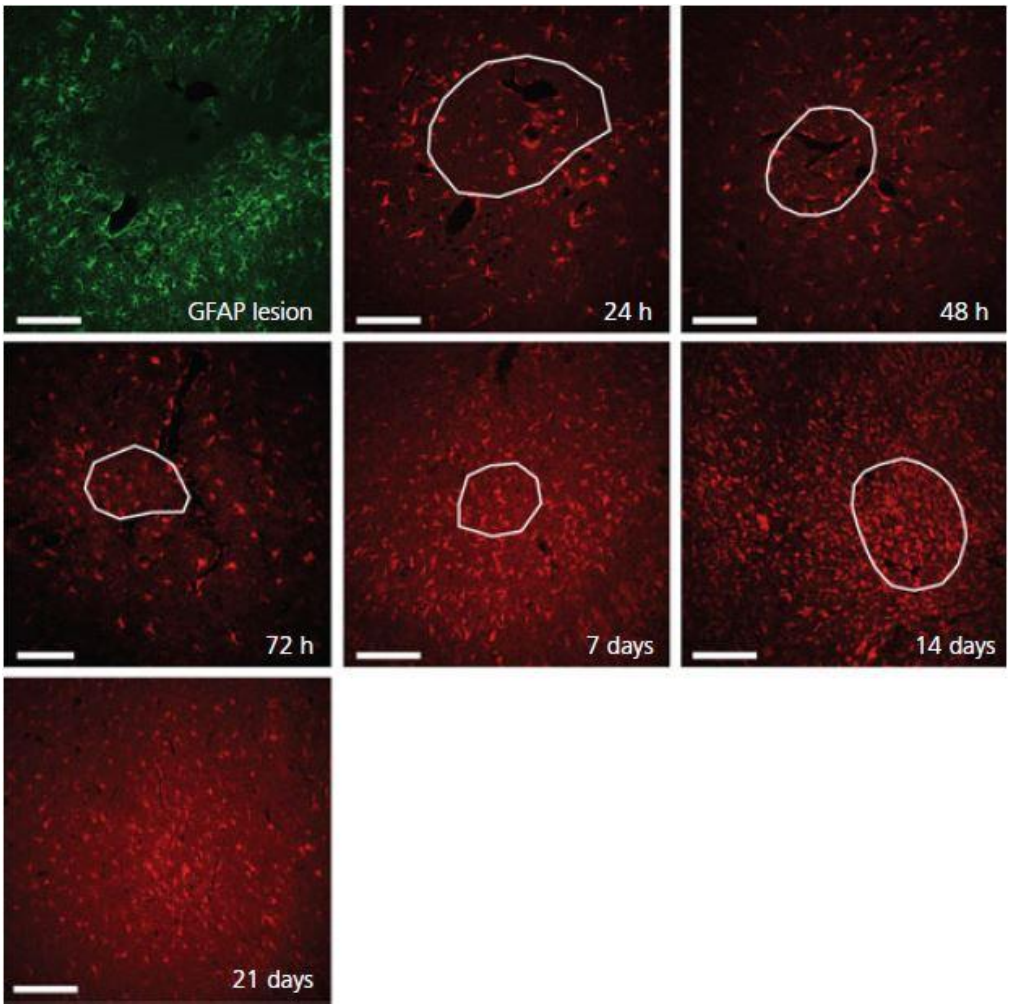

Figure 9. Time course of microglia activation following status epilepticus induced by pilocarpine [416]. Rats are the same described in Gualtieri et al. [417] (17). Microglia activation was followed at several time points during epileptogenesis, using an antibody against Iba-1 (43). During the first 72 h after SE, microglia activation remained circumscribed inside and around the astrocyte lesion, located in the CA3 stratum lacunosum molecolare (white boundaries), illustrated by an antibody against glial fibrillary acidic protein (GFAP). At 7 days after SE, microglia cells greatly increased and activation was clearly observed outside the lesion and even in the CA3 stratum pyramidalis, as also fully displayed 14 days after SE. Microglia activation still persisted at 21 days after SE, even though the lesion had disappeared. Scale bars = 50 µm.

10. Limbic systems

The limbic system primarily governs memory processing and emotional responses. Its key components include the hippocampus, which plays a crucial role in memory formation as part of the Papez circuit, and the amygdala, responsible for emotional responses, the formation of

emotional memories, and driving behaviors. Clinical manifestations of limbic system disorders commonly encompass epilepsy, states of confusion, and varying degrees of cognitive impairment [418, 419]. In cases of TLE, these two vital components, the hippocampus and amygdala, can be profoundly affected [420].

The hippocampus, located in the medial temporal lobe, plays a crucial role in various cognitive functions. Not only is it responsible for learning and memory, including the formation of new memories and the consolidation of previous ones, but it also contributes to spatial navigation [421, 422]. The hippocampal formation consists of several regions, including the CA, which is further subdivided into CA1, CA2, CA3, and CA4 (the latter also known as CA3c, with CA3a proximal to CA2). Additionally, it encompasses the DG along with its hilus, the subiculum, and the entorhinal cortex. These interconnected regions form a complex network essential for various aspects of cognition and memory processing.

The amygdala, located in the anterior temporal lobe, plays a vital role in processing emotions, particularly those related to fear and anxiety [423, 424]. This amygdaloid complex comprises various nuclei, divided into major groups:

1. Basolateral Amygdala (BLA): This group includes the lateral, basal, and accessory-basal nuclei of the amygdala.
2. Cortical-Like Nuclei Group.
3. Centromedial Nuclei Group [425].

The amygdala receives sensory input from both cortical and thalamic glutamatergic connections, providing information about the external environment. Additionally, it receives afferent projections from the hippocampus and brainstem [426]. The amygdaloid complex's nuclei are interconnected hierarchically, with multiple pathways converging at the central nucleus. This organizational structure allows sensory information to flow from the lateral nucleus of the amygdala through various nuclei within the amygdala and ultimately reach the central nucleus [424]. More specifically, sensory information can travel directly from the lateral nucleus to the central nucleus, or it can reach the central nucleus through the BLA. The BLA has glutamatergic projections to the sensory cortex, the nucleus accumbens, and the hippocampus [427, 428]. The central nucleus, in turn, plays a key role in generating emotional responses, such as immobilization or escape, by stimulating the brainstem or activating the hypothalamic nuclei and the bed nucleus of the stria terminalis.

10.1. Roles and interactions of specific limbic areas in seizures

The origin of limbic seizures can vary both between individual cases and within cases, whether in patients or experimental animals. Extensive experimental evidence, both in vitro and in vivo, and observations in human patients, indicate that several limbic structures can serve as the starting point for seizures. These structures include the hippocampus, entorhinal cortex, perirhinal cortex, and amygdala. In some instances, one of these structures may dominate as the seizure initiator, while in others, seizure onset can be multifocal. Furthermore, during the progression of individual seizures, neuronal activity spreads widely along synaptic pathways, often achieving high levels of synchronization between limbic and other regions in both brain hemispheres [429].

Early theories proposed that seizure propagation within "re-entrant loops" in the limbic system played a crucial role in sustaining and prolonging seizures beyond the initial few seconds of interictal discharges [430]. However, more recent and precise measurements of the activity cycles within each seizure have revealed near-zero mean phase lags between the various limbic structures [431, 432]. This suggests that these structures may behave more like coupled oscillators than traditional re-entrant loops. Supporting this perspective, studies on models, such as the intrahippocampal tetanus toxin model, have shown that the left and right hippocampi exhibit a near-zero phase lag, with the lead switching sides repeatedly during each seizure. On each side, the CA3 region consistently leads CA1, reflecting predominantly unidirectional transmission through the Shaffer collaterals [433]. These long-range networks appear to be crucial for sustaining seizures in vivo, as they allow regions to re-excite one another during recovery from periods of inhibition.

Specific components of the limbic system may make distinct contributions to seizures. For example, the DG has been likened to a "gate" that controls the spread of seizures within the network. As mentioned earlier, in vitro models of epileptiform synchronization suggest that the CA3 region is particularly effective at generating interictal discharges, while the entorhinal cortex excels at generating prolonged seizure-like events. The relatively frequent and brief interictal discharges may exert control over seizure initiation, as evidence has shown that lesioning slices between the hippocampus and entorhinal cortex can lead to seizures originating in the latter region. Moreover, stimulating the circuit at a low frequency (0.5–1.0 Hz) can block seizure initiation, reinforcing the concept that repetitive activity, akin to that produced by interictal discharges, can have anticonvulsant effects. In the intact brain, the circuitry becomes even more intricate, with midline

thalamic nuclei, integral to the limbic system, potentially playing a pivotal role in synchronizing limbic seizures through their diffuse and widespread connections [434-436].

10.2. Cyto-architectonic changes of limbic system in the mouse model of TLE

10.2.1. Hippocampus

The histopathological classification system for hippocampal cell loss in patients with mTLE was introduced in a prior study [437]. This classification identifies five distinct patterns:

1. Hippocampi with no significant difference in neuronal cell densities compared to age-matched autopsy controls (no MTS) (∼19%).
2. A classical pattern characterized by severe cell loss in CA1 and moderate neuronal loss in all other subfields except CA2 (19%).
3. Extensive neuronal cell loss in all hippocampal subfields (53%).
4. Severe neuronal loss confined to sector CA1 (10%, 6%).
5. Severe neuronal loss confined to the hilar region (7%, 4%).

These patterns correlate with clinical data, revealing that an early age of initial precipitating injury (IPI <3 years) is an important predictor of hippocampal pathology, particularly MTS type 2 and 3. For MTS type 4, documented IPIs occurred at a later age (mean 6 years). In MTS type 5 and normal-appearing hippocampus (no MTS), the first event appeared beyond the age of 13 and 16 years, respectively. Postsurgical outcomes were significantly worse in atypical MTS, especially in MTS type 5, where only 28% of patients experienced seizure relief after a 1-year follow-up period, compared to the successful seizure control observed in MTS types 2 and 3 (72% and 73%) [437].

Physiological studies have indicated two types of dynamic interactions between the hippocampus, amygdala, and entorhinal cortex, as revealed by intracranial recordings. This suggests that there might be two different mechanisms of epileptogenesis in the neuronal networks of patients with mTLE [438]. Vossler et al. [439] found that the location of seizure onset was related to the degree of hippocampal pathology, with low-grade hippocampal sclerosis being associated with initial ictal discharges in both the hippocampus and temporal cortex. In contrast, high-grade hippocampal sclerosis was associated with initial ictal discharges restricted solely to the hippocampus. It is plausible that the existence of two different types of neuronal loss in the mouse model of TLE may be linked to two types of dynamic interactions between the hippocampus, amygdala, and entorhinal cortex [440].

Three hypotheses, namely the 'mossy fiber sprouting hypothesis,' 'dormant basket cell hypothesis,' and 'irritable mossy cell hypothesis' [441], have been proposed based on kindling, KA, or brain trauma models to underlie the mechanisms of epileptogenesis in patients with mTLE. However, these hypotheses may not be applicable to Type 2 neuronal loss in the mouse pilocarpine model and in patients with similar pathological changes. This is because almost complete loss of CA1 and CA3 pyramidal neurons disrupts the traditional tri-synaptic neural pathways, making it unlikely for hyperactivity to propagate from the reorganized DG through a sclerotic CA3 and/or CA1 region to other brain regions unless neurons in the DG have established synaptic connections with remaining neurons in these areas, either directly or indirectly.

In Type 2 neuronal loss, the surviving neurons in CA1 and CA3 areas are primarily CB (calbindin), CR (calretinin), and PV-immunopositive interneurons [440, 442]. Some of these interneurons also display GluR1 immunopositivity [443]. Surviving GluR1 immunopositive neurons exhibit newly sprouted dendrites with growth cone-like dendritic spines in CA1 and CA3 areas [443]. Additionally, an anterograde study involving the injection of phaseolus vulgaris leucoagglutinin into the DG reveals the sprouting of MFs into the gliotic CA1 area, establishing potential synaptic contacts with CB, CR, PV, and GluR1 immunopositive neurons. This suggests that the surviving CB, CR, PV, and GluR1 immunopositive neurons in gliotic CA1 and CA3 areas may act as a bridge between the DG and subiculum, potentially playing a role in the propagation of abnormal epileptic activity. It is also plausible that the inhibitory nature of surviving CB, CR, PV-immunopositive interneurons could shift towards excitatory, as previously reported in the subiculum of patients with TLE [444], thereby directly participating in the generation of epileptiform activity.

10.2.2. Entorhinal cortex

In patients with TLE, a distinct loss of neurons has been observed in the anterior portion of the medial entorhinal cortex, even in the absence of apparent damage to the temporal neocortical gyri. This neuronal loss is most prominent in layer III of the EC but is also noticeable in layer II, particularly in the rostral field [445, 446]. Subsequent neurophysiological studies in patients have indicated that entorhinal-hippocampal interactions may work in concert to generate and propagate seizures [447].

In the mouse model, a significant loss of total neurons (including principal cells and interneurons) in layers II and III, as well as a drastic loss of interneurons in layers IV to VI, may lead to an imbalance

in interactions between principal cells and interneurons. This imbalance can result in the hyperactivity of surviving principal cells and contribute to the development of epileptogenesis originating from the entorhinal cortex [448]. Given that glutamate is a neurotransmitter in the lateral entorhinal cortex to piriform cortex/endopiriform nucleus pathway, stimulating the entorhinal cortex may trigger a feedforward inhibition of pyramidal cells in the piriform cortex. Therefore, neuronal loss in the entorhinal cortex may lead to a reduction in this feedforward inhibition, subsequently causing hyperactivity in pyramidal cells within the piriform cortex. This neuroanatomical alteration could help explain the importance of the piriform cortex in the progression and secondary generalization of limbic seizures, as previously reported.

10.2.3. Subiculum

It is generally assumed that subicular neurons are more resilient to epileptic injury compared to neurons in the hippocampus [449]. However, it has been observed that neuronal loss does indeed occur in the subiculum of patients with TLE [450]. In a study involving the mouse pilocarpine model of TLE, it was found that approximately 14% of subicular neurons were lost [451]. Notably, a higher percentage of CB-positive interneurons (46.5%) and PV-positive interneurons (34.4%) were lost, suggesting a disproportionate loss of pyramidal neurons and interneurons. In other words, the greater loss of interneurons may be associated with the generation of interictal epileptiform activity in the dorsal subiculum [451]. This finding highlights the complex interplay between different types of neurons in the subiculum and their potential roles in the generation of epileptic activity.

10.2.4. Amygdala

Neuronal loss and gliosis in the amygdala have been documented in patients with TLE [446]. In epilepsy patients with conditions such as Ammon's horn sclerosis or focal lesions in the temporal lobe, there was a significant reduction in neuronal density in the lateral amygdaloid nucleus when compared to normal controls. The mean volumetric density in epilepsy patients was reduced to 59% of that in individuals without epilepsy [452]. Additionally, it was observed that perineuronal satellitosis in the lateral amygdala appears to be a distinguishing feature of TLE with Ammon's horn sclerosis [453].

Interestingly, the extent of atrophy in the amygdala does not seem to be directly correlated with the frequency of seizures, the patient's age, or the age of epilepsy onset [454]. However, it was suggested that the loss of three subtypes of interneurons, specifically those expressing CB, CR, and

PV, in the lateral, basal, and accessory basal nuclei of the amygdala may lead to an imbalance between excitatory and inhibitory activities, subsequently contributing to the development of epilepsy [451]. This underscores the intricate nature of neural interactions within the amygdala and their potential role in the onset of epileptic activity.

11. Trilostane

Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile) is a synthetic steroid that was initially discovered 40 years ago for its ability to reduce the stimulation of adrenal corticoids by adrenocorticotropic hormone (ACTH) [455]. Further research revealed that trilostane is orally active and acts as a competitive inhibitor of steroid synthesis [456]. Scientists explored its potential applications in various human conditions, including hyperadrenocorticism (HAC), hyperaldosteronism, and breast cancer [457-461]. Trilostane was initially used in breast cancer treatment to inhibit adrenal steroid synthesis of androstenedione and testosterone. These androgens can potentially be converted to estrogens through aromatase activity, which can stimulate the growth of hormone-sensitive breast cancer cells. Trilostane's inhibition of androgen production was seen as a way to reduce estrogen levels in breast cancer patients. Recent research has uncovered an additional, unique mode of action for trilostane. This discovery suggests that trilostane could be a valuable endocrine therapeutic option, even when endocrine tumors have developed resistance to both antiestrogen treatments and aromatase inhibitors. This finding implies that trilostane may have a role in the treatment of endocrine-related conditions beyond its original application in breast cancer [462].

While trilostane showed some efficacy in certain cases of human HAC, it was not as effective as other available treatments [463]. In a recent consensus statement, it is no longer recommended as a treatment option for HAC. However, it is still used in the treatment of human breast cancer [462]. The use of trilostane in canine HAC was first reported by Hurley and colleagues [464], who successfully treated a series of 15 dogs, including two with adrenal-dependent disease. Trilostane was initially authorized for use in the United Kingdom in 2005 for the treatment of canine HAC and has since gained authorization in many other countries, including the United States.

A study investigated the potential therapeutic effects of Trilostane in rodent models of inflammation and nociception [465]. Trilostane, a drug initially developed for breast cancer and

Cushing's disease, was repurposed in this study to explore its anti-inflammatory and analgesic properties. The study was important because it addressed the unmet need for new treatments for inflammatory diseases and pain, especially for patients who do not respond well to current therapies or cannot tolerate their side effects.

The researchers conducted a series of experiments to evaluate Trilostane's effects in different rodent models:

1. **Anti-Inflammatory Effects:**

- ✓ Trilostane was found to inhibit the production of TNF-α and monocyte chemoattractant protein-1 (MCP-1) in models of LPS-induced systemic and pulmonary inflammation. TNF-α is a key mediator in inflammatory diseases, and MCP-1 is a biomarker of pulmonary inflammation.
- ✓ In a delayed-type hypersensitivity model, trilostane significantly reduced ear swelling induced by 2,4-dinitrofluorobenzene, further confirming its anti-inflammatory activity.

2. Analgesic Effects:

- ✓ Trilostane was tested in a hot plate nociception model, where it increased the latency of paw-licking behavior. This suggests potential analgesic effects.
- ✓ In a formalin-induced nociception model, Trilostane reduced the duration of pain-related behaviors, particularly in the late phase of the model.

However, it is important to note that the analgesic effects of Trilostane were comparatively mild when compared to the standard pain medication, oxycodone.

The researchers also discussed the mechanisms behind trilostane's effects, highlighting that the drug is an inhibitor of 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD), which plays a role in corticosterone and aldosterone production. While trilostane's exact mechanism of action in inflammation and nociception remains unclear, the study indicated that it may work through novel pathways. The study concluded that trilostane has potential anti-inflammatory and analgesic properties in mouse models, which have not been reported previously. Its use in pain and inflammatory diseases may be limited due to its relatively mild analgesic effects and the superiority of current standard-of-care treatments. Additionally, the long-term safety of trilostane for these conditions is uncertain. However, for life-threatening diseases like cancer, trilostane's unique properties may justify its use as an alternative approach.

Another research delved into the antidepressant-like properties of trilostane in mice [466]. This study focused on its effects on neurosteroids and monoamine levels in the brain. Trilostane was found to influence the levels of neuroactive steroids such as pregnenolone (PREG), PROG, dehydroepiandrosterone (DHEA), and androstenedione. These neuroactive steroids were known to play a significant role in regulating neurotransmitters, including glutamate, GABA, and serotonin (5-HT), which, in turn, impacted mood and depression.

The research built on prior findings that trilostane exhibited antidepressant-like properties in the forced swimming test (FST) and concurrently regulated plasma ACTH and corticosterone levels, markers of stress-induced hypothalamus-pituitary-adrenal axis activation. It also revealed that peripheral steroid levels influenced the effects of trilostane, as adrenalectomy and castration blocked its antidepressant effects. Notably, trilostane treatment altered the levels of neurosteroids in the hippocampus and frontal cortex, affecting PROG and PREG contents.

The study investigated the co-administration of trilostane with various neuroactive steroids and antidepressants. It demonstrated that trilostane enhanced the antidepressant activity of dehydroepiandrosterone sulfate (DHEAS) but exhibited only additive effects when combined with pregnenolone sulphate (PREGS), PROG, or ALLO. Trilostane treatment also led to an increase in 5-HT and norepinephrine turnover in the hippocampus, which was likely related to its antidepressant actions. When co-administered with selective serotonin reuptake inhibitors (SSRIs) as fluoxetine, sertraline, and imipramine, trilostane further reduced immobility in the FST, underlining its potential as an adjunct to SSRIs.

11.1. Mode of action

Trilostane acts as a competitive inhibitor of the 3β-HSD (in the adrenal cortex), which is a crucial enzyme system involved in the synthesis of various steroids, including cortisol and aldosterone. This enzyme catalyzes the conversion of 3β-hydroxysteroids (such as PREG, 17-hydroxypregnenolone, and DHEA) into 3-ketosteroids (such as PROG, 17α-hydroxyprogesterone (17α-OHP), and androstenedione). Trilostane itself does not have direct hormonal activity and does not interact with the primary sex hormone receptors [467].

Most studies on the mode of action of trilostane have been conducted in vitro, in laboratory rats, or in humans. There is limited information available on the effects of trilostane in healthy dogs. In dogs with HAC, trilostane has been shown to significantly increase the concentrations of 17-

hydroxypregnenolone and DHEA, indicating its inhibitory effect on the 3β-HSD system. However, while cortisol concentrations decreased in dogs treated with trilostane, 17α-OHP concentrations did not change significantly. Researchers have postulated that in addition to its 3β-HSD inhibitory effect, trilostane may influence 11β-hydroxylase, leading to a reduction in the conversion of 17α-OHP to cortisol and potentially affecting the interconversion of cortisol and cortisone by 11β-hydroxysteroid dehydrogenase (11β-HSD) [468]. However, canine 11β-HSD appears to behave differently from its human counterpart. Further studies demonstrated that cortisone concentrations in normal dogs increase following ACTH stimulation, which is different from the human response [469]. Additionally, in dogs with pituitary-dependent HAC, cortisone concentrations are consistently elevated, while trilostane treatment reduces cortisone concentrations, albeit to a lesser extent than cortisol concentrations [469]. This indicates that the effects of trilostane in dogs may differ from its effects in humans due to subtle species differences in steroid synthesis.

11.2. Pharmacology

There have been limited pharmacokinetic studies conducted on trilostane. In various species such as rats, monkeys, and humans, trilostane is orally absorbed relatively quickly. In rats, peak blood concentrations are reached within 0.5 to 1 hour, in monkeys between 2 and 4 hours, and in human volunteers between 2 and 4 hours. In dogs, peak concentrations of trilostane are observed within about 1.5 hours after administration, and it typically returns to baseline levels within approximately 18 hours. The variability in systemic trilostane levels following oral administration may be influenced by its limited water solubility. Notably, administering trilostane immediately after a meal can enhance its absorption.

The elimination half-life of trilostane varies among species, with a relatively short duration in rats (7 hours), humans (6 to 8 hours), and a longer period in monkeys (48 hours). Trilostane is rapidly metabolized into its active metabolite, ketotrilostane, in rats, which exhibits about 1.7 times the inhibitory activity against steroid synthesis compared to trilostane. Conversely, administering ketotrilostane to rats results in the rapid formation of trilostane, indicating that these compounds coexist in vivo. Trilostane and ketotrilostane further undergo metabolism into one of four additional metabolites. While fecal excretion is the primary route of elimination in rats, urinary excretion is more significant in monkeys.

12. GABA Receptors

Gamma-aminobutyric acid, or GABA, is a pivotal neurotransmitter in the adult forebrain. Its role as a primary inhibitory neurotransmitter is well-established. When released from interneuron terminals, GABA acts on both pre- and postsynaptic GABA receptors, eliciting a range of inhibitory effects [470, 471]. GABA receptors are classified into three primary types: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_A$ receptors are ionotropic in nature and function as receptor-operated channels. This means that when they are activated, they allow ions to flow through them, rapidly altering the membrane potential. In contrast, $GABA_B$ receptors are metabotropic, modulating signaling through slower and more prolonged mechanisms. $GABA_B$ receptors activate intracellular second messengers, which subsequently mediate cellular responses. It is noteworthy that $GABA_B$ receptors are well-established in controlling the release of neurotransmitters from both excitatory and inhibitory terminals [472]. However, the role of $GABA_A$ receptors in regulating neurotransmitter release remains a topic of debate among researchers, as it lacks a clear consensus. Initially, $GABA_C$ receptors were thought to be primarily confined to the retina within the adult CNS. Nevertheless, subsequent research revealed their inhibitory functions in the adult rodent hippocampus [473-475]. Here, $GABA_C$ receptors are thought to be located extrasynaptically, meaning they are not closely associated with the synapses themselves. Instead, they are activated through the spillover of GABA that is released at synapses. This distinct role underscores the complexity of GABA receptor function and the diverse mechanisms by which they modulate neural signaling in the adult brain [475]. The classification and functional diversity of GABA receptors have significant implications for our understanding of inhibitory neurotransmission and its regulation in the CNS.

12.1. $GABA_A$ Receptors

$GABA_A$ receptors, part of the GABA receptor family, are key players in the modulation of inhibitory neurotransmission in the CNS. These receptors exist in multiple molecular forms and have widespread distribution in both the CNS and the peripheral nervous system (PNS) [476, 477]. They play a critical role in controlling neuronal excitability and have garnered significant attention due to their relevance in the pharmacological modulation of inhibitory neurotransmission. $GABA_A$ receptors are members of a superfamily of ligand-gated ion channels, which includes other well-known receptors such as nicotinic-cholinergic, glutamate, and glycine receptors (GlyRs) [478]. These receptors are integral to the regulation of Cl^- channels in neurons. The $GABA_A$ receptor complex is a

heterooligomeric protein, typically composed of five subunits arranged in a pentameric structure. These subunits consist of multiple homologous yet unique membrane-spanning components, including alpha, beta, gamma, delta, and others [476].

The intriguing aspect of $GABA_A$ receptors lies in the diversity of these subunits, as various isoforms of alpha, beta, and gamma subunits are differentially expressed throughout the CNS. This differential expression gives rise to an array of receptor isoforms, often referred to as isoreceptors. These isoreceptors exhibit markedly different affinities for GABA, the primary inhibitory neurotransmitter in the brain, as well as for drugs that modulate GABA receptor-mediated chloride conductance [479, 480].

Notably, the subunit composition of $GABA_A$ receptors plays a central role in determining their pharmacological properties. Among the various subunits, the gamma2 subunit stands out as crucial for benzodiazepine binding and receptor modulation. $GABA_A$ receptors lacking gamma2 subunits, while retaining alpha and/or beta subunits, can still be modulated by certain drugs like barbiturates but do not bind or respond to benzodiazepines. Additionally, the specific isoform of the alpha subunit expressed in a given receptor complex significantly influences its sensitivity to GABA, the rate of agonist-induced desensitization, and the relative binding affinities for both benzodiazepine and nonbenzodiazepine drugs [480].

This subunit diversity is associated with marked variations in the pharmacological profiles of $GABA_A$ receptors. For instance, the cerebellum and hippocampus are characterized by the presence of two distinct benzodiazepine/$GABA_A$ receptor types, known as type I and type II [481, 482] . These receptor types are predominantly found in these respective brain regions and differ in their response to pharmacological agents. The underlying reason for this diversity lies in the differential expression of alpha subunits. More recently, the discovery of the alpha6 subunit, which is exclusively expressed in cerebellar granule cells, has added another layer of complexity. Receptors containing the alpha6 subunit, in combination with beta and gamma subunits, exhibit unique pharmacological characteristics. These receptors are selectively modulated by inverse agonists like Ro15-4513, often referred to as an "alcohol antagonist." Intriguingly, they do not bind benzodiazepine agonists [483, 484].

These findings highlight the remarkable diversity of $GABA_A$ receptors within the CNS and underscore the regional differences in their distribution and allosteric modulation by various drugs. Furthermore, it emphasizes the significant role of receptor subunits in determining the

pharmacological properties of $GABA_A$ receptors. Additionally, these receptors are sensitive to modulation by natural compounds, which further adds to their complexity and the subtleties of their pharmacological responses. The functional implications of this diversity in receptor subunit composition are a subject of ongoing research and provide valuable insights into the modulation of inhibitory neurotransmission in the brain.

13. Neurosteroids

Neurosteroids, originally termed by French physiologist Etienne Baulieu, are steroids synthesized within the brain, exerting rapid non-genomic actions to modulate neuronal excitability. Precursors for neurosteroid synthesis are circulating steroid hormones, with local production occurring in brain structures such as the hippocampus [485]. Structurally, neurosteroids can be categorized as pregnane neurosteroids (e.g., ALLO, THDOC), androstane neurosteroids (e.g., androstanediol, etiocholanone), and sulfated neurosteroids (e.g., PREGS and DHEAS) (Figure 10). Recognized for their sedative, anesthetic, and antiseizure properties in animals and humans [486-489], steroid hormones, specifically PROG and deoxycorticosterone, serve as precursors for endogenous neurosteroids ALLO and THDOC, respectively [490]. Androgens derived from testosterone, such as androstanediol and estradiol, are also considered neurosteroids [491].

Distinct from classical steroid hormone receptors regulating gene transcription, the acute effects of neurosteroids are not related to such interactions. Neurosteroids do not directly activate intracellular steroid receptors; instead, they primarily modulate brain excitability through interactions with neuronal membrane receptors and ion channels, particularly $GABA_A$ receptors [492, 493]. As endogenous regulators of neuronal excitability, neurosteroids offer significant therapeutic potential [494, 495].

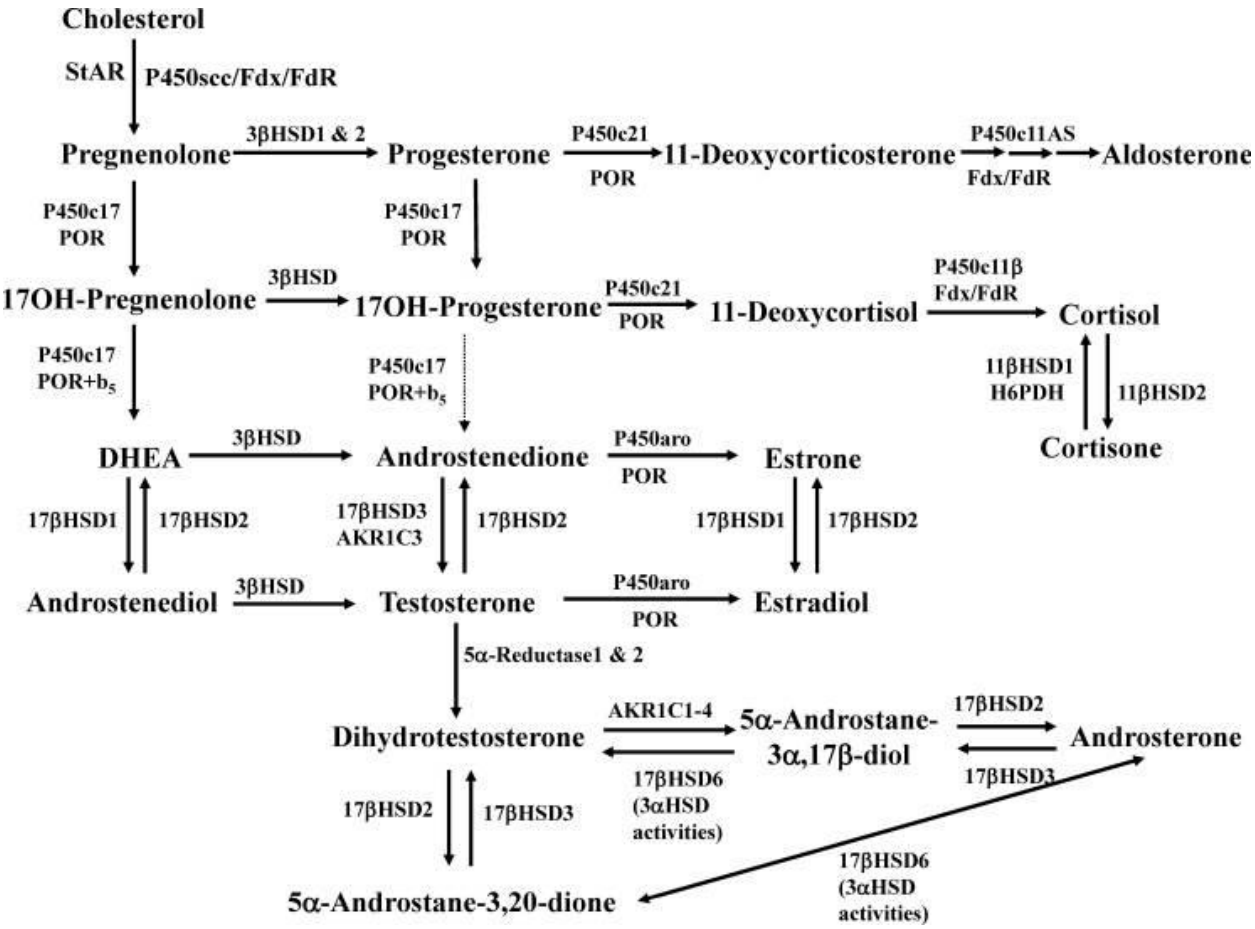

Figure 10. Major human steroidogenic pathways. Key enzymes and cofactor proteins are shown near arrows indicating chemical reactions. cytochrome P450 cholesterol side chain cleavage (P450scc) cleaves cholesterol to pregnenolone, the first committed intermediate in steroid biosynthesis. The steroids in the first column are Δ^5-steroids, which constitute the preferred pathway to C19 steroids in human beings. The dashed arrow indicates poor flux from 17α-hydroxyprogesterone to androstenedione via P450c17, and the three small arrows below P450c11AS emphasize the three discrete steps with intermediates corticosterone and 18-hydroxycorticosterone. Not all intermediate steroids, pathways, and enzymes are shown.

The burgeoning field of neurosteroidogenesis originated at the crossroads of research in the neuropharmacology of ligand-gated ion channel receptors and investigations into steroid hormone synthesis. The revelation that steroids could be synthesized de novo in the brain, coupled with experiments delineating novel functions for specific steroidal compounds at nonclassical $GABA_A$ and NMDA receptors, brought this nascent field into focus. Pioneering studies by Harrison and Simmonds, as well as Majewska, elucidated that certain steroids, notably PROG derivatives, could modulate $GABA_A$ receptor function by increasing the duration and frequency of channel opening, acting at a site distinct from the GABA site [496, 497]. Conversely, other steroids functioned as negative modulators, inhibiting GABAergic function [498-502]. Endocrinologists, in parallel,

conducted experiments situating these neuroactive steroids at their action site in the brain. The discovery of steroids, such as PREG and DHEA, at higher concentrations in nervous system tissues than in plasma, initially observed by Baulieu and colleagues in the 1980s, suggested de novo synthesis within the CNS and PNS [503, 504]. The term "neurosteroids" was coined to emphasize their unconventional origin, distinguishing them from steroids derived from classical steroidogenic organs like gonads, adrenals, and placentae.

Crucial questions arose regarding the origin and accumulation of neurosteroids in the nervous system. Studies, reviewed extensively [505, 506], directly examined whether enzymes implicated in steroidogenesis in classic organs (adrenals, gonads, and placentae) were responsible for neurosteroid synthesis. Unquestionably, these studies confirmed the presence of steroidogenic enzymes in the nervous system. Depending on the synthesized steroid, their effects ranged from modulating gene expression through classic intracellular nuclear receptors to influencing neurotransmission via membrane glucocorticoid-gated and other neurotransmitter receptors. While the modulation of neurotransmitter receptors by neurosteroids is well-established, understanding the consequences of this action and potential differences between developmental and adult stages remains a focus of inquiry. In adults, neurosteroid stimulation of neurotransmitter receptors induces behavioral effects associated with those receptors, such as reduced anxiety, sedation, and decreased seizure activity. Additional developmental roles for neurosteroids include involvement in neuronal modeling, where DHEA and DHEAS stimulate embryonic axonal and dendritic growth, and ALLO induces neurite regression [507, 508]. Furthermore, neurosteroids exert effects on neurotransmitter receptor expression during development, influencing their ability to mediate effects [509-514].

13.1. Biosynthesis of neurosteroids

Steroid hormones synthesized from cholesterol through a series of enzymatic reactions orchestrated by both P450s and non-P450s. These enzymes collaborate to direct the synthesis of distinct steroids within specific cells. Tissue-specific steroid synthesis depends on the expression levels of a cohort of enzymes and potential competition among enzymes for specific substrates. In the adrenals and gonads, the synthesis of androgens, estrogens, glucocorticoids, and progestins follows a defined scheme. In the adrenals, the expression of P450c11β in the zona fasciculata/zona

glomerulosa facilitates glucocorticoid synthesis, while P450c11AS expression in the zona glomerulosa enables mineralocorticoid synthesis. Notably, in the human adrenal, P450c17 serves as a crucial branch point in steroidogenesis. The absence of its expression in the zona glomerulosa allows for the conversion of PROG to 11-deoxycorticosterone. Together with P450c11AS expression, this pathway ultimately leads to mineralocorticoid synthesis. Conversely, P450c17 expression with its 17α hydroxylase activity facilitates 17α hydroxylation of PREG. This, in conjunction with P450c21 and P450c11β, leads to glucocorticoid (cortisol) synthesis in the zona fasciculata. Additionally, P450c17 expression with both its 17α hydroxylase and 17,20 lyase activities in the zona reticularis results in the cleavage of the C17,20 bond, converting C21 to C19 steroids and leading to androgen synthesis [515].

While the regulation of specific steroid synthesis in the adrenal gland is governed by the expression and activity of a specific set of steroidogenic enzymes, the ovary presents a more intricate scenario. Here, estrogen synthesis involves the collaboration of two distinct cell types: granulosa and theca cells. Notably, granulosa cells lack P450c17 expression, whereas theca cells express P450c17. Consequently, there is a necessity for coordinated expression of steroidogenic enzymes and the seamless shuttle of steroid precursors and products between both cell types to achieve efficient estrogen synthesis. This intricate process is facilitated by the physical arrangement of theca cells surrounding granulosa cells. In the brain, the situation becomes even more complicated. Steroidogenic enzymes are expressed in discrete cell types, specifically neurons and glia. Furthermore, their distribution in the brain may not necessarily overlap, in terms of both region and developmental stage. Additionally, enzyme activity may vary between cell bodies and fibers extending from those cell bodies. Consequently, steroid precursors and products may exist at sites far removed from their cell body of origin.

The initial, rate-limiting, and hormonally regulated step in the synthesis of all steroid hormones is the conversion of cholesterol to PREG, as depicted in Figure 10. This pivotal reaction is catalyzed by the mitochondrial enzyme cytochrome P450 cholesterol side chain cleavage (P450scc), through three successive chemical reactions: 20α-hydroxylation, 22-hydroxylation, and scission of the C20–C22 carbon bond in cholesterol. P450scc, a singular species, is found in all steroidogenic tissues, including the brain [516, 517]. Initial ribonucleic acid analysis aimed to determine the presence of enzymes involved in steroid synthesis in the brain, confirming region-specific gene expression through real-time and Polymerase chain reaction analysis. However, due to the low abundance of

mRNAs, subsequent focus shifted to the detection of proteins, a more easily discernible aspect through immunocytochemistry [518, 519].

The molecular intricacies of steroid hormone biosynthesis and the involved enzymes have been comprehensively reviewed [520]. In this context, the following serves as a concise summary of steroidogenesis pathways, as depicted in Figure 10, with detailed information available elsewhere [505] [10]. Following the synthesis of PREG, specific steroidogenic enzymes play crucial roles in transforming it into various types of steroid hormones. PREG can undergo conversion to PROG, facilitated by 3β-HSD, or be subject to 17 hydroxylation by P450c17, a microsomal P450 enzyme. Notably, the 17 hydroxylase reaction exhibits substrate specificity, with certain species favoring Δ5 steroids (e.g., PREG) and others preferring Δ^4 steroids (e.g., PROG) [520]. The resultant 17-hydroxypregnenolone can be further converted to DHEA by P450c17. Subsequently, DHEA is transformed into androgens through the mediation of tissue-specific 17-βHSDs, ultimately giving rise to androstenediol and then testosterone, facilitated by 3β-HSD. Further modifications include the conversion of testosterone to estradiol by P450aro (aromatase), and the conversion of testosterone to dihydrotestosterone (DHT) through the action of 5α-reductase.

PROG undergoes metabolism to yield glucocorticoids, mineralocorticoids, or neuroactive steroids through the involvement of various enzymes. The synthesis of glucocorticoids (corticosterone in rodents, cortisol in humans) and mineralocorticoids (aldosterone) involves a sequence of enzymatic steps. This includes 21 hydroxylation, mediated by microsomal P450c21, followed by the action of a zone-specific P450c11. In the zona fasciculata/reticularis, P450c11β catalyzes the conversion of 21-hydroxypregnenolone to corticosterone, while in the zona glomerulosa, P450c11AS facilitates the production of aldosterone. In humans, glucocorticoid synthesis necessitates 17 hydroxylation of PREG. Notably, this process does not involve 17,20 lyase activity, which would lead to the formation of DHEA. The regulatory balance between the 17 hydroxylase and 17,20 lyase activities of P450c17 dictates whether the human adrenal will synthesize glucocorticoids (solely through 17 hydroxylase activity) or DHEA (involving both 17 hydroxylase and c17.20 lyase activities). This intricate regulation of enzymatic activities plays a pivotal role in determining the specific steroid products synthesized by the human adrenal gland [515].

The synthesis of additional neuroactive steroids involves the activity of two enzymes: 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD). PROG, deoxycorticosterone, or testosterone serve as substrates for 5α-reductase, resulting in the conversion to their 5α reduced derivatives, namely

5α-DHP, 5α-dihydrodeoxycorticosterone (also known as DHDOC), or DHT, respectively. Subsequently, these steroids can be transformed into neuroactive steroids through conversion to their 3α reduced derivatives, facilitated by 3α-HSD. In rodents, a single 3α-HSD enzyme can mediate all these reactions, providing a unified mechanism. In contrast, humans possess several 3α-HSD genes with distinct substrate specificities [518]. Consequently, the developmental and cell-specific expression patterns of these steroidogenic enzymes play a crucial role in determining the synthesis of specific steroids and neurosteroids in a particular tissue at a specific developmental stage. The intricate regulation of these enzymes contributes to the diversity of neuroactive steroids produced in different tissues and developmental contexts.

A pervasive distribution of steroidogenic enzymes is evident across key brain regions, encompassing the cortex, hippocampus, olfactory bulb, basal ganglia, hypothalamus, thalamus, and cerebellum. Additionally, select enzymes exhibit expression in the tectum, pons, medulla, pituitary, spinal cord, and various PNS regions. The consistent presence of steroidogenic enzymes in specific brain areas across diverse species implies a conserved role in neurosteroid synthesis and function throughout evolutionary processes. The co-localization of specific steroidogenic enzymes has been demonstrated in both whole brain sections and cultured neuronal or glial cells [518].

In the periphery, the steroid precursors are primarily synthesized in the gonads, adrenal gland, and feto-placental unit. However, the synthesis of these neurosteroids likely occurs in the brain, either directly from cholesterol or from intermediates derived peripherally. Due to their high lipophilicity and the ability to readily cross the blood–brain barrier, neurosteroids synthesized in peripheral tissues accumulate in the brain [521].

13.2. Mechanisms of neurosteroid actions

There is compelling evidence suggesting that the acute effects of neurosteroids are not associated with interactions with classical steroid hormone receptors, which typically regulate gene transcription. However, chronic effects of neurosteroids manifest through a dual mechanism involving both genomic actions, mediated by classical intracellular steroid receptors, and non-genomic rapid actions, targeting ion channels and membrane receptors in the brain. Notably, the genomic effects of neurosteroids primarily arise from their metabolic conversion to traditional steroids [522]. Neurosteroids, in themselves, do not exhibit direct activity at intracellular steroid receptors. Instead, they primarily modulate brain excitability by interacting with neuronal

membrane receptors and ion channels [523]. Several observations support this notion. Firstly, the effects of neurosteroids manifest rapidly, occurring within minutes, in contrast to the slower onset and prolonged duration typical of steroid hormone actions via intracellular steroid receptors [524]. Secondly, neurosteroids display low-affinity interactions with nuclear steroid hormone receptors [522, 525]. While metabolites of neurosteroids generated through intracellular oxidation of the 3α-hydroxyl group may bind to steroid receptors, the direct interaction of neurosteroids with these receptors is limited. Thirdly, recent studies in PROG receptor knockout mice conclusively demonstrate that the classical steroid receptor is not necessary for the sedative, anxiolytic, and anticonvulsant activities of PROG and related neurosteroids [526, 527]. Lastly, neurosteroids have been shown to directly modulate the activity of ligand-gated ion channels, particularly $GABA_A$ receptors [492]. These collective findings underscore the intricate and multifaceted mechanisms through which neurosteroids exert their effects on neuronal function and behavior.

13.3. Neurosteroids as modulators of neuronal excitability

Neurosteroids synthesize through the enzymatic conversion of cholesterol to PREG by the P450scc enzyme [528]. Subsequent transformation of PREG results in the formation of either 17α-hydroxypregnenolone or PROG, serving as precursors to a diverse array of steroid derivatives that interact with various neurotransmitter systems. Notably, neurosteroids exhibit the capability to engage with $GABA_A$, NMDA, glycine, and opioid σ1 receptors, as extensively reviewed elsewhere [523].

The categorization of neurosteroids into two classes hinges on their metabolism, involving the addition of sulfate residues. Nonsulfated neurosteroids, exemplified by ALLO and THDOC, enhance $GABA_A$ receptor function, as discussed in the subsequent section [529]. Conversely, sulfated neurosteroids manifest more intricate modulatory properties, potentially increasing neuronal excitability through negative modulation of $GABA_A$ receptors and/or augmentation of glutamatergic activity. However, the variable effects of sulfated neurosteroids on glutamate-mediated neurotransmission, influenced by GluR subunit composition [530], necessitate further investigation to elucidate their pathophysiological relevance in epileptic disorders.

Given the established role of GABAergic inhibition in epileptic disorders, this review predominantly focuses on the impact of neurosteroids on $GABA_A$ receptor function. Nonsulfated neurosteroids exhibit concentration-dependent modulatory mechanisms on $GABA_A$ receptors [531]: at nanomolar

concentrations (e.g., during stress and oestrus), they act as modulators, while at micromolar concentrations (as observed during parturition), they directly open $GABA_A$ channels. The interaction of neuroactive steroids with $GABA_A$ receptors involves the potentiation of $GABA_A$ currents via the α1 subunit at lower concentrations, and direct activation relies on the interaction with both α1 and β2 subunits [532].

Figure 11 illustrates that $GABA_A$ receptor activation results in two types of currents, dictated by their location and subunit composition. Synaptic receptors generate phasic 'transient' currents in response to GABA release from synaptic vesicles, while extrasynaptic and perisynaptic receptors produce a tonic 'always on' current in response to low ambient GABA levels [533]. The predominant contribution to the tonic GABAergic current comes from $GABA_A$ receptors containing α5 and δ subunits [534]. Intriguingly, mice lacking the $GABA_A$ δ-subunit exhibit an attenuated response to neurosteroids [535], aligning with the perspective that tonic rather than phasic inhibition may represent the preferential target for neurosteroid modulation [536].

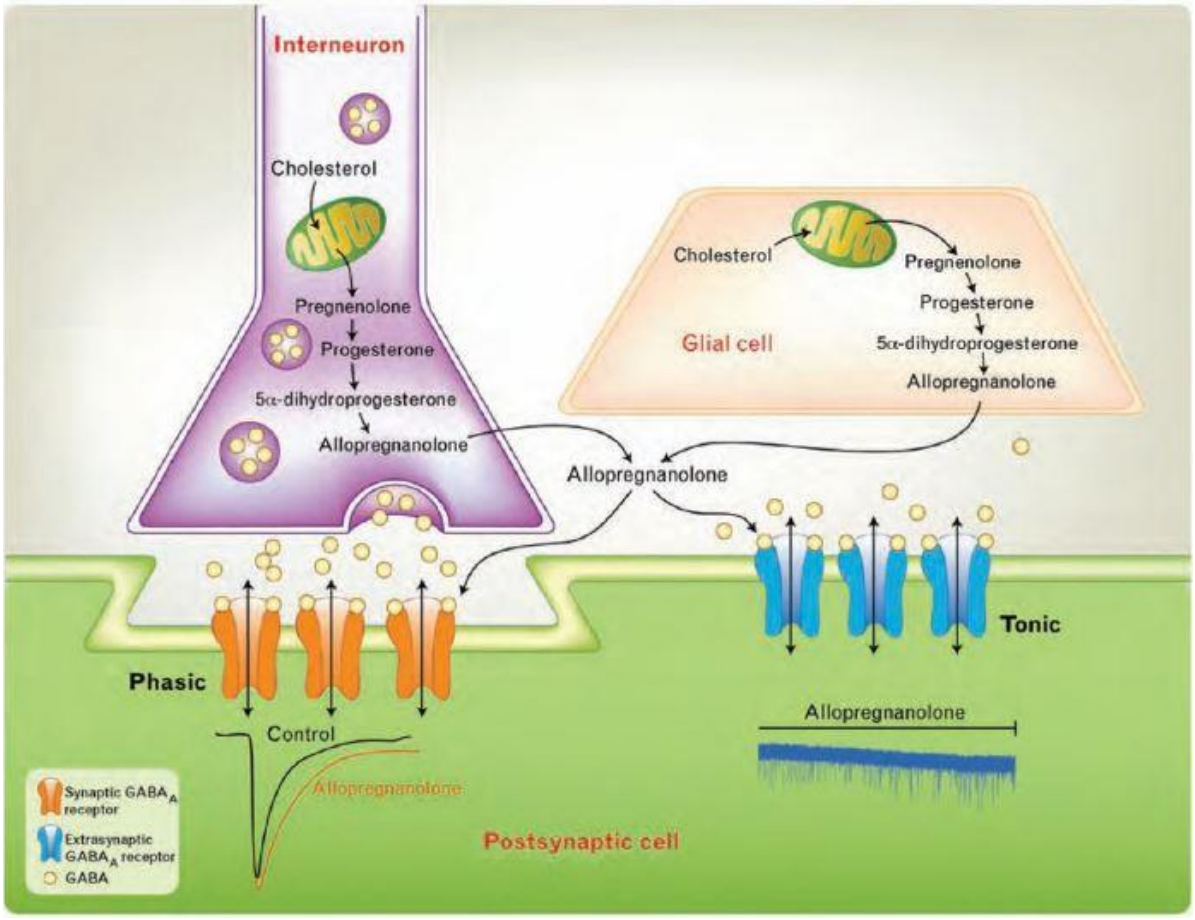

Figure 11. Neuroactive steroids and their actions on GABA released from an interneuron interacts with $GABA_A$ receptors at synaptic (orange) and extrasynaptic (blue) locations, generating phasic and tonic inhibitory currents, respectively. The schematic drawings below the two receptors illustrate the corresponding effect of ALLO on $GABA_A$-mediated currents, showing an increase in the decay time constant of synaptic events and an increase in the inhibitory tone as revealed by the downward shift in the holding current [537].

13.4. Excitatory neuroactive steroids are $GABA_A$ receptor antagonists

The recognition of certain steroids possessing convulsant and proconvulsant properties dates back to the early 1950s. During these investigations, high doses of DHEA and 17a,21-dihydroxypregn-4-en-3-one consistently elicited seizure activity in laboratory animals [538, 539]. In an extensive screening of 168 pregnane steroids, Atkinson and colleagues [539] reported that the majority of behaviorally active steroids exhibited sedative-hypnotic activity. However, a subset of approximately 20 compounds induced seizures when administered intravenously to mice. Similar to the rapid sedative or anesthetic actions of 3α-hydroxysteroids, the excitatory effects of these steroids also occur swiftly and are unlikely to involve classical genomic mechanisms. This historical evidence underscores the diverse neuroactive properties exhibited by different steroids, ranging

from sedation to excitatory effects, and highlights the complex interplay between steroids and neural function.

The synthetic amidine RU5135 stands out as the extensively studied convulsant steroid. This 3α-hydroxysteroid incorporates an amidine function in ring D and, akin to anesthetic 3α-hydroxysteroids, lacks hormonal activity. Notably, RU5135 exhibits potent convulsant properties, eliciting electroencephalographic spiking in the cerebral cortex of rats even at low doses ranging from 2-4 mg/kg [540]. Numerous laboratories have delved into the actions of RU5135 on $GABA_A$ receptors, employing both biochemical (ligand binding) and electrophysiological techniques. RU5135 emerges as one of the most potent $GABA_A$ receptor antagonists to date, boasting an affinity several hundred times greater than that of the prototypic receptor antagonist bicuculline [541-543]. The kinetic profile of RU5135's inhibitory actions led Simmonds and Turner to conclude that RU5135 and bicuculline likely interact with similar, if not identical, binding sites [542]. Intriguingly, despite its remarkably high affinity for $GABA_A$ receptors, with reported K values in the low nanomolar range, RU5135's inhibitory effects extend beyond this receptor (or receptors). It serves as an even more potent antagonist of strychnine-sensitive GlyRs [541, 542]. This apparent lack of specificity may hold significance, considering the reported structural similarities, as well as protein and nucleotide sequence identity, between $GABA_A$- and glycine-gated Cl- channels. These findings underscore the multifaceted pharmacological actions of RU5135 on neurotransmitter receptors, contributing to our understanding of its convulsant effects.

PREGS and DHEAS, both occurring naturally, are recognized as excitatory neuroactive steroids. Studies have reported their proconvulsant effects in laboratory animals [538] and their ability to enhance neuronal excitability, as evidenced by increased firing rates when directly applied to septal-preoptic neurons in guinea pigs [544]. Notably, these naturally occurring excitatory neuroactive steroids have been investigated for their impact on inhibitory synaptic events mediated by GABA. Research conducted by Majewska and colleagues sheds light on the effects of PREGS and DHEAS on the ligand binding and functional properties of $GABA_A$ receptors. Their findings indicate that at low micromolar concentrations, both steroids act as antagonists, inhibiting GABA receptor-mediated 36Cl uptake in synaptoneurosomes and Cl conductance in cultured neurons [545, 546]. Interestingly, PREGS, akin to benzodiazepine receptor inverse agonists, has been reported to antagonize $GABA_A$ receptor-mediated currents by reducing channel opening frequency [547]. More recent studies have characterized saturable low-affinity binding sites (Ka = 3 ELM) for DHEAS in rat

brain synaptoneurosomes, and the specific binding of this steroid has been reported to be inhibited by barbiturates [546]. However, further research is needed to establish whether these binding sites are indeed associated with $GABA_A$ receptors.

Despite their excitatory actions, it is noteworthy that these natural neuroactive steroids, particularly PREGS, lack selectivity for $GABA_A$ receptors, as similar steroid concentrations also block glycinemediated inhibitory events [548]. This underscores the complex pharmacological profile of these neuroactive steroids and suggests that their excitatory effects may be attributed, at least in part, to the antagonism of GABA. Similar to RU5135, the non-selectivity of PREGS for $GABA_A$ receptors adds an additional layer of complexity to our understanding of the interactions between neuroactive steroids and neurotransmitter systems.

It is crucial to note that, unlike GABA-activated Cl^- currents, glycine-activated Cl^- currents remain unaffected by ALLO. Additionally, reports indicate that PREGS exhibits selective augmentation of glutamate-induced depolarizations mediated by the NMDA subtype of the GluR in chick spinal cord neurons [549]. Studies in cultured rat hippocampal neurons further revealed that PREGS significantly enhances NMDA receptor-mediated elevations in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$. Despite its potentiation of NMDA-induced inward currents and intracellular $[Ca2+]_i$, PREGS does not potentiate the neurotoxic effects of NMDA in cultured rat cortical or hippocampal neurons. This suggests that the excitatory properties of PREGS may stem from both a reduction in GABA-mediated inhibitory events and an enhancement of glutamate-mediated excitation. However, further research is required to elucidate the precise sites and mechanisms of action of excitatory neuroactive steroids in order to deepen our understanding of their complex modulatory effects. In general, glucocorticoids are known to elevate CNS excitability and reduce seizure threshold in animals [550]. However, the underlying mechanisms responsible for these excitatory actions are not fully understood. Majewska [551] has reported "antagonist-like" interactions of corticosterone, cortisol, and cortisone, as well as their reduced metabolites, with [^{35}S] TBPS binding sites in the rat cerebral cortex. Nevertheless, the functional consequences of these interactions remain unclear. Notably, there is a single report indicating that low concentrations of cortisol potentiate GABA-mediated contractions of the guinea pig ileum, while high concentrations inhibit them [552]. However, most investigations have failed to observe either inhibitory or augmentary effects of glucocorticoids on $GABA_A$ receptor-mediated responses [553]. Furthermore, a unique membrane-bound corticosteroid receptor has been identified in the amphibian brain, which

appears to mediate the ability of corticoids to rapidly suppress male reproductive behavior in this species [554]. Importantly, this steroid binding site is not associated with $GABA_A$ receptors, even though amphibians possess $GABA_A$ receptors modulated by 3α-hydroxysteroids [554]. The existence of similar corticosteroid receptors in the mammalian brain and their potential contribution to the intricate neuroactive properties of glucocorticoids are subjects that warrant further investigation.

13.5. Receptor-active neurosteroids in brain

The well-established interactions of natural 3α-hydroxysteroids with $GABA_A$ receptors, combined with the presence of both 5α-reductase and 3α-hydroxysteroid oxidoreductase in the brain and many peripheral tissues, have led to speculation about the potential role of these steroids as endogenous modulators of central and/or peripheral $GABA_A$ receptors [497, 555, 556]. To explore this further, it was developed radioimmunoassays for quantifying ALLO and allotetrahydroDOC in various tissues, including the brain [557, 558]. The levels of ALLO in plasma and the brain generally mirror those of PROG. Although both steroids can be detected in the male rat brain and plasma, their levels are typically lower under most conditions. Notably, exposure of male rats to brief ambient-temperature swim stress induces rapid (within 5 minutes) and robust (4-20-fold) increases in ALLO and allotetrahydroDOC levels in both the brain (cerebral cortex and hypothalamus) and plasma [557]. The brain levels of these 3α-hydroxysteroids measured after swim stress fall within the range of concentrations (approximately 3-10 ng/g or 10-30 nM) previously demonstrated to enhance GABA-activated Cl^- currents in electrophysiological experiments [559].

In an experiment [557] aimed at pinpointing the tissue source of these steroids, male rats were adrenalectomized several weeks before being subjected to swim stress. In adrenalectomized rats, neither steroid could be detected in plasma, both before and after swim stress, with an assay sensitivity of 85 pg/ml. Interestingly, even after adrenalectomy, appreciable levels (>3 ng/g) of ALLO (but not allotetrahydroDOC) were still present in the cerebral cortex of rats. Similar levels of ALLO were observed in brain tissue from female rats that underwent adrenalectomy and oophorectomy. Additionally, the brain level of ALLO after swim stress matched or exceeded that of PROG. These findings indicate that, following exposure to stress, the adrenal gland serves as the primary source of allotetrahydroDOC in the brain and plasma. Nevertheless, a significant fraction of ALLO may be synthesized de novo in the brain, likely through the conversion of PREG to PROG [557].

The term "neurosteroid" was introduced by Baulieu in 1981 to describe a steroid hormone intermediate, DHEAS, discovered in the brain at concentrations independent of its plasma levels [560]. DHEAS is part of a range of neurosteroids, including PREGS, now believed to be synthesized de novo in the mammalian brain through the classical mevalonate pathway to cholesterol [485, 504]. Cytochrome P-450-catalyzed side-chain cleavage of cholesterol to PREG has been demonstrated in mitochondria prepared from glial cultures of oligodendrocyte-rich embryonic rat brain [561]. Enzymes found in limbic regions of the brain [562], as well as in glial cultures, have been shown to catalyze the oxidation of PREG to PROG [563]. Mixed cultures of neurons and glia can also reduce PROG to ALLO via the intermediate 5a-pregnane-3,20-dione [564, 565], formed by 5a-reductase activity present in both neurons and glial cells [566]. The subsequent reduction of 5a-pregnane-3,20-dione to ALLO by 3a-hydroxysteroid oxidoreductase [567] reportedly occurs in astrocytes from rat brain [568]. Additionally, the epimeric 3β-hydroxy-5α-pregnan-20-one is formed from PROG by cultured fetal cells (neurons and glia) from the rat brain [565].

It has been established [569] that deoxycorticosterone (DOC), formed from PROG in the zona fasciculata of the adrenal cortex but not in the brain, can undergo reduction by 5α-reductase in the brain to 21-hydroxy-5α-pregnane-3,20-dione. Subsequently, it can be converted by 3α-hydroxysteroid oxidoreductase to allotetrahydroDOC. There is no evidence of 21-hydroxylation of steroids with a saturated ring A in the brain. Therefore, it is not surprising that no detectable in vivo conversion of ALLO to allotetrahydroDOC was found in the rat [501]. Other potential biosynthetic pathways for the formation of $GABA_A$ receptor-active neurosteroids include the generation of alfaxalone from 11β-hydroxyprogesterone and the formation of 3α,21-dihydroxy-5α-pregnane-11,20-dione from corticosterone. These potent $GABA_A$ receptor-active metabolites have been isolated from human urine [570], especially in 17α-hydroxylase-deficient patients [571]. Therefore, both alfaxalone and alfadolone could possibly be formed from ring A-reduced 11β-hydroxysteroids that are oxidized by 11β-HSD in the brain [572, 573]. However, it is crucial to note that corticosterone itself is only metabolized to its 11-ketone in the adult rat brain [573].

An intriguing hypothesis, put forth by Costa and colleagues [574, 575], suggests that peripheral benzodiazepine receptors situated on adrenal and glial mitochondria may regulate the synthesis of neuroactive steroids, such as PREGS, ALLO, and allotetrahydroDOC.

In humans, PROG undergoes reduction by 5α-reductase to ALLO and by 5β/3α-reductase to PREG, both of which are metabolites active at $GABA_A$ receptors. The occurrence of these bioreductions in

the human brain has not been conclusively demonstrated to date and is the subject of extensive investigation, particularly in the fetal-placental unit, where these anxiolytic metabolites of PROG are known to be formed. In a recent study of 40 premenopausal women, it was observed that two normal women had no detectable circulating levels of ALLO in the luteal phase of the menstrual cycle. However, all women studied thus far have demonstrable circulating levels (>0.1 ng/ml) of PREG when the plasma PROG level is above 1 ng/ml. It is presumed that the 5α-reduced $GABA_A$ receptor-active neurosteroids in the rat brain will also have their 5β/3α-reduced (termed normal) neurosteroid counterparts in the human brain. Notably, plasma levels of both ALLO and PREG are highly correlated with PROG levels in normal women during the menstrual cycle and pregnancy. In the third trimester of pregnancy, average plasma levels of ALLO and PREG reach approximately 30 ng/ml or approximately 100 nM [576].

13.6. Molecular targets of inhibitory neurosteroids

Neurosteroids can directly modulate various receptors. Inhibitory neurosteroids demonstrate activity at $GABA_A$ receptors, as well as other members of the Cys-loop family. Additionally, they affect various types of ligand-gated ion channels and voltage-gated ion channels [577, 578].

In contrast, potentiating neurosteroids such as ALLO and THDOC exhibit higher sensitivity at $GABA_A$ receptors compared to other members of the Cys-loop receptor family [579]. The homomeric ρ1 $GABA_A$ receptor, however, displays lower sensitivity to potentiating neurosteroids than heteromeric $GABA_A$ receptors [580]. On the other hand, the Drosophila Resistance to Dieldrin $GABA_A$ receptors is insensitive to these neurosteroids but has been instrumental in identifying their binding site at mammalian $GABA_A$ receptors [532]. GlyRs also exhibit little or no sensitivity to potentiating neurosteroids [581-583].

At $GABA_A$ receptors, potentiating neurosteroids exhibit activity in the nanomolar concentration range. Although their potency is similar across different receptor subtypes, these steroids demonstrate greater efficacy at extrasynaptic-type receptors containing the δ subunit [164]. In receptors with the δ subunit, ALLO can enhance the response to GABA EC10 beyond that achieved by a saturating concentration of GABA, and the maximum effect (macroscopic efficacy) is higher than at receptors containing the γ2 subunit. Consequently, a more substantial effect is likely to be observed at low concentrations of neurosteroids at extrasynaptic-type receptors, suggesting that

potentiating neurosteroids may have a more pronounced impact on GABA tonic than on synaptic currents.

Inhibitory neurosteroids (PREGS and DHEAS) exhibit lower selectivity compared to potentiating neurosteroids. GlyRs are inhibited by PREGS and DHEAS in the low micromolar range [584, 585], while the GABA ρ1 receptor shows weak sensitivity to PREGS (IC50 > 300 μM) [586, 587]. Some evidence also suggests that PREGS inhibits nicotinic acetylcholine receptors (nAChRs) at micromolar concentrations in bovine adrenal chromaffin cells [588], and it has been demonstrated to activate homomeric α7 nAChRs [589, 590]. The C. elegans $GABA_A$ receptor homologue UNC-49B/C is antagonized by both PREGS and DHEAS at micromolar concentrations [591, 592].

PREGS has been demonstrated to modulate other receptor families as well. Recombinant NMDA receptors expressed in Xenopus oocytes can be positively and negatively modulated by PREGS depending on subunit composition (EC50, IC50 > 10 μM): receptors comprising the GluN1 subunit expressed with GluN2A or GluN2B are potentiated by PREGS, while those comprising GluN1 with the GluN2C or GluN2D subunit are inhibited [530, 593]. However, there is some evidence that PREGS may increase glutamate release from presynaptic terminals via potentiation of receptors containing GluN2D subunits in hippocampal slices from P3-4 rats [594], suggesting the effect of PREGS on NMDA receptors may be dependent on various factors.

PREGS exhibits diverse effects on various receptor families. It acts as a non-competitive antagonist at α- AMPA and kainate receptors expressed in oocytes (IC50s > 10 μM) [549, 595]. The transient receptor potential melastatin 3 (TRPM3) receptor is directly activated by PREGS (EC50 ~ 20 μM) [596]. PREGS also directly activates sigma1 (σ1) receptors in hippocampal neurons [597]. Additionally, PREGS can potentiate currents through voltage-gated Ca^{2+} channels [598] and inwardly-rectifying K^+ channels containing the Kir2.3 subunit [599]. In contrast, voltage-gated Na^+ channels are inhibited by PREGS [600]. Therefore, PREGS acts on multiple target proteins in the brain, with its highest potency observed at $GABA_A$ receptors. PREGS can also modulate various receptors in the presynaptic terminal, influencing neurotransmitter release. PREGS has been reported to affect acetylcholine release [601, 602], glutamate release [603, 604], glycine release [604], and GABA release [597, 604].

13.7. Inhibitory neurosteroids and the $GABA_A$ receptors

The molecular determinants for the interaction with and inhibition by inhibitory neurosteroids at $GABA_A$ receptors are not fully understood. In contrast to potentiating neurosteroids, inhibitory neurosteroids are less potent [605]. Moreover, these two classes of neurosteroids do not compete for common binding sites at $GABA_A$ receptors [606-608]. Inhibitory steroids act as non-competitive inhibitors at $GABA_A$ receptors with respect to GABA [497, 609] and exhibit state-dependent block, with greater inhibition observed at higher agonist concentrations [610].

Despite similarities in blocking profiles, including activation-dependence and sensitivity to a mutation in the M2 α-helix ($\alpha1^{V256S}$), between sulphated neurosteroids and 3β-hydroxypregnane steroids, these two classes of neurosteroids do not compete for a single binding site [611-613]. Furthermore, inhibition by PREGS shows only weak voltage-dependence, suggesting an unlikely binding to a site, such as in the open channel, experiencing the membrane electric field [609, 610].

Single-channel recordings have demonstrated that PREGS reduces the mean cluster duration of GABA single-channel currents, representing the average time between the first opening and the last closing transition of a channel during bursts occurring between sustained periods of desensitization. Notably, PREGS does not affect intracluster open or closed time distributions [611]. The development of block by PREGS is gradual and occurs at similar rates for open or closed GABA channels. In whole-cell recordings, PREGS has minimal impact on GABA peak currents but results in increased block of steady-state currents, resembling an apparent elevation in the rate of desensitization [614].

The inhibitory effect of PREGS is independent of the presence of a γ2 subunit, as demonstrated by Wang et al. [613], although it exhibits greater potency at receptors containing the γ2 subunit compared to the δ subunit [615]. However, the full characterization of the $GABA_A$ receptor subtype selectivity of PREGS remains incomplete. Prior investigations suggest similar potency of PREGS at recombinant α1β2γ2L and α5β2γ2L receptors expressed in oocytes. Nevertheless, variations are observed between α1, α2, and α3 containing αβγ receptors, with PREGS being 10-fold more potent at receptors containing α3 than α1 and 2.5-fold more potent than at receptors containing α2 [616].

The potentiating neurosteroids exhibit activity at $GABA_A$ receptors at low nanomolar concentrations, inducing direct activation only at higher submicromolar to micromolar concentrations, as reported by Lambert et al. [492] and Hosie et al. [532]. The binding site for the potentiation of $GABA_A$ receptors by neurosteroids is situated at glutamine 241 in the α subunit, a

position conserved among the α1-6 subunits [532, 617]. Mutations at glutamine 241 do not impact PREGS binding, suggesting an unlikely shared binding site for potentiating and inhibitory neurosteroids [608]. Glutamine 241 resides at the base of a water-filled cavity between the M1-M4 interface, a location expected to expand in depth and volume after receptor activation, facilitating neurosteroid binding and potentially sustaining the channel in an open state [532]. Although the binding site for potentiating neurosteroids exists exclusively in the α subunits, introducing M1 from α to β2 or γ2 subunits in receptors with a mutated (and removed) binding site in the α subunit ($\alpha1^{Q241L}$) makes them sensitive to neurosteroids [618].

14. Neurosteroids and epilepsy

14.1. Progesterone and Its Metabolite

Numerous studies suggest a potential role for neurosteroids in epileptogenesis, specifically during the latent period characterized by a seizure-free interval. The latent period is intricately defined as the time span between the impact of the initial brain insult and the emergence of the inaugural spontaneous seizure. Throughout this interval, the brain undergoes pathological alterations, transitioning from its typical state to a seizure-prone condition [619].

In investigations utilizing the pilocarpine model of SE in rats, heightened expression of the P450scc enzyme was observed during the latent period of epilepsy [416]. This enzyme plays a crucial role in the synthesis of neurosteroids in the brain. The translocator protein facilitates the transport of cholesterol into the mitochondria, where P450scc is situated on the inner mitochondrial membrane. Subsequently, cholesterol undergoes cleavage, yielding PREG, which is further metabolized into tissue-specific steroids by enzymes operating in the endoplasmic reticulum [416, 620]. The disruption of neurosteroid synthesis through the administration of finasteride, a 5α-reductase and neurosteroid synthesis inhibitor, following pilocarpine-induced SE in rats, resulted in the cessation of the latent period. Intriguingly, finasteride exhibited a proconvulsant effect in rats with spontaneous seizure activity occurring post the latent period [416]. Additionally, in the mouse hippocampus kindling model of epileptogenesis, where the anti-epileptogenic activity of PROG was assessed, finasteride, acting as a neurosteroid synthesis inhibitor, completely abrogated the PROG anti-epileptogenic effect. This was evident through the retardation of kindled seizures, suggesting a potential dependence of this particular PROG action on ALLO [621]. Seyle et al. [622] were among

the pioneers in conducting this type of study back in 1942, where they assessed the anticonvulsant properties of PROG in the pentylenetetrazol animal model using immature male rats. Subsequently, researchers over the years have consistently affirmed these findings in studies involving both male and female rodents. Various doses of PROG were administered in convulsive animal models, including the amygdala kindling model [623, 624], hippocampal kindling model [625], WAG/Rij rats, the genetic absence model (demonstrating an increase in the number and duration of spike-wave discharges) [626], and the kainate model [627]. Billiar et al. [628] conducted an evaluation of the distribution and metabolism of PROG, along with the products of its metabolism and estradiol. In this instance, a continuous infusion of $[3H]^-$ or [14C] PROG and estradiol [3H] was administered to female rhesus monkeys. The experiments revealed significantly higher hormone levels in cerebral tissues compared to carotid arterial blood, with the estradiol concentration being notably highest in the anterior pituitary (20 times). Regarding PROG, diminished concentrations were noted in the "central gray," while concentration levels remained consistent for the amygdala, hippocampus, preoptic-anterior hypothalamus, cerebellum, hypothalamus, thalamus, and anterior pituitary. Higher concentrations were observed in the cervical spinal cord, optic chiasm, mesencephalon, medulla oblongata, and pons when compared with the control group [628]. Extensive research over the years has consistently shown that the administration of exogenous PROG leads to a threefold increase in its concentration in cerebral tissue compared to peripheral tissue levels. In the case of administering both PROG and its metabolite, 5α-DHP, to rats via IV administration, the compounds accumulated in the brain, with the highest concentration in the hypothalamus and anterior pituitary regions. However, the concentration in the cerebral cortex was notably low [629]. While the high concentration in the anterior pituitary and hypothalamus is associated with gonadotropin release [629], the PROG metabolite may possess anticonvulsive potential. IV 5α-DHP, in fact, demonstrated a reduction in generalized and focal seizures in female fully kindled rats [630], and this effect was linked to its interaction with $GABA_A$ receptors [631].

Akula et al. [632] conducted a comparison of the anticonvulsant effects of PROG (at doses ranging from 20-80 mg/kg s.c.) and ASMs on the IV pentylenetetrazol-induced seizure threshold in mice. All the compounds investigated exhibited anticonvulsant effects in a dose-dependent manner. Notably, PROG demonstrated a more potent effect compared to tiagabine, GABA, adenosine, and gabapentin. However, its effect was found to be less pronounced than that of clonazepam, diazepam, chlordiazepoxide, phenobarbital, carbamazepine, pentobarbital, pregabalin, and

phenytoin [632]. Clinical data suggests that SE may be linked to a significant reduction in CSF concentration of PROG, reaching levels 64% lower than those observed in matched controls [633].

14.2. Allopregnanolone

Allopregnanolone (ALLO), among the recognized neurosteroids, stands out as the potent and extensively investigated natural endogenous positive modulator of $GABA_A$ receptors [634]. This compound plays a crucial role in the maturation of the CNS and influences various behaviors in adult life, as substantiated by Mòdol et al. [635]. Notably, ALLO has been shown to promote myelinization, synaptogenesis, and exhibit protective and trophic properties concerning neurons, both during developmental stages and in the context of disorders [636-638]. Extensive research underscores the significant role of disturbances in ALLO levels in the pathomechanism of various diseases, including neurological and psychiatric disorders [639]. Altering neurosteroid levels in rat neonates, particularly ALLO, has been shown to modify exploratory and anxiety-like behavior, as well as disrupt aversive learning in adult animals [635].

In a study by Lévesque et al. [640], the impact of ALLO (administered at doses of 9.6-12.8 mg/kg/day) on interictal jumps and high-frequency oscillations (ripples: 80-200 Hz, rapid ripples: 250-500 Hz) was assessed in the pilocarpine model of mTLE. The neurosteroid significantly reduced the frequency of interictal spikes and fast ripples in the hippocampal CA3 field compared to the control group. Further investigations demonstrated the anticonvulsant properties of ALLO, mitigating both behavioral and electrographic seizures in a model of SE, even in the presence of benzodiazepine resistance [169]. Notably, hippocampal ALLO concentration showed a significant decrease in rats surviving kainate-induced SE, as measured nine weeks later [641].

Consistent with findings in rodents, human data support these observations. Meletti et al. [633, 642] analyzed ALLO concentrations in CSF samples from patients experiencing SE, revealing a significant approximately 30% reduction in ALLO levels. Additionally, two cases of adults with super-refractory SE in 2017 reported positive outcomes with the introduction of ALLO treatment (administered at doses of 5.6 mg/h for 5 days through a 120 h continuous infusion) [643].

14.3. Deoxycorticosterone

Deoxycorticosterone (DOC), a neurosteroid recognized as a mineralocorticoid precursor, exhibits anticonvulsant properties through its enzymatic conversion to THDOC. This converted neurosteroid acts as a positive allosteric modulator of $GABA_A$ receptors. Stress-induced fluctuations in DOC levels

can occur due to its synthesis regulation by ACTH hormone, suggesting a connection between stress-dependent alterations in susceptibility to seizures, particularly notable in children [644].

Notably, in adult rats subjected to gamma-hydroxybutyric acid challenge, a model simulating generalized absence seizures, THDOC, when administered systemically or locally into the thalamic ventrobasal nucleus, unexpectedly potentiated seizure activity [645].

ACTH demonstrates anticonvulsant efficacy in various childhood seizure types; however, its utilization is accompanied by numerous side effects. Despite the unknown mechanism underlying ACTH's anticonvulsant actions, it is established that ACTH induces the release of DOC alongside cortisol from the adrenal cortex. It has been proposed that DOC may, at least partially, mediate the anticonvulsant effects of ACTH. In an experiment [646] it was aimed to evaluate the anticonvulsant properties of DOC in infant rats, examining age-related alterations in DOC's actions. The assessment of DOC's anticonvulsant effects encompassed hippocampal-kindled seizures, maximal pentylenetetrazol test, and maximal electroshock seizures in 15-day-old rats. Age-related changes in responsiveness to DOC were also explored using the maximal pentylenetetrazol test model. DOC exhibited suppression of generalized convulsions in all three seizure models. Notably, focal spiking in the hippocampal-kindling model remained incompletely suppressed, even at elevated doses. The manifestation of ataxia escalated proportionately with the administered dose, with the time of peak seizure suppression roughly corresponding to the zenith of ataxia in all models. Anticonvulsant efficacy was observed in both infant and adult rats; however, the adult population exhibited significantly higher ED50s. Young rats displayed ataxia at the time of testing (15 minutes), whereas adults did not, although ataxia became apparent at later time points. In conclusion, DOC emerged as a potent anticonvulsant against generalized seizures, particularly in infants, warranting further clinical evaluation for its efficacy against generalized seizures in this age group [646].

Edwards et al. [647], in their investigations, delved into the underlying mechanisms responsible for the immediate anticonvulsant effects of DOC in juvenile rats. Fifteen-day-old rats underwent pretreatment with various compounds, encompassing (a) agonists targeting the receptors binding DOC, specifically mineralocorticoid receptors; (b) the 5α− and 5α-3α–reduced metabolites of DOC, along with agonists binding to the receptors of the 5α−reduced metabolite of DOC (progesterone receptors); and (c) DOC itself, both in the presence and absence of metabolism and receptor blockers. Subsequently, pentylenetetrazol was administered after fifteen minutes, and the ensuing maximal pentylenetetrazol seizure responses were evaluated. Agonists targeting mineralocorticoid

receptors augmented the latency to forelimb flexion in PTZ seizures, occasionally resulting in complete seizure suppression. Notably, at low, nonconvulsant doses, spironolactone (a mineralocorticoid-receptor antagonist) hindered the anticonvulsant effects of a nonsedating, though not a sedating, dose of DOC. These results hint at the plausible direct involvement of mineralocorticoid receptors in DOC's anticonvulsant effects. Additionally, at low, nonconvulsant doses, finasteride (impeding the metabolism of DOC) partially impeded the protective effects of DOC, indicating the potential contribution of metabolites to DOC's anticonvulsant actions. DHDOC, the primary metabolite of DOC, acting as an agonist at progesterone receptors and an allosteric modulator of the $GABA_A$ receptor, as well as THDOC, a secondary metabolite of DOC serving as an allosteric modulator of the $GABA_A$ receptor, both demonstrated the capacity to block maximal pentylenetetrazol seizures. These findings posit that both DOC and its metabolites may collectively contribute to the observed anticonvulsant effects in juvenile rats, potentially exerting their influence through interactions with various receptors [647].

14.4. Pregnenolone Sulphate

Pregnenolone Sulphate (PREGS), in contrast to other neurosteroids, appears to exhibit proconvulsive activity based on available research findings. Maciejak et al. [648] demonstrated that an elevated level of PREGS led to an increase in alanine concentration, a precursor of glutamate known to contribute to seizure development. Similarly, Reddy and Kulkarni, in their study investigating the impact of long-term administration of PREGS and DHEAS (both at a dose of 10 mg/kg/day for 4 weeks) on convulsive activity in the mouse pentylenetetrazol model, observed a distinct decrease in the seizure threshold. Notably, the proconvulsive effects induced by long-term DHEAS administration were prevented by pre-treatment with PROG (at a dose of 5 mg/kg) or ALLO (at a dose of 0.1 mg/kg) [648].

14.5. Ganaxolone

Ganaxolone (3alpha-hydroxy-3beta-methyl-5alpha-pregnan-20-one; GNX) is classified as an exogenous neurosteroid, serving as the 3betamethylated exogenous analogue of ALLO. GNX shares a mechanism similar to its natural counterpart, acting as an allosteric modulator of $GABA_A$ receptors by binding to both synaptic and extrasynaptic $GABA_A$ receptors [649, 650]. The activation of synaptic $GABA_A$ receptors is associated with specific effects, while the activation of extrasynaptic

$GABA_A$ receptors is linked to persistent or tonic inhibition [651]. Importantly, studies have confirmed that this neurosteroid does not activate classic nuclear PROG receptors [652].

GNX demonstrates notable anticonvulsive properties across various animal seizure models, encompassing limbic seizures in the 6-Hz model, clonic seizures induced by pentylenetetrazol, and bicuculline or amygdala-kindled seizures [652]. Gasior et al. [653], in their investigations, compared GNX's activity with diazepam and valproate. Their findings indicated that GNX, with an effective dose predicted to protect 50% of the mice (ED50) at 3.45 mg/kg against the clonic phase, proved to be the most effective anticonvulsant. It not only reduced convulsive activity, including clonic and tonic seizures, but also mitigated the lethal effects of pentylenetetrazol. In contrast, diazepam exhibited anticonvulsant activity targeting tonic seizures and lethality, while valproate specifically suppressed tonic attacks [653]. These results were further validated in studies involving behavioral and electrographic seizures in fully amygdala-kindled mice, a representative model of mTLE [653]. Mares and Stehĺíková [654] explored the activity of GNX in cortical epileptic afterdischarges using rats of different ages (12, 18, and 25 days old). GNX administration at 40 mg/kg inhibited the progressive prolongation of cortical epileptic afterdischarges in 25-day-old rats and delayed it in 12-day-old rats. However, no significant protective effects were observed in 18-day-old rats [521]. GNX was also assessed in WAG/Rij rats, a genetic model of absence epilepsy, through intracerebral injections [654]. Bilateral administration into thalamic nuclei exacerbated the occurrence of epileptic spike-wave discharges. Conversely, microinjection into the peri-oral region of the primary somatosensory cortex suppressed spike-wave discharges. Notably, ALLO produced a very similar response, and the effects of PREGS were dose-dependent, exhibiting proconvulsant effects at low doses and anticonvulsant effects at higher doses when microinjected into thalamic nuclei or the somatosensory cortex [655].

GNX, administered at a daily dose of 1500 mg, underwent scrutiny as an adjunctive therapy in randomized, placebo-controlled trials involving adults with partial onset epilepsy, with or without secondary generalization. A Phase II trial included 147 refractory adults (100 females and 47 males, aged 18-69 years). Of the 131 participants completing the trial, 124 entered the open-label extension study. The outcomes were promising, with GNX leading to an 18% reduction in mean weekly seizure frequency, compared to a 2% increase in the placebo group. Responder rates, indicating a reduction of seizures by at least 50%, were 26% and 13% in the GNX and placebo groups, respectively. The positive efficacy of GNX appears to be sustained long-term, as

demonstrated in the open-label extension [656]. Despite some adverse effects, such as dizziness, fatigue, and somnolence, leading to treatment discontinuation in 7% of GNX and 6% of placebo subjects, 36 patients continued GNX treatment for over a year in the open-label extension, and adverse effects did not differ significantly from those reported in the Phase II trial [655]. Additional results from the open-label extension, published in 2013, highlighted adverse effects observed in more than 10% of patients, including headache (21%), convulsion (16%), fatigue (16%), fall (14%), nasopharyngitis (14%), dizziness (13%), contusion (12%), and nasal congestion (10%) [657]. A detailed analysis in 2017 emphasized that adverse effects in the GNX group were generally mild to moderate. Among untoward events leading to discontinuation in the GNX group were headache and lethargy, while in the placebo group, postictal psychosis, headache, and convulsion occurred. Clinical laboratory tests did not reveal major disturbances, with mild thrombocytopenia found in only one patient on GNX, and it did not result in treatment discontinuation [658].

14.6. Role of neurosteroids in epileptogenesis

As it was written before, neurosteroids are steroids synthesized within the brain, exerting unconventional and rapid effects on neuronal excitability. Well-established steroid hormones, such as PROG and DOC, are known to possess anticonvulsant actions [659, 660]. The anticonvulsant properties of PROG and DOC primarily stem from their conversion in the brain into neurosteroids, specifically ALLO and THDOC, respectively [523]. Emerging evidence suggests a role for endogenous neurosteroids in regulating epileptogenesis [252, 661]. Previous studies in our lab demonstrated that P450scc, a crucial enzyme in steroid synthesis, was upregulated in the CA3 hippocampal subfield following pilocarpine-induced SE. These changes predominantly occurred in glial cells, although there was also a transient increase observed in neurons. Importantly, the induction of P450scc was more pronounced with longer episodes of SE. Similar increases in P450scc staining were evident in other limbic areas, including the DG, subiculum, and entorhinal cortex, in the pilocarpine-treated rats. Furthermore, P450scc was induced by SE in a diverse population of hippocampal glia. This induction of P450scc was linked to the delayed onset of spontaneous seizures. Conversely, inhibiting neurosteroid synthesis accelerated the onset of spontaneous seizures, but this effect was observed only in animals with significant increases in P450scc. These

findings imply that the induction of neurosteroid synthesis in reactive glial cells is associated with a delayed onset of spontaneously recurrent seizures [661].

The kindling model demonstrated impaired development and persistence of limbic epileptogenesis in mice lacking PRs [662]. To investigate the mechanisms behind the observed seizure resistance, the role of neurosteroids was explored using finasteride, a 5α-reductase inhibitor blocking the synthesis of progesterone-derived neurosteroids. The rate of rapid kindling was assessed in both control animals and those receiving PROG injections with or without concurrent finasteride treatment [621]. PROG significantly delayed the rate of kindling, and pretreatment with finasteride blocked PROG's inhibition of kindling epileptogenesis [621]. These findings indicate a contributory role of neurosteroids in limbic epileptogenesis, suggesting that inhibiting neurosteroids may incite mechanisms promoting epileptogenesis.

The induction of P450scc in glial cells provided suggestive but not necessarily predictive evidence of increased neurosteroid synthesis in the brains of pilocarpine-treated rats [663]. To further investigate this, the enzyme 5α-reductase was inhibited using the specific and irreversible inhibitor finasteride (100 mg/kg). This intervention aimed to assess whether inhibiting 5α-reductase could influence the onset of seizures in pilocarpine-treated rats, potentially by reducing the synthesis of neurosteroids such as ALLO known to enhance inhibition through interaction with $GABA_A$ receptors [531]. Finasteride at 10 mg/kg was demonstrated to acutely deplete ALLO without affecting 3α-dihydroprogesterone, PROG, or DOC [664]. Repeated administration at 25 mg/kg significantly decreased both ALLO and THDOC brain levels [509]. To eliminate the possibility that the effects of finasteride on the latent period could be attributed to other variables, various paradigms of SE duration were tested, and the correlation between 5α-reductase inhibition, P450scc induction, and the latent period duration was explored. In rats experiencing at least 180 minutes of SE, finasteride (100 mg/kg) led to an early onset of stage 5 seizures, consistent with the correlation among SE duration, P450scc induction, and the latent period duration [665]. In contrast, the same finasteride treatment was ineffective in rats exposed to 90 minutes of pilocarpine-induced SE, where only limited changes in P450scc expression were observed [661]. Finasteride also failed to alter the duration of the latent period in adult (8-week-old) rats exposed to 60 minutes of SE, which exhibited SRSs approximately 10 days after SE [537]. However, stage 5 seizures appeared in around 50% of young (3-week-old) rats exposed to 60 minutes of SE and treated with finasteride during the first week of treatment when P450scc levels were notably high. No further stage 5 seizures were

observed in subsequent finasteride treatment days when P450scc immunoreactivity reached its peak level [537]. Importantly, the experimental groups exposed to 60 minutes of SE differed in the extent and duration of P450scc induction, with markedly higher levels in the CA3 hippocampal subfield of young rats. These findings suggest a relationship between neurosteroid synthesis, the extent of P450scc induction in glial cells after SE, and the influence of neurosteroids on the latent period [666].

The development of epilepsy is intricately linked to complex alterations in neuroplastic mechanisms, and dysregulation of neurosteroid synthesis may play a role, as explored in various epileptogenic models [662]. The role of the prototype endogenous neurosteroid ALLO in controlling limbic epileptogenesis was investigated. Treatment with finasteride, a neurosteroid synthesis inhibitor, resulted in a significant increase in epileptogenesis in the hippocampus kindling model [621]. Exogenous administration of ALLO at doses producing levels similar to gonadotropins markedly inhibited epileptogenesis. In female epilepsy rats, finasteride treatment exacerbated seizure frequency [667]. Neurosteroid-mediated increase in tonic inhibition in the hippocampus could inhibit the spread of the seizure discharge from the hippocampal focus, thereby suppressing the rate of development of behavioral kindled seizure activity without affecting the focal electrographic discharges. The exact mechanisms are unclear, but increased tonic inhibition by ALLO is shown to impair NMDA receptor-mediated excitability in the hippocampus [668]. It is likely that such a mechanism may underlie PROG's disease-modifying effect in the kindling model. Based on these pilot studies, it is suggested that augmentation of neurosteroid synthesis may represent a unique strategy for preventing or retarding epileptogenesis.

We obtained evidence that the competitive inhibitor of 3β-HSD trilostane has a modulatory effect on epileptogenesis, so as to be considered a potential AED. We recently discovered that, after inducing SE, rats treated with trilostane or a vehicle for 6 days exhibited a significant increase in neocortical levels of PREG, PREGS, PROG, 5α-DHP, and ALLO. Specifically, this change was dependent on the effects of trilostane, as assessed 6 hours after the last injection, in comparison to all other treatment groups. Additionally, neocortical levels of PREG were affected by trilostane, leading to a noteworthy reduction in the amount.

Trilostane also induced significant changes in the hippocampus by increasing PREG, PROG, 5α-DHP, and ALLO levels. Post hoc comparisons revealed that PREG increased after daily trilostane treatment compared to the vehicle-treated group with the same dosage interval or the vehicle-

treated group after 1 week of washout. PREG levels returned to basal levels 1 week after the last trilostane injection (6 days of trilostane treatment). PROG levels significantly increased after trilostane administration but returned to normal after the washout (6 days of trilostane treatment). Six hours after the last injection of trilostane, 5α-DHP significantly increased, along with a marked increase in ALLO ($p = 0.035$ vs. the vehicle-treated group at the same time, $p = 0.015$ vs. the sham-treated vehicle). In the hippocampus, trilostane did not affect the levels of PREGS or PREG. PREG was undetectable 6 hours after the last injection of trilostane but returned to normal after the washout. In this experiment, we assessed the latency for rats (treated with trilostane for 6 days) to develop the first electrocorticographic (ECoG) SRS and the first convulsive SRS, with or without loss of posture. Following repeated administration of trilostane ($n = 12$) or vehicle ($n = 13$), the latency to develop the first ECoG SRS was significantly increased in the trilostane group. Additionally, the latency to develop the first convulsive SRS was also significantly increased by trilostane. Consistently, the latency to develop the first convulsive SRS with loss of posture was significantly longer after treatment with trilostane. To explore the differences found in the time course of epileptogenesis, we hypothesized that the vehicle- and trilostane-treated rats could have developed a different SE. However, this hypothesis was not confirmed by analyzing the video-ECoG of vehicle- or trilostane-treated rats, which exhibited similar latencies to develop seizures and analogous SE duration. To further investigate whether trilostane administration during SE could modify the time course of epileptogenesis, we conducted a second experiment characterizing the impact of a single trilostane administration on the onset of SRSs. Again, trilostane ($n = 12$) or vehicle ($n = 12$) was administered 10 minutes after the IP administration of KA. In contrast with the first experiment, this protocol did not change the characteristics of SE. However, the latency to develop SRSs was similar in both treatment groups.

14.7. Neurosteroids and the chronic period of epilepsy

Multiple lines of evidence suggest that neurosteroids play a modulatory role in epilepsy during the chronic phase of the disease, characterized by the manifestation of motor seizures. The catamenial exacerbation of epileptic seizures, which could be interpreted as evidence of persisting epileptogenesis in TLE, provides compelling support for the involvement of steroids in this chronic neurological disorder. This phenomenon is explained by the influence on $GABA_A$ receptor plasticity

exerted by fluctuations in steroid production and their conversion into neurosteroids [669]. The modulatory properties of peripheral steroids and their relationship with physiological fluctuations in neurosteroids during the ovarian cycle have been elucidated by analyzing changes in δ-$GABA_A$ receptor subunits in mice, with seizure threshold assessed by KA administration [670]. The study revealed that the amplitude of tonic $GABA_A$ receptor-mediated current is two-fold higher during late diestrus in DG granule cells. This change was correlated with a 43% increase in hippocampal δ-$GABA_A$ subunit levels, and both findings were associated with fluctuations in PROG plasma levels. Remarkably, female mice injected with kainate during diestrus, when PROG levels peak, exhibited a more pronounced latency to seizure appearance, and the mean seizure duration was significantly shorter. These findings strongly suggest a role for PROG in producing $GABA_A$-mediated antiseizure effects during the perimenstrual period.

15. Neurosteroids and status epilepticus[1]

Abstract

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Status epilepticus (SE) is a common neurological emergency with considerable associated health-care costs, morbidity, and mortality. In about one-third of cases, SE is refractory toward first-line intravenous benzodiazepines. Allopregnanolone, a neurosteroid that positively modulates synaptic and extrasynaptic g-aminobutyric acid type A receptors, has been evaluated as a possible novel treatment for SE. Notwithstanding the positive results obtained in the animal models of SE, the use of allopregnanolone for the treatment of benzodiazepine-resistant SE in humans resulted in controversial findings. Here, we summarize the main preclinical and clinical evidence about the effects of neurosteroids on SE to provide a possible pathophysiological background for their rational use.

Keywords: Allopregnanolone, g-aminobutyric acid type A receptor, Ganaxolone, Neurosteroids, Status epilepticus.

Introduction

Status epilepticus (SE) is a potentially fatal neurological disorder that affects approximately 41 people per 100,000 adults, with a mortality rate of 0-3% in children [142, 671-674] and 20-30% in adults [675, 676]. SE is characterized by persistent seizures, caused by either a failure to terminate them or by mechanisms that result in abnormally protracted epileptic activity that may be resistant to antiepileptic drugs or other pharmacological treatments. Current medical guidelines recommend a rapid stepwise approach, beginning with benzodiazepines in monotherapy, followed by intravenously administered antiepileptic drugs as second-line medications if SE persists and the third by general anesthetic to induce pharmacologic coma [672, 677-679]. Despite an increasing number of available drugs, approximately one-third of SE cases are refractory SE (RSE) or super-refractory (SRSE) to benzodiazepines and second-line medications [138]. For this reason, new therapeutic approaches are needed. To this aim, it is important to improve our understanding of the mechanisms by which seizures can evolve into a fully developed SE.

Involvement of γ-aminobutyric acid type A receptors in the pathophysiology of status epilepticus

During the SE, intrinsic brain mechanisms are unable to terminate the seizure, and abnormal excitatory mechanisms are activated to maintain a prolonged generation of seizures. Several changes such as protein phosphorylation, neurotransmitter release, and modification in ion channel

configuration occur and could allow a seizure to progress into the SE. In particular, clinical and experimental evidence suggests that changes in the localization and subunit composition of γ-aminobutyric acid type A ($GABA_A$) receptors are implicated in the pathophysiology of SE [150]. Thus, understanding the role of these receptors could lead to innovative and effective therapeutic approaches.

Most $GABA_A$ receptors are expressed in neuronal tissue and represent the principal mediators of inhibitory transmission within the CNS. $GABA_A$ receptors are formed by the assembly of multiple subunit subtypes (α1-α6, β1- β3, γ1-γ3, δ, ε, θ, and ρ1- ρ3) into a pentamer to form chloride ion channels that can be differently modulated by several allosteric regulators, including benzodiazepines and neurosteroids [531, 680]. Although the subunit composition affects its functional and pharmacological properties, also cellular localization is crucial. During the SE, $GABA_A$ synaptic receptors (β 2/3 and γ 2 subunits) internalize from the cell surface, causing a reduction in inhibition at the synaptic level [156]. These receptors, which are found throughout the brain, typically contain γ2 subunit and are essential for the response to benzodiazepines, the first-line therapy for seizure termination [155]. Their internalization reduces neuronal postsynaptic inhibition and determines a progressive loss of sensitivity to benzodiazepines. Surprisingly, extrasynaptic $GABA_A$ receptors (δ and/or α4 subunits) are not affected by this phenomenon [156]. They are mostly found in the hippocampus, thalamus, amygdala, hypothalamus, and cerebellum, and the presence of d subunit makes these receptors insensitive to a range of benzodiazepines, suggesting that they must be differently targeted [157].

The neurosteroids allopregnanolone (ALLO), pregnanolone (PREG), and THDOC are known to modulate the extrasynaptic $GABA_A$ receptors. In the CNS, progesterone is actively converted to 5αdihydroprogesterone (5αDHP) and subsequently, by the action of the enzyme 3a-hydroxysteroid oxidoreductase, to ALLO. Notably, ALLO acts as a potent positive allosteric modulator of GABAergic transmission by enhancing GABA-mediated opening of the Cl^- channel in the $GABA_A$ receptor, hence prolonging inhibitory postsynaptic currents [162, 163]. This neurosteroid acts not only on extrasynaptic but also on synaptic $GABA_A$ receptors and, thus, enhances both phasic (synaptic) and tonic (extrasynaptic) inhibition [164-166]. However, extrasynaptic $GABA_A$ receptors are more sensitive to ALLO modulation, thanks to the presence of the δ subunit (Figure 12) [167]. Similar to ALLO, THDOC also exhibits a greater potency for δ-containing extrasynaptic receptors [168]. Indeed, the extrasynaptically expressed δ $GABA_A$ receptors in the dentate gyrus and other regions

contribute to tonic inhibition, network shunting, and seizure susceptibility reduction. Natural and synthetic neurosteroids such as ALLO and related pregnane analogs maximize tonic inhibition in the hippocampus and provide effective protection against a variety of limbic seizures and SE. Owing to its peculiar activity throughout the $GABA_A$ receptors and the higher sensitivity to extrasynaptic receptors, ALLO has been proposed for the treatment of benzodiazepine-resistant SE [169].

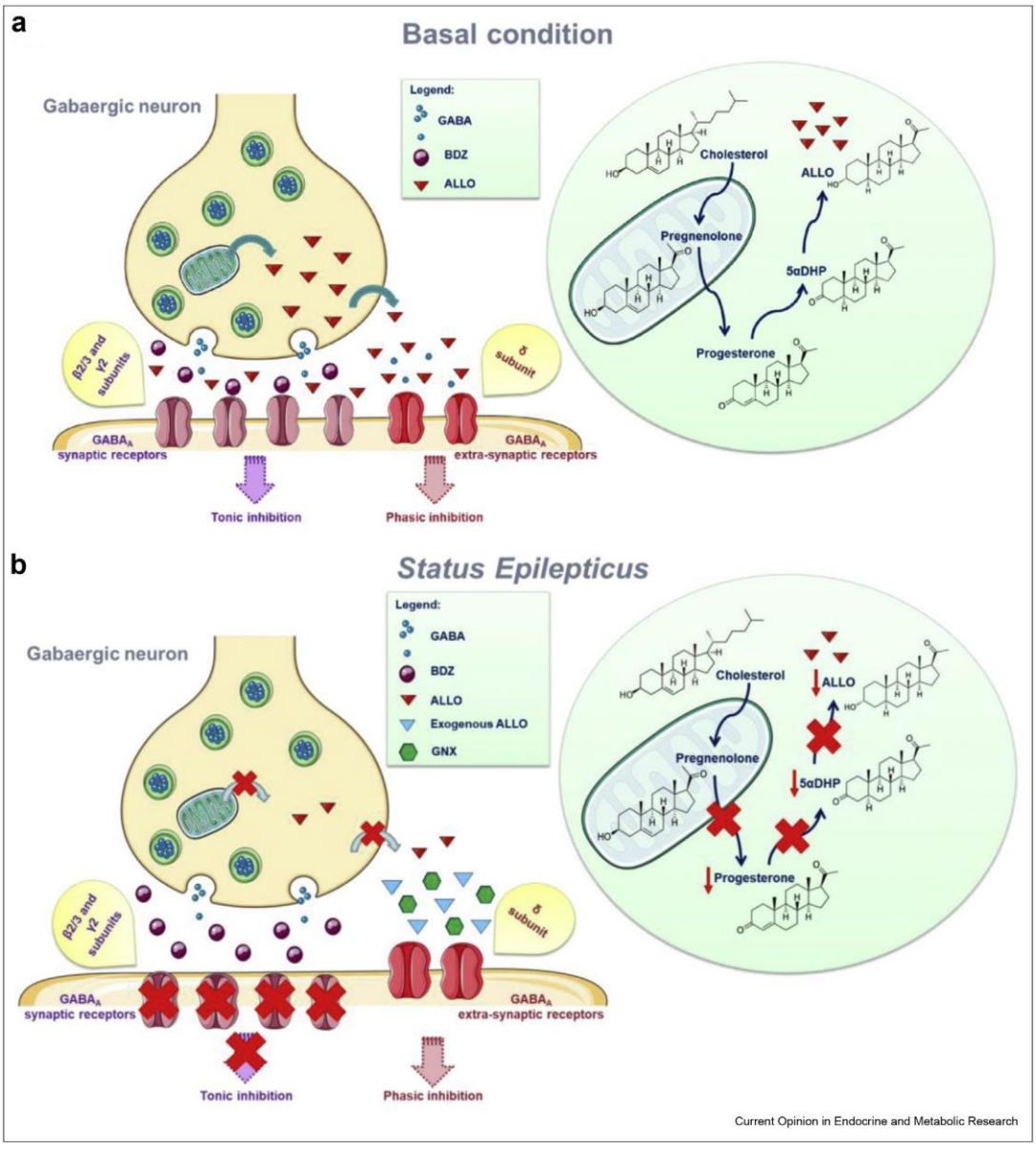

Figure 12. Allopregnanolone (ALLO) biosynthesis and action on g-aminobutyric acid type A ($GABA_A$) receptors in the brain. (a): In physiological conditions, the release of β-aminobutyric acid (GABA) from vesicles rapidly activates postsynaptic $GABA_A$ receptors, resulting in a transient tiny inhibitory postsynaptic current (phasic response). In addition, benzodiazepines (BDZs) produce most of their pharmacological actions by specifically enhancing postsynaptic $GABA_A$ receptors. ALLO is synthesized from progesterone and released in the synapse cleft by GABAergic neurons. Particularly, ALLO acts as a potent positive allosteric modulator of GABAergic transmission by enhancing both synaptic and extrasynaptic $GABA_A$ receptors, enhancing phasic and tonic inhibition. However,

because of the presence of the δ subunit, extrasynaptic $GABA_A$ receptors are more sensitive to ALLO modulation. (b): During the status epilepticus (SE), $GABA_A$ synaptic receptors (β2/3 and γ2 subunits) internalize from the cell surface, leading to a reduction in synaptic inhibition and a progressive loss of BDZ sensitivity. Extrasynaptic $GABA_A$ receptors (δ subunit) are not affected by this phenomenon, but both the decrease in ALLO synthesis and the lack of interaction with these receptors contribute to the persistence of this pathological condition. Because ALLO and ganaxolone (GNX) have similar activity on synaptic and extrasynaptic $GABA_A$ receptors, their exogenous administration can be effective in the treatment of BDZ-resistant SE. Other abbreviations: 5αDHP, 5α-dihydroprogesterone.

Efficacy of neurosteroids in animal models of status epilepticus

Based on reports showing that both ALLO and its synthetic 3β-methyl analog, ganaxolone (GNX), exhibit antiseizure activity in different in vivo seizure models, these molecules were tested also in animal models of SE.

In 2017, Le´vesque et al. [640] showed that 67% of untreated male rats exhibited seizures compared with 29% of ALLO-treated animals in the model of pilocarpine-induced SE. This experiment showed that ALLO reduces the frequency of interictal spikes and fast ripples in cornu ammonis 3 (CA3), a structure that plays an important role in ictogenesis and epileptogenesis.

Zolkowska et al. [681] found that intramuscular administration of ALLO or GNX (each at 3 mg/kg), 40 min after the onset of SE caused by tetramethylenedisulfotetramine, terminated the SE in, respectively, 92% and 75% of male mice, so preventing death in 85% and 50% of animals; the mean time intervals to termination of behavioral seizures were, respectively, 172 and 447 s. ALLO's beneficial effect is most likely owing to its more potent activity on $GABA_A$ receptors. According to others, intravenous (IV) injection of GNX in male rats reduced seizure severity in a dose and time-dependent manner, and its pharmacological profile was suggested to be advantageous concerning ALLO and other existing SE treatments [682]. Specifically, the longer duration of action of GNX as compared with ALLO was attributed to the lower rate of elimination of GNX: when administered at the same dose, GNX had higher exposure levels and a longer half-life (contains a 3β-methylation substitution conferring improved metabolic stability) than ALLO. The first study was conducted with intramuscular administration that produced higher initial ALLO plasma and brain levels which can be the reason why ALLO produced a faster onset of action and a better antiepileptic response than GNX.

Althaus et al. [683] found that administration of 5.6, 7.5, and 10 mg/kg SGE-516 doses, a novel synthetic neuroactive steroid with a similar mechanism of action to that of ALLO, significantly reduced aberrant spiking 20 min after commencement of SE in the soman-intoxication model of organophosphorus nerve agent-induced SE, and 10 mg/kg of SGE-516 reduced seizure activity even when administered 40 min after SE onset. Furthermore, all cohorts of male rats treated with SGE-516 exhibited significantly reduced neuronal cell death compared with the control treatment. Interestingly, IV administration of SGE-516 allows for faster action and higher brain exposures per dose than intraperitoneal administration. Indeed, the time between SGE-516 administration and reduction in SE was longer after intraperitoneal administration than IV.

In addition, Rogawski et al. [684] have recently published an article in which combinations of midazolam (MDZ), ALLO, and the a-amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid antagonist perampanel (PPL) were assessed for antiseizure activity in a diisopropylfluorophosphate SE model, using male rats. The combination of MDZ with either ALLO (6 mg/kg) or PPL (2 mg/kg) terminated the SE, but the onset of the PPL effect was slow. The combination of MDZ ALLO PPL caused a rapid and persistent suppression of electroencephalogram amplitude and largely prevented late rhythmic spike activity and, at the same time, consistently eliminated behavioral seizures. Animals that received MDZ alone exhibited spontaneous recurrent seizures, whereas those that received the combination of three drugs did not, suggesting the prevention of epileptogenesis. All combination treatments reduced neurodegeneration to a greater extent than MDZ alone, and most reduced astrogliosis but had mixed effects on markers of microglial activation. It was concluded that ALLO, a positive modulator of the $GABA_A$ receptor, and PPL, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist, are potential adjuncts to MDZ in the treatment of benzodiazepine-refractory organophosphate nerve agent-induced SE. In agreement, when compared with rats treated with delayed diazepam monotherapy, delayed dual therapy with PREG and diazepam reduced time in SE in sarin-exposed male rats. At one month and three months after exposure, the combination therapy of PREG and diazepam prevented impairment in the Morris water maze and reduced neuronal loss and degeneration [685].

Excessive production of reactive oxygen species (ROS) in abnormal conditions like SE can cause severe oxidative damage, resulting in altered neuronal function and neuronal cell death. Recently,

the neuroprotective effect of ALLO on ROS-mediated neuronal damage was evaluated in the pilocarpine-induced SE mouse model [686]. Hippocampal cell death, DNA fragmentation, oxidative DNA damage, and ROS production were significantly reduced in mice treated with ALLO after SE compared with mice treated with the vehicle. The antioxidant properties of ALLO were probably related to the induction of superoxide dismutase 2, which is one of the most powerful antioxidant enzymes and represents the first line of defense against the effects of ROS; thus, superoxide dismutase 2 induction is reasonably involved in the mediation of ALLO-related neuroprotection from the SE.

Recently, we found that injection of trilostane to inhibit all isoforms of the enzyme 3b-hydroxysteroid dehydrogenase induces a remarkable increase in the hippocampal and neocortical levels of neurosteroids such as ALLO and PREG [687]. Although trilostane-treated male rats had shorter electrocorticographic traces, the increase in ALLO and PREG levels did not affect SE cessation but were able to modulate it. In another experiment [641], we found that ALLO and PREG were reduced in the hippocampus of epileptic male rats, and linear regression analysis indicated that only ALLO levels were modulated by seizures. This suggests that ALLO synthesis could be stimulated in the presence of epileptic activity to represent a possible compensatory but insufficient mechanism to recover the tissue levels of this neurosteroid. The importance of this neurosteroid was further highlighted in an experiment in which levetiracetam was abruptly withdrawn in epileptic male rats. In such a condition, seizures markedly increased in frequency, and ALLO hippocampal levels positively correlated with the seizure frequency [688].

Translational approaches

In contrast with the numerous preclinical investigations showing that ALLO and other neurosteroids have a beneficial effect in various animal models of SE, only a few human studies reported promising results. In 2013, at the 4th Colloquium on SE in Salzburg, Vaitkevicius et al. [689] reported for the first time the use of ALLO infusion in a human patient. A 23-year-old man was admitted to the hospital with a new-onset RSE. Because the seizures were refractory to any pharmacological intervention, IV general anesthesia was established. It is well known that prolonged exposure to barbiturates and benzodiazepines results in systemic side effects owing to tolerance and physical dependence. Furthermore, these drugs cause a high propensity for the recurrence of epileptic

seizures. A 5-day continuous infusion of ALLO (5.6 mg/h) allowed the patient to successfully wean from pentobarbital without recurrence of SE. His clinical recovery was excellent.

In 2014, Broomall et al. [690] reported the first use of ALLO infusion in the treatment of pediatric SRSE. SRSE is a neurologic emergency with high morbidity and mortality. In addition, SRSE is managed with benzodiazepines and barbiturates or general anesthesia, but treatment is limited by side effects and pharmacoresistant. In this clinical study, healthy 11-year-old and 2-year-old girls were successfully treated with a continuous infusion of ALLO (86 mg/kg/h). In particular, ALLO treatment resulted in the cessation of SE and their cognitive improvement.

Another successful ALLO treatment was described in a 28-year-old adult man with SRSE [643]. After several days of ineffective treatment, the patient was administered ALLO over five days. The electroencephalogram (EEG) gradually resembled a normal waveform shape from roughly 60 h after the initial infusion till 7 days after ALLO was terminated. At a 3-year follow-up, the patient demonstrated good cognitive function with occasional seizures.

Regarding GNX, the synthetic derivative of ALLO, a phase II study evaluated its IV efficacy in patients (8 men and 9 women) with RSE. The results are encouraging. In particular, none of the 17 patients involved required IV anesthetic within 24 hours (h) of starting GNX. There was no extra intervention to treat SE recurrence in all patients after GNX termination.

In 2017, the first clinical trial of brexanolone, a proprietary formulation of ALLO, was performed by Rosenthal et al. [691] for the treatment of SRSE. The response assessment population included 22 patients; of whom, 16 received the standard dose (86 mg/kg/hour) regimen, and 6 received the high-dose (156 mg/kg/hour) regimen of brexanolone. Results revealed that 17 patients (77%) previously unable to be successfully weaned off a third-line agent (TLA) had a clinical response and were successfully weaned off all TLAs during the brexanolone maintenance phase (13 patients received the standard brexanolone dose, and 4 patients received the high-dose brexanolone regimen). In addition, 16 patients (73%) were successfully weaned off TLAs and brexanolone within five days of starting the brexanolone infusion without the need to reinstate anesthetic agents in the following 24 h. Among the 16 responders, 4 patients (23.5%) had a recurrence of SE within the 3-

week extended follow-up period. The mechanism of action of brexanolone, which is a selective positive allosteric modulator of $GABA_A$ receptors, offers a novel mechanism and therapeutic target for development as a potential treatment for SRSE.

Conversely, SAGE therapeutics started a randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of SAGE-547 (also known as brexanolone) injection in the treatment of subjects with SRSE [692]. In the phase III trial, 132 patients were randomized 1:1 to receive either brexanolone or placebo in addition to standard-of-care third-line anti-seizure agents for six days. The findings revealed that brexanolone failed to distinguish itself from placebo. Only 44% of patients receiving brexanolone were effectively weaned from other pharmacological treatments without seizures returning within 24 h, compared with 42% of patients receiving a placebo.

As mentioned previously, synaptic $GABA_A$ receptors internalize during SE, decreasing benzodiazepine efficacy. Extrasynaptic receptors, on the other hand, are unaffected by this phenomenon. Because ALLO has a higher sensitivity to extrasynaptic receptors, its success may be related to an increase in tonic inhibition, which contributes to the termination of benzodiazepine-RSE. For the first time, we recently demonstrated a significant reduction in the overall biosynthetic pathway leading to ALLO in the cerebrospinal fluid (CSF) of patients affected by SE [633, 642]. Our results showed that various neurosteroids able to positively modulate $GABA_A$-mediated inhibition such as progesterone, 5αDHP, PREG, and ALLO are reduced in the CSF of patients affected by SE. This defect probably precedes the appearance of seizures, representing a possible predisposing factor for SE. This finding supports the use of ALLO in the treatment of SE because its reduction potentially determines a diminished regulatory function of GABA in the CNS of these patients. On the other hand, not only ALLO but also other various neurosteroids able to positively modulate $GABA_A$-mediated inhibition are reduced in these patients, raising the question of whether simply administering ALLO is sufficient to restore the central levels of anticonvulsant neurosteroids in the presence of SE.

Conclusions

Understanding the pathophysiology of SE and its associated comorbidities is crucial for developing well-targeted novel drugs and improving the management approach of this condition. The involvement of neurosteroids in the SE pathophysiology has been suggested by reporting the reduction in progesterone, 5αDHP, ALLO, and PREG availability in patients with SE. However, the tentative attempt to increase the synthesis of these neurosteroids with trilostane in a preclinical model of SE resulted only in the attenuation of the SE, at variance with the positive findings obtained with specifically administered neurosteroids by others in different models of SE. This evidence together with negative results of the brexanolone randomized, double-blind, placebo-controlled trial suggests that further knowledge on the involvement of neurosteroids and their targets in SE is required to fully evaluate the possible therapeutic use of these molecules. Indeed, steroidogenic pathways could not be completely conserved in humans and rodents so to explain the inconsistencies. In addition, species-related differences in the SE characteristics may explain the reported contrasting findings. Anyway, the presence of a reduction in levels of important molecules such as neurosteroids in the CSF of patients with SE suggests that the recovery to normal levels of these molecules could be a rational goal for a therapeutic intervention aimed at preventing the appearance and recurrence of dramatic events such as the SE, in those patients with a predisposing factor[2].

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Chapter 2

Overview and Aims

Despite persistent efforts to uncover novel antiseizure medications (ASMs), approximately 30% of patients remain resistant to any class of ASMs [693]. Consequently, alternative medicine has garnered increased attention in recent years. Neurosteroids are steroids synthesized within the brain, exerting unconventional and rapid effects on neuronal excitability. Well-established steroid hormones, such as PROG and DOC, are known to possess anticonvulsant actions [659, 660]. Their anticonvulsant properties stem from their conversion in the brain into ALLO and THDOC, respectively [523]. Emerging evidence suggests a possible role for endogenous neurosteroids in regulating epileptogenesis [252, 661].

Epileptogenesis, the complex process leading to SRSs after an initial precipitating injury, remains a pivotal focus in epilepsy research. While neuroinflammation and glial cell reactions have been implicated in this process, the involvement of neurosteroids, particularly ALLO, presents an intriguing avenue for exploration.

Interestingly in 2020, we reported that two injections of the 3β-HSD inhibitor trilostane induce a remarkable increase in the levels of different neurosteroids in particular ALLO in both hippocampus and neocortex of healthy rats [687]. In light of this result, I aimed to elucidate the neurosteroid-mediated mechanisms of epileptogenesis and investigate the therapeutic potential of trilostane.

Utilizing the KA model as a screening platform offers a unique opportunity to investigate drugs with antiepileptogenic or disease-modifying potential. The temporal progression of seizures in this model allows for the evaluation of trilostane's effects at different stages, from the acute phase to

the chronic period. By administering trilostane during the latent period and chronic phase, we aim to unravel its impact on neurosteroid levels, specifically ALLO, and assess its therapeutic potential in modulating epileptogenesis and disease progression.

Hypothesis 1: Elevating Allopregnanolone Delays Epileptogenesis?

Building on evidence suggesting that blocking ALLO synthesis accelerates epileptogenesis, we hypothesize that the treatment with trilostane, by increasing ALLO levels, can potentially delay the onset of spontaneous seizures. This hypothesis is grounded in the observed neurosteroid alterations induced by trilostane in healthy rats, providing a rationale for investigating its impact on epileptogenesis during the latent period.

Hypothesis 2: Trilostane as an Antiepileptogenic and Disease-Modifying Agent?

Given the challenges inherent in developing drugs with antiepileptogenic or disease-modifying properties, our hypothesis posits that trilostane, recognized for its modulatory impact on neurosteroids, may function as a dual-action agent. Building upon previous observations regarding trilostane's influence on neurosteroid levels, we propose that its administration during the chronic phase in the KA model could potentially enhance neurosteroid availability and exert antiseizure effects. This, in turn, may lead to a modification in the trajectory of epilepsy progression. The rationale for this hypothesis is grounded in the intricate relationship between neurosteroids and epileptogenesis, and our aim is to investigate the potential therapeutic impact of trilostane in altering the course of epilepsy development.

Overall Aims:

1. Investigate the role of ALLO in epileptogenesis and test the hypothesis that elevating ALLO levels through trilostane delays the onset of spontaneous seizures.
2. Explore trilostane's potential as an antiepileptogenic and disease-modifying agent by studying its effects on neurosteroid levels during the chronic phase in the KA model.

3. Contribute valuable insights into the neurosteroid-mediated mechanisms of epileptogenesis and assess trilostane as a potential therapeutic intervention for modifying epilepsy onset and progression.

This thesis endeavors to deepen our understanding of the intricate relationship between neurosteroids and epileptogenesis and epilepsy, offering novel perspectives for therapeutic interventions in epilepsy management.

Chapter 3

Result 1

Antiepileptogenic Effects of Trilostane in the Kainic Acid Model of Temporal Lobe Epilepsy[3]

Abstract

Objective: Epileptogenesis after status epilepticus (SE) has a faster onset in rats treated to reduce brain levels of the anticonvulsant neurosteroid allopregnanolone with the 5α-reductase inhibitor finasteride; however, it still has to be evaluated whether treatments aimed at increasing allopregnanolone levels could result in the opposite effect of delaying epileptogenesis. This possibility could be tested using the peripherally active inhibitor of 3β-hydroxysteroid dehydrogenase/ Δ^{5-4} isomerase trilostane, which has been shown repeatedly to increase allopregnanolone levels in the brain.

Methods: Trilostane (50 mg/kg) was administered subcutaneously once daily for up to six consecutive days, starting 10 min after intraperitoneal administration of kainic acid (15 mg/kg).

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Seizures were evaluated by video-electrocorticographic recordings for 70 days maximum, and endogenous neurosteroid levels were assessed by liquid chromatography–electrospray tandem mass spectrometry. Immunohistochemical staining was performed to evaluate the presence of brain lesions.

Results: Trilostane did not alter the latency of kainic acid-induced SE onset or its overall duration. When compared to the vehicle-treated group, rats receiving six daily trilostane injections presented a remarkable delay of the first spontaneous electrocorticographic seizure and subsequent tonic–clonic spontaneous recurrent seizures (SRSs). Conversely, rats treated with only the first trilostane injection during SE did not differ from vehicle-treated rats in developing the SRSs. Notably, trilostane did not modify neuronal cell densities or the overall damage in the hippocampus. In comparison to the vehicle group, repeated administration of trilostane significantly decreased the activated microglia morphology in the subiculum. As expected, allopregnanolone and other neurosteroid levels were remarkably increased in the hippocampus and neocortex of rats treated for 6 days with trilostane, but pregnanolone was barely detectable. Neurosteroids returned to basal levels after a week of trilostane washout.

Significance: Overall, these results suggest that trilostane led to a remarkable increase in allopregnanolone brain levels, which was associated with protracted effects on epileptogenesis.

Keywords: epileptogenesis, kainic acid, neurosteroids, status epilepticus, temporal lobe epilepsy, trilostane

Key Points

- Trilostane did not alter the development of status epilepticus or subsequent damage in the hippocampus
- Trilostane caused a remarkable increase in allopregnanolone brain levels
- Trilostane suppressed the synthesis of preg- nanolone
- Trilostane consistently delayed the development of both electrographic and motor seizures
- Trilostane modulated epileptogenesis in kainic acid-treated rats

Introduction

Epileptogenesis is usually defined as the latent period separating an initial precipitating injury from the appearance of spontaneous recurrent seizures (SRSs) [694]. The key causal mechanisms of epileptogenesis are largely undetermined [695]; however, neuroinflammation and glial cell reaction have been repeatedly investigated as possibly involved factors [314, 696-698]. Interestingly, the glial reaction that follows status epilepticus (SE) is accompanied, especially in the hippocampus, by an increased expression of CYP11A1, the cytochrome P450 cholesterol side chain cleavage enzyme that produces pregnenolone, which is known as the precursor of progesterone and other neurosteroids [661].

Neurosteroids are so defined because they are produced in the nervous system and cooperate with peripherally born neuroactive steroids as regulators of neuronal excitability [576, 650, 699]. Their impact on different pathological conditions is well known not only for epilepsy but also for stress, anxiety, depression, psychosis, ataxia, and pain [700-704]. These different conditions share the most characterized target of neurosteroids, namely, γ–aminobutyric acid type A ($GABA_A$) receptor, which is positively modulated by allopregnanolone, pregnanolone, and THDOC to enhance inhibition [705]. Conversely, the neurosteroid pregnenolone sulfate reduces $GABA_A$ inhibitory currents, resulting in a proconvulsant effect [611].

Epileptogenesis can be accelerated by blocking the synthesis of allopregnanolone with finasteride, which is a 5α-reductase irreversible inhibitor [252]. In view of this evidence, we hypothesized that epileptogenesis could be delayed by increasing allopregnanolone levels. This effect could be obtained by administering trilostane, a 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase reversible inhibitor that targets steroid production prevalently in the adrenal cortex [466]. Interestingly, the changes in peripheral steroid production attained with trilostane effectively reduced forced swimming-induced immobility in rats and this result was prevented by the surgical removal of adrenal glands and gonads. All of this occurred because the reported behavioral changes were a consequence of the compensatory stimulation of pregnenolone production in the adrenal glands and gonads, which were promoted by the respective hypophyseal hormone [466].

In line with these findings, we recently reported that two injections of trilostane could be sufficient to induce a remarkable increase in pregnenolone, progesterone, 5α-dihydroprogesterone, and allopregnanolone levels in the hippocampus and neocortex of healthy rats [687]. Because trilostane is used therapeutically to control hyperadrenocorticism in dogs and is generally well tolerated [456, 706-708], we considered the possibility of administering this drug to rats during the latent period,

to investigate whether neurosteroids, especially allopregnanolone, could be associated with a delay in epileptogenesis.

Materials and Methods

Animals

Adult male Sprague Dawley rats (n = 103, Charles River), with an initial weight of 175−200 g, were housed in a specific pathogen-free facility providing a controlled environment with ad libitum access to water and food. All efforts were made to refine procedures, improve the welfare, and reduce the number of animals used for the experiments. The study protocol was authorized by the Italian Ministry of Health (323/2015-PR and 544/2020-PR). All experiments were performed in agreement with European Directive 2010/63/EU.

Experimental design

The effects of daily [709] subcutaneous injections of trilostane (50 mg/kg in sesame oil, Cayman Chemical) were investigated in the kainic acid (KA) model [688, 710, 711]. An intraperitoneal injection of KA (15 mg/kg in saline, Sigma-Aldrich) was performed 1 week after electrode implantation. Figure 13 illustrates the experimental design, which consisted of four distinct experiments. Rats were euthanized with isoflurane 6, 7, 13, or 70 days after KA administration.

Electrode implantation and video-electrocorticography

Electrode implantation, recording, and analysis of video-electrocorticographic (ECoG) traces were performed as previously described [710]. Briefly, rats were implanted with epidural electrodes in the frontal (bregma 0 mm, 3.5 mm lateral from midline) and occipital cortices (bregma −6.5 mm, 3.5 mm lateral from midline). One electrode was implanted below lambda in the midline and used as a reference (Figure 13). Offline ECoG traces were digitally filtered (band-pass: high, 50 Hz; low, 1 Hz) and manually analyzed using LabChart 8 PRO (ADInstruments). Seizures were identified in the ECoG traces, and convulsions were analyzed by synchronized video recordings. In particular, convulsions were scored as Stage (ST.) 0 if a clear epileptiform ECoG signal was observed without behavioral changes in the video; ST. 1−2 in the presence of absence-like immobility, “wet-dog shakes,” facial automatisms, and head nodding; ST. 3 when presenting with forelimb clonus and lordosis; ST. 4

corresponding to generalized seizures and rearing; and ST. 5 when seizures consisted of rearing with the loss of posture and/or wild running, followed by generalized convulsions.

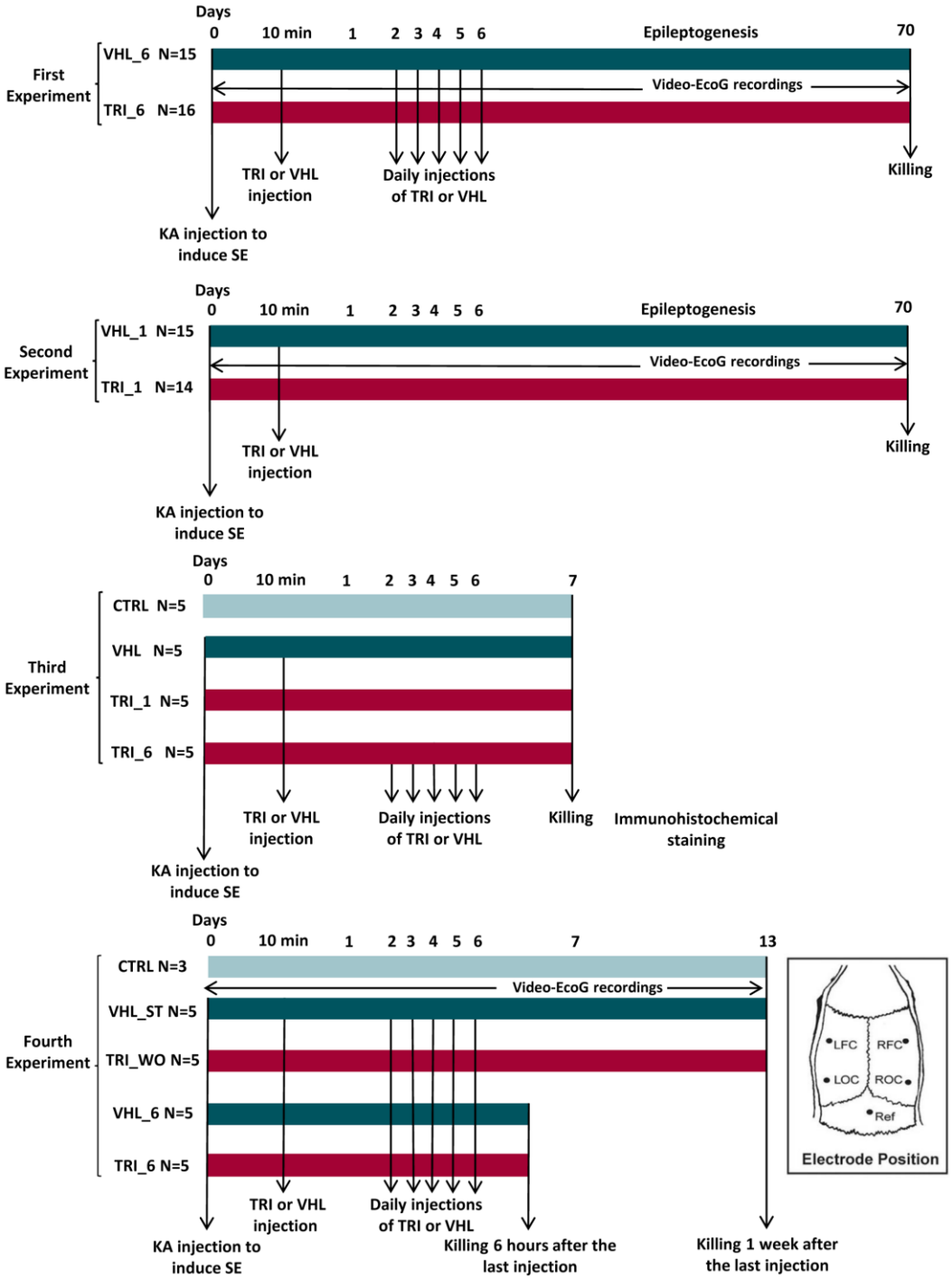

Figure 13. Experimental design. Rats were used in four different experiments. The first experiment consisted of six daily injections of trilostane (50 mg/kg, subcutaneously) or sesame oil. In the second experiment, rats were injected with trilostane or vehicle only one time, approximately 10 min from the kainic acid (KA; 15 mg/kg) intraperitoneal administration. A third experiment included untreated control

rats and KA-treated rats receiving sesame oil or trilostane, once or repeatedly. In the fourth experiment, the hippocampal and neocortical levels of neurosteroids were measured in untreated control rats, or after six daily injections of trilostane or vehicle, and considering 6 h and 7 days as the time intervals following the last injection of trilostane or vehicle. CTRL, control; ECoG, electrocorticographic; LFC, left frontal cortex; LOC, left occipital cortex; Ref, reference electrode; RFC, right frontal cortex; ROC, right occipital cortex; SE, status epilepticus; TRI_1, trilostane single injection; TRI_6, trilostane repeated injections; TRI_WO, trilostane washout; VHL, vehicle; VHL_ST, sham-treated vehicle; VHL-1, vehicle single injection; VHL-6, vehicle repeated injections.

Immunohistochemistry

Animals were deeply anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (pH 7.4), followed by fixation in Zamboni's fixative (pH 6.9) 24 h after the last injection. Brains were kept at 4° C in the same fixative for 24 h, cryoprotected in 15% and 30% sucrose solutions, and stored at −80° C until used. A freezing stage and sliding microtome (Leica SM2000R) were used to obtain 5−6 horizontal sections (50 µm thick) from bregma level −8.04 mm to −5.04 mm.

The immunohistochemical staining was performed as previously described [710]. More specifically, we used mouse anti-neuron- specific nuclear protein (NeuN; #MAB377 clone A60, 1:200, Millipore), mouse anti-glial fibrillary acidic protein (GFAP; #G3893, 1:500, Sigma-Aldrich), and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1; #019-19741, 1:1000, Wako) antibodies. Images were acquired using an Eclipse CiL (Nikon Instruments).

Fluoro-jade B

The brain sections were mounted on gelatin-coated slides and dried at room temperature. The following day, staining was performed as previously described [710], using fluoro-jade B (FJB; Millipore, # AG310-30MG). Images were acquired with a Leica SP2 AOBS confocal microscope.

Image analysis

All brain sections from −8.04 mm to −5.04 mm bregma levels were magnified (10×) and the different areas of interest (cornu ammonis 3 [CA3] stratum pyramidalis [Py], subregion B; CA3 lacunosum-moleculare [LMol]; CA1 Py; subiculum [Sub]) were analyzed.

NeuN-immunoreactive cells and FJB-positive cells were counted per square millimeter using the image analysis software NIS-Elements and ImageJ, respectively. The measured area (region of interest [ROI]) was kept unchanged for each hippocampal area regardless of the animal being analyzed. For NeuN, ROI values were .080 mm2 in the CA3 Py, .033 mm2 in the CA1 Py, and .113 mm2 in the Sub. For FJB, ROI values were .119 mm2, .047 mm2, and .115 mm2 in CA3 Py, CA1 Py, and Sub, respectively.

GFAP immunostaining was used to determine regions characterized by a loss of astrocytes. The image analysis software NIS-Elements was used to manually trace the unstained area (mm2) upon GFAP detection. This unstained area was determined for each section, then the mean value of the unstained area was calculated for each animal.

Microglial activation was assessed with the same image analysis software (NIS-Elements) by calculating the binary area fraction (BinaryArea/MeasuredArea). More precisely, the BinaryArea corresponded to the sum of areas of all binary objects, whereas the MeasuredArea represented the area of the measurement frame, which was kept constant in all analyzed sections.

Liquid chromatography–electrospray tandem mass spectrometry

The brains were carefully removed after euthanasia (isoflurane) and chilled on ice to dissect both the hippocampi and neocortices for liquid chromatography−electrospray tandem mass spectrometry analysis. Details about the performed protocol were published previously [688]. Only values above the limit of quantification were used for the statistical comparisons.

Statistics

The results were analyzed by using Student t-test or one-way analysis of variance (ANOVA) followed by the Holm−Šidak test, depending on the number of groups and normality assessment (Shapiro−Wilk test). If required, one outlier per dataset was identified using Grubbs test and removed using SigmaPlot 13 (Systat Software). All data are presented as mean and SEM, and they were regarded as significantly different at $p < .05$.

Results

Multiple injections but not a single injection of trilostane delayed the onset of SRSs Following KA administration (n = 95), 3 of 45 rats in the vehicle-treated group (7%) and 5 of 50 rats in the trilostane-treated group (10%) died during or after SE. In the vehicle-treated group, two rats (4%) did not develop SE and were discarded. Moreover, two trilostane-treated rats and one vehicle-treated rat lost the ECoG implant before the first SRS and were disregarded for the analysis of SRSs. In animals monitored for SE duration, trilostane did not reduce the duration of SE at the ECoG analysis (9.121 ± .221 h, n = 36), as it was similar to that of the vehicle-treated rats (9.398 ± .321 h, n = 34; p = .476, Student t-test). After SE, the rats were used in four different experiments.

In the first experiment, we evaluated the latency for the rats to develop the first ECoG SRS and first convulsive SRS, with or without loss of posture. After repeated administration of trilostane (n = 12) or vehicle (n = 13), the latency to develop the first ECoG SRS was significantly increased (p = .021, Student t-test) in the trilostane group (Figure 14A). Moreover, the latency to develop the first convulsive SRS was also significantly increased by trilostane (p = .038; Figure 14B). Consistently, the latency to develop the first convulsive SRS with loss of posture (Figure 14C) was significantly longer after treatment with trilostane (p = .018). To explain the differences found in the time course of epileptogenesis, we hypothesized that the vehicle- and trilostane-treated rats could have developed a different SE. This hypothesis was not confirmed by analyzing the video-ECoG of vehicle-or trilostane-treated rats, which presented similar latencies to develop the seizures (Figure 14D–F) and analogue SE duration (Figure 14G–I).

Then, to evaluate whether trilostane administration during SE could modify the time course of epileptogenesis, we performed a second experiment in which we characterized the impact of a single trilostane administration on the onset of SRSs. Again, trilostane (n = 12) or vehicle (n = 12) was administered 10 min after the intraperitoneal administration of KA (Figure 15A–I). Also this protocol did not change the characteristics of SE (Figure 15D–I) but, at variance with the first experiment, the latency to develop SRSs was similar in both treatment groups (Figure 15A–C).

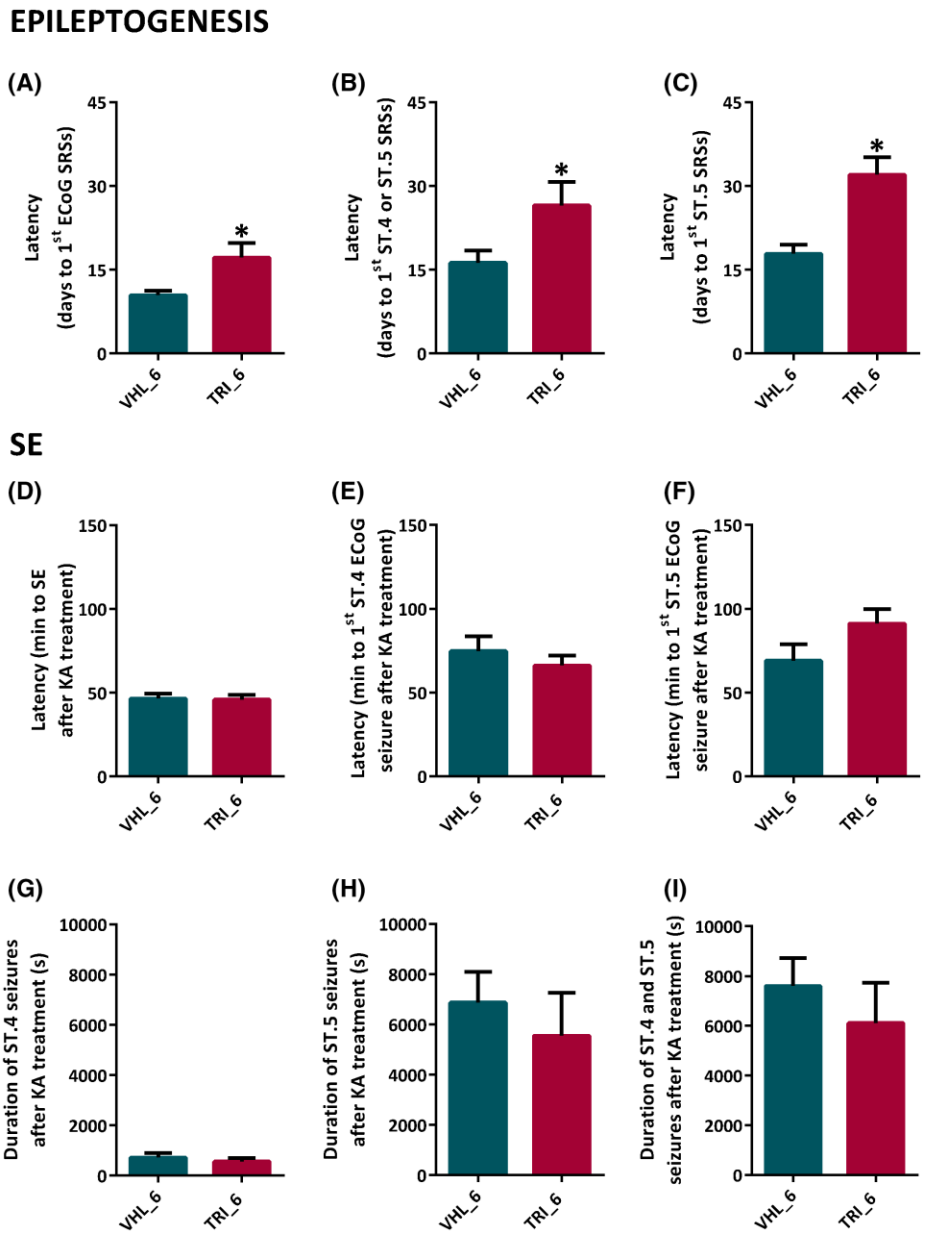

Figure 14. Time course of epileptogenesis and features of status epilepticus (SE) in rats undergoing a repeated administration of trilostane (50 mg/kg for 6 days) or vehicle. In comparison to the vehicle-treated group, the latency to develop the first electrocorticographic (ECoG) spontaneous seizure and then convulsive spontaneous recurrent seizures (SRSs) was significantly increased in the trilostane-treated group; trilostane was administered once daily for six consecutive days, starting 10 min after the intraperitoneal administration of kainic acid (KA; 15 mg/kg) to induce SE (A–C). No changes in the latencies to develop SE (D), or stage (ST.) 4 (E) or ST. 5 seizures (F) during SE were found. Similarly, no changes in the total duration (in seconds) of ST. 4 (G), ST. 5 (H), and a combination of ST. 4 and 5 seizures (I) were found during SE. Statistical analysis was performed using Student t-test. Results are shown as mean and SEM, and they are considered significant at $p < .05$. *$p < .05$. TRI_6, trilostane repeated injections; VHL_6, vehicle repeated injections.

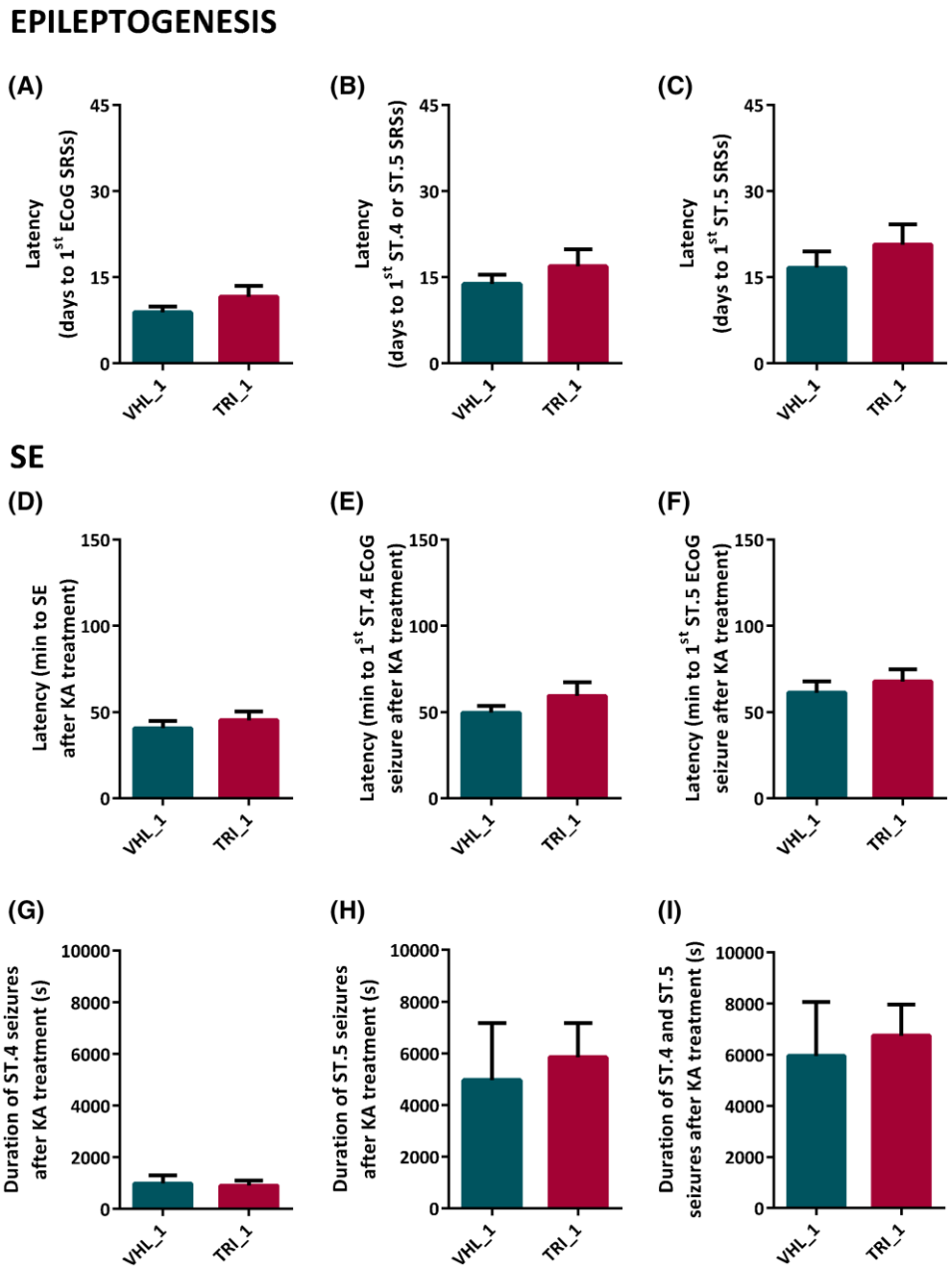

Figure 15. Time course of epileptogenesis and features of status epilepticus (SE) in rats undergoing a single administration of trilostane (50 mg/kg) or vehicle. In comparison to the vehicle-treated group, the latencies to develop the first electrocorticographic (ECoG) spontaneous seizure and then convulsive spontaneous recurrent seizures (SRSs) were unchanged in the trilostane-treated group when the injection was only 10 min after the intraperitoneal administration of kainic acid (KA; 15 mg/kg) to induce SE (A–C). No changes in the latencies to develop SE (D), or stage (ST.) 4 (E) or ST. 5 seizures (F) during SE were found. Similarly, no changes in the total duration (in seconds) of ST. 4 (G), ST. 5 (H), and a combination of ST. 4 and 5 seizures (I) were observed during SE. Statistical analysis was performed using Student *t*-test. Results are shown as mean and SEM, and they are considered significant at $p < .05$. TRI_1, trilostane single injection; VHL_1, vehicle single injection.

Trilostane did not protect neurons in the CA3 Py, CA1 Py, and Sub

Hippocampal damage in five animals per group. The animals were sacrificed on Day 7 after SE. Unfortunately, one rat in the trilostane group prematurely died. The results were analyzed by one-way ANOVA, which showed no effect of trilostane treatment on FJB positivity (cells/mm2) in the CA3 Py ($F_{2, 10} = .268$, $p = .770$), CA1 Py ($F_{2, 10} = .760$, $p = .493$), and Sub ($F_{2, 10} = .503$, $p = .619$). No FJB-positive cells were found in healthy rats (Figure 16A). Thus, FJB staining showed that trilostane did not reduce the number of damaged neurons in the abovementioned brain areas (Figure 16B–D).

Neuronal survival was also assessed by using NeuN immunostaining (Figure 16E). One-way ANOVA showed that KA had a significant effect on NeuN immunopositivity (cells/mm2) in the CA3 Py ($F_{3, 15} = 7.185$, $p = .003$), CA1 Py ($F_{3, 15} = 21.061$, $p < .001$), and Sub ($F_{3, 15} = 13.470$, $p < .001$). In comparison to healthy rats, a significant reduction in NeuN-immunopositive cells was evidenced in the CA3 Py (Figure 16F) of vehicle-treated rats ($p = .004$, Holm–Šídák test) and rats receiving a single ($p = .035$) or repeated trilostane administration ($p = .012$). Significant differences, compared to healthy rats, were also found in the CA1 Py (Figure 16G) for all the groups of rats receiving KA and, subsequently, vehicle or trilostane ($p < .001$ for all comparisons). Similarly, in the Sub (Figure 16H), the number of NeuN-immunopositive cells was significantly reduced in vehicle-treated rats ($p < .001$) and in rats treated with a single ($p = .001$) or repeated injections of trilostane ($p < .001$), in comparison to controls. Moreover, there were no beneficial effects of trilostane in any of the examined brain regions.

Trilostane did not affect the loss of astrocytes in the CA3 LMol and Sub

The impact of KA on astrocytes was characterized as a disappearance in the area of GFAP immunostaining. As expected, the GFAP immunostaining was consistent in healthy rats (Figure 17A), whereas GFAP-immunonegative areas were evident in all treatment groups where the rats were treated with KA (Figure 17B,C). The single or repeated administration of trilostane in rats did not significantly affect the damage in the examined brain regions (CA3 LMol: $F_{2, 9} = .933$, $p = .428$; Sub: $F_{2, 11} = .321$, $p = .732$).

Repeated administration of trilostane reduced the microglia-activated morphology in the Sub

When analyzed by a one-way ANOVA, a significant increase of Iba1 associated with the microglia-activated morphology was found in both the CA3 LMol ($F_{3, 13} = 30.767$, $p < .001$) and Sub ($F_{3, 14} = 112.570$, $p < .001$) of KA-treated rats when compared to healthy controls ($p < .001$, Holm–Šídák test; Figure 17D–F). Nevertheless, the microglia-activated morphology in the Sub was significantly attenuated in rats treated with multiple injections of trilostane ($p = .004$), in respect to vehicle-treated rats (Figure 17F).

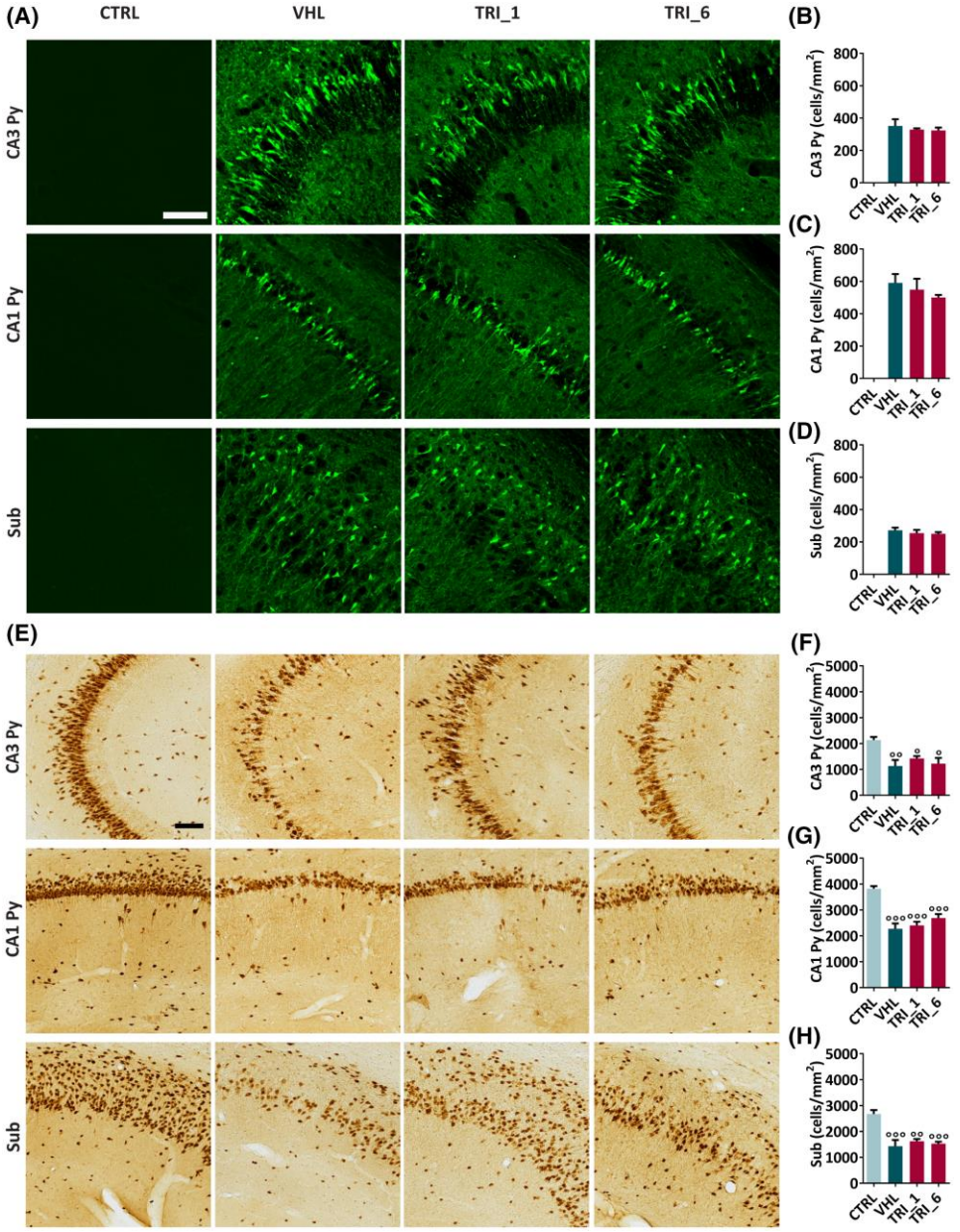

Figure 16. Effect of trilostane (50 mg/kg) or vehicle on neuronal cell death and survival in the cornu ammonis 3 stratum pyramidalis (CA3 Py, subregion B), cornu ammonis 1 stratum pyramidalis (CA1 Py), and subiculum (Sub) of rats after kainic acid-induced status epilepticus. Sections were stained for fluoro jade B to evaluate neuronal cell death (A–D) or against mouse anti-neuron- specific nuclear protein to evaluate neuronal cell survival (E–H). Both a single and repeated injections of trilostane did not display neuroprotective effects, in comparison to treatment with the vehicle. Quantification was performed using ImageJ. Statistical analysis was performed using one-way analysis of variance and the Holm–Šídák test. Data are shown as mean and SEM. °$p < .05$, °°$p < .01$, °°°$p < .001$; Scale bars = 100 μm. CTRL, control; TRI_1, trilostane single injection; TRI_6, trilostane repeated injections; VHL, vehicle.

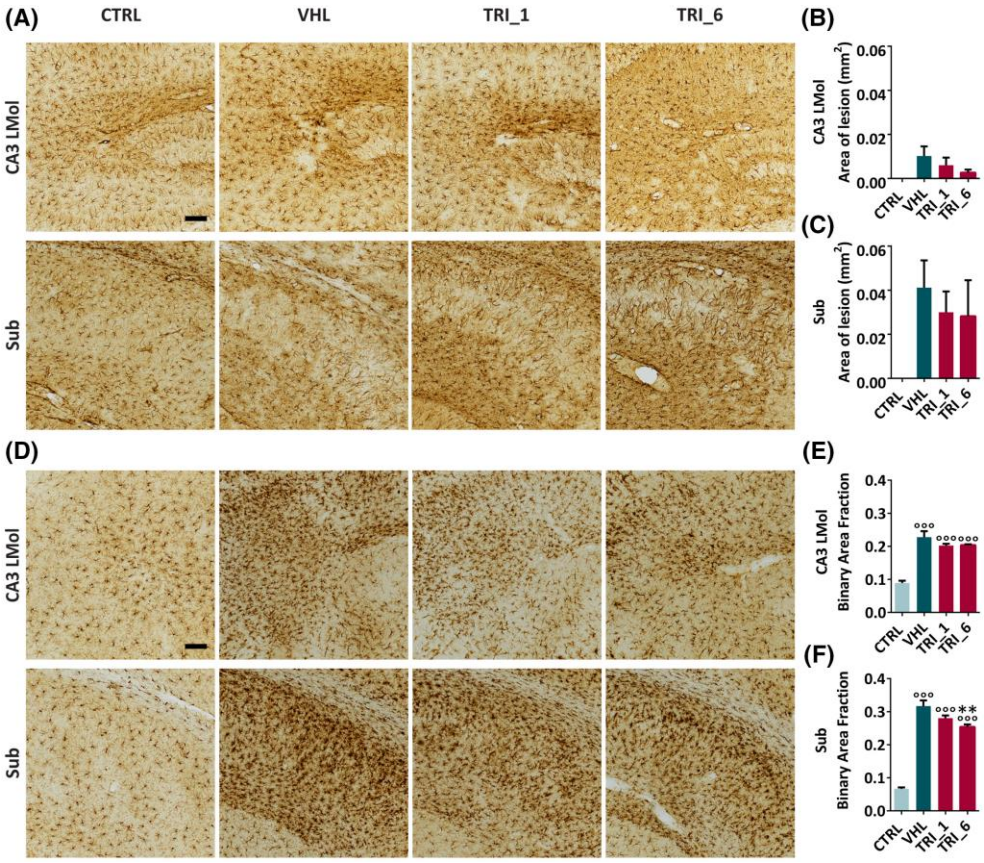

Figure 17. Effect of trilostane (50 mg/kg) or vehicle on loss of astrocytes and microglia morphology in the CA3 stratum lacunosum-moleculare (CA3 LMol) and subiculum (Sub) of rats after kainic acid-induced status epilepticus. Brain sections were stained against glial fibrillary acidic protein (A), a marker of astrocytes that was absent in the lesion occurring in the CA3 LMol (B) and Sub (C). The treatment with trilostane did not prevent the development of the lesion. Furthermore, brain sections were stained against rabbit anti-ionized calcium-binding adapter molecule 1 (D). In comparison to the healthy control group, the activated microglia morphology was significantly induced in the CA3 LMol and Sub after trilostane or vehicle administration. In Sub, a significant change was also observed in activated microglia morphology by comparing rats repeatedly treated with trilostane with those treated with the vehicle (E–F). Quantification was performed using the image analysis software NIS-Elements. Statistical analysis was performed using one-way analysis of variance followed by the Holm–Šídák test. Results are shown as mean and SEM, and they are considered significant at $p < .05$. Scale bars = 100 µm. **$p < .01$, vehicle (VHL) versus trilostane repeated injections (TRI_6); °°°$p < .001$ versus control (CTRL). TRI_1, trilostane single injection.

<u>Significant changes in neocortical and hippocampal neurosteroid levels were found in rats repeatedly treated with trilostane</u>

In the fourth experiment, rats were treated daily with trilostane (n = 10) or vehicle (n = 10) for 6 days after the induction of SE, and compared to healthy controls (n = 3). Five animals per treatment

group were sacrificed 6 h after the last injection. The remaining animals were sacrificed 1 week later. These animals were also monitored by ECoG and, consistently with the previous experiments, no significant effect of trilostane was evident for all the analyzed parameters, namely, the latency of SE development ($F_{3, 14} = 1.495$, $p = .259$, one-way ANOVA), the latency to the first ST. 4 seizure ($F_{3, 11} = 3.574$, $p = .050$), and the latency to the first ST. 5 seizure ($F_{3, 12} = 2.968$, $p = .075$). Similarly, no significant effects were observed for the total duration of ST. 4 ($F_{3, 14} = .562$, $p = .649$), ST. 5 ($F_{3, 14} = .577$, $p = .639$), and both ST. 4 and ST. 5 seizures during SE ($F_{3, 14} = .591$, $p = .631$; data not shown).

In these animals, we found a statistically significant increase of the neocortical (Figure 18A–F) levels of pregnenolone ($F_{4, 17} = 132.779$, $p < .001$, one-way ANOVA), pregnenolone sulfate ($F_{4, 16} = 17.544$, $p < .001$), progesterone ($F_{4, 15} = 53.841$, $p < .001$), 5α-dihydroprogesterone ($F_{4, 17} = 50.051$, $p < .001$), and allopregnanolone ($F_{4, 17} = 24.087$, $p < .001$). In particular, this change depended on the effects of trilostane, assessed 6 h after the last injection, when compared to all the other treatment groups ($p < .001$, Holm–Šídák test; Figure 18A,B,D–F). Also, neocortical levels of pregnanolone were affected by trilostane ($F_{4, 14} = 3.257$, $p = .044$), but in this case we found a remarkable reduction in the amount (Figure 18C).

Trilostane also produced significant changes in the hippocampus (Figure 18G–L) by increasing pregnenolone ($F_{4, 16} = 4.781$, $p = .010$), progesterone ($F_{4, 16} = 13.408$, $p < .001$), 5α-dihydroprogesterone ($F_{4, 16} = 30.712$, $p < .001$), and allopregnanolone ($F_{4, 15} = 5.122$, $p = .008$) levels. Post hoc comparisons showed that pregnenolone increased after daily trilostane treatment, in respect to the vehicle-treated group with the same dosage interval ($p = .048$), or the vehicle-treated group after 1 week of washout ($p = .019$), and went back to basal levels 1 week after the last trilostane injection ($p = .034$ vs. 6 days of trilostane treatment; Figure 18G). Progesterone levels significantly increased after trilostane administration ($p < .001$ vs. all the other groups) but returned to normal after the washout ($p = .002$ vs. 6 days of trilostane treatment; Figure 18J). Six hours after the last injection of trilostane, 5α-dihydroprogesterone ($p < .001$ vs. all the other groups; Figure 6K), as well as allopregnanolone ($p = .035$ vs. the vehicle-treated group at the same time, $p = .015$ vs. the sham-treated vehicle), were also markedly increased (Figure 18L). In the hippocampus, trilostane did not affect the levels of pregnenolone sulfate ($F_{4, 17} = .552$, $p = .700$) or pregnanolone ($F_{4, 15} = 2.551$, $p = .082$; Figure 18H,I). Pregnanolone was not detectable 6 h after the last injection of trilostane but returned to normal after the washout (Figure 18I).

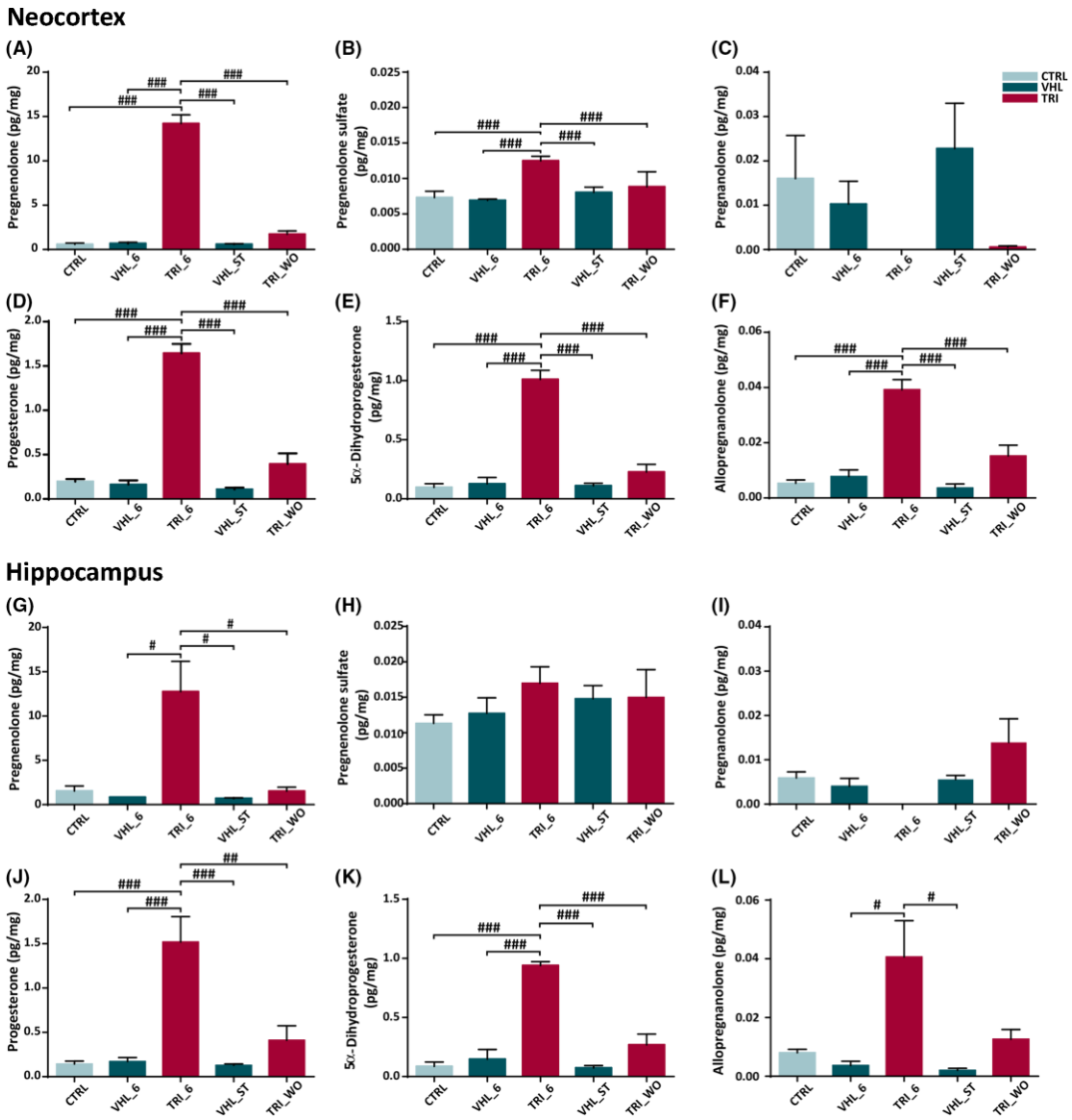

Figure 18. Effect of trilostane (50 mg/kg) or vehicle treatment on neurosteroid brain levels. The repeated daily injection of trilostane determined a significant augmentation of almost all of the studied neurosteroids when compared to the other treated groups (A, B, D–G, J–L). Only pregnenolone sulfate (H) did not increase its levels in the hippocampus, whereas pregnanolone was not detectable 6 h after the last injection of trilostane in both the hippocampus and neocortex (C, I). Statistical analysis was performed using one-way analysis of variance followed by the Holm–Šídák test. Data are shown as mean and SEM, and they are considered significant at $p < .05$. #$p < .05$, ##$p < .01$, ###$p < .001$, trilostane repeated injections (TRI_6) versus other groups. CTRL, control; TRI_WO, trilostane washout; VHL_6, vehicle repeated injections; VHL_ST, sham-treated vehicle.

Discussion

This study was aimed at assessing the effects of multiple trilostane injections posttreatment on SE dynamics, brain damage, neocortical and hippocampal neurosteroid levels, and duration of epileptogenesis as evaluated by the time interval that preceded the appearance of SRSs. The main outcomes of our study were the (1) lack of trilostane effects on SE dynamics and damage to the hippocampus; (2) remarkable increase in tissue levels of various neurosteroids, with the notable exception of pregnanolone, whose synthesis was suppressed; and (3) consistent delay in the appearance of both electrographic and motor seizures in rats repeatedly treated with trilostane, but not in those receiving a single injection. Overall, these findings support a modulatory effect of trilostane on epileptogenesis.

We did not observe any effect of trilostane administration on the investigated features of SE induced by KA. This finding was at odds with our previously reported result, in which KA-treated rats that received two trilostane injections before the SE induction presented an anticipated disappearance of convulsive seizures [687]. The difference between the present and previous studies suggests that trilostane could not be effective in counteracting the seizures when administered after SE onset. Other investigators reported that allopregnanolone or its analogue ganaxolone were effective in terminating SE induced by tetramethylenedisulfotetramine [681] or lithium–pilocarpine [682], even when administered after SE induction. This suggests that trilostane is probably unsuitable for the acute treatment of seizures and SE. A single trilostane injection during SE, not followed by other administrations, was also unable to modify the time course of epileptogenesis, thus suggesting that trilostane should be repeatedly administered to be effective.

Another important point was the lack of trilostane effects on brain damage, with a limited but significant reduction in microglia reactivity in the subiculum. This was surprising in view of the multiple mechanisms involved in neuroprotection, which could be activated by progesterone, 5α-dihydroprogesterone, and allopregnanolone, which involve both membrane and intracellular progesterone receptors, and also $GABA_A$ receptors [712]. It is well known that the potentiation of the $GABA_A$ receptor by diazepam [713] can produce protective effects in the pilocarpine model of SE [714, 715]. Conversely, diazepam administered 3 h after the onset of SE in KA-treated rats did not afford neuroprotection in the hippocampus, but only in some extrahippocampal regions [716]. This result was still associated with the successful termination of SE by diazepam, whereas trilostane was not effective in modifying the course of SE in our animals. For this reason, we believe

that the absence of neuroprotective effects exerted by trilostane could be due to its inefficacy in modulating SE dynamics.

Despite the lack of effects in controlling seizures during SE, and in producing neuroprotective effects, the repeated administration of trilostane consistently modified the time course of epileptogenesis. This apparently surprising result is consistent with the observation that finasteride could anticipate the appearance of SRSs in pilocarpine-treated rats, as reported by two independent groups [661, 717]. Finasteride is known to reduce the availability of allopregnanolone in the brain [664], so as to exert an effect opposed to that produced by trilostane. Thus, we interpreted the delayed epileptogenesis observed in rats treated with trilostane as the consequence of increased allopregnanolone availability; however, only indirect evidence supporting this hypothesis is found in the literature. Specifically, progesterone administration in mice was followed by a remarkable delay in the progression of epileptogenesis induced by kindling, an effect completely prevented by administering finasteride [621]. Interestingly, other investigators reported that allopregnanolone administered for 12 days to pilocarpine-treated rats after SE was able to reduce the occurrence of ripples and interictal spikes [640], but these authors did not systematically investigate the allopregnanolone effects on epileptogenesis. For these reasons, the leading role of allopregnanolone in the modulation of epileptogenesis remains to be fully demonstrated.

We tried to clarify the role of allopregnanolone by evaluating its hippocampal and neocortical levels, along with the levels of other allopregnanolone-related neurosteroids, in rats at the end of the period of trilostane administration and after a week of drug washout. The remarkable 12-fold increase in allopregnanolone hippocampal levels found in the trilostane group compared to vehicle-treated rats supports a major role for this neurosteroid in the effects observed for epileptogenesis; however, changes similar to those observed for allopregnanolone were also found for pregnenolone, progesterone, and 5α-dihydroprogesterone, all neurosteroids whose levels were probably not affected by the drug finasteride in the mentioned previous experiments. Unfortunately, we could not include a treatment group with finasteride to block the effects of trilostane, because finasteride also modifies testosterone metabolism, with obvious consequences on levels of the other neurosteroids [718] such as dihydrotestosterone, and the respectively targeted receptors. No effects of pregnenolone and 5α-dihydroprogesterone on $GABA_A$ receptors have been reported until now, and progesterone effects are known to be mediated by its conversion to allopregnanolone [719].

Trilostane could produce additional metabolic effects, because this drug was also reported to inhibit 11β-hydroxysteroid dehydrogenase in dogs, so as to impair the inactivation of cortisol, a hormone that is not produced in rats [706]. Unexpectedly, we found a complete inhibition of pregnanolone production, which was recovered after the trilostane washout, suggesting an inhibitory effect of trilostane on 5β-reductase. This activity is indirectly suggested by the finding that allopregnanolone, which shares the metabolic activity of 3β-hydroxysteroid dehydrogenase with pregnanolone [720], increased greatly. In view of this finding, a question could be raised on the overall effect of trilostane on the balance of anticonvulsive neurosteroids produced in the brain of rats with epilepsy, which include pregnanolone and THDOC; however, the reduction in pregnanolone levels was more than compensated for by the massive increase in allopregnanolone production observed in the hippocampus. The main role of allopregnanolone was also indicated by the absence of changes in the levels of the proconvulsant agent pregnenolone sulfate in the hippocampus of trilostane-treated rats, as well as by the limited increase of this neurosteroid observed in the neocortex. The change in pregnenolone sulfate neocortical levels was not so large as to possibly overcome the effects of allopregnanolone, whose levels were 52-fold higher than those found in the vehicle group.

We could not exclude a possible contribution of other hormones involved in the adrenal cortex and/or gonadal regulation to the findings of our experiments. By blocking 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase to reduce steroidal production in the adrenal cortex, adrenocorticotropic hormone (ACTH) would be expected to increase [721]. ACTH notoriously affects seizures, but its activity is thought to be dependent on the synthesis of deoxycorticosterone by the adrenal gland [646], which was impaired by trilostane in our rats. Similarly, a role for corticotropin-releasing hormone could also be considered, but this neuropeptide is a proconvulsant [722] and, to the best of our knowledge, could have opposed the delay in epileptogenesis found in trilostane-treated rats. Also, the gonadal axis [723] would need to be considered to interpret our findings. Testosterone levels were reported to be reduced by trilostane in rats [724]. The effects of testosterone on seizures were investigated in both the pilocarpine and KA models of SE, with the authors reporting [725] increased seizure occurrences in animals that received testosterone replacement after castration; however, these changes were observed only in the course of KA-induced SE, and no study has yet to establish a role for testosterone in epileptogenesis.

The KA model of temporal lobe epilepsy has been suggested to be suitable for the screening of putative disease-modifying/ antiepileptogenic agents. In this regard, it was shown that oral administration of everolimus (2–3 mg/kg) or an intraperitoneal injection of phenobarbital (60 mg/ kg), at several time points after SE onset, were not able to prevent the development of SRS [726]. On the other hand, our results suggest that trilostane displays potential as an antiepileptogenic drug, possibly by increasing allopregnanolone levels in the brain. Our study was based on a relatively short-term period of administration, so we cannot exclude that a longer period of treatment may result in more beneficial effects. It is nonetheless interesting to note that a delay in the onset of convulsive seizures occurred following the drug washout and the return of neurosteroid levels to the baseline, suggesting that these agents produced a change in the development of epileptogenesis rather than having exerted a transient antiseizure effect[4].

[4] Concept and design of the study: Anna Maria Costa, Chiara Lucchi, Giuseppe Biagini. Experiments, data acquisition, and analysis: Anna Maria Costa, Mohammad Gol, Chiara Lucchi. Drafting the manuscript and figures: all authors. All authors read and approved the final version of the manuscript.

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Conflict Of Interest Statement :None of the authors has any conflict of interest to disclose.

Chapter 4

Result 2

Seizure progression is slowed down by enhancing neurosteroid availability in the brain of epileptic rats[5]

Abstract

Trilostane is a 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase inhibitor able to produce a many-fold increase in brain levels of various neurosteroids, including allopregnanolone. We previously found that treatment with trilostane can slow down epileptogenesis in the kainic acid (KA) model of temporal lobe epilepsy. It is unknown if trilostane may have a similar effect on the progression of epilepsy severity, as observed in KA-treated rats. Consequently, we investigated the effects of trilostane (50 mg/kg/day, one week) in epileptic rats, given 64 days after KA administration. Seizures were monitored by video-electrocorticographic recordings before and during the treatment with trilostane or vehicle (sesame oil), and neurosteroid levels were measured in serum and cerebral tissue using liquid chromatography–electrospray tandem mass spectrometry after treatment. Pregnenolone sulfate, pregnenolone, progesterone, 5α-dihydroprogesterone, and allopregnanolone peripheral levels were massively increased by trilostane. With the only exception of hippocampal pregnenolone sulfate, the other neurosteroids augmented in both the neocortex and hippocampus. Only pregnanolone levels were not upregulated by trilostane. As expected, a significant increase in the seizure occurrence was observed in rats receiving the vehicle, but not in the trilostane group. This suggests that the increased availability of neurosteroids produced a disease-modifying effect in the brain of epileptic rats.

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1. Introduction

A variety of antiseizure medications (ASMs) are currently available to symptomatically treat epilepsy. Instead, it is challenging to develop drugs able to interfere with the pathophysiological mechanisms of epilepsy, so as to produce healing from this major neurological disorder or, at least, to reduce the impact of the disease. Two different categories of drugs able to exert an antiepileptic action have been defined: (i) drugs with an antiepileptogenic activity, and (ii) drugs with a disease-modifying effect. In the first case, the drug should ideally preclude the onset, or at least reduce the severity of epilepsy when this outcome could be predicted on the basis of risk assessment (e.g., post-traumatic epilepsy). In the second case, a drug is expected to modify the course of the disease [727].

The kainic acid (KA) model of temporal lobe epilepsy has been recently proposed as a screening platform to disclose drugs with antiepileptogenic properties or able to produce a modification in the disease progression [728]. In male rats, a single KA intraperitoneal injection is followed by a self-limiting status epilepticus (SE) accompanied by widespread cerebral lesions, which pave the way to the development of epilepsy [710]. We previously found that the onset of spontaneous convulsive seizures after the SE requires 18 days on average, as observed by electroencephalographic (ECoG) recordings. Moreover, long-term video ECoG monitoring of KA-treated rats evidenced a sigmoidal increase in the seizure frequency [729], reaching a plateau at approximately 122 days after the KA administration [730]. This characteristic makes the KA model attractive to test putative disease-modifying treatments.

We obtained evidence that the competitive inhibitor of 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase trilostane has a modulatory effect on epileptogenesis, so as to be considered a potential antiepileptogenic drug.[329] This result was related to the notable capability of trilostane to increase the concentration of various neurosteroids in the hippocampus and neocortex of healthy rats, and also immediately after the SE. Indeed, various experiments showed that different neurosteroids, especially allopregnanolone, exert antiseizure effects via the γ-aminobutyric acid type A receptors ($GABA_A$) and can also modify the development of epilepsy (i.e., epileptogenesis) by unclear mechanisms [731].

In view of the antiepileptogenic properties of trilostane revealed by its early administration in the KA model of temporal lobe epilepsy, which we related to a remarkable increase in allopregnanolone brain levels, we hypothesized that this drug could also increase the availability of neurosteroids in the brain of epileptic rats (i.e., in the chronic period of the model) so as to produce an antiseizure effect and, possibly, a change in the progression of epilepsy in a model in which seizures increase in frequency for many weeks.

2. Materials And Methods

2.1 Animals and experimental design

All experimental procedures were approved by the Italian Ministry of Health (544/2020-PR and 729/2021-PR) in agreement with European Directive 2010/63/EU.

Twenty-six adult male Sprague Dawley rats (Charles River, 175-200 g) were randomly assigned to the vehicle or trilostane group. All rats firstly received a single dose of KA (intraperitoneal injection at a dose of 15 mg/kg) [329] to induce SE, and then subcutaneous injections of sesame oil (the vehicle for trilostane) or trilostane (50 mg/kg) were administered for one week on days 64-70 after KA injection. This timing was selected because corresponds to the rising period of seizure occurrence, as found by others [732].

Video ECoG recordings were performed one week before and during treatments for both groups (n=13) [329]. For this purpose, electrode implantation, recording, and analysis of video ECoG traces were performed as previously described [710]. In brief, rats were implanted with epidural electrodes in the frontal (bregma 0 mm, 3.5 mm lateral from midline) and occipital cortices (bregma −6.5 mm, 3.5 mm lateral from midline). An additional electrode was used as a reference and implanted below lambda in the midline. Offline ECoG traces were digitally filtered (band-pass: high, 50 Hz; low, 1 Hz) and manually analyzed using LabChart 8 PRO (ADInstruments). The number of convulsive spontaneous recurrent seizures (SRSs) without or with loss of posture (stage, ST., 4-5 according to the Racine's scale), their total duration, and mean duration were assessed for each rat. Then, rats were euthanized by isoflurane on day 70, 6 hours after the last injection, and neurosteroid levels were assessed by liquid chromatography-electrospray tandem mass spectrometry in sera, hippocampi, and neocortices [710]. In particular, details about the performed protocol are fully available in our previously published article [641].

2.2 Statistical analysis

The seizure occurrence and characteristics were analyzed by repeated measures two-way analysis of variance (ANOVA) and the post hoc Holm–Šídák test. The Mann-Whitney test was used to analyze levels of neurosteroids, which were non-normally distributed. One outlier per dataset was identified using the Grubbs test and removed from each group of treatment. Results were summarized by mean ± standard error of the mean (SEM) values, or median and interquartile range values, and $p<0.05$ was considered as the threshold for statistically significant differences (SigmaPlot 13, Systat Software).

3. Results

3.1. Trilostane increased the levels of various neurosteroids in the brain and serum of epileptic rats

The neocortical levels of pregnenolone ($p<0.05$, Mann-Whitney test), pregnenolone sulfate ($p<0.01$), progesterone ($p<0.01$), 5α-dihydroprogesterone ($p<0.01$), and allopregnanolone ($p<0.01$) significantly increased in trilostane-treated rats compared to the vehicle group. No changes were found in the amount of neocortical pregnanolone (Figure 19A-F).

Trilostane significantly augmented also the hippocampal levels of pregnenolone ($p<0.01$), progesterone ($p<0.01$), 5α-dihydroprogesterone ($p<0.01$), and allopregnanolone ($p<0.05$). Consistently with the neocortex, pregnanolone was not modified by trilostane. In the hippocampus, pregnenolone sulfate was unchanged (Figure 19G-L).

By measuring the peripheral neurosteroid levels in sera, we observed a massive increase in pregnenolone ($p<0.01$), pregnenolone sulfate ($p<0.01$), progesterone ($p<0.01$), 5α-dihydroprogesterone ($p<0.01$), and allopregnanolone ($p<0.01$) in trilostane-treated rats compared to the vehicle group of epileptic rats, whereas pregnanolone did not change (Figure S1 in the Supporting Information).

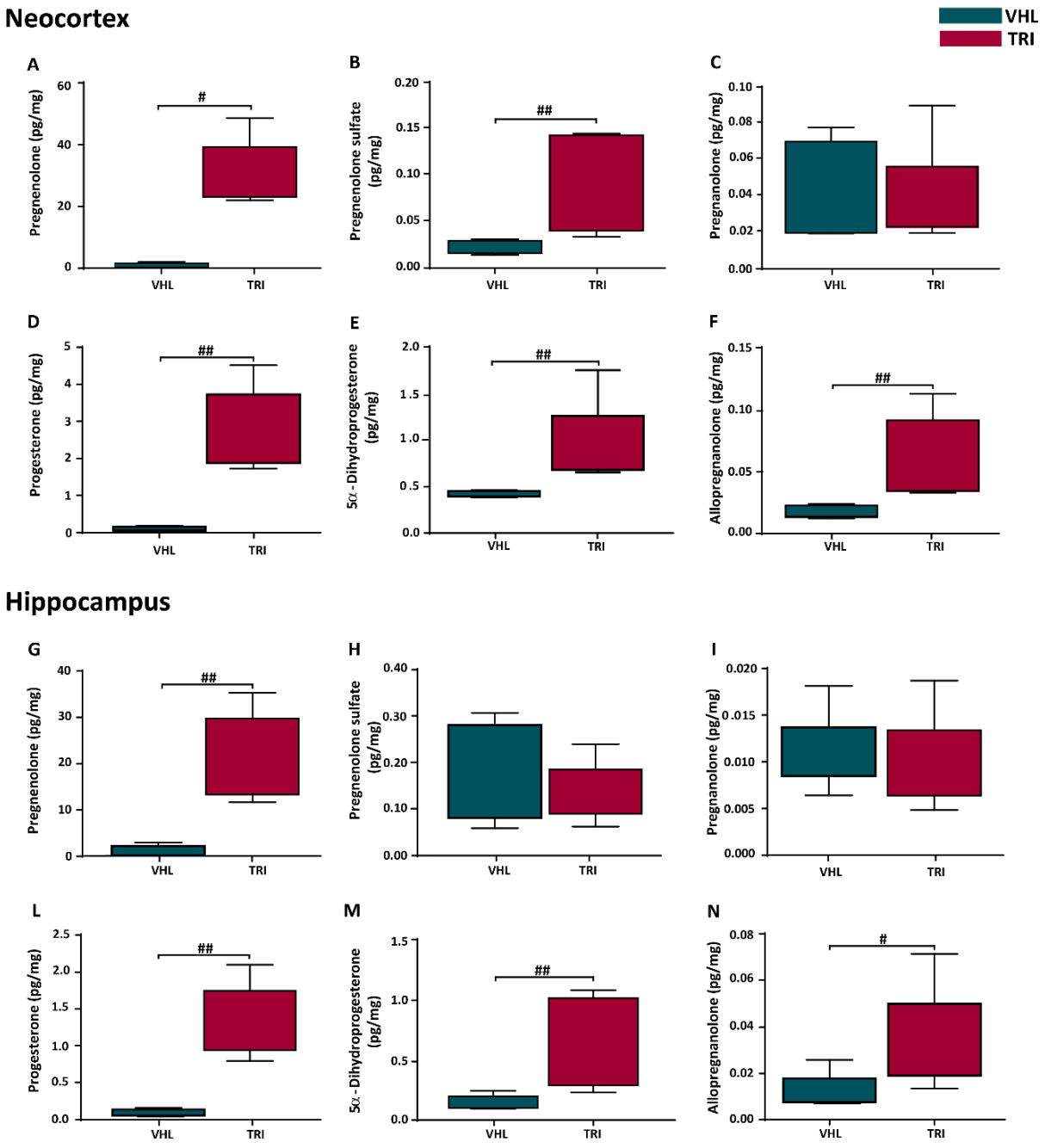

Figure 19. Effects of trilostane on neurosteroid levels in neocortex and hippocampus. The repeated daily administration of trilostane (TRI) resulted in a significant increase (A, B, D-F, G, J-L) of almost all of the examined neurosteroids when compared to the vehicle group (VHL). Only pregnenolone sulfate (H) levels in the hippocampus, and pregnanolone in both the hippocampus and neocortex (C, I) were not increased in the trilostane group compared to the vehicle group. Statistical analysis was performed by the Mann-Whitney test. Results are shown as median ± interquartile range values; *p<0.05 and **p<0.01 vs the vehicle.

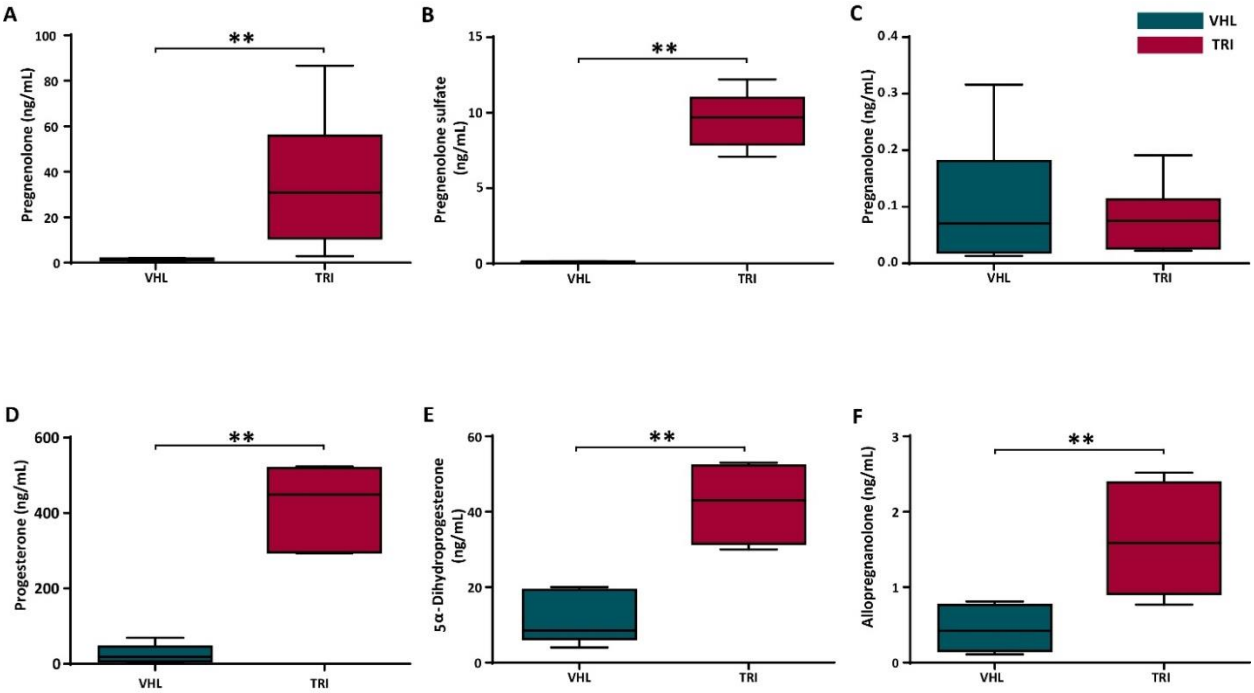

Figure S1. Effects of trilostane on neurosteroid levels in serum. The repeated daily administration of trilostane (TRI) resulted in a significant increase (A, B, D-F) of almost all of the examined neurosteroids, when compared to the vehicle group (VHL). Only pregnanolone (C) levels did not significantly increase in the trilostane group compared to the vehicle group. Statistical analysis was performed by the Mann-Whitney test. Results are shown as median ± interquartile range values; **p<0.01 vs. the vehicle.

3.2. The effect of trilostane on SRSs

There was no significant difference between the two experimental groups in the total number of ST. 4-5 SRSs observed in the week preceding treatments. As expected, the weekly number of convulsive SRSs significantly increased in rats receiving the vehicle compared to the pretreatment values of the same animals (p=0.025, Holm–Šídák test). In comparison to the vehicle-treated group, the administration of trilostane resulted in a small nonsignificant change in the occurrence of convulsive SRSs (Figure 20A). With respect to pretreatment values, trilostane did not modify convulsive SRSs.

In this regard, it was further observed that the number of non-convulsive SRSs was reduced or remained stable in all animals, except for 3 vehicle-treated rats and 1 trilostane-treated rat in which a remarkable increase greater than or equal to 50% of the pre-treatment number of ST. 0-3 SRSs occurred. A significant increase was also observed for the convulsive SRSs in 5 rats of the vehicle-treated group and in 3 rats of the trilostane-treated group. However, only in the trilostane-treated group were 2 rats also recorded displaying a reduction greater than or equal to 50% of the pre-

treatment number of ST. 4-5 SRSs. Accordingly, it was determined an increase of 256% in the total number of SRSs of the trilostane-treated group compared to 412% reached from the vehicle-treated group (See Supplementary Material for further details).

Analysis of total duration or mean duration of ST. 4-5 SRSs revealed that there was no difference between groups in the pretreatment week. Furthermore, there was no significant difference between vehicle-treated and trilostane-treated rats during the treatment. Also the intragroup comparisons between pretreatment and treatment values did not result in significant changes (Figure 20B-C).

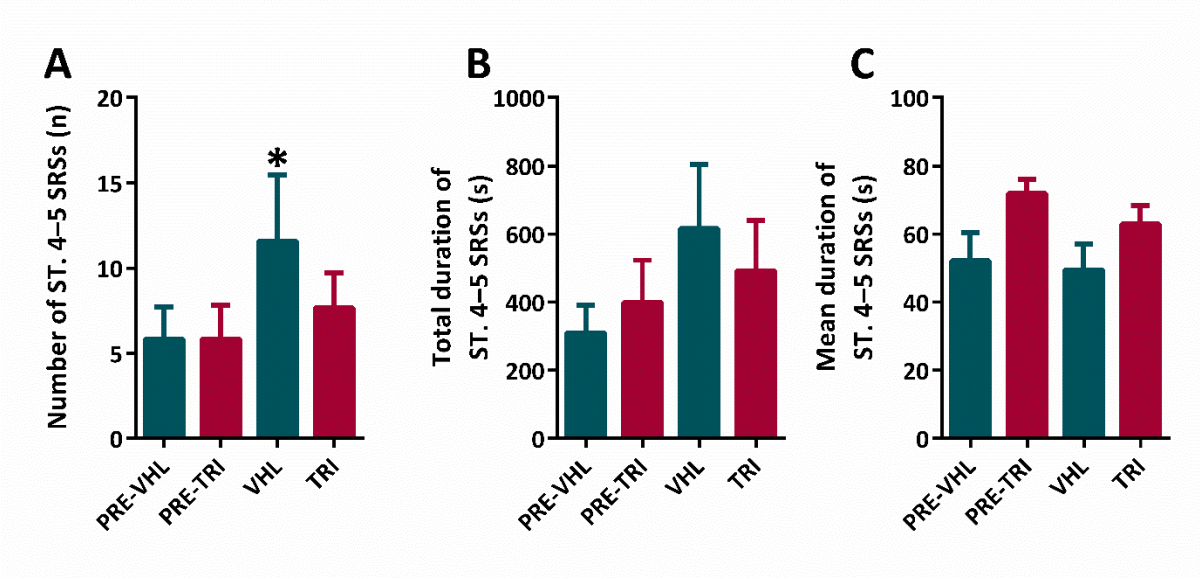

Figure 20. The effect of trilostane (TRI) on (A) number of stages (ST.) 4-5 spontaneous recurrent seizures (SRSs), (B) total duration, and (C) mean duration of ST. 4-5 SRSs. In panel A, the number of convulsive seizures was significantly increased in rats receiving the vehicle (VHL) compared to pretreatment (PRE-VHL). No changes in total duration (B) or mean duration of ST. 4-5 SRSs (C) were found in both groups. *$p<0.05$ compared to the PRE-VHL. Statistical analysis was performed by two-way repeated measures analysis of variance and the Holm–Šídák test. Results are shown as mean ± standard error of the mean (SEM) and $p<0.05$ were considered statistically significant.

It was further observed that the occurrence of non-convulsive SRSs was reduced or remained stable in all animals, except for three vehicle-treated rats and one trilostane-treated rat, in which a remarkable increase (greater than or equal to 50% of the pre-treatment value) of ST. 0-3 SRSs occurred. For convulsive SRSs, a significant increase was observed in five rats of the vehicle-treated group and in three rats of the trilostane-treated group. However, only in the trilostane-treated

group two rats displayed a reduction greater than or equal to 50% of pre-treatment values of ST. 4-5 SRSs. Accordingly, it was determined a 256% increase in the total number of SRSs in the trilostane-treated group compared to the 412% increase found in the vehicle-treated group (Figure S2).

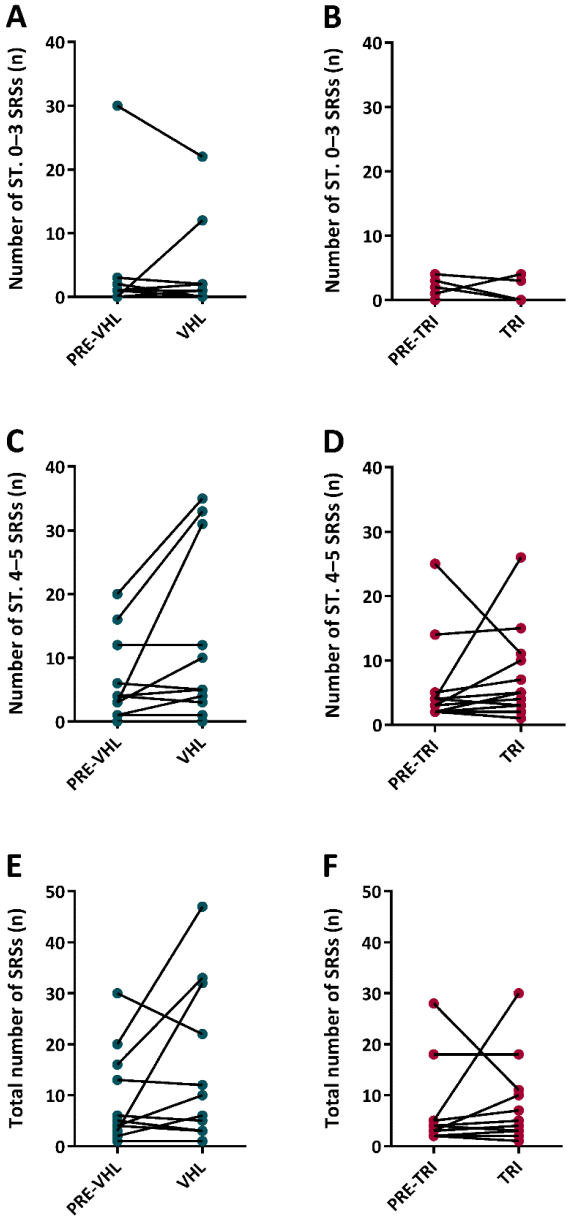

Figure S2. Weekly occurrence of spontaneous recurrent seizures (SRSs) in epileptic rats treated with trilostane (TRI) or sesame oil (VHL). All rats (n = 24) were recorded for one week (PRE-) before being treated with TRI (n = 12) or VHL (n = 12) the following week. In (A) and (B), the number of stage (ST.) 0-3 SRSs was reported respectively in each rat treated with TRI or VHL. The number of ST. 4-5 SRSs for each rat belonging to both groups was shown in (C) and (D). The total number of SRSs for all animals was represented in (E) and (F).

4. Discussion

This study resulted in two major findings: (i) a consistent induction elevation of almost all the investigated neurosteroid production levels also in the injured brain of KA-treatedepileptic rats trated with trilostane, with the exception of pregnenolone sulfate in the hippocampus, and (ii) the slowing down in the occurrence of convulsive SRSs in trilostane-treated rats. Indeed, we observed a statistically significant increase in the occurrence of SRSs only in the VHLvehicle-treated group, with respect to pretreatment values.

Trilostane is a drug able to potently increase the brain levels of various neurosteroids in healthy rats [687] as well as in those that received a KA injection to induce the SE and analyzed during epileptogenesis [329]. This could not be the case ofsame phenomenon has not been assessed in KA-treated epileptic rats, in which we previously found to present a reduction in allopregnanolone and pregnanolone hippocampal levels was previously found, suggesting an impaired capability to produce some neurosteroids in the sclerotic hippocampi [641]. Interestingly, the hypothesized impairment we previously found in basal conditions in epileptic rats did not preclude the possibility of can be overcome by stimulating the synthesis of neurosteroids in epileptic rats by administeringwith trilostane, a drug that increases the availability of neurosteroid precursors in the brain by promoting their synthesis from the periphery. The observed magnitude of trilostane stimulatory activity was roughly the same observed in our previous experiments [329, 641], thus suggesting a major role of the peripheral source of neurosteroids detected in the brain, i.e. adrenal glands.This observation highlights the role of peripheral pregnenolone in supporting the synthesis of neurosteroids in the brain, since the major effect of trilostane is to increase the production and release of pregnenolone in the adrenal cortex to compensate for the reduced availability of glucocorticoids caused by the partial block of peripheral steroidogenesis [466, 706]. However, this was not the case for pregnanolone, which levels were not modified by trilostane both peripherally and centrally. Intriguingly, we found that pregnenolone sulfate was not increased in the hippocampus in spite of the massive increase observed in the sera of trilostane-treated epileptic rats, whereas in the neocortex a significant elevation of this neurosteroid levels was evident. This finding might suggest a reduced transport of pregnenolone sulfate in the hippocampus of KA-treated rats, or its enhanced local metabolization.

The KA model represents a very useful tool to investigate epileptogenesis and the progression of epilepsy after brain damage [219, 733]. Using this model, myo-inositol administered 4 hours after

KA injection and continued for 4 weeks reduced the occurrence and duration of SRSs without affecting the onset of epilepsy, thus documenting a disease-modifying effect independent of any effect activity on epileptogenesis [734]. Interestingly, this effect result was ascribed to the modulation of $GABA_A$ receptors, which notoriously are modulated potentiated by neurosteroids such as allopregnanolone and pregnanolone, whereas pregnenolone sulfate displays opposite properties [735].

Other, different mechanisms could also be involved in the modification of epilepsy in KA-treated rats, for instance neuroinflammation. This was suggested by another investigation showing that simvastatin, administered 30 min after KA and then for 2 additional weeks, reduced the epileptic activity in video ECoG recordings of epileptic rats, which also presented a reduction in interleukin-1β and tumor necrosis factor-α hippocampal levels [736]. Interestingly, also neurosteroids are anti-neuroinflammatory agents, especially progesterone and allopregnanolone [737].

Finally, neuroinflammation is linked to oxidative damage, and the increase of endogenous antioxidant activity effectively modified the occurrence of weekly seizures in KA-treated rats, to suggest that multiple factors may participate in the modulation of epileptogenesis to determine a different epilepsy phenotype in the KA model. In this regards, neurosteroids such as allopregnanolone are involved in the microglial response to oxidation and may counteract the consequences of the oxidative stress [738, 739].

To conclude, the main outcome of our study is that the increase in brain neurosteroid availability can slow down the progression of seizure occurrence in the KA model, suggesting a possible disease-modifying effect of trilostane administration. To be definitely demonstrated, this possibility requires confirmation of the slow down for a longer period of observation and possibly after the interruption of trilostane administration. The present findings and the previous ones suggest that trilostane could be eligible as a drug able to deeply influence the course of temporal lobe epilepsy, as modeled in KA-treated rats.[6]

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Chapter 5

Summary, conclusion, and future perspectives

Epilepsy, a multifaceted neurological disorder, is defined by the ILAE as the occurrence of a minimum of two unprovoked seizures, each separated by a minimum interval of 24 hours. Alternatively, a diagnosis may be warranted with a solitary unprovoked seizure, provided there is a substantial probability exceeding 60% of experiencing a subsequent seizure within the ensuing decade. Additional diagnostic pathways involve identifying specific epilepsy syndromes, highlighting the complexity and varied manifestations of this condition [1]. Epileptogenesis, the latent period between an initial precipitating injury and the onset of SRSs, remains a subject of ongoing exploration. While largely undetermined, causal mechanisms have led researchers to investigate neuroinflammation and glial cell reactions as potential contributing factors [314, 693-695]. Particularly intriguing is the glial reaction observed, especially in the hippocampus, following SE.

It is demonstrated that temporally distinct activation profiles of microglia and astrocytes collaboratively contribute to epileptogenesis in a drug-induced SE model. The initial manifestation involves reactive microglia, succeeded by the appearance of reactive astrocytes, resulting in an increased susceptibility to seizures. Reactive astrocytes exhibit larger Ca^{2+} signals mediated by IP3R2. Interestingly, the deletion of this specific type of Ca^{2+} signaling leads to a reduction in seizure susceptibility following SE. Immediate pharmacological inhibition of microglial activation, particularly during the early phase, successfully prevents the subsequent development of reactive astrocytes, abnormal astrocyte Ca^{2+} signaling, and the augmented seizure susceptibility. These findings underscore the significance of the sequential activation of glial cells as a causal factor in epileptogenesis following SE.

Interestingly, neurosteroids, defined by their production in the nervous system, act as regulators of neuronal excitability and extend their impact beyond epilepsy to conditions such as stress, anxiety, depression, psychosis, ataxia, and pain [700-704]. The modulation of the $GABA_A$ receptor by neurosteroids, including ALLO, pregnanolone, and THDOC, enhances inhibition. Conversely, the neurosteroid pregnenolone sulfate exerts a proconvulsant effect by reducing $GABA_A$ inhibitory

currents. The synthesis of ALLO, a critical neurosteroid in epileptogenesis, can be impeded by finasteride, a 5α-reductase irreversible inhibitor [252]. Drawing from this evidence, researchers hypothesized that delaying epileptogenesis might be achievable by elevating ALLO levels. Trilostane, a reversible inhibitor primarily targeting steroid production in the adrenal cortex, emerged as a potential candidate for this intervention [466]. Interestingly, changes in peripheral steroid production with trilostane demonstrated efficacy in reducing forced swimming-induced immobility in rats. Recent research reported a notable increase in PREG, progesterone, 5α-DHP, and ALLO levels in the hippocampus and neocortex of healthy rats following two injections of trilostane [687]. Given its therapeutic use in controlling hyperadrenocorticism in dogs and its general tolerability [456, 706-708], the prospect of administering trilostane during the latent period in rats was considered. The aim was to explore whether neurosteroids, particularly ALLO, might be associated with a delay in epileptogenesis.

In this thesis I addressed the following questions:

1. Could trilostane change the SE dynamics and delay the onset of SRSs?

SE is a neurological emergency marked by prolonged seizures. Timely intervention is crucial, with critical time points (t1 and t2) defined by the ILAE. T1 is the point at which intervention should begin (set at 5 minutes for convulsive SE), while t2 marks when prolonged seizure activity may lead to lasting consequences. Epileptogenesis, the development of tissue capable of generating SRSs, contributes to the progression of epilepsy. The study investigated the impact of trilostane on SE dynamics and the onset of SRSs and explored the hypothesis that trilostane, a 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase reversible inhibitor, could delay epileptogenesis by increasing ALLO levels. Trilostane targets steroid production, primarily in the adrenal cortex. The study involved multiple trilostane injections in rats, leading to a significant increase in pregnenolone, PROG, 5α-DHP, and ALLO levels in the hippocampus and neocortex. Trilostane is a competitive inhibitor of 3β-HSD, a crucial enzyme system involved in steroid synthesis.

Multiple injections of trilostane, but not a single injection, delayed the onset of SRSs. Trilostane did not affect SE duration, and video-electroencephalogram analysis revealed similar latencies and SE characteristics between trilostane and vehicle-treated rats. Trilostane did not influence SE dynamics

directly after SE onset. While trilostane showed no immediate effects on SE dynamics or neuroprotection, it consistently modified the time course of epileptogenesis. The delay in the appearance of electrographic and motor seizures suggests a modulatory effect, particularly when administered repeatedly. Trilostane's inefficacy in altering SE dynamics contradicted previous findings where it anticipated the disappearance of convulsive seizures when administered before SE induction. This discrepancy indicates that trilostane might not be effective in countering seizures when given after SE onset. The observed delay in epileptogenesis may be attributed to increased ALLO availability, but the exact mechanisms require further investigation. In summary, trilostane, administered multiple times, delayed the onset of SRSs, indicating potential antiepileptogenic effects. It proposes that the increase in ALLO, among other neurosteroids, plays a major role in this delayed epileptogenesis. However, it did not directly influence SE dynamics or provide neuroprotection during SE. The study emphasizes the need for further research to elucidate the specific mechanisms underlying trilostane's impact on epileptogenesis.

2. Could trilostane exert a neuroinflammatory effect on different areas of the hippocampus by changing the neurosteroid levels in the hippocampus and neocortex?

Trilostane's potential to exert neuroinflammatory effects on different areas of the hippocampus, mediated by alterations in neurosteroid levels in both the hippocampus and neocortex, is a complex aspect illuminated by the study. The hippocampus, a region vital for learning and memory, may experience a nuanced impact due to altered neurosteroid levels. Trilostane's modulation of neurosteroids in the neocortex and hippocampus might lead to region-specific variations in neuroinflammatory responses. Neurosteroids, especially ALLO, have been associated with anti-inflammatory properties in the CNS. The study implies that trilostane, primarily recognized as a 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase reversible inhibitor, can intricately modulate neurosteroid levels. While the primary focus is on its role in epileptogenesis, the impact of these neurosteroid fluctuations on neuroinflammation is a crucial aspect to explore. The study found that trilostane did not significantly affect the loss of astrocytes in the CA3 LMol and Sub regions of the hippocampus, even with repeated administration. However, it demonstrated a notable reduction in microglia activation in the Sub region compared to vehicle-treated rats. These results suggest a potential anti-inflammatory effect of trilostane in specific hippocampal areas, contributing to the

understanding of its role in neuroinflammation associated with epileptogenesis. Trilostane, by significantly elevating the levels of neurosteroids like ALLO in both the hippocampus and neocortex, could potentially contribute to an anti-inflammatory milieu. ALLO, acting as a positive allosteric modulator of $GABA_A$ receptors, may enhance inhibitory neurotransmission, thereby suppressing neuroinflammatory responses. Trilostane's influence on neuroinflammation might be linked to its modulation of neurosteroid levels, particularly ALLO. ALLO 's anti-inflammatory effects could potentially mitigate neuroinflammatory processes in different hippocampal regions. In summary, trilostane's alteration of neurosteroid levels, particularly ALLO, may contribute to a neuroprotective environment with potential anti-inflammatory effects in different areas of the hippocampus. However, the specific mechanisms and regional nuances of this interaction warrant dedicated investigation to elucidate the full spectrum of trilostane's impact on neuroinflammation in the hippocampus and neocortex.

3. Could trilostane change the neurosteroids levels during the chronic phase of epilepsy, and what is its effect on SRSs?

Our experiments provide evidence that trilostane, administered during the chronic phase of epilepsy in the KA model, has a significant impact on neurosteroid levels and influences the occurrence of SRSs. Trilostane administration results in a consistent induction and elevation of almost all investigated neurosteroid production levels in the injured brain of KA-treated epileptic rats. Notably, trilostane significantly increases the levels of pregnenolone, pregnenolone sulfate, PROG, 5α-DHP, and ALLO in both the neocortex and hippocampus. The observed increase in neurosteroid levels, especially ALLO, suggests that trilostane positively influences the availability of neurosteroids during the chronic period of epilepsy in the KA model.

Furthermore, our results showed that trilostane has a notable effect on the occurrence of SRSs during the chronic phase. The vehicle-treated group demonstrates a statistically significant increase in the occurrence of SRSs compared to pretreatment values, indicating the expected progression of seizure frequency. In contrast, trilostane-treated rats show a small, nonsignificant change in the occurrence of convulsive SRSs. The number of non-convulsive SRSs either remains stable or decreases in most animals receiving trilostane. Total duration and mean duration of ST. 4-5 SRSs do

not exhibit significant differences between the trilostane-treated and vehicle-treated groups. Trilostane does not induce significant changes in convulsive SRSs compared to pretreatment values. The observed slowing down of SRSs in the trilostane-treated group compared to the vehicle-treated group suggests a potential disease-modifying effect of trilostane during the chronic phase of epilepsy in the KA model.

In summary, trilostane administration during the chronic phase of epilepsy in the KA model induces significant changes in neurosteroid levels, particularly increasing ALLO. This alteration in neurosteroid availability is associated with a slowing down of SRSs, highlighting trilostane's potential as a modulator of epilepsy progression in this experimental model.

Conclusion and future perspectives

Our studies examined trilostane's impact on epileptogenesis and revealed intriguing findings regarding its potential as an antiepileptogenic agent. Despite the lack of observed effects on SE dynamics and acute neuroprotection, repeated administration of trilostane consistently modified the time course of epileptogenesis, evidenced by a delay in the appearance of both electrographic and motor seizures in rats. Furthermore, trilostane administration consistently elevated most investigated neurosteroid production levels in the injured brains of KA-treated epileptic rats, except for pregnenolone sulfate in the hippocampus. Also, a noteworthy slowing down in the occurrence of convulsive SRSs was observed in rats treated with trilostane.

Trilostane, known for its ability to increase neurosteroid levels in healthy and SE-induced rats, was found to mitigate the reduction in ALLO and pregnanolone hippocampal levels observed in epileptic rats. This suggests that trilostane, by promoting neurosteroid synthesis from peripheral sources, overcomes the impaired capability of epileptic rats to produce certain neurosteroids in the brain.

Perspectives for the Future:

1. **Extended Observation Periods:** Further investigations should extend observation periods to validate the sustained impact of trilostane on slowing down seizure occurrence. This would

provide a more comprehensive understanding of its disease-modifying potential over longer durations.

2. **Post-Treatment Effects:** Exploring the effects of trilostane after treatment cessation is crucial to assess its lasting impact on neurosteroid levels and seizure progression. This post-treatment analysis could unveil whether trilostane induces a transient or lasting antiseizure effect.
3. **Mechanistic Clarifications:** The underlying mechanisms of trilostane's effects on neurosteroid synthesis and its relationship with seizure modulation need in-depth exploration. Mechanistic studies may unravel the specific pathways through which trilostane exerts its disease-modifying influence.
4. **Comparative Analyses:** Comparative studies with other antiepileptic drugs and interventions could help contextualize trilostane's efficacy in the broader landscape of epilepsy treatment. Understanding how trilostane compares with established therapies will aid in evaluating its clinical potential.
5. **Influence of Neuroinflammation:** Given the potential connection between trilostane, neurosteroids, and neuroinflammation, future research should delve into the drug's impact on inflammatory processes. This could offer insights into trilostane's broader effects on brain health and its role in mitigating inflammation-induced epileptogenic changes.

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