Mgr inż. Marek Pawlik

Methionine sulfoximine as a modulator of initial seizures in the lithium-pilocarpine model in juvenile rats

Metionino-sulfoksymina jako modulator inicjacji napadów padaczkowych u młodych szczurów w modelu litowo-pilokarpinowym

Doctoral thesis in the discipline of Medical Sciences,
in the form of monothematic collection of articles

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IMDiK
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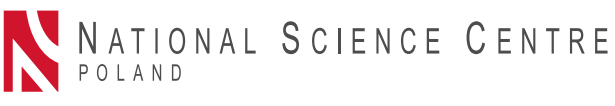
Defense of the doctoral dissertation before Scientific Council
of Mossakowski Medical Research Institute
Polish Academy of Sciences

Warsaw, 2022

This doctoral research was supported by the National Science Centre (NCN) of Poland (2016/23/B/NZ4/03678) and by ESF (POWR.03.02.00-00-I028/17-00).



First and foremost I would like to thank Prof. Jan Albrecht for his comprehensive guidance, countless fruitful discussions, brilliant anecdotes, i.e. being the best mentor I could imagine.

I would also like to thank Dr. Marta Obara-Michlewska for her always keen aid in experiments and being the person I could always seek guidance from, Dr. Anna Maria Czarnecka for introducing me to the mysteries of brain microdialysis and her recommendations of the best mountain trails, Dr. Mariusz Popek for his valuable practical help and bringing a lot of sarcasm and humor to the Department of Neurotoxicology, and Karolina Orzeł-Gajowik for encouraging me to work in MMRI PAS five years ago, echoing Mariusz in sarcastic sneers and being the best friend this whole time.

I also wish to extend my thanks to all my other colleagues from the Department of Neurotoxicology, those still present and those whom I had the pleasure of meeting during these five years, for creating an exceptional work environment.

Finally, I wish to thank my partner Daria, my parents and grandparents for always being there for me.

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List of abbreviations used in the dissertation:

[^{3}H]D-Asp – tritium-labelled D-aspartate

ACh – acetylcholine

GC-MS – gas chromatography-mass spectrometry

GGC – glutamine-glutamate cycle

Gln – glutamine

Glu – glutamate

GS – glutamine synthetase

GSH – glutathione

HPLC – high-pressure liquid chromatography

KA – kainic acid

MSO – L-methionine-D,S-sulfoximine

Pilo – pilocarpine

SE – *status epilepticus*

TLE – temporal lobe epilepsy

Streszczenie (abstract in Polish)

Zachodząca w cyklu glutamina-glutaminian (GGC) przemiana glutaminy (Gln) do glutaminianu (Glu) odpowiedzialna jest za syntezę ok. 70 procent puli tego neuroprzekaźnika. Choć wzmożona transmisja glutaminergiczna jest stałym elementem napadów padaczkowych, mechanizm mobilizacji przekaźnikowej puli Glu w inicjacyjnej fazie choroby nie został dobrze poznany. Postawiono hipotezę, że zahamowanie aktywnej syntezy Gln, bezpośredniego prekursora Glu, mogłoby osłabić lub zatrzymać aktywność napadową mózgu w tej fazie. Aby zbadać tę hipotezę, młodym szczurom w litowo-pilokarpinowym modelu padaczki, podawano L-metionylo-D,S-sulfoksyminę (MSO) – swoisty inhibitor syntetazy glutaminy, lub sól fizjologiczną, po czym wywoływano napady drgawkowe przy użyciu agonisty receptora muskarynowego, pilokarpiny (Pilo). Przebieg napadów analizowano przez 1 godzinę od iniekcji Pilo, z wykorzystaniem behawioralnej skali Racine'a, EEG, EMG oraz biosensorów Glu umieszczonych w hipokampie; część zwierząt otrzymała również znakowane izotopowo astrocytarne lub neuronalne prekursory cyklu Krebsa, odpowiednio [1,2-^{13}C]octan lub [U-^{13}C]glukozę, w celu zbadania wpływu MSO i/lub Pilo na zmiany w przebiegu GGC. Po zakończeniu obserwacji zwierzęta były uśmiercane, a ich tkanka mózgowa wykorzystana była do oznaczeń biochemicznych i molekularnych. Przeprowadzono również oddzielną serię doświadczeń, z wykorzystaniem skrawków mózgowych, pobranych od zwierząt poddanych działaniu MSO, w których badano zmiany w wychwycie i wyrzucie [^{3}H]D-asparaginianu ([^{3}H]D-Asp), znakowanego trytem, niemetabolizowanego analoga Glu. Ekspresję mRNA kodującego białko wczesnej odpowiedzi, c-Fos, mierzono metodą PCR w czasie rzeczywistym.

Wykazano, że zastosowanie MSO łagodzi napady drgawkowe, a także wyraźnie opóźnia ich wystąpienie, zarówno na poziomie behawioralnym, jak i elektrograficznym, nie łagodzi jednak współwystępującego z napadami wzrostu poziomu zewnątrzkomórkowego Glu w hipokampie. Analiza przemiany *in vivo* znakowanych ^{13}C prekursorów metabolicznych ([U-^{13}C]glukozy i [1,2-^{13}C]octanu) do Gln i Glu przy użyciu chromatografii gazowej - spektrometrii masowej (GC-MS) nie wykazała istotnego wpływu MSO na ten proces. Zaobserwowano, że MSO znacząco osłabia wyrzutu ([^{3}H]D-Asp), nie wpływając wyraźnie na jego wychwyt. Badania molekularne wykazały silną ujemną korelację pomiędzy poziomem ekspresji mRNA c-Fos, a czasem dzielącym podanie Pilo od wystąpienia uogólnionego napadu drgawkowego, przy jednoczesnym braku zależności ekspresji od intensywności napadów. MSO wykazało tendencję do osłabienia odpowiedzi c-Fos na Pilo.

W sumie, przedstawione wyniki sugerują, że mechanizm opóźnienia i osłabienia napadów drgawkowych w modelu pilokarpinowym przez MSO nie jest związany z obniżeniem syntezy glutaminy, a raczej z bezpośrednim oddziaływaniem na mechanizmy uwalniania Glu z synapsy. Skuteczność przeciwdrgawkowego działania MSO sugeruje, że ten związek (lub jego pochodna) może stać się skutecznym narzędziem w ew. zapobieganiu i terapii najwcześniejszych napadów padaczkowych, zaś c-Fos pożytecznym markerem biochemicznym modulacji najwcześniejszej fazy inicjacji napadu.

Abstract (in English):

Glutamate (Glu) is the main excitatory neurotransmitter in the brain, and about 70 percent of its neurotransmitter pool is generated in the glutamine-glutamate cycle (GGC). Although increased glutamatergic transmission is a persistent element of epileptic seizures, neither the metabolic origin of the Glu surplus nor the mechanisms underlying its enhanced release in initial phase of the disease are known. It appeared reasonable to hypothesize that, inhibition of the active synthesis of glutamine (Gln), a direct precursor of Glu, would decrease or even stop the initial ictal activity of the brain. To test this hypothesis, juvenile rats in a lithium-pilocarpine model of temporal lobe epilepsy were administered a low, non-convulsive dose of L-methionine-D,S-sulfoximine (MSO), a specific glutamine synthetase inhibitor, or saline, and convulsive seizures were subsequently induced using the chemoconvulsant pilocarpine (Pilo). The course of the seizures was analyzed for 1 hour after the Pilo injection, using the Racine behavioral scale, EEG, EMG and intrahippocampal Glu biosensors; some of the animals also received ^{13}C-labelled astrocytic or neuronal precursors of the TCA cycle, [1,2-^{13}C]acetate or [U-^{13}C]glucose, respectively, to assess metabolic changes under the action of MSO and/or Pilo. After observation, the animals were sacrificed and their brain tissue was used for biochemical and molecular assays. A separate series of experiments was also performed using acutely isolated brain slices isolated from MSO-pretreated animals, to examine changes in the uptake and efflux of a non-metabolizable Glu analogue, [^{3}H]D-Aspartate ([^{3}H]D-Asp). Expression of mRNA coding for the early response protein, c-Fos, was analyzed using real time PCR.

Pretreatment with MSO alleviated seizures and significantly delayed their onset, both at the behavioral and electrographic levels, but did not attenuate the seizure-associated accumulation of extracellular Glu in the hippocampus. In metabolomic studies, MSO did not markedly affect the *de novo* synthesis of Gln and Glu. As analyzed in brain slices, MSO significantly attenuated the release of [^{3}H]D-Asp, but not its uptake. Expression level of c-Fos mRNA was negatively correlated with the latency onset to the first generalized seizure, but not with seizure intensity, and MSO tended to attenuate the Pilo-dependent c-Fos response.

The results demonstrate the effectiveness of MSO in attenuating the initial Pilo-induced seizures, especially in delaying their onset. However, metabolomic data indicate that the seizure-attenuating effects of MSO are not due to inhibition of glutamine synthase activity, but rather to its direct action on the Glu-releasing machinery. In perspective, the results suggest that MSO or its derivative(s) may become a useful tool in prevention or therapy of the very initial seizures and that, c-Fos appears to be a useful, therapy-sensitive biochemical marker of the very initial stage of epileptogenesis.

Novelty of the dissertation

In this dissertation the following findings were demonstrated for the first time:

- Pretreatment with a low, non-convulsive dose of the glutamine synthase inhibitor MSO delays the onset of electrographic and behavioral seizures in the juvenile rat lithium-pilocarpine (Li-Pilo) model of TLE, and alleviates symptoms of the initial Pilo-induced seizures.

- Low-dose MSO does not affect conversion of metabolic precursors to Glu and Gln, but decreases Glu efflux in acute brain slices. Collectively, the results indicate that the seizure-modulating effect of MSO occurs by a non-canonical mechanism, reflecting interference with the Glu-releasing machinery.

- Expression of mRNA coding for the early response protein c-Fos is negatively correlated with the period from convulsant administration to the onset of seizure, but shows no correlation with seizure intensity.

- MSO tends to attenuate the c-Fos mRNA expression evoked by Li-Pilo.

1. Introduction

1.1. Temporal lobe epilepsy – basic disease characteristics

Epilepsy, one of the most common neurological diseases affecting around 65 million people worldwide,[1] is characterized by recurrent, unprovoked or reflex seizures.[2] Its most common form is temporal lobe epilepsy (TLE)[3,4] featured by seizures originating in limbic structures, such as the hippocampus, entorhinal cortex and amygdala, or in the temporal neocortex.[3,5] It is also the most pharmacoresistant type of epilepsy; despite the plethora of available medications[6–9] (Article III),[10] estimates show that 51 to as much as 75 percent of people with advanced stage of disease still suffer from recurrent seizures.[11,12] In addition to a substantial impediment to quality of life and daily functioning, chronic epilepsy can also lead to many severe comorbidities, such as cardiovascular disorders, dementia, depression, fractures,[13] and often leads to 'sudden unexpected death in epilepsy'.[14,15]

TLE is characterized by a triphasic course: epileptogenesis, usually occurring in childhood or adolescence,[16] begins with initial seizure(s), defined as the initiation phase or initial *status epilepticus* (SE). After the initial seizures extinguish, the disease progresses to the latent, seizure-free phase which can last a few days or even several years. Despite the asymptomatic character of this phase, astrocytosis and neuronal loss in the hippocampus develop gradually. These changes in neuronal and astroglial organization finally lead to the chronic phase in which spontaneous seizures reappear, and to hippocampal sclerosis, a morphological degeneration that is a hallmark of developed TLE.[17,18]

1.2. Li-Pilo model of TLE

Over the years, many animal models have been developed to reflect the three phases of human epileptogenesis, the most frequently used involve systemic application of chemical convulsants – pilocarpine (Pilo)[19] or kainic acid (KA).[20,21] The two models are characterized by relatively low invasiveness (no surgery or direct interference with the brain needed) and labor-intensity, while still maintaining high reliability[22] and effectiveness, allowing the use of numerous animals in a short time.[23] Both models replicate the main features of clinical TLE with clearly defined latent and chronic periods of similar length, and comparable rates of pharmacoresistance among experimental animals.[22] However, Pilo has the ability to induce the SE faster than KA (respectively ~1 h and ~2 h after convulsant administration),[24] leading to a higher frequency of seizures, and a much earlier, more pronounced neuronal loss,[22,24] making it the preferred model for research into early changes in TLE. Initially, a significant drawback of the Pilo model was the high mortality rate, but a modification consisting in pretreatment with lithium (Li-Pilo) allowed one to reduce the dose of Pilo needed to induce SE, and thus notably reduced mortality, as well as many other cholinomimetic side effects of

Pilo.[25–27] The studies described in this dissertation employed this model adapted to young immature rats, to best reflect the most common, juvenile variant of TLE.[27,28]

1.3. Glu and mechanism of seizures: role of Glu recycling to Gln

Glutamate (Glu), the major excitatory transmitter in the brain, appears to play a crucial role in evoking and maintaining epileptic seizures. Extracellular accumulation of Glu in the brain as shown using the microdialysis technique is thought to represent its pool active in neurotransmission.[29] Increased extracellular Glu is observed immediately before and during the seizure in the human epileptogenic hippocampus[30,31] and neocortex,[32] and in pertinent brain structures in a plethora of acute and chronic animal models of TLE.[33–36] Of note, the magnitude of the increase of extracellular Glu seems to correlate positively with the ictal activity.[31] A study involving a group of 72 chronic epileptic patients demonstrated that Glu, but not the inhibitory amino acid GABA or their precursor Gln, increases markedly and remains high throughout a seizure.[32] In the initial phase of TLE the status of extracellular neurotransmitters appears to be more complicated. Meurs *et al.*,[37] using microdialysis in three different rat models of the intrahippocampal administration of convulsive agents, showed an immediate increase in Glu levels following seizure induction. In the latter study, the increase of Glu was followed by elevation of GABA, which contrasts with the observation made in the chronic phase.[31] However, even an increase of Glu level is not necessary a rule in the initial phase – for contrasting results see: Millan *et al.*,[33] Smolders *et al.*[38]

About 70% of the Glu in the neurons originates from Gln generated in the glutamate/glutamine cycle (GGC)[39] (Figure 1), suggesting that the Glu precursor glutamine (Gln), which in the GGC is the product of the astrocytic enzyme glutamine synthetase (GS) may play a role in the maintenance of paroxysmal excitation elicited by Glu. Indeed, Tani *et al.*[40] showed that even though GGC is not absolutely required for maintaining basic levels of glutamatergic transmission, it is critical to sustaining higher rates of excitatory neurotransmission. Data from the TLE kainate model obtained by Kanamori and Ross[35,36] support the association of seizures with a pool of Glu which is directly derived from Gln. An increase in extracellular Glu during seizures was accompanied by a decrease in extracellular Gln,[36] whereas inhibition of neuronal Gln uptake resulted in a reduction in seizure frequency.[35] Furthermore, a recent study on MSO rat model of TLE has shown that increased blood Gln evoked by oral Gln supplementation, significantly increased the frequency of convulsive seizures during the early phase of GS inhibition.[41]

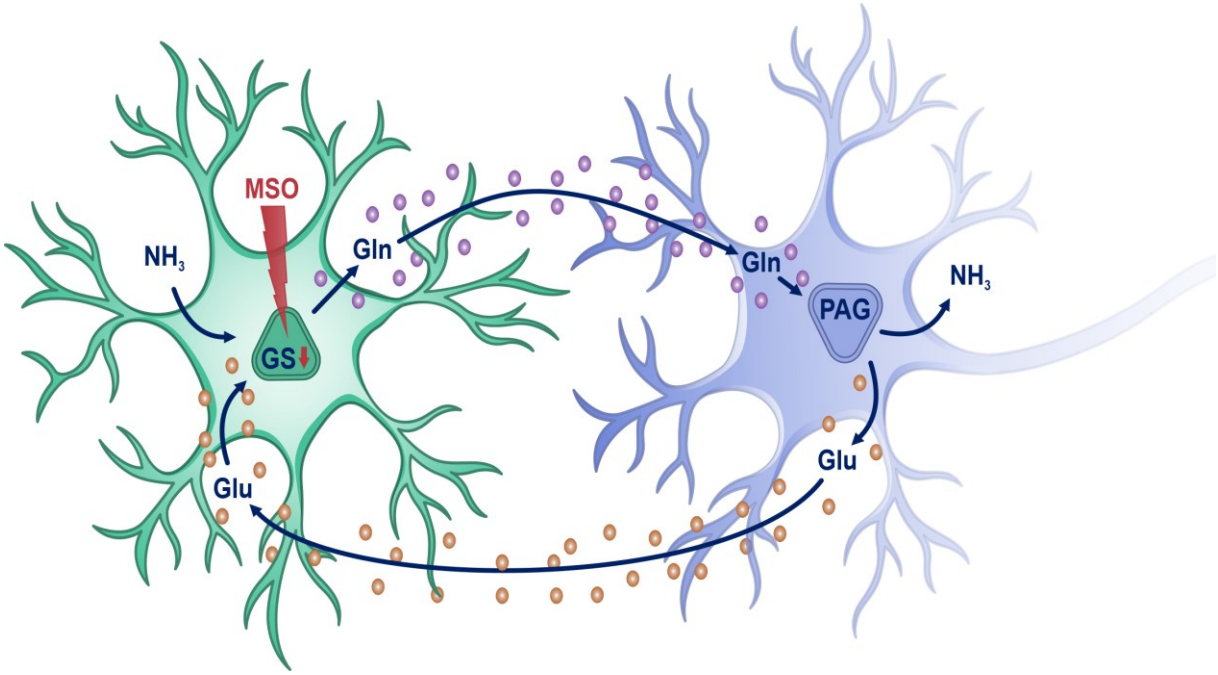

Figure 1. Glutamine-Glutamate Cycle and the backbone of the original hypothesis for methionine sulfoximine (MSO) pretreatment. During epileptic seizure, glutamatergic transmission is enhanced. MSO inhibits glutamine synthetase (GS) activity in astrocyte (green) and consequently glutamine (Gln) synthesis. As a result, the recycling of Gln to glutamate (Glu) reduces in glutamatergic neuron (blue), and subsequently the perisynaptic accumulation of Glu decreases, thus mitigating the seizure.

Chronic TLE is characterized by reduced GS expression and activity [42,43] (see extensive review by Eid et al.[44]), which critically contributes to the observed high levels of extracellular Glu in interictal periods[45,46] and thus to recurrent seizures.[47,48] Accordingly, epileptic seizures can be evoked by prolonged chemical inhibition of GS with the GS inhibitor MSO[48–50] and have also been reported to concur with mutations in the GS gene.[51,52]

We hypothesize that the initiation phase involves a reverse sequence of events compared to the chronic period, namely, that the initial seizures are elicited and maintained by Glu derived from newly synthesized Gln.

1.4. MSO – the glutamine synthetase inhibitor

The action of methionine sulfoximine (MSO) as a potent, irreversible GS inhibitor was first reported by Pace and McDermott,[53] the GS-inhibitory effect of MSO was later shown to be exclusive to the S-sulfoximine isomer of MSO.[54,55] The effect of MSO depends on the dose – higher doses cause convulsive seizures[47,55] and upon prolonged treatment lead to hippocampal sclerosis and, consequently, chronic TLE.[47,48,56] While in the chronic setting MSO contributes to epileptogenesis by inactivating GS, its direct convulsive effect appears to be associated with the methylation mechanism rather than with GS inhibition.[57,58]

Experimental data from *in vitro* studies[59,60] and *in vivo* amyotrophic lateral sclerosis model[61] showed that inhibition of GS results indeed in decreased cerebral levels of both Glu and Gln, however, it was not known what effect this would have on convulsant-induced neurotransmitter dyshomeostasis. Blin and colleagues[62] have shown that even low doses of MSO are able to notably decrease GS activity, down to ~15 % of basal level, without causing serious side effects.

Therefore, in the studies included in this dissertation, we employed a moderate, subconvulsive dose of MSO, to assess whether it is capable of diminishing the neurotransmitter pool of Glu and, thus, weakening or stopping propagation of initial Pilo-evoked seizures.

1.5. Biomarkers of epileptogenesis

With regard to TLE, the term "marker" refers to two different, not ideally overlapping notions. One is that of "clinical" marker, which serves to identify an early manifestation of the disease and to design an adequate treatment modality to prevent the advanced symptoms. The other, best coined a "mechanistic marker", would help to unravel an aspect of mechanism underlying epileptogenesis. Ideally, a clinical marker should be present in plasma and its level should not be affected by the release from peripheral tissues.[63] Though several clinical markers have been identified to date, none of them has attained clinical application yet. A serum protein – neurofilament light,[64] DNA chaperone HMBG1,[65] calcium-binding astrocytic protein S100β[66] and matrix metalloproteinase 9[67] are four candidate molecules for epileptogenesis biomarkers detected in blood early after SE, with a potential diagnostic and prognostic value; however, only the two former molecules are not released in significant amounts from peripheral sources. Moreover, they were tested in the chronic phase of TLE, so it is not possible to predict their usefulness as markers in the initiation phase.

With regard to mechanistic markers, a FOS proto-oncogene (c-Fos), an immediate early gene and one of the earliest markers of neuronal activity appears to be a plausible candidate.[68–71] It was previously used to map the activity of specific brain regions during seizures induced in animals by 4-aminopyridine[70,72] and pentylenetetrazol.[68,69,73,74] Its expression has also been found to be increased in the human epileptic neocortex[75] and correlated with epileptiform activity in human brain slices.[72]

In this dissertation we investigate how hippocampal c-Fos mRNA expression correlates with the intensity and the time to onset of first Pilo-evoked seizures. We specifically asked if expression of this marker is modulated by pretreatment with MSO.

2. The objective of dissertation

2.1. General objective

The main objective of the studies included in this dissertation was to evaluate the role of GS and GGC in the initial stage of TLE in the juvenile rat Li-Pilo model. The evaluation was based on the analysis of the effects of the GS inhibitor MSO on the development of the first Pilo-induced seizures. In due course, this objective evolved into more specific aims encompassing untoward effects of MSO. The specific aims are described in the articles I, II and III.

2.2. Specific objectives

Article I. Analysis of the effect of pretreatment with non-convulsive dose of MSO on the course of the initial convulsive seizure, in relation to GS inhibition, behavioral symptoms, electroencephalographic and electromyographic parameters, and extracellular Glu level in the hippocampus.

Article II. Evaluation of the impact of MSO pretreatment on: i) conversion of metabolic precursors (glucose, acetate) to Gln and its neuroactive metabolites Glu and GABA during the initial Pilo-induced seizure development; ii) Glu efflux and uptake in acute brain slices subjected to depolarizing conditions.

Article III. The correlation analysis of c-Fos mRNA expression with behavioral parameters of seizure in Li-Pilo model rats after pretreatment with MSO. Results of the experiments were published as part of an extensive review describing state of the art of molecular antiepileptogenesis targets.

Together, the listed articles present a coherent study describing how pretreatment with a non-convulsive MSO dose modulates a spectrum of parameters characterizing the initial seizures in the Li-Pilo model of TLE.

3. Materials and Methods

3.1. Animals

All animal experiments were approved by the local ethical committee in Warsaw (consent no. 405/2017) and conducted in accordance with national guidelines and the EC Directive 2010/63/EU. Male Sprague Dawley rats (the animal colony of the Mossakowski Medical Research Institute, Polish Academy of Sciences in Warsaw – MMRI PAS) were housed individually at constant temperature and 12-h light/dark cycling with free access to standard rat food and tap water. Animals undergoing surgery for stereotactic implantation of electrodes and biosensors were housed starting at postnatal day 19 (P19); those that have not undergone surgery – starting at P23.

3.2. Juvenile rat Li-Pilo TLE model

The procedure was essentially as described by van der Hel *et al.*[28] with minor modifications. At P23, were injected intraperitoneally (i.p.) with lithium carbonate (222 mg/kg) dissolved in saline (pH equalized to 7.4) – the use of Li^+ increases the sensitivity of rats to Pilo, thereby reducing its side effects and mortality rates.[25] At P24, 18–20 h after Li^+ treatment, animals were injected i.p. with methyl-scopolamine (1 mg/kg), an agent that reduces the peripheral cholinomimetic effects of Pilo, and finally thirty minutes later with the chemoconvulsant – M1 receptor agonist Pilo (40 mg/kg). From then on, animal's behavior was continuously monitored for either 15 or 60 minutes, utilizing five-stage Racine scale[76] to determine the severity of seizures. After a given period, rats were anesthetized with isoflurane and immediately decapitated. Control rats that did not receive Pilo were given equal volumes of saline at the same time.

3.3. Pretreatment with MSO

MSO was dissolved in saline and administered i.p. (75 mg/kg). Rats that were used in experiments with Pilo received MSO 150 min before Pilo (or before saline, in the case of control groups). Rats used for *ex vivo* experiments with brain slices received MSO 150 min before decapitation. Control rats that did not receive MSO were given equal volumes of saline at the same time.

3.4. Surgical procedures

Rats at P19 were anesthetized with ketamine (75 mg/kg) and dexmedetomidine (0.5 mg/kg) for stereotactic implantation of electrodes and biosensors. EEG electrodes were made of stainless steel screws, implanted intracranially, their end resting on the cerebral cortex, and also acting as an anchor for the sensor headmounts. EMG electrodes were placed below the neck muscles. For biosensor experiments, guide cannula was implanted over the left CA1 hippocampal region. Headmount and cannula were secured using dental acrylic cement and the incision was sutured below and above the

headmount. Following the surgery, rats were woken up from anesthesia with atipamezole hydrochloride (1 mg/kg) and treated with ketoprofen (5 mg/kg) once a day up to P21.

3.5. EEG, EMG and extracellular Glu recording on free moving animals

One day prior to the experiment, the rats at P23 were placed in a round transparent cage to reduce their stress related to the new environment. The next day rats (P24) were connected to the data acquisition system – in animals with only EEG/EMG sensors, the procedure did not require any special treatment, however, in those with implanted cannulas, mild isoflurane anesthesia was necessary to safely insert the biosensor. From then on, the EEG, EMG, and biosensor data were continuously acquired (sampling frequency 2 kHz for EEG/EMG and 1 Hz for a biosensor) up to 60 min after Pilo administration. Baseline recordings were acquired for at least 30 min prior to MSO administration (or saline in control groups).

3.6. Metabolic studies

Rats received a single i.p. dose of metabolic precursors, [1,2-^{13}C]acetate or [U-^{13}C]glucose: i) either at the same time as Pilo or ii) 45 min after Pilo. In both cases, rats were decapitated 15 min after the precursor administration. Hippocampi and entorhinal cortex samples were immediately dissected and frozen in liquid nitrogen. Amino acids were extracted from tissue samples and analyzed using gas chromatography coupled to mass spectrometry (GC-MS) and high-pressure liquid chromatography (HPLC).

3.7. [^{3}H]D-Asp uptake and efflux in acute brain slices

Rats anesthetized with isoflurane were decapitated, the brain was immediately removed, and cortices were cut into 350 μm thick slices, using McIlwain tissue chopper. The slices were pre-incubated for 30 min in the Krebs buffer and then used for measuring uptake or efflux of radioactive [^{3}H]D-Aspartate ([^{3}H]D-Asp)which is a non-metabolizable analogue of glutamate.

The efflux of [^{3}H]D-Asp was measured after 15 minutes of incubation in Krebs buffer containing [^{3}H]D-Asp (1.4 μCi / mL) and unlabeled D-Asp (100 μmol / L). Slices were moved to a chamber perfusion system, rinsed with Krebs buffer (0,5 mL / min rate), and the initial fraction was collected for 20 min to establish the baseline efflux. The perfusate samples were then collected for 20 min, at 1-minute intervals. A depolarizing pulse was introduced by raising KCl concentration in the Krebs buffer to (75 mM), with simultaneous reduction of NaCl (47.7 mM) for 2 min, at the time point corresponding to fractions 5. and 6. Scintillation fluid (2 mL) was added to each sample and brain slice and radioactivity was measured in the liquid scintillation counter (LSC).

The uptake of [^{3}H]D-Asp was initiated by adding [^{3}H]D-Asp (0.1 μCi / mL) to vary the extracellular concentrations of unlabelled D-Asp (50–2000 μM). Incubation with the radioisotope was

continued for 3 min and was terminated by rapid vacuum filtration through nitrocellulose filter discs, followed by flushing four times with 2 mL of ice-cold Krebs buffer. Slices were weighed, immersed in scintillation fluid (4 mL), and the radioactivity was measured in the LCS.

3.8. GS activity assay

GS activity was determined using the γ-glutamyl transferase assay.[77] Immediately after decapitation, brain tissue samples were dissected, frozen, and homogenized in ice-cold buffer composed of sucrose and HEPES. Next, brain homogenates were incubated for 20 min at 37 ºC with the reaction buffer, containing L-Gln, hydroxylamine-HCl, Na-arsenite, ADP, $MnCl_2$, and imidazole-HCl. The reaction was terminated by adding the stop-solution, containing trichloroacetic acid, HCl, and $FeCl_3$. The solution was cleared by centrifugation, and the reaction product, γ-glutamyl hydroxamate, was determined colorimetrically.

3.9. RT-qPCR

Total RNA was extracted from hippocampus using Trizol. Extracted RNA was reverse transcribed using RT-PCR kit. The mRNA expression was determined with TaqMan probe assays. The fold change in gene expression was determined by the $2^{-\Delta\Delta C_T}$ method.[78]

3.10. Western Blotting

Immediately after decapitation, rat hippocampus was isolated on ice, and frozen in liquid nitrogen. Frozen tissue samples were homogenized in buffer and centrifuged, then the content of protein was assessed by Western Blotting as previously described.[79,80] Briefly, membranes were incubated overnight at 4℃ with primary antibody, washed, incubated with secondary, HRP-conjugated IgG, and the chemiluminescence was measured. After stripping, membranes were incubated with the reference antibody (HRP-conjugated anti-GAPDH) developed as described above.

3.11. Data evaluation

Initial data curation was performed in Microsoft Excel 2016, statistical analysis in Prism 7.0, and EEG/EMG data processing using Matlab. Detailed information on the particular statistical analyzes can be found in the figure captions and in the Materials and Methods sections of Article I (pages 9-10) and II (pages 13-14).

4. Summary of the most important results with reference to the current state of knowledge

4.1. Pretreatment with MSO delays onset and alleviates symptoms of initial seizure

Administration of non-convulsive dose of MSO substantially delays the onset of initial seizure in juvenile rats subjected to Li-Pilo treatment, both on behavioral (Article I, Figure 3; Article II, Figure 1) and electrographic (Article I, Figure 4 B) level. Animals that received MSO showed significantly diminished behavioral symptoms of seizures as measured by the Racine scale (Article I, Figure 2; Article II, Figure 1), as well as markedly reduced muscle contraction power in EMG (Article I, Figure 5). Pretreatment with MSO noticeably increased the number of seizure-free animals (Article I, Figure 3; Article II, Figure 1).

4.2. The delayed onset of seizures is not due to decreased Glu synthesis

Measurements of extracellular Glu performed with intrahippocampal biosensors did not show any change caused by pretreatment with MSO throughout whole observation period (Article I, Figure 6 A). The metabolic studies failed to demonstrate any substantial alteration in *de novo* Glu synthesis between MSO-treated and untreated groups (Article II, Figure 2). Considering that during the period of the highest symptom variability, GS activity was not reproducibly decreased (Article I, Figure 1 A), these results indicate that MSO exerted its effects by a mechanism other than GS inhibition.

MSO inhibits not only GS but also γ-glutamylcysteine synthetase, thus lowering glutathione (GSH) levels.[81] GSH in the brain acts as a neuromodulator, binding to NMDA receptors,[82] and its deficit has been shown to impair field excitatory postsynaptic potentials in brain slices,[83] which could partially explain the lower sensitivity of rats to Pilo. However, available evidence does not support this interpretation. In the study by Steullet *et al*.,[83] low GSH levels induced an increase in excitability of CA1 pyramidal cells, an effect opposite to that observed by us. Decreased GSH has also been shown to be correlated with higher frequency of spontaneous seizures[84] and associated with severe cognitive impairment,[85] the effects not observed even with long-term chronic administration of MSO.[62]

Since alterations in the GGC do not appear to play a key role here, involvement of other neurotransmitter systems appears to be likely. Pilo stimulates not only glutamatergic but also cholinergic transmission,[86,87] which may be essential for maintaining seizures.[86] MSO at a non-convulsive dose has also been shown to increase acetylcholine (ACh) concentration in the brain,[88] which could strengthen a seizure. However, evidence towards this end is ambiguous. While an

antiepileptic drug, ketamine, stimulates ACh release when administered alone,[89] it lowers its levels when given during seizure.[86]

4.3. Pretreatment with MSO reduces the release of Glu

Ex vivo experiments have shown a distinctly diminished efflux of non-metabolized Glu analog, [^{3}H]D-Asp, during K^+-induced depolarization in acute brain slices from rats pretreated with MSO (Article II, Figure 4 A), however, MSO did not significantly affect the uptake of [^{3}H]D-Asp (Article II, Figure 4 B). These results suggest that MSO can directly interfere with mechanisms underlying the Glu release. One possibility is that MSO affects the expression/activity of VGLUT1, the principal vesicular Glu transporter responsible for loading synaptic vesicles with Glu.[90] Another factor that may be affected is SV2A, a neuronal membrane protein that participates in synaptic vesicle formation and neurotransmitter release,[91] which is a target of the widely used antiepileptic drugs levetiracetam[92] and brivaracetam.[93] It is also possible that none of these proteins are altered, but their co-expression is disturbed, a phenomenon recently observed in Pilo-treated rats not responding to levetiracetam.[94]

In conclusion, further study will have to resolve whether the Glu release is reduced due to changes in the synthesis or degradation of vesicular proteins directly, by methylation,[58] or whether the response is secondary to the aforementioned, MSO-induced alterations in ACh and/or GSH levels.

4.4. c-Fos mRNA expression is negatively correlated with a time lapse to the onset of seizure

The level of c-Fos mRNA expression showed a strong negative correlation with a period elapsing between Pilo injection and the initial seizure occurrence, with a tendency of MSO to attenuate c-Fos response (Article III, Figure 1 A). This observation, to our knowledge not reported before, can be interpreted to mean that the longer the animal stays resistant to the ictal stimulus, the lower the c-Fos expression in the brain. The correlation with the intensity of the seizure was also tested, but was not apparent there (Article III, Figure 1 B-C), suggesting that the expression of c-Fos may be a result of the susceptibility of animals to seizures rather than its severity.

The above observations leave open the question whether c-Fos should be considered only as an early biochemical biomarker of epileptogenesis, or whether it plays a causative role in seizure protection. Further studies on other TLE models and analysis of blood c-Fos levels in relation to ictal activity are necessary to resolve the question.

There is evidence bespeaking the protective role of c-Fos. In the rat pentylenetetrazol TLE model severity and duration of seizures significantly decreased and the number of c-Fos positive cells significantly increased, as a result of trigeminal nerve stimulation, an experimental neuromodulation method.[73] Interesting data were also obtained in studies on *Proechimys guyannensis*, an Amazonian

rodent species, in which seizures do not develop into SE and afterwards, into chronic TLE.[95] Its extraordinary resistance to seizures appears to be associated with very limited neurodegeneration in the hippocampus, where an unusually high level of c-Fos expression was maintained for several hours after the administration of convulsant.[96] Similarly to our findings, the authors of that article did not find any correlation between c-Fos expression and seizure intensity.

4.5. Summary of the results

The studies presented in Articles I and II provide a comprehensive analysis of the role of the GS inhibitor, MSO, as a modulator of the early ictal phase in a juvenile rat Li-Pilo model of TLE. MSO at a single subconvulsive dose, has been shown to be effective in delaying the onset of both convulsive and electrographic seizures, as well as in alleviating behavioral symptoms. However, MSO did not affect the incorporation of metabolic precursors into the GGC, the principal route of Glu synthesis. This observation indicated that MSO acted by a non-canonical mechanism. *Ex vivo*, MSO significantly inhibited Glu efflux from brain slices upon their depolarization with a generic stimulus (high K^+), indicating that its seizure-attenuating effect results from interference with an as yet unidentified element of the Glu-releasing machinery. Another finding, presented in the Article III, was that the level of c-Fos mRNA is negatively correlated with a time to onset of generalized seizure, but not with its intensity.

The results raise hope that MSO or its derivative(s) may be a potential tool for novel antiepileptogenic therapies, most likely in combination with already working anti-epileptic drugs, however, it would require further research into its mechanisms of action, role in early epileptogenesis, and subsequently, associated isobolographic studies. c-Fos may become a valuable biochemical marker of the very initial stage of epileptogenesis and the seizure susceptibility.

5. Conclusions

I. Pretreatment with a non-convulsive dose of MSO delays the onset of behavioral and electrographic seizures and mitigates the intensity of seizures by a mechanism bypassing its canonical mode of action, i.e. inhibition of GS.

II. In accordance with conclusion I, MSO at doses inhibiting GS activity does not modulate the seizure-related increase of extracellular Glu levels in hippocampus of Pilo-treated rats, nor affects de novo Gln and Glu synthesis during the initial seizure period.

III. Pretreatment with MSO significantly decreases K+-induced Glu release in acute brain slices, indicating that the seizure modulating effect of MSO may be due to interference with mechanisms of synaptic Glu release.

IV. The level of c-Fos mRNA expression in the hippocampus shows a strong negative correlation with the time lapse to the first generalized seizure, and MSO tends to decrease Pilo-related c-Fos response;

V. MSO or its properly designed derivatives may in the future become novel therapeutic modalities at the initial stage of epileptogenesis.

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7. Reprints of articles included in the collection

7.1. Certified by the MMRI PAS Library, five-year impact factor (IF) of publications included in the collection.

List of publications included in the collection:

Article I — Pawlik M, Obara-Michlewska M, Popek M, Czarnecka AM, Czuczwar SJ, Łuszczki J, Kołodziej M, Acewicz A, Wierzba-Bobrowicz T, Albrecht J. 2021. Pretreatment with a glutamine synthetase inhibitor MSO delays the onset of initial seizures induced by pilocarpine in juvenile rats. *Brain Research*. Feb 15;1753:147253. doi: 10.1016/j.brainres.2020.14725 (Five-Year **IF=3.582**).

Article II — Pawlik M, Aldana BI, Belfiori-Carrasco LF, Obara-Michlewska M, Popek M, Czarnecka AM, Albrecht J. 2021. Inhibition of Glutamate Release, but Not of Glutamine Recycling to Glutamate, Is Involved in Delaying the Onset of Initial Lithium-Pilocarpine-Induced Seizures in Young Rats by a Non-Convulsive MSO Dose. *International Journal of Molecular Sciences*. 22(20):11127. doi: 10.3390/ijms222011127 (Five-Year **IF=6.132**).

Article III — Pawlik M, Miziak B, Walczak A, Konarzewska A, Chrościńska-Krawczyk M, Albrecht J, Czuczwar SJ. 2021. Selected molecular targets for anti-epileptogenesis. *International Journal of Molecular Sciences*. 22(18):9737. doi: 10.3390/ijms22189737 (Five-Year **IF=6.132**).

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7.2. Reprints of articles

7.2.1. Article I – Pawlik M, Obara-Michlewska M, Popek M, Czarnecka AM, Czuczwar SJ, Łuszczki J, Kołodziej M, Acewicz A, Wierzba-Bobrowicz T, Albrecht J. 2021. Pretreatment with a glutamine synthetase inhibitor MSO delays the onset of initial seizures induced by pilocarpine in juvenile rats. *Brain Research*. Feb 15;1753:147253. doi: 10.1016/j.brainres.2020.14725

Brain Research 1753 (2021) 147253

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Pretreatment with a glutamine synthetase inhibitor MSO delays the onset of initial seizures induced by pilocarpine in juvenile rats

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ARTICLE INFO

Keywords:
Convulsive seizures
Glutamatergic transmission
Glutamine synthesis
Methionine sulfoximine
EEG

ABSTRACT

The contribution of glutamatergic transmission to generation of initial convulsive seizures (CS) is debated. We tested whether pretreatment with a glutamine synthetase (GS) inhibitor, methionine sulfoximine (MSO), affects the onset and progression of initial CS by cholinergic stimulus in juvenile rats. Male rats (24 days old, Sprague Dawley) sequentially received i.p. injections of lithium-carbonate, MSO, methyl-scopolamine, and pilocarpine (Pilo). Pilo was given 150 min after MSO. Animals were continuously monitored using the Racine scale, EEG/EMG and intrahippocampal glutamate (Glu) biosensors. GS activity as measured in hippocampal homogenates, was not altered by MSO at 150 min, showed initial, varied inhibition at 165 (15 min post-Pilo), and dropped down to 11% of control at 60 min post-Pilo, whereas GS protein expression remained unaltered throughout. Pilo did neither modulate the effect of MSO on GS activity nor affect GS activity itself, at any time point. MSO reduced from 32% to 4% the number of animals showing CS during the first 12 min post-Pilo, delayed by ~6 min the appearance of electrographic seizures, and tended to decrease EMG power during ~15 min post-Pilo. The results indicate that MSO impairs an aspect of glutamatergic transmission involved in the transition from the first cholinergic stimulus to the onset of seizures. A continuous rise of extracellular Glu lasting 60 min was insignificantly affected by MSO, leaving the nature of the Glu pool(s) involved in altered glutamatergic transmission undefined.

Abbreviations: CS, Convulsive seizure; Gln, Glutamine; Glu, Glutamate; GS, Glutamine synthetase; i.p, Intraperitoneal; MSO, Methionine sulfoximine; P19, Postnatal day 19; Pilo, Pilocarpine; *SE*, *Status epilepticus*; SRS, Spontaneous recurrent seizures; TLE, Temporal lobe epilepsy; WB, Western Blot.

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https://doi.org/10.1016/j.brainres.2020.147253
Received 21 August 2020; Received in revised form 26 November 2020; Accepted 16 December 2020
Available online 7 January 2021

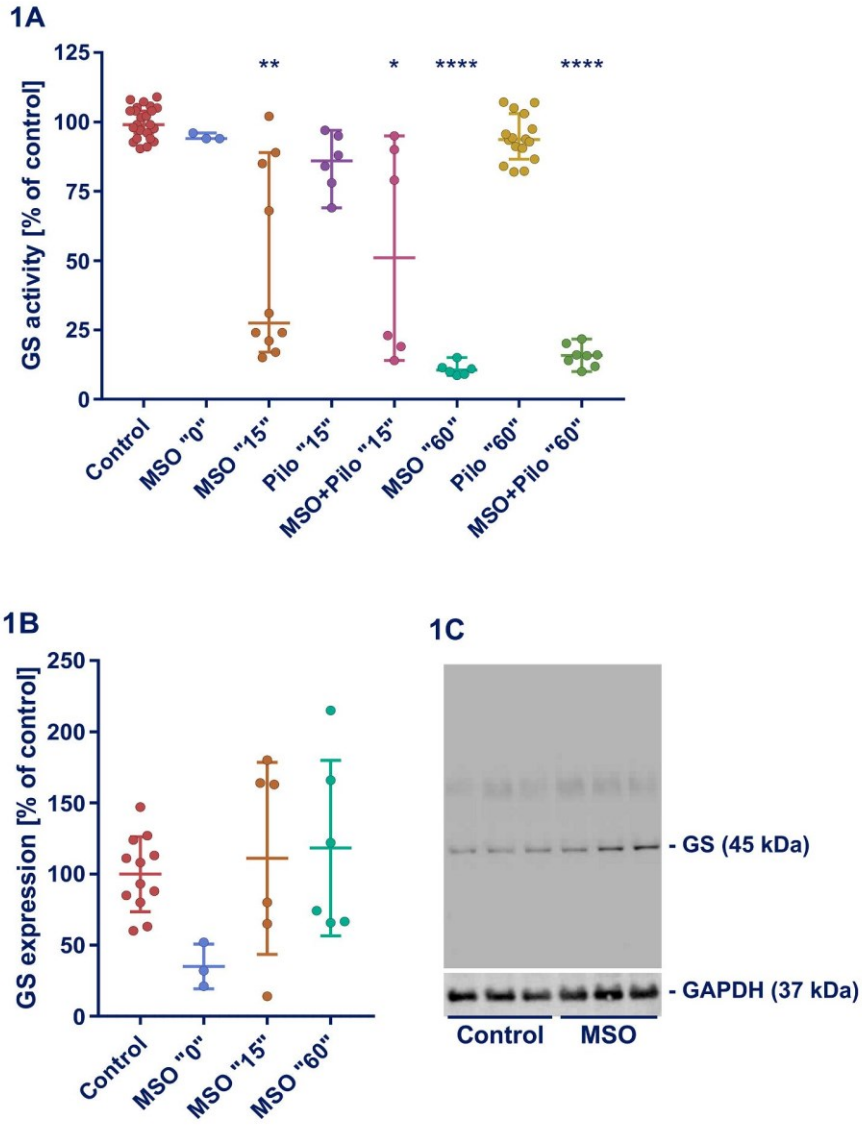

Fig. 1. Effects of methionine sulfoximine (MSO) on glutamine synthetase (GS) activity and expression. Rats were injected with MSO, and 150 min later pilocarpine (Pilo) was added. This point is here referred to as MSO "0"; measurements were further taken after 15 min (referred to as MSO "15", Pilo "15" and MSO + Pilo "15") and after 60 min (MSO "60", Pilo "60" and MSO + Pilo "60", respectively). (A) Effects of MSO and/or Pilo on GS activity in hippocampus. Results are median ± 95% CI for the following numbers of animals: Control = 23, MSO "0" = 3, MSO "15" = 10, MSO "60" = 6, Pilo "15" = 6, Pilo "60" = 16, MSO + Pilo "15" = 6, MSO + Pilo "60" = 8. * – $p < 0.05$, ** – $p < 0.01$, **** – $p < 0.0001$, Kruskal-Wallis test with Dunn's correction; (B) Effects of MSO on GS expression in hippocampus. Results are mean ± SD for the following numbers of animals: Control = 12, MSO "0" = 3, MSO "15" = 6, MSO "60" = 6. Statistical analysis: one-way ANOVA with Šidák correction; (C) Representative Western Blot images of GS in the rat hippocampus.

1. Introduction

Temporal lobe epilepsy (TLE) is the most common and therapy-resistant form of epilepsy (Engel and Salamon, 2015; Sheng et al., 2018). The disease commences with initial seizures defined as the initial *status epilepticus* (*SE*), evolves to the latent, asymptomatic phase, and progresses to the chronic phase characterized by spontaneous recurrent seizures (SRS), associated with hippocampal sclerosis (Tai et al., 2018). TLE has been studied in animal models in which the initial seizures are evoked by depolarizing agents, either acting non-specifically or stimulating excitatory neurotransmitter systems, the glutamatergic or the cholinergic system (Jefferys et al., 2016; Lévesque et al., 2016). Administration of pilocarpine (Pilo), either alone or in conjunction with the pilocarpine active dose-reducing agent, lithium (Li-Pilo model) (Turski et al., 1989) has turned out to be useful in reproducing the three phases of human TLE in rodents (Leite et al., 1990; Sloviter, 2008; van der Hel et al., 2014).

The mechanism involved in the onset of initial seizures has not been investigated in much detail. When initial seizures are induced by Pilo, the issue debated is the relative involvement of cholinergic vs glutamatergic transmission as their trigger. A Pilo treatment-induced increase of extracellular Glu has been documented in some (Khan et al., 1999; Millan et al., 1993; Santana-Gómez et al., 2015; Slais et al., 2008; Smolders et al., 1997a), but not all reports (Cavalheiro et al., 1994; Meller et al., 2019; Soukupova et al., 2015), showing variability with regard to the analyzed brain region (Meurs et al., 2008; Smolders et al., 1997b). Recent studies in which the involvement of both transmitters was assessed simultaneously, revealed predominance of massive accumulation of acetylcholine, with only negligible or substantially delayed accumulation of Glu (Brandt et al., 2015; Hillert et al., 2014; Meller et al., 2019). However, in the above quoted studies observations have started at time points at least 30 min remote from application of initiation stimulus, the period when initial seizures have already developed. In the present study we analyzed the events at the very onset, starting at post-stimulus time 0. Given that extracellular Glu levels depend on the multitude of factors other than synaptic Glu release (Obrenovitch et al., 1996; van der Zeyden et al., 2008), we evaluated the role of Glu in the onset of Pilo-induced seizures by inhibiting neurotransmitter Glu supply to synapses. Around 70% of the neurotransmitter pool of Glu is derived from glutamine (Gln) synthesized in astrocytes by glutamine synthetase (GS) and is transferred to neurons where part of it is converted to synaptic Glu (Albrecht et al., 2010; Billups et al., 2013; Kanamori and Ross,

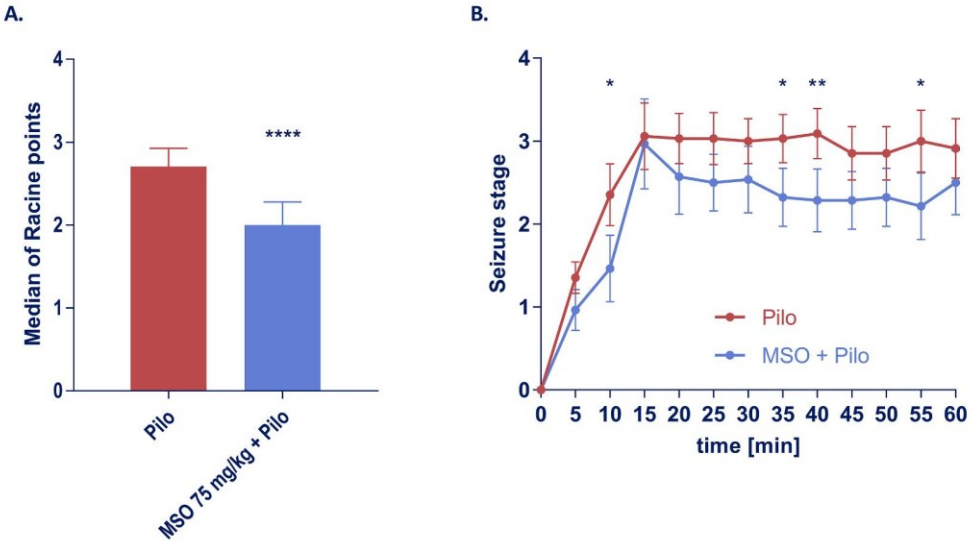

Fig. 2. Effects of pretreatment with MSO on the intensity of behavioral seizures. (A) Results are median ± 95% CI for the following numbers of animals: Pilo N = 34, MSO + Pilo N = 28; **** – $p < 0.0001$ vs. Pilo, Mann-Whitney test; (B) Progression in time of the effects of MSO. Results are median ± 95% CI, numbers of animals as indicated in Fig. 2A. * – $p < 0.05$, ** – $p < 0.01$ vs. Pilo. Multiple Mann-Whitney tests with Holm-Šidák correction.

2013). In the present study we tested how inhibition of GS impacts the onset and progression of initial seizures produced in juvenile rats using the Li-pilocarpine model of TLE, in which initial *SE* is well separated in time from the subsequent stages of the disease (van der Hel et al., 2014). We analyzed the effect of a selective GS inhibitor, methionine sulfoximine (MSO) on GS activity and the electrographic and behavioral manifestations of the seizures. MSO has been successfully employed as a tool to prevent the Gln-dependent aspect of stimulation of neuronal networks in vitro by ammonia (Schwarz et al., 2012), and to potentiate Gln deficit-dependent aspects of depression (Son et al., 2018) without producing significant untoward effects. However, MSO at high concentration (>150 mg/kg) acts as convulsant (Brusilow and Peters, 2017; Eid et al., 2008; Rowe and Meister, 1970), which upon prolonged administration induces subsequent phases of TLE, including hippocampal sclerosis and SRS (Eid et al., 2008). Therefore, we administered MSO at a relatively low dose (75 mg/kg) which fell short of causing convulsions throughout the whole observation period. We hypothesized that inhibition of GS at this low MSO dose, will limit the availability of Glu-derived pool of neurotransmitter Glu in a degree disclosing its role in the onset or propagation of the initial seizures. The degree of GS activity inhibition by MSO was monitored in control- and Pilo-treated rats starting at 150 min after its administration, i.e. at a time point at which Pilo was given to induce seizures.

In principle, the mechanism of GS inhibition by MSO relies on MSO binding to the active site of the GS molecule (Ronzio and Meister, 1968; Rowe and Meister, 1970). However, the relatively short half-life of GS in mammalian tissues (Miller and Carrino, 1980; Feng et al., 1990; Saini et al., 1990) suggested the possibility that >3 h treatment with MSO could affect GS activity also by influencing expression of GS protein. We therefore evaluated the effect of MSO on GS protein expression, a parameter to our knowledge never tested before. We focused the study on the hippocampus, a structure which basing on behavioral data and EEG analyses appears to be primarily involved in Pilo-induced cholinergic excitation (Friedman et al., 2007) and seizures (Toyoda et al., 2013). There is evidence that in the Pilo model, seizure propagation to amygdala and cortex is subsequent to their induction in hippocampus both in rats (Turski et al., 1983) and mice (Turski et al., 1984). Changes in extracellular Glu were measured using hippocampus-inserted biosensors, a procedure which allows continuous monitoring and relatively good spatial resolution (van der Zeyden et al., 2008). Of note, the GLU

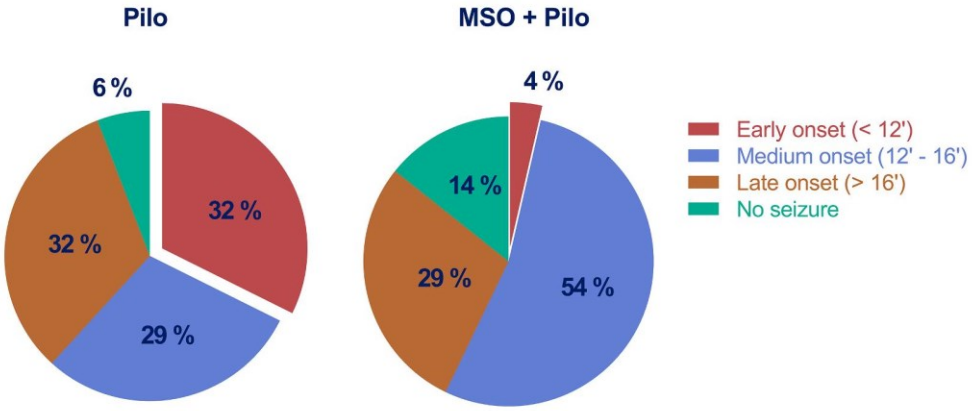

Fig. 3. Effects of MSO pretreatment on behavioral seizure inducibility by Pilo. Observations started directly after Pilo injection. The onset of behavioral seizures occurred early (<12 min after Pilo, "early onset"), at medium time (12–16 min, "medium onset") or later (>16 min, "late onset"). N = 28 (pretreated with MSO); N = 34 (no MSO).

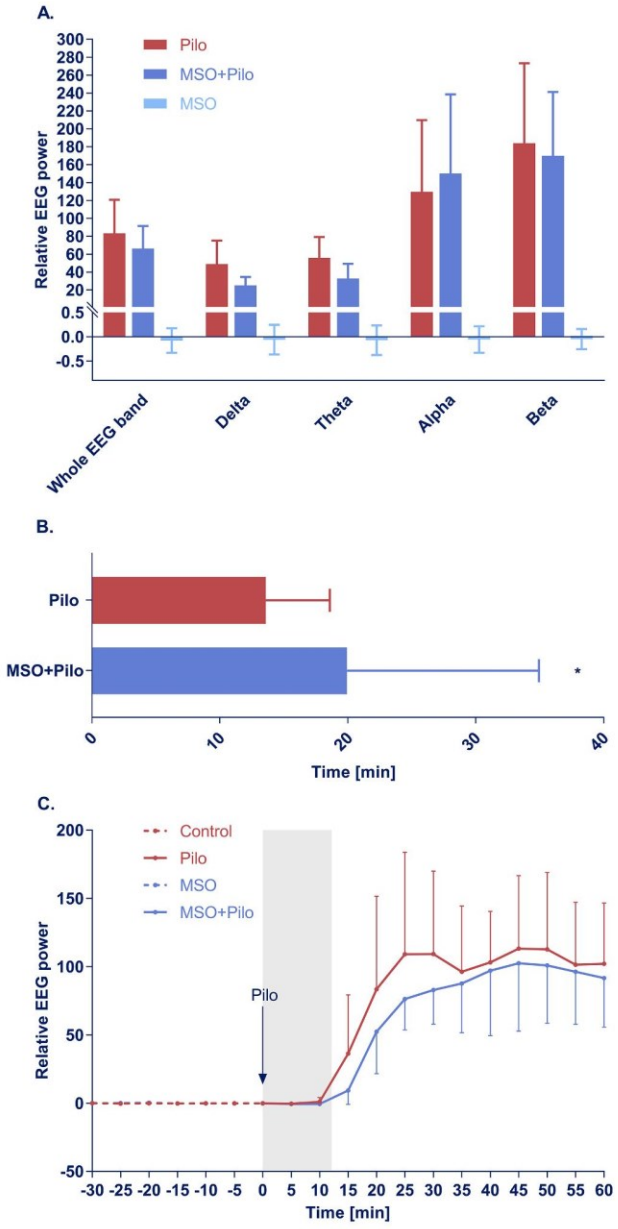

Fig. 4. Effects of MSO and/or Pilo administration on EEG. (A) Effects of MSO on relative EEG power during one hour after pilocarpine (Pilo) administration. The MSO group covers 1 h period before the administration of Pilo. Results are mean ± SD for the following numbers of animals: Pilo N = 12, MSO + Pilo N = 7. Statistical analysis: multiple *t*-test with Holm-Šidák correction; (B) Effect of pretreatment with MSO on latency to onset of electrographic seizures. Results are mean ± SD, for the following number of animals: Pilo N = 19, MSO N = 15; * – $p < 0.05$, Student's *t*-test; (C) Effects of MSO on progression in time of changes in relative EEG power. The shadowed area indicates the "early onset" period illustrated in Fig. 3. Results are mean ± SD for the number of animals indicated in the legend to Fig. 4A. Statistical analysis: multiple *t*-test with Holm-Šidák correction. Results for MSO alone and control cover the period between "-30" and "0".

biosensor only detects relative changes in extracellular GLU concentration. However, progression of relative changes of Glu, not their absolute values, constitute the critical determinant of excitation/inhibition imbalance underlying convulsive seizures.

2. Results

2.1. Brain GS activity and GS protein expression

Treatment of control rats with 75 mg/kg of MSO did not affect the hippocampal GS activity for the first 150 min. The activity was found decreased by on average 50% at 165 min after MSO; the decrease turned out to be statistically significant despite considerable rat-to-rat variability of the responses. At 210 min, inhibition of GS activity reached 83.7% with little variation between individual animals. Pilo, which was given at the time point corresponding to the 150th minute after MSO administration did not significantly alter the enzyme activity in either MSO-pretreated or non-treated rats throughout the observation period. Of note, the individual variability of the responses to MSO at 165 min (15 min Pilo administration) in the Pilo group mirrored that observed in animals treated with MSO alone (Fig. 1A).

Treatment with MSO did not affect the expression of GS protein in hippocampus, except for a statistically insignificant tendency towards decrease noted at 150 min after MSO administration (Fig. 1B). Hence, enzyme inhibition observed throughout the period of exposure to MSO followed the well-established mechanism of direct MSO binding to GS (Ronzio and Meister, 1968; Rowe and Meister, 1970).

2.2. Behavioral symptoms

Pretreatment with MSO reduced the intensity of CS caused by Pilo administration from 2.71 ± 0.63 to 2.00 ± 0.72 on the Racine scale (Fig. 2A). Analysis of the progression in time revealed multiphasic manifestation of seizure reduction by MSO: the reduction reached statistical significance during the first 10 min, then at 35–40 min, and for a brief period at 55 min after Pilo administration (Fig. 2B). Most interestingly, as shown in Fig. 3, pretreatment with MSO appeared to prevent CS in some animals ("no seizure"), and substantially reduced the number of animals in which the onset of seizures occurred during the first 12 min ("early onset"). Not earlier than between 12 and 16 min ("medium onset"), synchrony of the onset at 58–61% in both MSO-pretreated rats and non-pretreated ones could be observed.

2.3. EEG analysis

Total EEG power increased over 60-fold after injection of Pilo, the increase being relatively the lowest for the delta and the highest for the beta band (Fig. 4A). Pretreatment with MSO did neither significantly reduce the whole power nor the power measured at any particular wavelength, but significantly extended the latency of electrographic seizure from 13.58 to 19.93 min (Fig. 4B). The EEG power in Pilo-treated rats reached a maximum at about 25 min after Pilo administration; pretreatment with MSO extended this time to about 45 min. No change of EEG power was noted in Pilo-treated rats, whether or not MSO-pretreated during the first 10 min (Fig. 4C).

2.4. EMG analysis

EMG power increased substantially during seizures in pilocarpine-treated animals, the increase being lower in MSO + Pilo than in Pilo rats (4.30 ± 5.29 vs 11.48 ± 12.48 of baseline energy). However, although MSO pretreatment tended to decrease mean EMG power, the decrease did not reach statistical significance (Fig. 5A). Time course analysis revealed the absence of the effect of MSO pretreatment at any time point before Pilo administration, as well as during the first 12 min after Pilo treatment corresponding to the early onset period illustrated in (Fig. 5B).

2.5. Extracellular Glu concentration

Pilo treatment tended to increase gradually the extracellular Glu

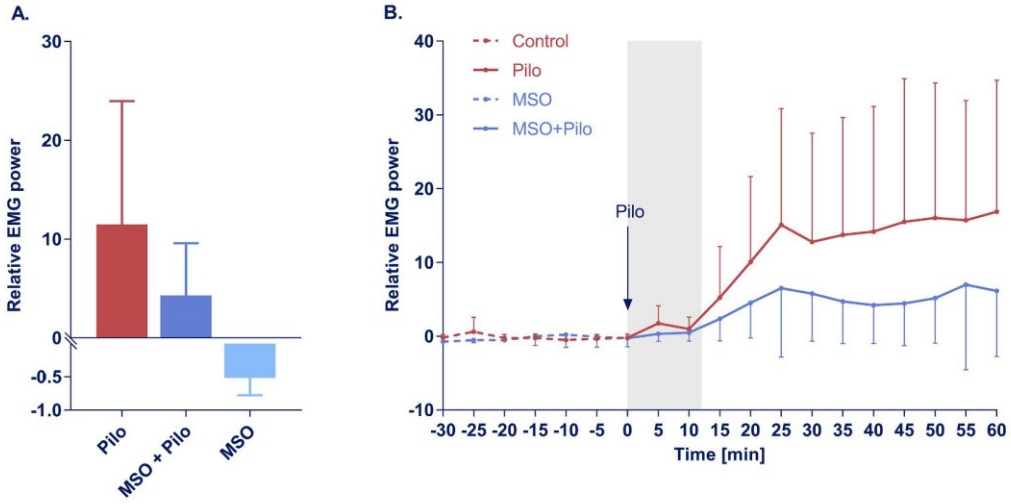

Fig. 5. Effects of MSO and/or Pilo administration on EMG. (A) Effects of MSO on relative EMG power during one hour after pilocarpine (Pilo) administration. Results are mean ± SD for 6 (MSO, MSO + Pilo) and 7 animals (Pilo). The MSO group covers 1 h period before the administration of Pilo. Statistical analysis: Student's *t*-test; (B) Effects of MSO on progression in time of changes in relative EMG power. The shadowed area indicates the "early onset" period illustrated in Fig. 3. Results are mean ± SD for the number of animals indicated in the legend to Fig. 5A. Statistical analysis: multiple *t*-test with Holm-Šidák correction. Results for MSO alone and control cover the period between "-30" and "0".

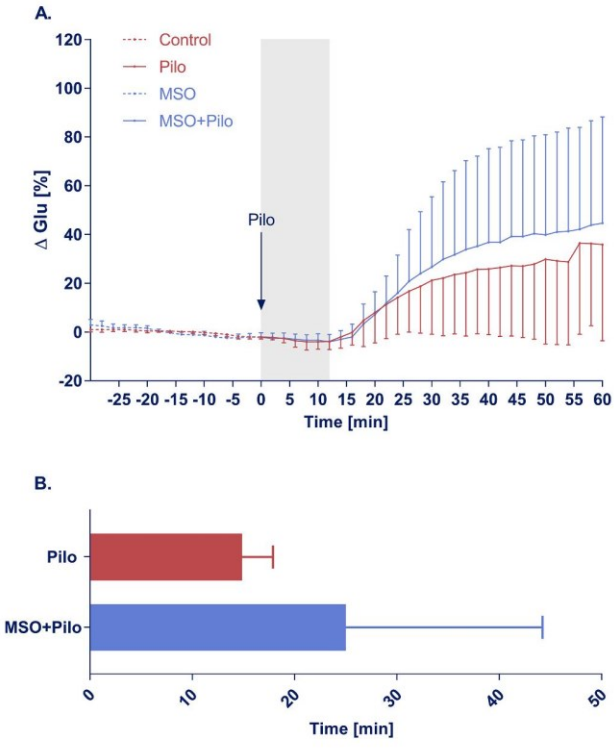

Fig. 6. Effects of MSO and/or Pilo administration on the extracellular glutamate (Glu) in hippocampus. (A) Effects of pretreatment with MSO on the extracellular level of Glu in pilocarpine-treated rats. The shadowed area indicates the "early onset" period illustrated in Fig. 3. Results are mean ± SD, for 7 (Pilo) and 6 (MSO + Pilo) animals. Statistical analysis: multiple *t*-test with Holm-Šidák correction; statistical analysis of differences the values obtained at subsequent time points, performed by one-way ANOVA with repeated measures on time with Tukey's correction, revealed no significant differences throughout. (B). Effect of pretreatment with MSO on the latency to Glu rise. Results are mean ± SD, for the number of animals as in the legend to Fig. 6A. Statistical analysis: Student's *t*-test;

concentration (Fig. 6A), albeit due to considerable individual variation the concentration change measured at subsequent time points never reached statistical significance (one-way ANOVA with repeated measures on time with Tukey's correction, test data not shown). The tendency towards increase became apparent starting about 14 min after Pilo administration that is, at the time point beyond the early seizure onset period defined in Fig. 3 (see Fig. 6A), and MSO tended to delay this apparent Glu rise further (Fig. 6B).

2.6. *Effects of MSO treatment in control rats*

Treatment of animals with MSO neither induced convulsive symptoms nor did it significantly affect total EEG power or power at the individual bands (Fig. 4A). MSO only very slightly reduced mean EMG power (−0.08 ± 0.21) (Fig. 5A), but the reduction was not apparent when individual time points were considered (Fig. 5B).

2.7. *Evaluation of correlation between the biochemical and physiological parameters studied*

Calculation of Spearman's coefficient values revealed that all the parameters determined as a function of time are statistically significantly correlated with each other. The correlation turned out to be moderate in 11% of cases, strong in 32% of cases and very strong in 57% of cases – the strongest between machine-collected data (EEG, EMG and glutamate level), the weakest between them and behavioral symptoms (Fig. 7).

2.8. *Verification of tissue damage*

EEG electrode placement induced moderate lesions consisting of focal disruption of the arachnoid mater with mild ischemic changes in the left hemisphere (Fig. 8C-D). Tissue damage typical of the procedure was recorded in the Glu biosensor placement site in the hippocampus (Fig. 8F-G).

3. Discussion

Cholinergic stimulation of rodent brain by the muscarinic agonist Pilo, often used in conjunction with the Pilo threshold dose-reducing

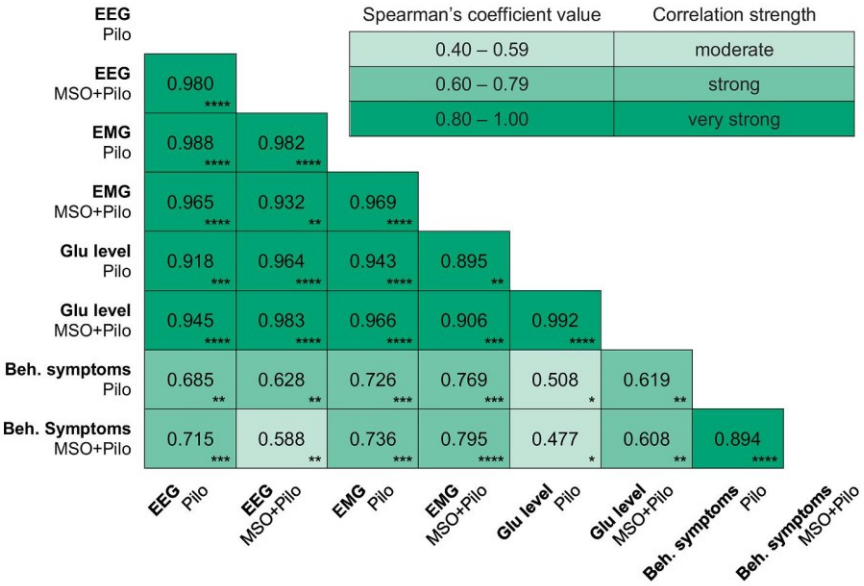

Fig. 7. Spearman correlation coefficient hemi-matrix for inter-relationships of EEG, EMG, Glu level and behavioral symptoms. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$, **** – $p < 0.0001$.

agent lithium, is a useful method to induce initial convulsive seizures, and their later progression through a period of latency toward SRS, resembling TLE in humans (Cavalheiro et al., 1991; Leite et al., 1990). The well-documented role of glutamatergic activation in chronic TLE both in humans (Çavuş et al., 2016; Eid et al., 2004; van der Hel et al., 2005) and in animal models (Kanamori and Ross, 2013; Perez et al., 2012; van der Hel et al., 2014), has become good enough reason to ask about the role of glutamatergic transmission in the onset and during the progression of initial seizures induced by Pilo. In view of the multitude of mechanisms accounting for the emergence of Glu in the extracellular space (van der Zeyden et al., 2008), here we decided to test how initiation of seizures is affected by the enforced reduction of the neurotransmitter Glu pool. Since no less than 70% of neurotransmitter Glu is derived from Gln synthesized by GS, inhibition of Glu conversion to Gln by pretreatment of animals with the GS inhibitor MSO appeared to be a useful option. Earlier, MSO has been demonstrated to block neuronal network activity in the spinal cord (Hülsmann et al., 2000) and hippocampal slices (Kam and Nicoll, 2007). MSO also moderated epileptiform activity induced in hippocampal slices by a GABA receptor antagonist, bicuculline (Bacci et al., 2002). The intracerebral administration of MSO reduced advanced, amygdala kindling–induced seizures which – as opposed to other models – originally were associated with increased GS activity (Sun et al., 2013).

The key finding of the present study is that pretreatment with MSO substantially delays the onset of initial convulsive and electrographic seizures induced by Pilo. GS plays a dual role in glutamate recycling: it i) inactivates Glu in the process of astrocytic Gln synthesis and ii) provides Gln serving as a precursor for neurotransmitter Glu (Bak et al., 2006). In this context, the results can most simply be interpreted as indicating that inhibition of GS at its onset (15 min post Pilo) impoverishes the Gln pool that serves as a precursor of neurotransmitter Glu in a degree sufficient to limit glutamatergic contribution to the initiation of seizures. The relatively high individual variation of the physiological benefits of MSO at this stage is likely to reflect considerable animal-to-animal differences in MSO sensitivity. At later times, growing GS inhibition may have reversed the beneficial effect by impairing Glu clearance by astrocytes. MSO-evoked accumulation of Glu in the extracellular space has been earlier observed in different experimental settings (Rodríguez Díaz et al., 2005; Trabelsi et al., 2017). In chronic TLE, substantial loss of astrocytic GS in disease-transformed astrocytes is a mechanism believed to underlie induction and propagation of SRS in a range of animal models (Bidmon et al., 2008; Perez et al., 2012; van der Hel et al., 2014) and in humans (Eid et al., 2013; van der Hel et al., 2005). However, other potential central or peripheral effects of MSO might interfere with seizure induction or progression. MSO-induced Gln depletion could limit the synthesis and availability of neurotransmitter GABA, leading to glutamatergic disinhibition (Liang et al., 2006) and seizure promotion (Naylor, 2010). In MSO-pretreated Pilo rats, GABA loss might thus counteract MSO-induced seizure delay. If present in MSO-pretreated Pilo rats as described in the present study, this effect would likewise facilitate neuroexcitation and seizures. Furthermore, MSO is known to inhibit glutathione synthesis in peripheral tissues (Palekar et al., 1975), which may decrease performance of muscles and their response to extrinsic stimuli (Morales et al., 1994). This phenomenon may have contributed to the here observed selective attenuation by MSO pretreatment of the behavioral (not the electrographic) seizures at later periods after Pilo.

The continuous rise of extracellular Glu was observed both in MSO-pretreated and MSO-untreated Pilo rats. The absence of statistically significant changes between the two groups of animals, but also absence of statistically significant progression of Glu accumulation assessed on the time point to time point basis, is likely due to the variability of responses of individual animals. However, not even a tendency towards change in extracellular Glu content in either group was observed in the first 12 min after Pilo administration, a period roughly corresponding to the MSO-induced delay in onset of seizures. In this critical period, changes in the neurotransmitter Glu pool may be too variably altered between animals, reflecting variation in GS inhibition by MSO. Clearly, identification of the Glu pool involved in the onset of Pilo-induced seizures and alterations of its status following MSO treatment can only be achieved by following conversion of radiolabeled precursors to-, and interconversion between Glu and Gln in this period, a strategy previously employed with regard to the kainate model of TLE (Alvestad et al., 2011; Walls et al., 2014). Whatever the nature of this early Glu pool, the relatively insignificant modulation by MSO of Pilo-induced changes of EEG, EMG and Racine score at later time period indicates that, Glu accumulating beyond ~15 min post-Pilo most likely represents the synaptic release-independent pool not involved in inducing seizures (Nyitrai et al., 2002; Obrenovitch et al., 1996; van der Zeyden et al., 2008).

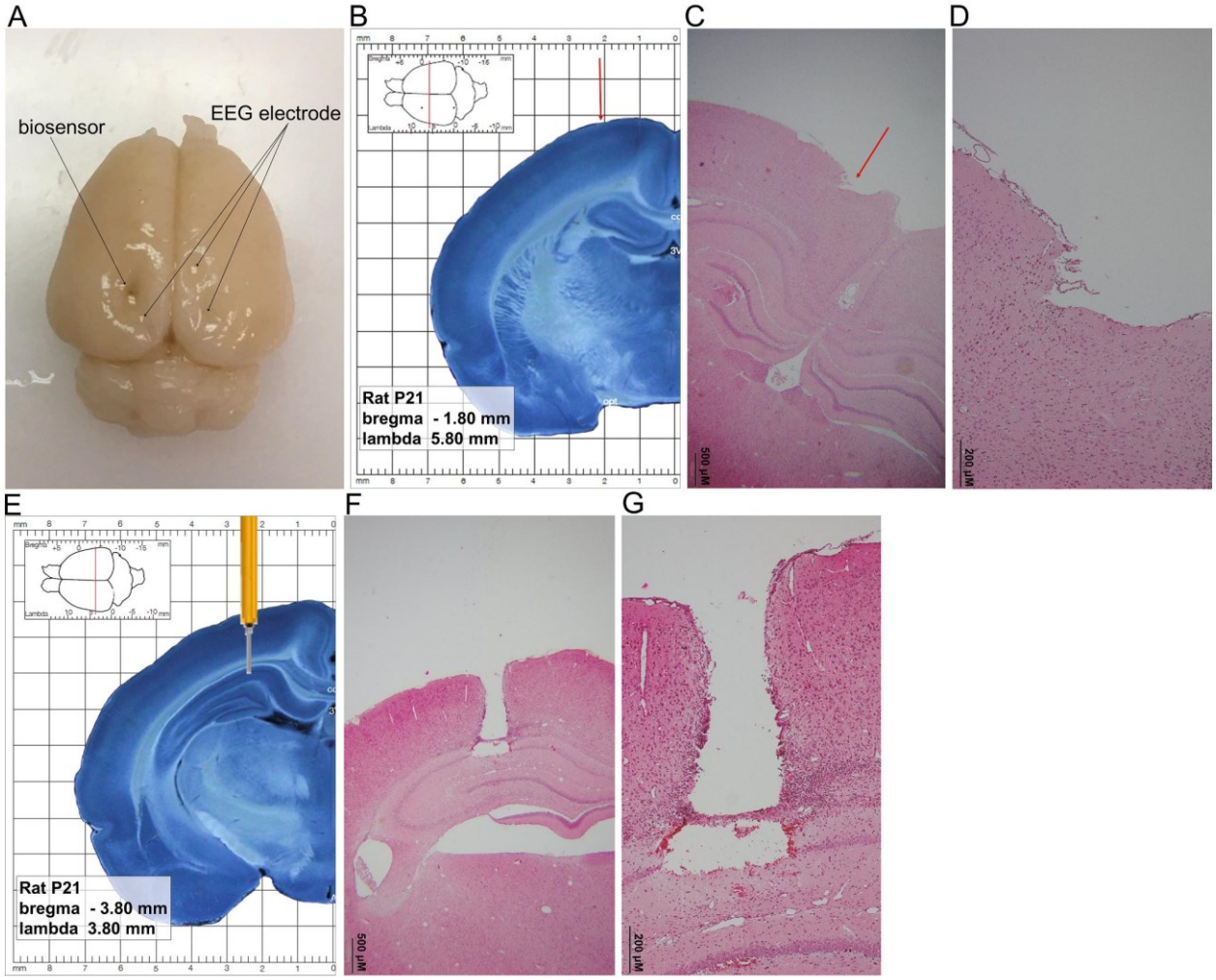

Fig. 8. Localization of EEG electrodes and biosensor. (A) The exemplary macroscopic view of the rat brain with biosensor and EEG electrodes placement sites indicated by arrows; (B) Graphical representation of the EEG electrode placement (stereotaxic coordinates – 1.8 mm anteroposterior, – 2.0 mm lateral); (C-D) Representative photomicrographs of coronal sections of the rat left hemisphere showing microscopic nature of cortical lesions consisting of focal disruption of the arachnoid mater with mild ischemic changes; (E) Graphical representation of the biosensor placement (stereotaxic coordinates – 3.8 mm anteroposterior, – 2.4 mm lateral, – 1.9 mm depth); (F-G) Representative photomicrographs of coronal sections of the rat left hemisphere verifies biosensor placement site in the hippocampus.

In conclusion, the overriding observation of the present study is that pretreatment with MSO delays the onset of behavioral and electrographic seizures in Pilo treated rats. This allows to define the role of glutamatergic transmission as an accelerator of the onset of seizures induced by cholinergic stimulation. However, in light of the present results, glutamatergic transmission would appear to play a relatively minor role in beyond-the-onset progression of Pilo-induced seizures, confirming recent reports pointing to the domination of cholinergic excitation in this capacity (Hillert et al., 2014; Meller et al., 2019). Interestingly in this context, domination of the cholinergic transmission in seizure onset appears to hold to seizures evoked by non-cholinergic stimuli (Hillert et al., 2014; Jope and Gu, 1991). It must be noted, however, that relative contribution of cholinergic and glutamatergic transmission to the initial seizures may differ between animals which do, and those which do not develop chronic epilepsy with SRS (François et al., 2011; Meller et al., 2019). Our experimental setting precluded follow-up of the animals' status beyond the initial 60 min of Pilo treatment, rendering beyond reach their *ex-post* grouping according to the propensity to develop SRS.

Most of the antiepileptic drugs that have been tried in rodents with Pilo-induced *SE* turned out to be ineffective in preventing SRS (Miziak et al., 2020). Therefore, it will be of interest to analyze the potential of MSO as a treatment modality.

4. Materials and methods

4.1. Animals

Animal experiments were performed according to the national guidelines on animal experimentation and were approved by the local ethical committee in Warsaw (consent no. 405/2017, 21.11.2017) in accordance with EC Directive 2010/63/EU. Male Sprague Dawley rats (the animal colony of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw) were housed individually in constant temperature and 12-h light/dark cycling with free access to standard rat chow and tap water. Animals undergoing surgery for stereotactic implantation of electrodes and biosensors were housed starting at postnatal day 19 (P19); those that have not undergone surgery – starting at P23.

4.2. Li^{+}-pilocarpine TLE model

The procedure was essentially as described by van der Hel et al.

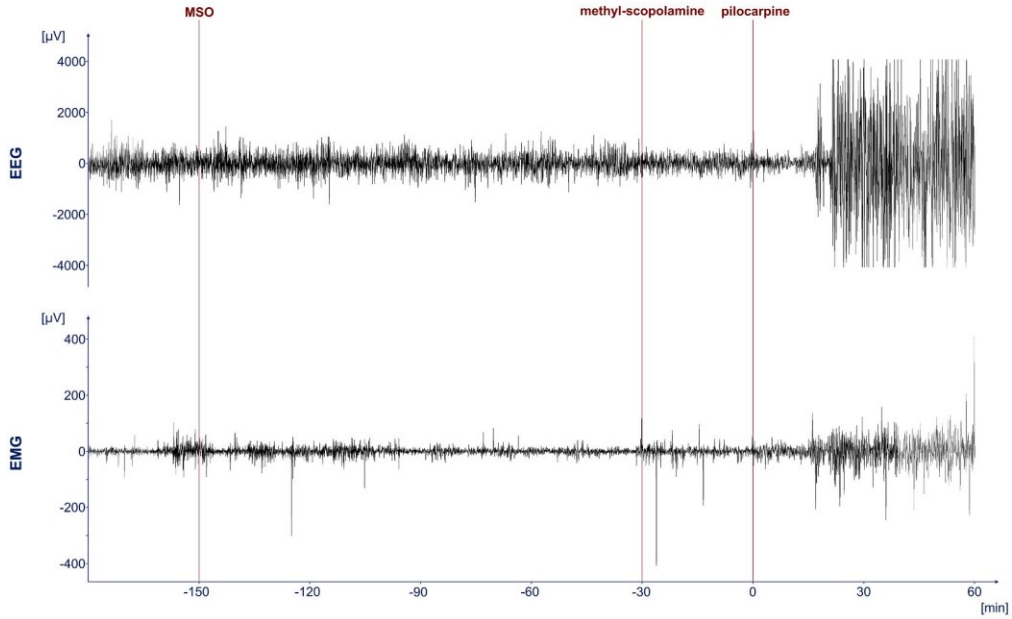

Fig. 9. An example of a representative EEG and EMG recording from a MSO + Pilo treated rat. The timelines of experiments were the same when saline alone was used instead of MSO.

(2014) with minor modifications. At P23 rats were injected intraperitoneally (i.p.) with lithium-carbonate (222 mg/kg; Sigma) dissolved in saline (pH equalized to 7.4). At P24, 18–20 h after Li^+ treatment, animals were injected i.p. with methyl-scopolamine (1 mg/kg; Sigma) to reduce peripheral side effects of pilocarpine. Thirty minutes later pilocarpine (40 mg/kg; Sigma) was administered i.p. and rat behavior was monitored continuously. Every five minutes the behavior was classified according to five Racine stages (Racine, 1972); the time period to reach generalized seizure (stage 4–5) was noted as well. After 1 h rats were decapitated and brain tissue was collected for histological and/or biochemical analyses.

4.3. Pretreatment with MSO

Rats received MSO (Sigma) dissolved in saline at 75 mg/kg, i.p., 2.5 h before administration of Pilo. Rats that did not receive MSO were given saline alone. All animals were decapitated 3.5 h after MSO administration. The timeline of MSO and Li-Pilo administration, and of EEG and EMG recordings (for procedure description see below) is illustrated in Fig. 9.

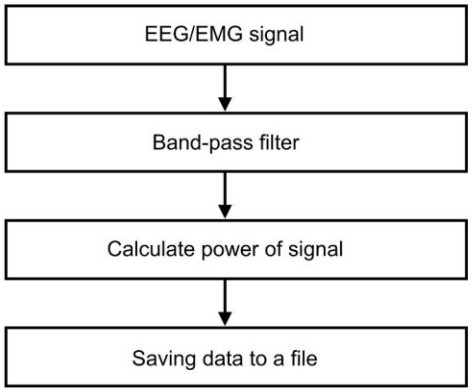

Fig. 10. Block diagram of the program in Matlab.

4.4. Surgical procedures

Rats at P19 were anesthetized with ketamine (75 mg/kg, Vetoquinol) and dexmedetomidine (0.5 mg/kg, Orion Pharma) for stereotactic implantation of electrodes and biosensors. For experiments aimed at monitoring EEG-EMG only, 2EEG/1EMG rat headmounts (8239 Rat Headmount, Pinnacle Technology, Lawrence, KS) were used; for experiments including Glu biosensor recordings, 2EEG/1EMG/1BIO mouse headmounts (8402 Mouse Headmount, Pinnacle Technology) were used. The headmount was affixed to the skull with stainless steel screws that also acted as EEG electrodes, all the screws resting on the cerebral cortex. Two parietal screws were used for EEG recording, one (mouse headmount) or two (rat headmount) frontal screws for reference and grounding. EMG electrodes were implanted below the neck muscles. For biosensor experiments, guide cannula was implanted into the left CA1 hippocampal region (coordinates vs. bregma: – 3.8 mm anteroposterior, – 2.4 mm lateral, – 1.9 mm depth; for a graphical presentation of EEG electrode and biosensor placement see Fig. 8. Head mount and cannula were secured using dental acrylic cement and the incision was sutured below and above the head mount. Following the surgery, rats were woken up from anesthesia with atipamezole hydrochloride (1 mg/kg, Orion Pharma) and treated with ketoprofen (5 mg/kg, Sandoz) once a day up to P21.

4.5. EEG, EMG recordings and video recording of seizure events

All recordings were conducted inside a transparent, round cage allowing animals to move freely, with free access to food and water. Rats were acclimated to these conditions for one day prior to the experiment (P23). On the day of experiment, rats were connected to the data acquisition system (8401 Data Conditioning & Acquisition System, Pinnacle Technology) and at least 30 min of baseline recording was acquired, using Sirenia software (Pinnacle Technology), prior to administration of MSO and/or methyl-scopolamine. Seizure events were verified by video recording synchronously with EEG/EMG. A representative EEG and EMG recording is illustrated in Fig. 9.

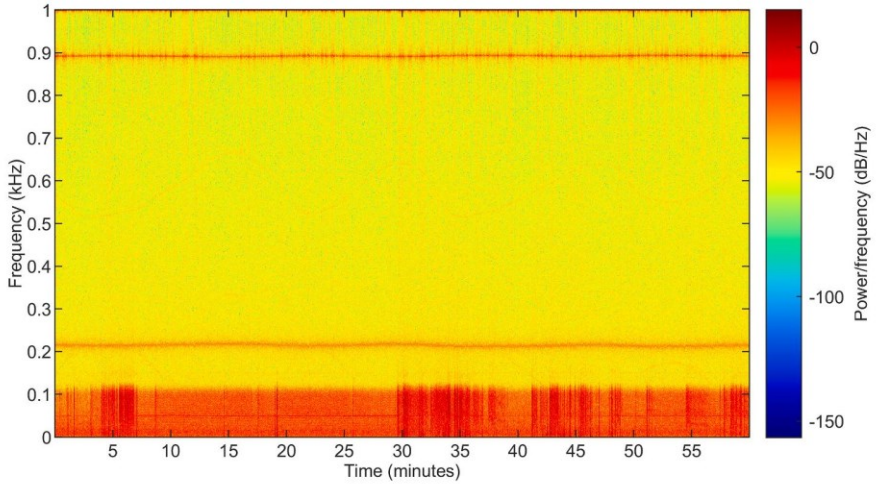

Fig. 11. Example of an EMG signal spectrogram registered throughout 60 min after Pilo administration.

4.6. Extracellular glutamate recording

Glutamate (Glu) biosensors (7004 Mouse Biosensor with Integrated Reference, Pinnacle Technology) were used for continuous intrahippocampal recordings of extracellular Glu concentrations *in vivo*. The biosensor was inserted into the hippocampus under the mild inhalation anesthesia using isoflurane (Baxter) and the recording procedure as described by the manufacturer was followed.

4.7. EEG, EMG and biosensor data analysis

The power of EEG signal for individual bands was calculated using Butterworth filters. For each data set signal power was calculated in particular frequencies (Kadam et al., 2017): total EEG band (0–100 Hz), delta (1–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), beta (13–30 Hz), gamma (30–100 Hz). Block diagram of the data elaboration scheme in Matlab program is illustrated in Fig. 10.

Fig. 11 presents an example of a spectrogram for the registered EMG signal. It shows that the highest energy is distributed in the 0–150 Hz frequency.

To account for the high variability of the power values between animals, all EEG and EMG data are standardized and presented as relative (Δ) power, calculated as:

$$\Delta power = \frac{power}{baseline\ power} - 1$$

The biosensor data were standardized using 30-minutes period before Pilo injection as a baseline and presented as relative Glu level, determined analogously to the calculation presented above. Data were averaged over 2-minute intervals.

Standardization and averaging calculations were performed in Microsoft Excel 2016.

4.8. Brain glutamine synthetase (GS) activity assay

The activity of glutamine synthetase (GS) was determined using the γ-glutamyl transferase assay as described by Bidmon et al. (2008). Immediately after dissection fragments of the hippocampi and entorhinal cortex were frozen on dry ice and stored at −80 °C. Frozen tissue samples were then homogenized (1:25 ratio of tissue weight to buffer volume) in ice-cold buffer composed of 0.32 M sucrose and 2.5 mM HEPES (pH ~7.5). Next, 50 µl portions of the brain homogenates were incubated (20 min at 37 °C) with 450 µl of the reaction buffer, containing (in mM): 60 L-glutamine, 15 hydroxylamine-HCl, 20Na-arsenite, 0.4 ADP, 3 $MnCl_2$, and 60 imidazole-HCl (pH 7.4). The reaction was terminated by adding 500 µl of the stop-solution, containing (in M): 0.2 trichloroacetic acid, 0.67 HCl, and 0.37 $FeCl_3$. The solution was cleared by centrifugation (5 min at 15,000 g) and the reaction product, γ-glutamyl hydroxamate, was determined colorimetrically at 500 nm (absorbance microplate reader SPECTROstar Nano, BMG Labtech). A standard curve was created by the use of γ-glutamyl hydroxamate dilutions ranging 0.3125–10 mM. The protein concentration of brain homogenates was measured with the Bradford assay (Bradford, 1976). The GS activity was expressed as millimolar γ-glutamyl hydroxamate per minute per mg of protein.

4.9. GS protein assay by Western Blotting

After decapitation, immediately removed rat hippocampus was isolated on ice and snap-frozen in liquid nitrogen. Frozen tissue samples were homogenized in buffer and centrifuged as described by Popek et al. (2020). Protein concentrations were performed using a BCA Protein Assay (Thermo Scientific). The content of GS was assessed by Western Blotting as previously described (Zielińska et al., 2016; Popek et al., 2020). Membranes were incubated overnight at 4 °C with anti-GS antibody (1:10,000, Sigma), washed, incubated with HRP-conjugated IgG (1:8000; Sigma) and developed using Chemiluminescent Super Signal West Pico Substrate (Pierce, Rockford, IL, USA). After stripping, membranes were incubated with HRP-conjugated anti-GAPDH antibody for 1 h (1:8000; Proteintech), which was used as a loading control, and developed as described above. Data were expressed as percentage change in chemiluminescent band intensity of GS antibody divided by GAPDH.

4.10. Verification of brain tissue damage

The brain was drop-fixed in 4% paraformaldehyde/PBS. Serially cut tissue sections (5 µm thick) were stained with hematoxylin and eosin and observed using Olympus BX43 microscope.

4.11. Data evaluation

Statistical analysis of data was performed employing Prism 7.0 software. Statistical differences were calculated by Student's *t*-test or Mann-Whitney test for, respectively, parametric or non-parametric data regarding two groups and by One-way ANOVA or for, respectively, parametric or non-parametric data regarding more than two groups. Multiple comparisons were always followed by adequate *post hoc* tests. A

P-value of 0.05 or less was considered statistically significant. The information as to which test was selected for characterization of each individual parameter is provided in the figure legends.

Correlation between the biochemical and physiological parameters evaluated in this study was analyzed using non-parametric Spearman's coefficient test. All data were averaged at equal 5-minute intervals.

CRediT authorship contribution statement

Marek J. Pawlik: Data curation, Formal analysis, Funding acquisition, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Marta Obara-Michlewska:** Funding acquisition, Investigation, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. **Mariusz P. Popek:** Investigation, Project administration. **Anna Maria Czarnecka:** Validation, Writing - review & editing. **Stanisław J. Czuczwar:** Conceptualization, Methodology, Writing - review & editing. **Jarogniew Łuszczki:** Conceptualization, Methodology, Writing - review & editing. **Marcin Kołodziej:** Data curation, Formal analysis, Software. **Albert Acewicz:** Visualization. **Teresa Wierzba-Bobrowicz:** Visualization. **Jan Albrecht:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.+

Acknowledgements

This work was supported by the National Science Centre (NCN) of Poland (2016/23/B/NZ4/03678) and by ESF (POWR.03.02.00-00-I028/17-00).

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7.2.2. Article II – Pawlik M, Aldana BI, Belfiori-Carrasco LF, Obara-Michlewska M, Popek M, Czarnecka AM, Albrecht J. 2021. Inhibition of Glutamate Release, but Not of Glutamine Recycling to Glutamate, Is Involved in Delaying the Onset of Initial Lithium-Pilocarpine-Induced Seizures in Young Rats by a Non-Convulsive MSO Dose. *International Journal of Molecular Sciences*. 22(20):11127. doi: 10.3390/ijms222011127

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Inhibition of Glutamate Release, but Not of Glutamine Recycling to Glutamate, Is Involved in Delaying the Onset of Initial Lithium-Pilocarpine-Induced Seizures in Young Rats by a Non-Convulsive MSO Dose

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Citation: Pawlik, M.J.; Aldana, B.I.; Belfiori-Carrasco, L.F.; Obara-Michlewska, M.; Popek, M.P.; Czarnecka, A.M.; Albrecht, J. Inhibition of Glutamate Release, but Not of Glutamine Recycling to Glutamate, Is Involved in Delay in Onset of Initial Lithium-Pilocarpine-Induced Seizures in Young Rats by a Non-Convulsive MSO Dose. *Int. J. Mol. Sci.* **2021**, *22*, 11127. https://doi.org/10.3390/ijms222011127

Academic Editor: Andreas Grabrucker

Received: 16 September 2021
Accepted: 12 October 2021
Published: 15 October 2021

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Abstract: Initial seizures observed in young rats during the 60 min after administration of pilocarpine (Pilo) were delayed and attenuated by pretreatment with a non-convulsive dose of methionine sulfoximine (MSO). We hypothesized that the effect of MSO results from a) glutamine synthetase block-mediated inhibition of conversion of Glu/Gln precursors to neurotransmitter Glu, and/or from b) altered synaptic Glu release. Pilo was administered 60 min prior to sacrifice, MSO at 75 mg/kg, i.p., 2.5 h earlier. [1,2-^{13}C]acetate and [U-^{13}C]glucose were i.p.-injected either together with Pilo (short period) or 15 min before sacrifice (long period). Their conversion to Glu and Gln in the hippocampus and entorhinal cortex was followed using [^{13}C] gas chromatography-mass spectrometry. Release of in vitro loaded Glu surrogate, [^{3}H]D-Asp from ex vivo brain slices was monitored in continuously collected superfusates. [^{3}H]D-Asp uptake was tested in freshly isolated brain slices. At no time point nor brain region did MSO modify incorporation of [^{13}C] to Glu or Gln in Pilo-treated rats. MSO pretreatment decreased by ~37% high potassium-induced [^{3}H]D-Asp release, but did not affect [^{3}H]D-Asp uptake. The results indicate that MSO at a non-convulsive dose delays the initial Pilo-induced seizures by interfering with synaptic Glu-release but not with neurotransmitter Glu recycling.

Keywords: epilepsy; glutamatergic transmission; glutamine synthesis; methionine sulfoximine; metabolomics

1. Introduction

In the mammalian brain, glutamine synthetase (GS) is bimodaly involved in the regulation of excitatory, glutamatergic transmission [1,2]. On the one hand, GS-mediated conversion of Glu and ammonia to Gln in astrocytes neutralizes Glu newly released from neurons. On the other hand, a portion of Gln newly formed in astrocytes is transferred back to neurons by specific Gln transporters catalyzing its astrocytic release (system N) and neuronal uptake (system A), respectively [3–5]. Here, it enriches the neurotransmitter pool of Glu: the above sequence of events are defined as the glutamine–glutamate cycle (GGC).

Methionine sulfoximine (MSO) is an irreversible GS inhibitor [6]. Acute GS inhibition by high MSO doses rapidly stimulates glutamatergic transmission, inducing convulsions and seizures [6–8], while GS inactivation associated with prolonged MSO treatment is accompanied by hippocampal sclerosis and spontaneous recurrent seizures (SRS) resulting from inefficient Glu uptake, a status manifesting chronic temporal lobe epilepsy (TLE) [7,9–11]. GS loss in brain-vulnerable regions (mainly hippocampus) has also been found to correlate with advanced TLE induced by other chemical stimuli, including pilocarpine (Pilo) [12–14] or kainic acid (KA) [15].

On the other hand, exogenously added Gln will induce a long-lasting, glutamatergic transmission-bolstering effect. This has been directly demonstrated in striatal cortical slices [16], but has also been recorded in epilepsy models: in entorhinal cortical slices derived from Pilo-treated chronic epileptic rats [17], in rats with KA-induced seizures [18], and in an in vitro model of post-traumatic epilepsy [19]. The role of Gln in promoting excitatory transmission and seizures has also been demonstrated in experiments employing low MSO doses causing moderate inhibition of GS. Low MSO depressed glutamatergic transmission and epileptiform discharges in cerebral cortical slices in vitro [20,21]. In vivo, intracerebral administration of MSO reduced amygdala kindling-induced seizures [22].

Pilocarpine is considered a particularly valuable model for examining the development of temporal lobe epilepsy both in adult [23,24] and juvenile rats [25–28]. We recently have demonstrated that in 24-day rats treated with lithium-pilocarpine (Li-Pilo) following exactly the procedure described in [26], pretreatment with MSO at a low dose (75 mg/kg b.w.) which produced no convulsions, delayed the onset of and attenuated the initial convulsive and electrographic seizures [29]. It appeared reasonable to speculate that MSO at this low dose inhibited an aspect of glutamatergic transmission critical for provoking the initial seizures. In the present study, we test two not mutually exclusive hypotheses regarding the mechanism by which MSO could induce the inhibition. The first was that MSO interferes with the GGC in a way affecting de novo Glu synthesis in this model. To test this hypothesis, [^{13}C] mass spectrometry was employed to follow incorporation in vivo of ^{13}C labelled metabolic precursors: acetate and glucose, to Glu and Gln. This procedure has previously been used to study Glu synthesis and metabolism in models of brain pathologies associated with enhanced glutamatergic transmission [30–36], including treatments with convulsive MSO dose in vivo [30] and in vitro [36–38].

The second hypothesis was that MSO interferes with the availability of Glu at the synapse by modifying its release and/or reuptake. Here, we took into consideration earlier published data demonstrating that MSO affects Glu transport in various brain preparations in vivo [39] and in vitro [40,41]. To account for this hypothesis, we tested the effect of MSO on the uptake and release of the newly taken up ^{3}H-labelled Glu surrogate, D-aspartate ([^{3}H]D-Asp) in ex vivo brain slices derived from rats treated with a low dose of MSO. High, depolarizing potassium concentrations were used as a generic release stimulus.

2. Results

2.1. Behavioral Assessment of Seizures

MSO significantly reduced behavioral symptoms of seizures throughout the whole 60-min observation period (Figure 1A) as well as during the short 15-min period (Figure 1D), the magnitude similar to that observed previously [29]. MSO significantly delayed the onset of generalized seizure in the "short period" group (Figure 1E,F) and tended to delay the onset in the "long period" group (Figure 1B,C), again, in a manner similar to that previously described [29]. As shown in the heat map (Figure 1G), the severity of seizures between individuals varied substantially throughout whole observation period.

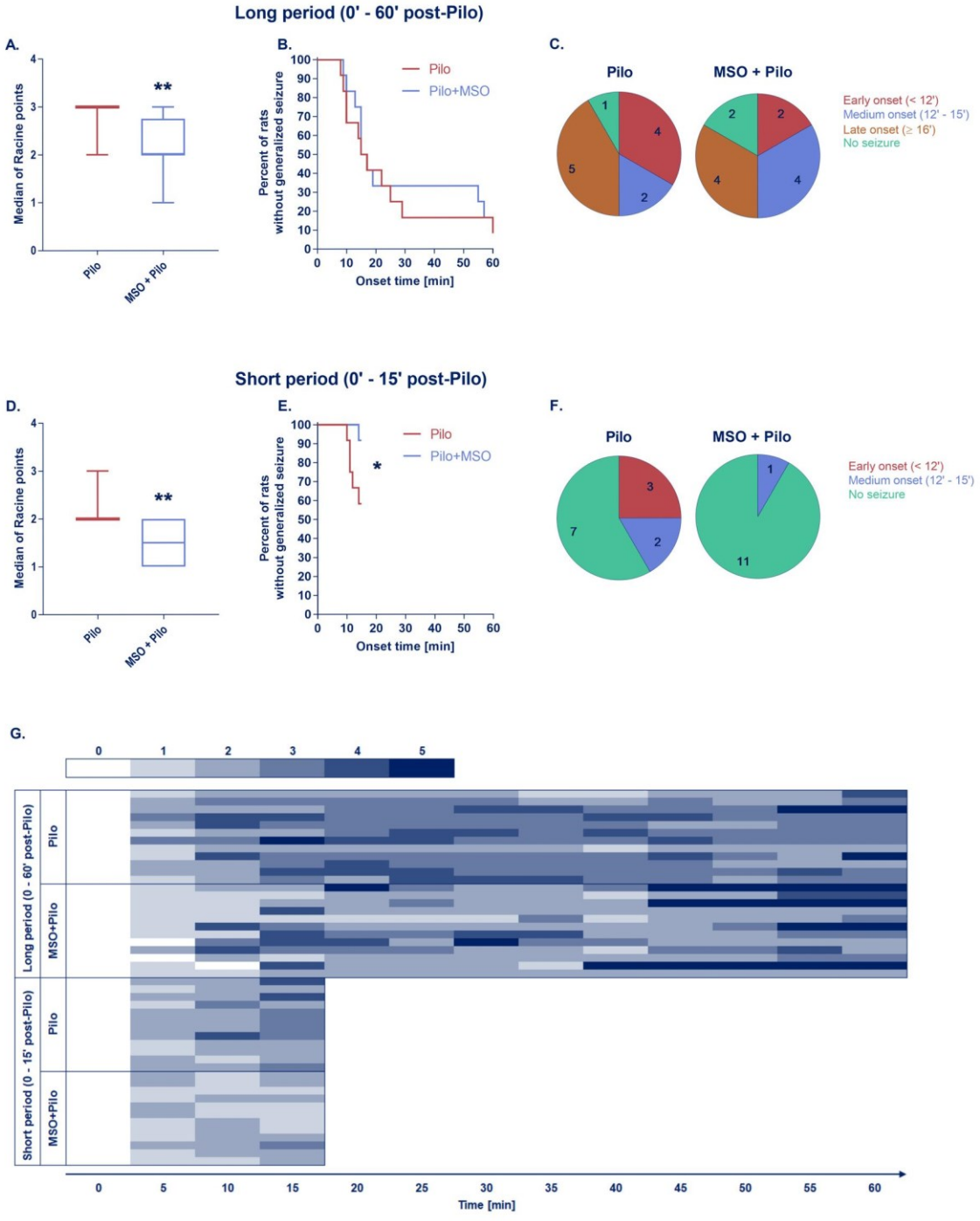


Figure 1. Box charts show the averaged magnitudes of seizures presented as a median of Racine points over a long period (**A**) or short period of observation (**D**), ** $p < 0.01$, Mann–Whitney test; survival plots show latency to the onset of the first generalized seizure among each group (**B**,**E**), * $p < 0.05$, Gehan–Breslow–Wilcoxon test; pie charts present the onset periods of the first generalized seizure—colors indicate distinct periods, numbers represent quantity of animals in each onset period (**C**,**F**); heat map shows detailed seizure dynamic for each animal in Racine scale (**G**); Pilo, MSO+Pilo N = 12 for both time periods.

2.2. Generation of Glu and Gln from [U-^{13}C]glucose or [1,2-^{13}C]acetate

2.2.1. Glutamate

At 15 min after Pilo administration, ^{13}C-enrichment of Glu from labelled glucose was drastically decreased by MSO both after the first (M+2) and the second turnover (M+4) (Figure 2A). In the latter, the levels of 5 out of 6 samples were below the detection limit of gas chromatography coupled to mass spectrometry (GC-MS). However, Pilo treatment alone did not induce changes in the enrichment (Pilo group), MSO failed to decrease the ^{13}C-enrichment from glucose in Pilo-treated rats (MSO+Pilo group). No changes after either combination of treatments were apparent in the entorhinal cortex, except for a tendency towards increase of M+4 labeling in Glu (Figure 2C). A slight tendency to decrease of Glu labelling from acetate by MSO became apparent in the entorhinal cortex (Figure 2D) but not in the hippocampus (Figure 2B). Again, MSO failed to decrease the ^{13}C-enrichment from acetate, leaving M+2 and M+4 Glu levels unchanged relative to the control in both brain structures. No noteworthy changes in Glu ^{13}C enrichment were observed at 60 min after Pilo administration (Figure 2E,H). In both time points and brain structures, and under either condition, ^{13}C-enrichment in Glu derived from labeled glucose was around two times higher than that derived from acetate.

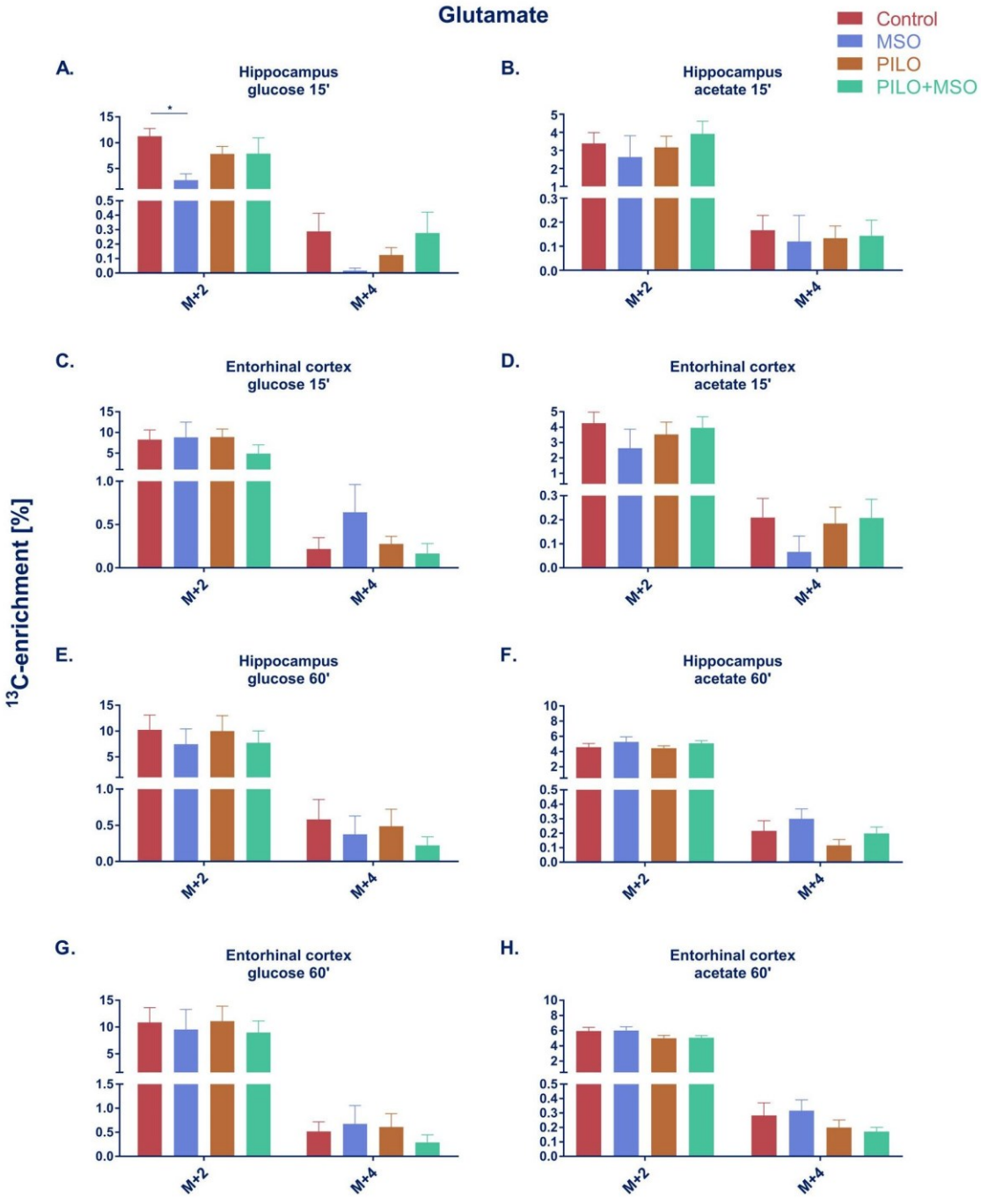


Figure 2. Percentage of ^{13}C-enrichment in glutamate derived from [U-^{13}C]glucose (**A**,**C**,**E**,**G**) or [1,2-^{13}C]acetate (**B**,**D**,**F**,**H**) in hippocampus and entorhinal cortex of TLE model rats. * $p < 0.05$, one-way ANOVA with Tukey's correction; Control, MSO, Pilo, MSO+Pilo N = 6; mean ± SEM. Designations "15'" and "60'" refer to the short (**A**–**D**) and long period (**E**–**H**) of Pilo action and correspond to 150-165 min or 195-210 min time of action of MSO, respectively, rendering in each case a 15 min presence of the isotope in the body.

2.2.2. Glutamine

Similarly to glutamate, glutamine enrichment from ^{13}C-labelled glucose in the hippocampus at 15 min was significantly reduced by MSO after the first turnover and almost

indeterminable after the second turnover (4 out of 6 samples below detection limit) (Figure 3A). At 15 min, MSO failed to significantly affect glutamine enrichment from glucose in the entorhinal cortex (Figure 3C). A slight tendency towards decrease of Gln labelling by MSO was observed in both structures at 60 min when glucose was used as a precursor (Figure 3E,G). At 15 min, a tendency of MSO to decrease Gln labelling was also observed in acetate-derived metabolites in both structures; however, the effect did not reach statistical significance in either of the groups (Figure 3B,D). No change was observed in either structure after either treatment at 60 min (Figure 3F,H). Unlike Glu, where the majority of the newly labelled neurotransmitter originated from glucose, more than twice as much Gln was labelled from acetate than from glucose.

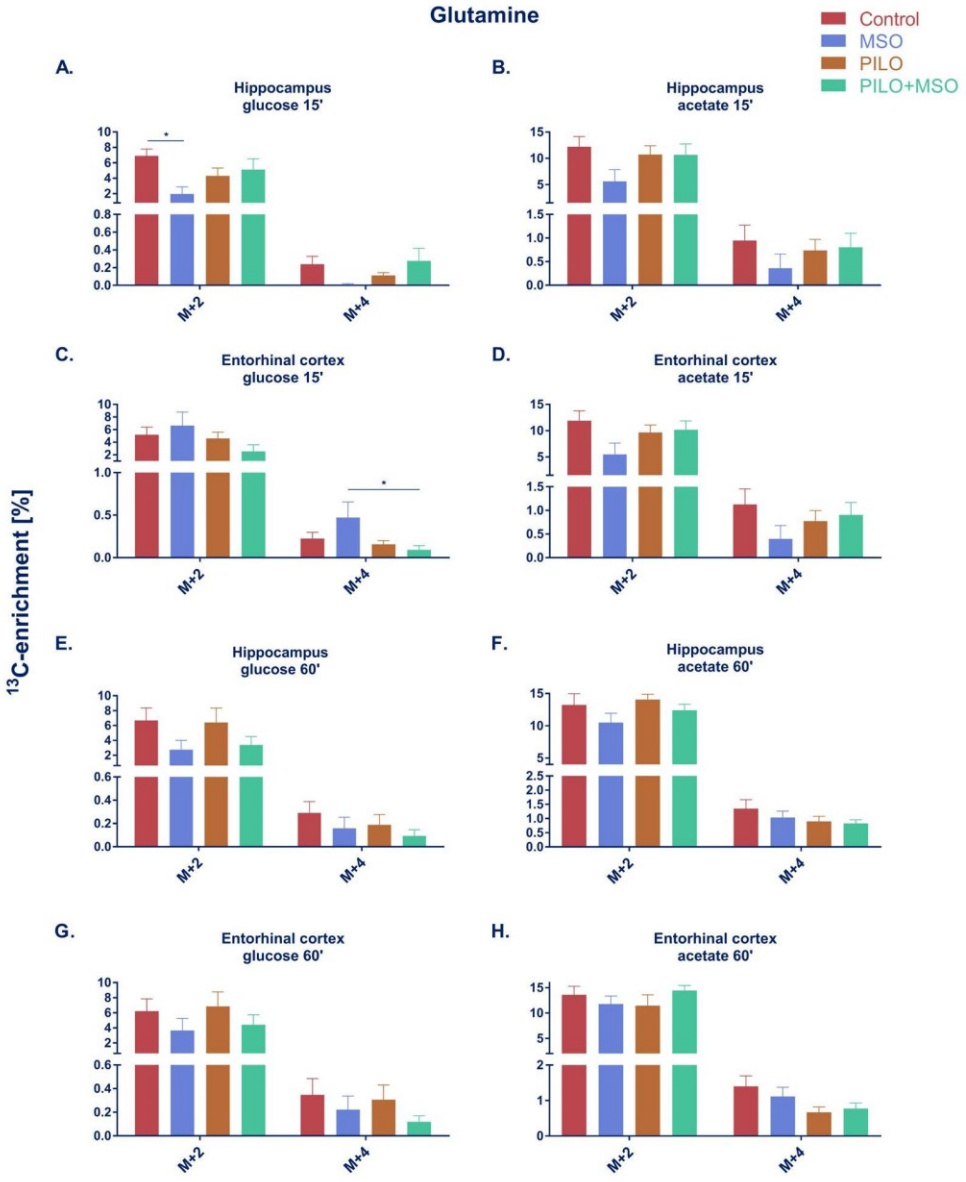


Figure 3. Percentage of ^{13}C-enrichment in glutamine derived from [U-^{13}C]glucose (**A**,**C**,**E**,**G**) or [1,2-^{13}C]acetate (**B**,**D**,**F**,**H**) in the hippocampus and entorhinal cortex of TLE model rats. * $p < 0.05$, one-way ANOVA with Tukey's correction; Control, MSO, Pilo, MSO+Pilo N = 6; mean ± SEM. Designations "15'" and "60'" refer to the short (**A**–**D**) and long period (**E**–**H**) of Pilo action and correspond to 150–165 min or 195–210 min time of action of MSO, respectively, rendering in each case a 15 min presence of the isotope in the body.

2.3. Glu and Gln Brain Tissue Content in MSO and/or Pilo-Treated Rats–A High Performance Liquid Chromatography (HPLC) Analysis

In line with a previous report [42], none of the treatments produced statistically significant changes in hippocampal or entorhinal cortical Glu at either time point. Considering all the experimental variants, the Glu content ranged from 531.1 ± 89.4 in the entorhinal cortex to 788.1 ± 187.8 in the hippocampus (nmol/mg of protein, mean ± SD) (Figure A1).

2.4. [³H]D-Asp Efflux

D-Asp release evoked by K^+-induced depolarization was markedly lowered due to administration of MSO (Figure 4A). For four consecutive minutes after potassium pulse [³H]D-Asp levels in individual fractions were found to be significantly decreased in comparison with control. Area under the curve was on average 37% smaller in the MSO group as well (2.445 ± 0.139 vs. 3.888 ± 0.239 in control), indicating that the whole released pool of D-Asp was reduced. Baseline level of D-Asp was slightly decreased (0.204 ± 0.006% in MSO vs. 0.309 ± 0.005% in control) which may suggest that D-Asp transport is affected by MSO in normal conditions as well, however, these changes were statistically insignificant.

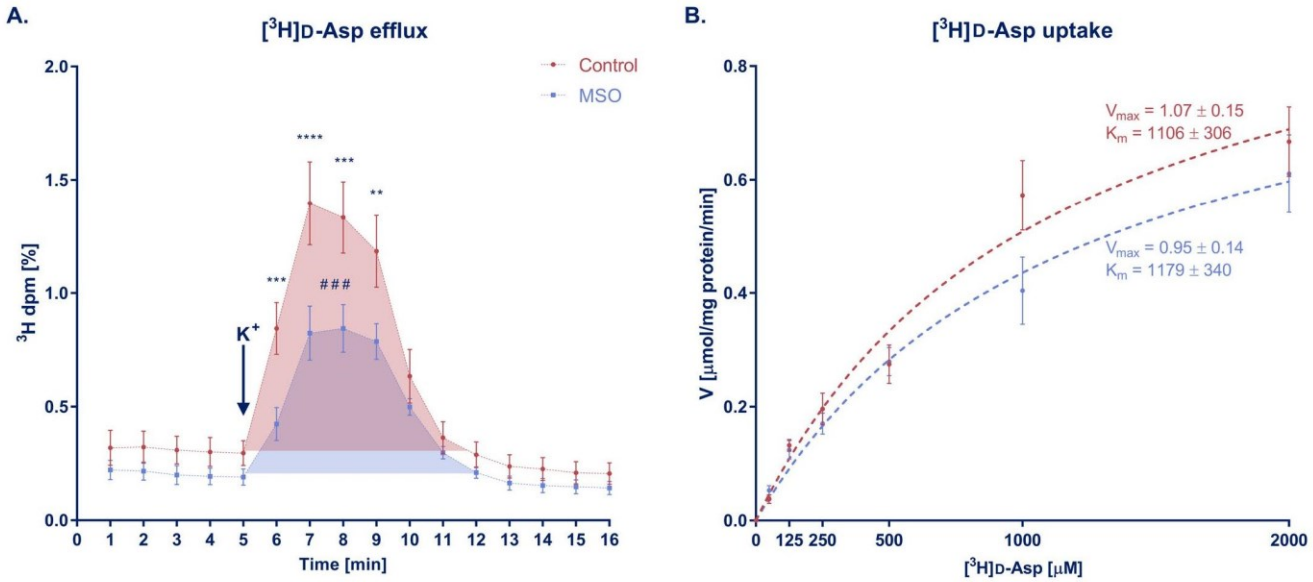


Figure 4. (**A**) [³H]D-Asp efflux from brain slices of rats that received MSO or vehicle (control group). Arrow indicates the moment when K^+ depolarizing pulse was introduced. * statistical significance between time points, multiple t-test with Holm–Šidák correction; **, ***, **** $p < 0.01$, 0.001, 0.0001 respectively; #—statistical significance between areas under curve (AUC), one-tailed t-test; baseline for AUC comparison was set as a mean of fractions 1-5; baselines were compared using linear regression but changes between the slopes were not significant; control N = 5, MSO N = 6; mean ± SEM. (**B**) [³H]D-Asp uptake from brain slices of rats that received MSO or vehicle (control group). The dashed lines represent Michaelis–Menten kinetic curves; V_{max} and K_m constants were compared using t-test; control, MSO N = 7; mean ± SEM.

2.5. [³H]D-Asp Uptake

There was no change in the kinetics of [³H]D-Asp uptake, especially at lower concentrations of D-Asp (50–500 µM) (Figure 4B). At higher concentrations (1000–2000 µM), a slight decrease of the uptake rate was observed in the MSO-treated group; however, the impact of MSO was not pronounced enough to significantly change V_{max} or K_m parameters. This lack of effect of MSO confirms an earlier observation of unchanged Glu uptake in MSO-pretreated rat brain synaptosomes [40].

3. Discussion

Our primary hypothesis regarding the mechanism by which low dose MSO attenuates the initial seizures in the Li-Pilo model was that MSO decreases the synthesis of neurotransmitter Glu by reducing the formation of its precursor Gln. The hypothesis was verified by measuring the effects of MSO treatment on the incorporation in vivo of ^{13}C labelled metabolic precursors: acetate and glucose to neurotransmitter Glu and Gln in the hippocampus and entorhinal cortex, the two structures involved in the propagation of pilocarpine-induced seizures [43]. The only statistically significant change observed throughout the study was a decrease of ^{13}C -enrichment of both Glu and Gln from glucose in hippocampus in the period of 150–165 min after MSO administration alone. However, at the very same time point, MSO failed to modulate in either direction the enrichment from glucose in rats with Li-Pilo-evoked seizures. Otherwise, MSO did not induce any significant effect on ^{13}C–enrichment from glucose nor acetate, in any animal group either at the short (15 min) or at the long period (60 min) of the development of initial seizures. It is intriguing that the decrease by MSO of ^{13}C–enrichment from glucose at 15 min was not observed at the later time point. Tentatively, this may be due to the biphasic effect of MSO on GS activity, leaving open the time brackets of the system in the long period. A study with human GS revealed that initial competitive inhibition is a reversible process and is followed by irreversible activation [44]. The general absence of the effects of extrinsic factors became apparent notwithstanding the predictable metabolic fates of ^{13}C glucose or ^{13}C acetate in this experimental setting. Both preferential ^{13}C–enrichment of Glu from glucose and of Gln from acetate are a good fit into the present views on the role of either of the compound as a metabolic precursor in neurons and astrocytes. Indeed, neurotransmitter pool of Glu is thought to be preferentially synthesized from glucose. The de novo synthesis may occur directly in neurons [45], or in astrocytes. The synthesis in astrocytes depends on anaplerosis, i.e., reactions providing a net increase in TCA cycle intermediates [46]. The primary anaplerotic enzyme in the brain, pyruvate carboxylase (PC), which serves as the main pathway of de novo glutamate synthesis is exclusively expressed in astrocytes [47,48]; reviewed by Schousboe et al. [49]. With regard to Gln, which is exclusively synthesized in astrocytes, its relatively high labelling from acetate supports the preferred [50], albeit not exclusive [36,38] astrocytic metabolism of acetate.

Most previous studies in which MSO was found to significantly affect generation of Glu from [^{13}C] metabolic intermediates (glucose and/or acetate) have analyzed effects of direct application of MSO to in vitro preparations, at concentrations ranging from 1 to 10 mM [36–38]. Significant reduction of ^{13}C glucose flux in the brain in vivo was reported upon 6 h after injection of 150 mg/kg MSO i.p. [30], which is a sub-acute dose reported to cause convulsion [51]. The here used 75 mg/kg i.p. dose of MSO administered for 165–210 min was comparatively low, likely below the threshold required to produce a significant effect. It is intriguing, however, that the reduction of ^{13}C–enrichment of Gln and Glu from glucose at the 15 min time point by MSO found in control rats was not observed in Pilo-treated rats. The mechanism by which Pilo abrogated the effect of MSO could be related to deregulation of glucose metabolism. Depending upon the experimental setting, Pilo-induced seizures in their initial phase are accompanied either by an increased [52–55] or decreased cerebral glucose uptake/consumption [56–58]. Whatever their direction, the changes in glucose metabolism inflicted by Pilo-induced seizures may interfere with the effects of MSO. It is likely that in the MSO+Pilo group, the effect of MSO is additionally masked by Pilo-evoked release of a large pool of unlabeled Glu, indeed, a small tendency towards decrease of a ^{13}C labelled Glu pool is noted at 15′ post-Pilo (Figure 2A).

The observation that MSO added at a low, non-convulsive dose did not significantly affect the ^{13}C–enrichment of Gln or Glu in Li-Pilo-stimulated brain slices substantiated investigation of GGC-bypassing effects of MSO on Glu release and uptake. The key observation made was that MSO markedly reduced the release of newly loaded [^{3}H]D-Asp from ex vivo brain slices subjected to the isotonic depolarization stimulus (75 mM K^+). [^{3}H]D-Asp release has been observed to mimic fairly well depolarization evoked release

of the synaptic vesicular pool of Glu in brain slices [59] and cultured neurons [60]. Earlier, this paradigm proved useful in explaining variable responses of glutamatergic transmission in different models of hepatic encephalopathy [61]. The present results suggest that in the Li-Pilo-treated rats in situ, MSO attenuated the glutamatergic aspect of the initial seizures by directly inhibiting Glu release, albeit interactive interference of MSO and Pilo with the metabolism of Glu precursors to Glu described in the previous paragraph cannot be excluded. Since induction and propagation of seizures by Pilo is subject to a complex mechanism encompassing a yet not fully resolved interplay of cholinergic and glutamatergic stimulation [62,63], it is clear that the reduction of the release, noted in brain slices obtained from MSO-treated rats in response to a single depolarizing stimulus, can only be considered as a rough approximation of its seizure-attenuating effect of Pilo in situ. Of note, basal, unstimulated [^{3}H]D-Asp release from the slices was not affected by MSO exposure (Figure 4), a finding contrasting with stimulation of Glu release observed in different experimental settings at pro-convulsive MSO doses [39,41]. The above discrepancy underscores the dose-dependent duality of the effects of MSO.

Clearly, caveats of the interpretation that potassium-stimulated [^{3}H]D-Asp release genuinely reflects seizure-induced synaptic release of endogenous Glu remain to be addressed. If the interpretation is correct and if MSO-evoked reduction of synaptic Glu release is sustained throughout development of TLE to its full-blown stage, MSO would be a valuable addition to the hotly debated list of antiepileptic drugs with antiepileptogenic activity [64,65]. The fact that MSO is relatively much less neurotoxic in primates than in rodents [8] substantiates further search into its antiepileptogenic potential. Clearly, studies towards this translational goal will have to encompass measurements of the effects of repeated reinjections of MSO in the time period following induction of the initial seizures.

The mechanism by which MSO reduces [^{3}H]D-Asp (Glu) release deserves separate studies. Synaptic Glu release is a complex phenomenon consisting of an array of tightly controlled steps including Glu transport, synaptic vesicle formation, fusion, neurotransmitter release and vesicle recycling via endocytosis at the active zone (for recent reviews see [66–68]); either of the above mentioned aspects deserves careful consideration.

It cannot be excluded that modulation by MSO of glutamatergic transmission involves Gln synthesis-bypassing mechanisms other than synaptic Glu release as well. MSO inhibits not only GS, but also γ-glutamylcysteine synthetase, an enzyme diverting a significant portion of Glu towards glutathione synthesis [69]. MSO was also found to variably affect Gln fluxes in cultured astrocytes [70], and brain cortex slices [71]. However, the abovementioned findings were obtained in experimental conditions not easily translatable to those recorded in ex vivo slices in the present study.

Collectively, notwithstanding a number of caveats to be resolved, the results of the present study may be best interpreted as indicating that MSO at a non-convulsive dose delays, and possibly attenuates, the initial Pilo-induced seizures by interfering with mechanism of Glu release. By contrast, the results do not support the view that the "canonical" glutamine synthetase-inhibiting activity of MSO plays an essential role in the attenuation of the seizures at this stage.

4. Materials and Methods

4.1. Juvenile Rat Li$^+$-pilocarpine TLE Model

The procedure was essentially as described previously [26,29] with minor modifications. At postnatal day 23 (P23), male Sprague Dawley rats (the animal colony of the Mossakowski Medical Research Institute, Polish Academy of Sciences in Warsaw) were injected intraperitoneally (i.p.) with lithium carbonate (222 mg/kg; Sigma-Aldrich, Steinheim, Germany) dissolved in saline (pH equalized to 7.4). At P24, 18–20 h after Li+ treatment, animals were injected i.p. with methyl-scopolamine (1 mg/kg; Sigma-Aldrich, Steinheim, Germany) and thirty minutes later with pilocarpine (40 mg/kg; Sigma-Aldrich, Steinheim, Germany). From then on, the animal's behavior was continuously monitored

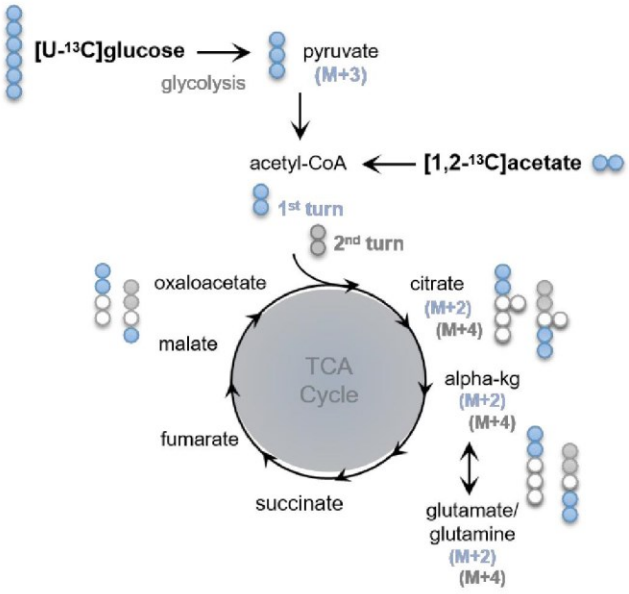
utilizing five-stage Racine scale [72]: 1: mouth and facial movement, 2: head nodding, 3: forelimb clonus, 4: rearing with forelimb clonus, 5: rearing and falling with forelimb clonus, considering stages 1–3 as a focal and 4–5 as a generalized seizure. Rats were decapitated either 15′ (short period) or 60′ (long period) after Pilo administration (see also paragraph *4.4*). Control rats that did not receive Pilo were given equal volumes of saline at the same time.

4.2. Pretreatment with MSO

MSO (Sigma-Aldrich, Steinheim, Germany) was dissolved in saline and administered i.p. at 75 mg/kg. Rats that were used in experiments with Pilo received MSO 150 min before Pilo; thus, 165 min or 210 min before decapitation. Rats that were used in experiments with Pilo received MSO 150 min before Pilo; thus, 165 min (Figures 2 and 3A–D) or 210 min (Figures 2 and 3E–H) before decapitation. Rats used for ex vivo experiments with brain slices received MSO 150 min before decapitation (Figure 4). Control rats that did not receive MSO were given equal volumes of saline at the same time.

4.3. Metabolic Studies In Vivo

The following procedures were based on previously published papers [33,73]. The main labelling patterns of the metabolites from [U-^{13}C]glucose or [1,2-^{13}C]acetate in the experimental setting of the present investigation are detailed in Scheme 1.



Scheme 1. Schematic representation of the main labelling patterns obtained after incubation with [U-^{13}C]glucose or [1,2 ^{13}C]acetate. [U-^{13}C]glucose is taken up by cells in the brain tissue and metabolized to [U-^{13}C]pyruvate (M+3) during glycolysis. [U-^{13}C]pyruvate which in turn can be metabolized in the tricarboxylic acid (TCA) cycle, entering as [1,2-^{13}C]Acetyl-Coenzyme A ([1,2-^{13}C]Ac-CoA) which reacts with unlabelled oxaloacetate and, hence, double-labelled (M+2) TCA cycle intermediates are produced, illustrated as blue circles. [1,2-^{13}C]acetate can also enter the TCA cycle via [1,2-^{13}C]Ac-CoA and generate double-labelled intermediates. The labelled neuroactive amino acid [4,5-^{13}C]glutamate (M+2) can be formed from α[4,5-^{13}C]ketoglutarate (α-kg M+2). From glutamate (M+2), [4,5-^{13}C]glutamine (M+2) can be synthesized in the astrocytes by glutamine synthase (GS). In the second turn of the TCA cycle, [1,2-^{13}C]Ac-CoA reacts with oxaloacetate M+2 formed in the first turn of the cycle, resulting in the production of M+3 and M+4 labelled metabolites (depicted with grey circles).

Rats divided into four groups (control, MSO, Pilo, MSO+Pilo) received a single i.p. dose of [U-^{13}C]glucose (543 mg/kg) or [1,2-^{13}C]acetate (504 mg/kg) (Cambridge Isotope Laboratories, Tewksbury, MA, USA) dissolved in saline, either at the same time as Pilo (short period) or 45 min after the injection of Pilo (long period). All animals were decapitated after 15 min. Therefore, the precursors were present in the body at the short and in the long period of Pilo action. Brain tissue was immediately dissected and frozen in liquid nitrogen. Frozen hippocampi and entorhinal cortex samples were homogenized in 2 mL of ice-cold 70% ethanol and then centrifuged for 20 min at 20,000x *g*. Supernatant was collected, lyophilized, and used for amino acids analysis in GC-MS and HPLC; pellets were used for protein determination with the BCA assay (Thermo Fisher Scientific, Waltham, MA, USA).

4.3.1. Metabolic Mapping Using GC-MS

Lyophilized extracts from hippocampus and entorhinal cortex tissue were resuspended, acidified to pH 1-2 with HCl and subsequently evaporated to dryness using nitrogen. With the use of 96% ethanol and benzene, the analytes were extracted, evaporated again to dryness and derivatized with 14% DMF/86% MTBSTFA [74]. Gas chromatography (GC, Agilent Technologies 7820A chromatograph, J&W GC column HP-5MS, parts no. 19091S-433, Santa Clara, CA, USA) coupled to mass spectrometry (MS, Agilent Technologies, 5977E, Santa Clara, CA, USA) was used to separate and analyze the samples. With the use of unlabeled standards, metabolites of interest were corrected to their ^{13}C natural abundance in order to assess their isotopic enrichment. Calculations were performed according to [75]. Data integration and enrichment determination was performed with the software MassHunter Quantitative Analysis software v.6.0.3881 (Agilent Technologies, Santa Clara, CA, USA). Data are presented as labelling (%) of M+ X, where M is the mass of the unlabelled molecule and X is the number of labelled C-atoms in a given metabolite.

4.3.2. Quantitative Determination of Intracellular Amino Acids by HPLC

Tissue extracts, previously resuspended in water, were separated by reverse-phase HPLC using an Agilent ZORBAX Eclipse plus C18 column (4.6 × 150 mm, particle size 3.5 μm; 959,963–902, Agilent Technologies, Santa Clara, CA, USA) in an Agilent 1260 Infinity system coupled to a 1260 Infinity fluorescence detector (Agilent Technologies, Santa Clara, CA, USA) as described previously [76]. Briefly, samples were derivatized with o-phthaldialdehyde and separation was performed with a mobile phase gradient consisting of a mixture of buffer A (10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 6.9; 5 mM NaN_3) and buffer B (acetonitrile 45%: methanol 45%: water 10%, v:v:v). Amino acids quantification was performed using standards containing a mixture of the amino acids of interest on increasing concentrations. The acquired data was normalized to the protein concentration in the dry tissue samples. Effects of MSO on the Gln content could not be accurately assessed because the position of the MSO peak which was in much excess over Gln largely overlapped with the position of the latter.

4.4.[^{3}H]D-Asp Studies Ex Vivo on Acute Brain Slices

Rats anesthetized with isoflurane (Baxter, Deerfield, IL, USA) were decapitated, the brain was immediately removed, and cortices were cut into 350 μm thick slices, using McIlwain tissue chopper. The slices were pre-incubated for 30 min in the Krebs buffer (37°C, aerated with 95% O2 and 5%CO2, pH 7.4), composed of [mM]: 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 10 glucose. After preincubation, slices were used either for uptake or efflux measurement protocols, employing the radioactive [^{3}H]D-Aspartate ([^{3}H]D-Asp) as the non-metabolised analogue of glutamate.

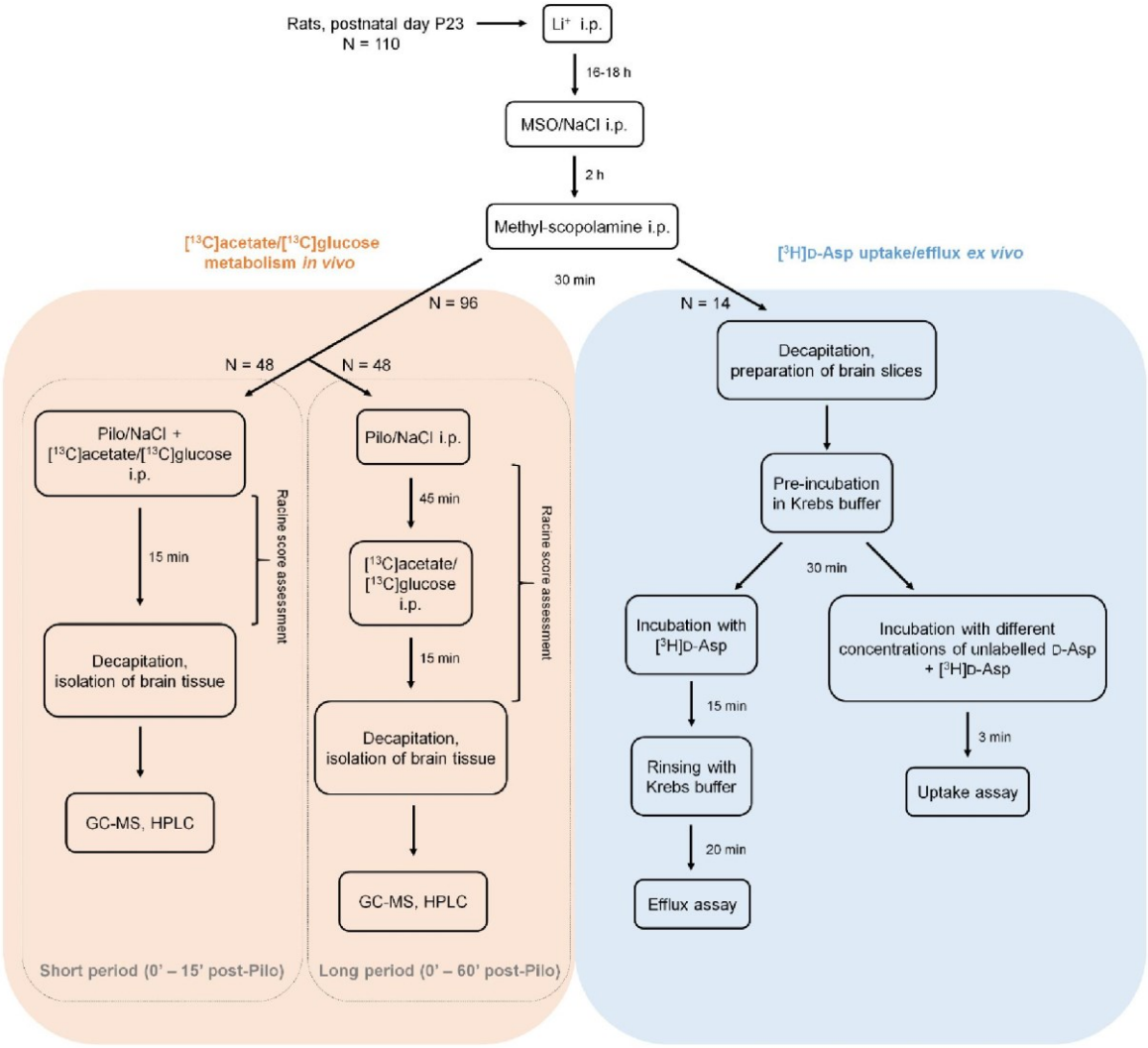
4.4.1. [^{3}H]D-Asp Efflux Assay

The [^{3}H]D-Asp efflux was assayed based on the method previously described [77], with modifications. [^{3}H]D-Asp efflux was measured after 15 min incubation in Krebs buffer containing 1.4 μCi/mL [^{3}H]D-Asp (Perkin-Elmer, Waltham, MA, USA) and unlabeled D-Asp (100 μmol/L). The slices were moved to a chamber perfusion system (Brandel, Gaithersburg, MD, USA) and rinsed with Krebs buffer at 0,5 mL/min rate. The initial fraction was collected for 20 min to establish baseline efflux. Perfusate samples were then collected for 20 min, at 1 min intervals. At the time period indicated in Figure 4A. (fractions 5 and 6), a depolarizing pulse was introduced by raising KCl concentration in the Krebs buffer to 75 mM, with simultaneous reduction of NaCl to 47.7 mM). Radioactivity contained in the preparation and released from brain slices were measured by a Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer, Waltham, MA, USA).

4.4.2. [^{3}H]D-Asp Uptake Assay

The [^{3}H]D-Asp uptake was assayed as previously described [78]. Briefly, the uptake was initiated by adding [^{3}H]D-Asp, 0.1 μCi/1 mL (Perkin-Elmer, Waltham, MA, USA), to varying extracellular concentrations of unlabelled D-Asp (50–2000 μM). The incubation with the radioisotope was continued for 3 min and was terminated by rapid vacuum filtration through nitrocellulose filter disks (Millipore, Billerica, MA, USA), followed by flushing four times with 2 mL of ice-cold Krebs buffer. The slices were weighed, immersed in 4 mL of InstaGel scintillation fluid (Perkin-Elmer, Waltham, MA, USA), and the radioactivity of slices on filter disks was measured in the Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer, Waltham, MA, USA).

A detailed diagram of experiments conducted in this study is presented in Scheme 2.



Scheme 2. A diagram of the in vivo and ex vivo experiments in the Li-Pilo model. Male Sprague Dawley rats were administered sequentially with lithium, MSO or saline and methyl-scopolamine, starting at postnatal day 23 (P23). Afterwards, animals intended for metabolic experiments (left, orange panel) received a dose of a convulsive agent–pilocarpine or saline and were observed for 15 (short period groups) or 60 min (long period groups) and then decapitated. All rats in in vivo experiments were also injected with ^{13}C-labelled metabolic precursor (acetate or glucose), 15 min prior to decapitation. Dissected brain tissue was homogenized and analyzed with gas chromatography coupled to mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC). Animals intended for ex vivo experiments with ^{3}H-labelled Glu surrogate, D-aspartate ([^{3}H]D-Asp) (right, blue panel) were decapitated 30 min after methyl-scopolamine injection, the brain was isolated and immediately cut into slices. Acute brain slices were pre-incubated in Krebs buffer for 30 min, then incubated with [^{3}H]D-Asp and subjected to efflux or uptake assays. In the metabolic studies (orange panel), a total of 96 animals were used: 6 animals in each group (Control, MSO, Pilo, MSO+Pilo): 6 × 4 groups × 2 ^{13}C-precursors × 2 settings (long and short period). A total of 14 animals were used in the uptake/efflux experiments (blue panel).

4.5. Data Evaluation

All statistical analysis were performed with Prism 7.0 (Graphpad Software Inc, La Jolla, CA, USA) software. T-tests or Mann–Whitney tests were used for comparisons of two groups (respectively for parametric and non-parametric data) and one-way ANOVA for more than two groups (all the data sets considered were parametric); the Gehan–Breslow–Wilcoxon test was used for comparison of survival curves. Multiple comparisons were followed by Holm–Šidák's (multiple t-tests) or Tukey's (ANOVA) post hoc tests. The

significance level was set at $p < 0.05$. Heat map was prepared with Excel 2016 (Microsoft, Redmond, WA, USA).

5. Conclusions

The major novel observation of the present study is that MSO at a non-convulsive dose delays the initial Pilo-induced in a mode not discernably related to its canonical, glutamine synthetase-inhibiting activity. This observation is unique in that, as mentioned in the Discussion, previous therapeutic interventions with MSO in other diseases were based on its canonical mechanism of action. The present results strongly indicate that the seizure-ameliorating effect of MSO is due to its interference with Glu release.

Author Contributions: Conceptualization, J.A.; methodology, J.A., B.I.A., M.O.-M., M.P.P., M.J.P.; validation, M.J.P., J.A.; formal analysis, M.J.P., M.O.-M.; investigation, M.J.P., M.O.-M., M.P.P., L.F.B.-C.; resources, J.A., B.I.A.; data curation, M.J.P.; writing—original draft preparation, J.A., M.J.P., M.O.-M., M.P.P., B.I.A.; writing—review and editing, J.A., M.J.P., M.O.-M., B.I.A., A.M.C., L.F.B.-C.; visualization, M.J.P., B.I.A.; supervision, J.A.; project administration, J.A., M.J.P., M.O.-M.; funding acquisition, J.A., M.J.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Science Centre (NCN) of Poland (2016/23/B/NZ4/03678) and by ESF (POWR.03.02.00-00-I028/17-00).

Institutional Review Board Statement: Animal experiments were conducted according to the guidelines of the Declaration of Helsinki and the national guidelines on animal experimentation, and they were approved by the local ethical committee in Warsaw (consent no. 405/2017, 21.11.2017) in accordance with EC Directive 2010/63/EU.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

Acknowledgments: The authors would like to thank Jolanta Dyniewicz and Katarzyna Kosińska from the Department of Neuropeptides of MMRI PAS for their invaluable help with samples lyophilization.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

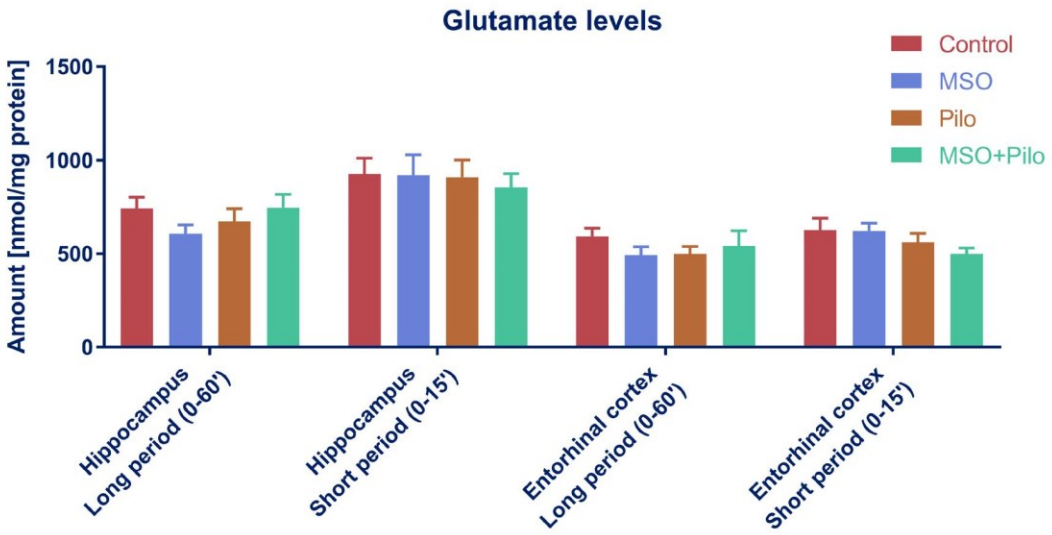


Figure A1. Glutamate levels in hippocampus and enthorhinal cortex of TLE model rats. One-way ANOVA with Tukey's correction; Control, MSO, Pilo, MSO+Pilo N = 12; mean ± SEM.

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7.2.3. Article III – Pawlik M, Miziak B, Walczak A, Konarzewska A, Chrościńska-Krawczyk M, Albrecht J, Czuczwar SJ. 2021. Selected molecular targets for anti-epileptogenesis. *International Journal of Molecular Sciences*. 22(18):9737. doi: 10.3390/ijms22189737

International Journal of
Molecular Sciences

MDPI

Review

Selected Molecular Targets for Antiepileptogenesis

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Abstract: The term epileptogenesis defines the usually durable process of converting normal brain into an epileptic one. The resistance of a significant proportion of patients with epilepsy to the available pharmacotherapy prompted the concept of a causative treatment option consisting in stopping or modifying the progress of epileptogenesis. Most antiepileptic drugs possess only a weak or no antiepileptogenic potential at all, but a few of them appear promising in this regard; these include, for example, eslicarbazepine (a sodium and T-type channel blocker), lamotrigine (a sodium channel blocker and glutamate antagonist) or levetiracetam (a ligand of synaptic vehicle protein SV2A). Among the approved non-antiepileptic drugs, antiepileptogenic potential seems to reside in losartan (a blocker of angiotensin II type 1 receptors), biperiden (an antiparkinsonian drug), nonsteroidal anti-inflammatory drugs, antioxidative drugs and minocycline (a second-generation tetracycline with anti-inflammatory and antioxidant properties). Among other possible antiepileptogenic compounds, antisense nucleotides have been considered, among these an antagomir targeting microRNA-134. The drugs and agents mentioned above have been evaluated in post-status epilepticus models of epileptogenesis, so their preventive efficacy must be verified. Limited clinical data indicate that biperiden in patients with brain injuries is well-tolerated and seems to reduce the incidence of post-traumatic epilepsy. Exceptionally, in this regard, our own original data presented here point to c-Fos as an early seizure duration, but not seizure intensity-related, marker of early epileptogenesis. Further research of reliable markers of early epileptogenesis is definitely needed to improve the process of designing adequate antiepileptogenic therapies.

Keywords: epileptogenesis; antiepileptic drugs; losartan; nonsteroidal anti-inflammatory drugs; antioxidative drugs; antagomirs; c-Fos; epileptogenesis markers

Citation: Pawlik, M.J.; Miziak, B.; Walczak, A.; Konarzewska, A.; Chrościńska-Krawczyk, M.; Albrecht, J.; Czuczwar, S.J. Selected Molecular Targets for Antiepileptogenesis. *Int. J. Mol. Sci.* **2021**, *22*, 9737. https://doi.org/10.3390/ijms22189737

Academic Editor: Fabio Altieri

Received: 5 August 2021
Accepted: 2 September 2021
Published: 8 September 2021

1. Introduction

Antiepileptic drugs (AEDs; also named antiseizure drugs) efficiently control epileptic seizures in no more than ca. 65–70% of patients with epilepsy; this limitation holds for AEDs of the second and third generations as well, which substantiate a search for alternative treatment approaches [1]. Considering further that AEDs are only administered to patients with developed epilepsy, their antiepileptogenic potential has been a matter of dispute [2].

Epileptogenesis is a durable process that converts a normal mammalian brain into an epileptic one [3,4]. It therefore seems very likely that stopping or at least modifying the progress of epileptogenesis may eventually prevent the occurrence of epilepsy.

2. Mechanisms of Epileptogenesis

An initial insult, which may comprise status epilepticus, stroke or head trauma, is required to initiate epileptogenesis [3,4]. Interestingly, this process may persist after

the onset of seizures and may negatively affect the seizure frequency [4]. Following the initial insult, two subsequent stages may be considered: (i) an acute one lasting from hours to weeks and encompassing neurodegeneration, elevated inflammatory activity and transcriptional events and (ii) a chronic one, taking even months, characterized by a sequence of events typically including neurogenesis, the sprouting of mossy fibers, reorganization of neuronal circuits and gliosis [5]. There is a correlation between mossy fiber sprouting (to the brain locations they never exist in) and the expression of seizure activity. Additionally, dentate granule cells present aberrant locations in the dentate gyrus (so-called ectopic granule cells), forming abnormal connections within the molecular layer of the dentate gyrus and in neurons located in the hippocampal CA3 field, which eventually results in the formation of excitatory circuits [6]. After all, gliosis may participate in the progress of epileptogenesis, as astrocytes may release a number of neurotransmitters and modulators, glutamate being the main excitatory neurotransmitter involved in the generation of epileptiform discharges [5]. In spite of the initial insult (for instance, acute seizure activity) that elevates the number of newborn neurons, neurogenesis within the hippocampus is decreased when chronic epilepsy develops. This is also often the case during epileptogenesis [5].

The stage of epileptogenesis has also been analyzed in terms of the expression of a number of genes. Interestingly, the genes responsible for the control of many signaling pathways undergo substantial alterations in their expression. For example, the genes for mTOR (mammalian target of rapamycin), insulin-like growth factor 1, transforming growth factor β, p38MAPK (p38 mitogen-activated protein kinases) and Jak-STAT (Janus kinases-signal transducer and activator of transcription proteins) may be given [6].

3. AEDs—Do They Modify Epileptogenesis?

AEDs have been in clinical use for decades, and their efficacy in inhibiting seizure activity in circa 65–70% of patients has been confirmed [7]. Notably, apart from patients surviving traumatic brain injury or stroke or those with initial febrile seizures, their probable antiepileptogenic potential in clinical conditions has not been studied. As for traumatic brain injury, the preventive use of carbamazepine, phenobarbital, phenytoin or valproate to stop the development of epilepsy has failed [8]. The same holds true for diazepam and phenobarbital in cases with febrile seizures and phenytoin or valproate in patients with brain tumors [8]. On the other hand, the prospects for their antiepileptogenic activity have been evaluated in a number of animal models—post-status epilepticus models induced by pilocarpine, kainate or electrical stimulations of discrete brain areas (for instance, the amygdala and hippocampus) seem to yield data possessing a predictive potential [9].

A fundamental question arises whether AEDs may be given to patients who are at risk of epilepsy, i.e., after status epilepticus [2] or brain infection [10]. A preventive treatment with AEDs could be implemented in vulnerable patients only, where markers of epileptogenesis tend to appear. This has been best illustrated in the case of EEG markers in epileptic seizures following cerebral malaria; around 10% of children presenting the markers developed epilepsy [10].

The neuroprotective potential of AEDs against status epilepticus-induced brain damage in rodents has been widely documented (for review, Reference [2]). For instance, diazepam (in the low- and high-dose ranges), gabapentin, pregabalin, topiramate or valproate protected the vulnerable brain areas in animals that survived status epilepticus. There are also AEDs whose neuroprotective potential is not significant, and good examples are carbamazepine or phenytoin. As already stated above, neurodegeneration is encountered during the process of epileptogenesis, so the possibility exists that neuroprotective AEDs may at least reduce its intensity, which would eventually positively affect the frequency and intensity of seizures. In animal post-status epilepticus models, the best probable antiepileptogenic effect would be the total blockade of seizure activity following the silent period after status epilepticus. In control animals, distinct spontaneous seizure activity develops. AEDs were generally administered for a couple of days or weeks after status

epilepticus—there are also examples of much longer AED administration (117 days for phenobarbital). The obtained results are mainly discouraging. Although valproate given at high daily doses for 40 days after kainate-induced status epilepticus in rats prevented hippocampal neurodegeneration and totally inhibited spontaneous seizures [2], the results obtained by other groups of investigators were the opposite. Following status epilepticus induced by pilocarpine in rats, valproate was given at 600 mg/kg daily for 3 weeks, and no neuroprotection or inhibition of spontaneous convulsions were recorded [2]. The results reported by a third group concerned status epilepticus produced by electrical stimulation of the basal amygdala in rats. Although valproate (at 600 mg/kg for 4 weeks) exerted clear-cut neuroprotection in the hippocampal area, no protection against spontaneous seizures was observed [2]. Some protective effects of gabapentin (a reduction of acquired epilepsy) or pregabalin (an extended latency to the onset of spontaneous convulsions) after chemically induced status epilepticus were noted [2]. Many conventional or newer AEDs were totally ineffective in this respect. Interestingly, diazepam at a high dose of 20 mg/kg administered as a single injection 2 h after status epilepticus evoked by electrical stimulation of the amygdala significantly reduced the number of rats exhibiting spontaneous convulsions [2]. Additionally, in the lithium-pilocarpine-induced status epilepticus in female rats, phenobarbital was administered i.p. for 2 weeks at 15 mg/kg twice daily, and then, the spontaneous seizure activity was studied for 7 days between 8 and 9 weeks after status epilepticus. Not only a reduction in the number of rats with spontaneous seizure activity was observed but the median frequency of convulsions sharply diminished from 7.5 to 1 seizure per week [11]. Another positive example might be levetiracetam (a ligand of synaptic vehicle protein SV2A [2]), however, when administered at a very high dose of 500 mg/kg (p.o. twice daily) for 4 weeks in mice after pilocarpine-produced status epilepticus [12]. The seizure activity was assessed in the presence of levetiracetam within four weeks after status epilepticus. In these circumstances, levetiracetam effectively reduced the number of spontaneous seizures, mortality and exerted neuroprotective effects [12].

Although voltage-operated sodium channel inhibitors (carbamazepine and phenytoin) seem ineffective in terms of epileptogenesis (for review, Reference [2]), a third-generation AED, eslicarbazepine, apart from the sodium channel blockade is also an effective blocker of T-type voltage-dependent calcium channels [13]. Interestingly, this drug proved an efficient inhibitor of epileptogenesis in the pilocarpine model of status epilepticus in mice. The drug was given i.p. at 150 and 300 mg/kg once daily for 6 weeks. Eventually, the spontaneous seizure activity was significantly reduced when evaluated 8 weeks after the status and the mossy fiber sprouting was considerably inhibited [14]. Thus, as a target for antiepileptogenesis, T-type voltage-dependent calcium channels could be considered. The beneficial effects of eslicarbazepine in this respect seem to support this assumption [14]. However, more preclinical studies are required on this issue. Remarkably, one other T-type calcium channel inhibitor, ethosuximide [13] at 25 and 50 mg/kg, was in the same experimental approach devoid of an antiepileptogenic activity (see below, Reference [14]).

Lamotrigine is a relatively new AED blocking voltage-operated sodium channels [13]. There are also data available suggesting the blockade of AMPA glutamate receptors at pharmacologically relevant concentrations [15], although recent data by Fukushima et al. [16] pointed to a blockade of NMDA glutamate receptors. When given i.p. 24 h after lithium-pilocarpine status epilepticus in rats at 10 or 20 mg/kg daily for one week, lamotrigine very distinctly inhibited the post-status epilepticus spontaneous seizure activity evaluated in weeks 5 and 6. In the hippocampus, neurodegeneration and astrogliosis were reduced by a lamotrigine pretreatment [17]. Ethosuximide (a T-type calcium channel blocker [13]) at 25 and 50 mg/kg in the same experimental conditions proved completely ineffective [17].

4. Approved Non-AEDs with an Antiepileptogenic Potential

Employing approved non-AEDs, showing a potent antiepileptogenic potency, would reduce the time necessary for implementing a novel antiepileptogenic drug compared to completely new compounds, requiring full approval procedures. No doubt, such drugs are available, and losartan seems a very promising one. Interestingly, this antihypertensive drug (a blocker of angiotensin II type 1 receptors) was evaluated in a rat model of acquired epilepsy in which sodium deoxycholate, via a craniotomy window, was administered to the brain surface. This procedure resulted in an extravasation of albumins into the brain due to vascular injury [18]. The extravasation of albumin complexes into the brain tissue may be encountered in a stroke, head trauma or infection [19–21]. This process is responsible for neuroinflammation involving TGF-β signaling and subsequent epileptiform activity [22]. Losartan at 100-mg/kg i.p. was administered 40 min following deoxycholate, and its relevant plasma concentration was maintained through drinking water (2 g/L) for 3 weeks. The spontaneous seizure activity was evaluated for 2 weeks, starting from the 7th day after losartan was stopped. It turned out that the losartan treatment effectively affected epileptogenesis, which was reflected by a significant reduction of rats exhibiting spontaneous convulsions. While all control rats presented clear-cut seizure activity, 60% of the animals receiving losartan were seizure-free. A sharp reduction in the average number of convulsions was also recorded, with 8/week in the control group vs. 2.25/week in the losartan group [18]. The authors carried out an additional experiment in the absence of blood–brain barrier damage. Toward this aim, they perfused albumin over the brain tissue and started to record the electrocorticographic activity for 110 days. At least two spontaneous seizures (appearing 2 days after albumin exposure) were noted in 85% of rats, and the average number of seizures reached 6.08 per week. In contrast, the proportion of seizing rats in the group perfused with albumin + losartan was reduced to 25%, the average number of seizures being 0.23 seizures/week.

The antiepileptogenic activity of losartan was also reported in the kainate post-status epilepticus-induced epileptogenesis model in rats [23]. The angiotensin receptor 1 blocker was initiated s.c. at 10 mg/kg 2 h after the onset of status epilepticus and continued up to the 3rd day, and then, the animals were switched to losartan in drinking water up to 4 weeks. Spontaneous seizure activity was evaluated for 3 months. Apart from seizures, possible behavioral deficits and hippocampal neurodegeneration were also taken into consideration. Evidently, a pretreatment with losartan increased the latency of the onset of seizures and provided distinct neuroprotection to the CA1 hippocampal subfield. In other hippocampal regions, neuroprotection was also observed, although less expressed. Importantly, in a number of behavioral tests, losartan pretreated rats exhibited considerably less deficits [23]. When losartan was evaluated in an identical experimental approach in spontaneously hypertensive rats, the only difference observed was associated with the behavioral deficits not affected by the losartan pretreatment [24]. Some antiepileptogenic activity of losartan was confirmed in amygdala-kindled rats, because this antihypertensive drug extended the latency time of the development of fully kindled seizures [25]. The drug was either administered i.c.v. or peripherally and significantly elevated the number of stimulations needed to obtain fully kindled rats. After all, no damage to the blood/brain barrier was observed in these animals. However, some seizure parameters (threshold for after discharge induction, after discharge duration or seizure severity in fully kindled animals) were not modified by losartan pretreatment [25].

Rapamycin (an immunosuppressant drug) has been documented to block mTOR complex 1 (mammalian target of rapamycin), which is a serine/threonine protein kinase responsible for neuronal protein synthesis [26]. This drug has been tested for its potential antiepileptogenic properties in rats subjected to status epilepticus induced by kainate at 10 mg/kg [27]. In rats given rapamycin at 6 mg/kg every 4 days, a considerable reduction in the number of spontaneous convulsions was evident on days 17 and 21 after status epilepticus. Following status epilepticus produced by the electrical stimulation of the rat angular bundle, rapamycin (6 mg/kg daily for a week and then every other day for 6 weeks

following status epilepticus) totally inhibited the occurrence of spontaneous seizure activity in 25% of the animals. In the remaining 75%, a significant reduction of seizure activity was shown [28]. This antiepileptogenic activity of rapamycin was associated with its neuroprotective effect in the hippocampus and a reduction in the increased permeability of the blood/brain barrier. Nevertheless, inflammation markers in the hippocampus were not affected by the pretreatment with rapamycin [28]. However, there are also data available on the lack of antiepileptogenic activity of this mTOR blocker. After 24 h following pilocarpine-induced status epilepticus in mice, the rapamycin administration (10 mg/kg) was started and continued up to 2 months. The monitoring of spontaneous motor seizures began 1 month after status epilepticus and was carried out for a month, and after 2 months, mossy fiber sprouting was evaluated. Whilst mossy fiber sprouting and dentate gyrus hypertrophy were suppressed in mice receiving rapamycin, no difference in the seizure frequency was found. Additionally, the loss of hilar neurons was not prevented. The seizure frequency reached 0.137 seizures per hour in the control group and 0.133 seizures per hour in the rapamycin group. There was a large number of subjects in both groups (N = 64) [29]. In a very similar experimental approach, rapamycin was given in a lower dose of 3 mg/kg in mice [30]. No effect of rapamycin on the spontaneous seizure frequency was noted, however, mossy fiber sprouting was reduced by 42% and the hypertrophy of the dentate gyrus was decreased compared with the control group. Nevertheless, in rapamycin-treated mice, the generation of ectopic granule cells, loss of hilar neurons or granule cell proliferation were still observed [30].

Potential antiepileptogenic agents may be searched for among nonsteroidal anti-inflammatory drugs affecting diverse inflammatory pathways. A good example is celecoxib, blocking the cyclooxygenase 2 and HMGB1/TLR-4 pathways, which was evaluated in rats surviving lithium-pilocarpine status epilepticus [31]. The drug was started at 20 mg/kg p.o. one day after status epilepticus and stopped at day 28th, thus covering the latent period. The parameters of spontaneous convulsions (frequency and duration observed between 28 and 42 days) were considerably reduced by the celecoxib pretreatment. Concomitantly, a potent neuroprotection was observed in the hippocampus with aberrant neurogenesis/gliogenesis being significantly decreased [31].

N-acetylcysteine is a drug approved by the FDA for the management of liver toxicity resulting from an overdose of acetaminophen, and it may also be applied as an agent loosening the thick mucus encountered in patients with chronic obstructive lung diseases [32]. The main antioxidant activity of this drug is due to its chemical structure of a reduced glutathione precursor [32]. When given an i.v. dose of 30 mg/kg immediately after systemic kainate-induced status epilepticus in rats, an increased seizure threshold of flurothyl ether was noted 12 weeks later, and mossy fiber sprouting was inhibited [33]. In another experiment, N-acetylcysteine was supplemented at 100 mg/kg p.o. for 5 weeks following brain trauma induced in rats by fluid percussion injury. Rats supplemented with the antioxidant did not react to the subthreshold dose of pentylenetetrazol (30 mg/kg i.p.), which, in control rats, induced generalized tonic-clonic seizures. Moreover, the latency of the first myoclonic jerk was considerably reduced in the vehicle-treated group, whilst the pretreatment with N-acetylcysteine brought it back to the control value of the rats without brain injury [34].

Minocycline (a second-generation tetracycline with anti-inflammatory and antioxidant properties [35]) was shown to possess some antiepileptogenic potential in lithium-pilocarpine-induced status epilepticus in rats. The authors of this study [36] provided evidence that the status epilepticus produced a prolonged activation of both astrocytes and microglia. Minocycline was given at 45 mg/kg for 2 weeks after the status epilepticus. Then, after a period of 6 weeks after minocycline was withdrawn, spontaneous recurrent convulsions were monitored for 2 weeks. The seizure activity was considerably suppressed in minocycline-pretreated animals in terms of its frequency, severity and duration. Further, this drug also mitigated the activation of microglia and reduced the elevated production of tumor necrosis factor-α and interleukin-1β in the hippocampal

CA1 subfield and the neighboring cortex. However, the activation of astrocytes was not affected by the minocycline pretreatment [36]. When the status epilepticus was induced electrically in rats, minocycline failed to modify the spontaneous recurrent seizures [37]. Nevertheless, the pretreatment with this drug abolished the spatial memory deficit and normalized locomotion. No anti-inflammatory effects of minocycline were shown [37].

5. Combined Treatments

Considering a number of diverse mechanisms present during epileptogenesis, some authors have represented the point of view that combinations of drugs or agents sharing complementary mechanisms might be especially useful in this regard. The first attempt assumed there were beneficial effects of two combinations with AEDs, i.e., levetiracetam + topiramate and levetiracetam + phenobarbital, in mice after intrahippocampal kainate [38]. The former combination of levetiracetam (200 mg/kg i.p.) and topiramate (30 mg/kg i.p.) was given for 5 days (latent period), starting after 6 h from the status induction. Video/EEG monitoring for 1 week was brought about 4 and 12 weeks after intrahippocampal kainate. Brain histology was evaluated 6 and 12 weeks later. The authors distinguished electrographic seizures (observed in the EEG) and electroclinical seizures (recorded both in the EEG and videos), the former being more frequent. The combined treatment was very effective in that it reduced the frequency of spontaneous recurrent electroclinical seizures by 80% when compared to nontreated mice. The number of animals with spontaneous electroclinical convulsions was also diminished, indicating that some mice were completely protected. Moreover, the severity of the electroclinical convulsions was distinctly reduced, as manifested by less-generalized seizures (stages 4 and 5 according to a Racine scale). However, the electrographic seizure activity was not affected by the combined treatment. Moreover, no effects on the neurodegeneration or inflammatory reactions were found. The second drug combination (phenobarbital, initiated by a bolus dose of 25 mg/kg and then 3 times daily at 15 mg/kg i.p.) + levetiracetam (other details of the experiment identical to the first combination) did not modify any parameter evaluated in this study [38]. In the same model of status epilepticus in mice, a number of combinations, consisting of two to four different drugs, was evaluated in a comparable experimental approach [39]. The most beneficial combination of levetiracetam (60 mg/kg i.p.) + atorvastatin (3 mg/kg i.p.) + ceftriaxone (60 mg/kg i.p.) inhibited not only the incidence of electroclinical seizures (by 100%) but the incidence of electrographic seizures (by 60%) as well. Hippocampal neurodegeneration was not affected [39].

6. Antioxidative Dietary Supplements

Resveratrol (a natural phytoalexin polyphenol that may be extracted from grapes and other food products) possesses a strong antioxidative potential [40]. Apart from its antioxidative properties, the compound also exhibits anti-inflammatory and anticarcinogenic activities [41]. These mechanisms of action may be quite encouraging in terms of epileptogenesis inhibition, considering that not only inflammatory processes but also oxidative stress may be involved in this process [40]. Indeed, resveratrol proved effective in inhibiting spontaneous seizures after intrahippocampal kainate-induced status epilepticus in rats [42]. Whilst, in the control group, 75% (N = 12) of the rats exhibited seizure activity, only 14.3% (N = 7) did so in the resveratrol group. Additionally, in the latter group, a considerable reduction in the number of spontaneous recurrent seizures was shown. As regards the histological evaluation, the resveratrol group presented neuroprotection in some hippocampal areas (CA1 and CA3a), and mossy fiber sprouting was distinctly inhibited [42]. This antioxidative compound also extended the seizure latency and reduced the seizure score in pentylenetetrazol-kindled convulsions in rats, along with the neuroprotection and inhibition of oxidative stress induced by seizure activity [43].

Another compound, curcumin (an active component of turmeric), has been documented in vitro to possess, apart from an antioxidant activity, anti-inflammatory and neuroprotective properties [44]. Interestingly, the in vitro activity was not confirmed in the

hippocampal tissue one week after status epilepticus in the post-electrical rat model for temporal lobe epilepsy [44]. Nevertheless, when applied during the silent period to rats following kainate-induced status epilepticus, while it did not prevent the development, it did reduce the severity of the subsequent spontaneous convulsions and offered a significant protection against cognitive impairment [45].

Bioactive phytochemical sulforaphane (available in broccoli sprout supplements) is an activator of the transcription factor (nuclear factor erythroid 2-related factor 2; Nrf2) responsible for the stimulation of various cellular defense lines through a number of cytoprotective genes [46]. Pauletti et al. [47] induced status epilepticus in rats via the electrical stimulation of the ventral hippocampus and then applied a combination of sulforaphane (5 mg/kg i.p.) with N-acetylcysteine (500 mg/kg i.p.) for seven days, followed by sulforaphane alone for another 7 days. Evidently, the combined treatment effectively inhibited the epileptogenesis progression as measured between 2 and 5 months, which was reflected by a significant reduction in the frequency of spontaneous seizures evaluated 5 months after the status epilepticus. Moreover, in rats receiving both antioxidants, the hippocampal neurodegeneration was considerably less expressed, and they performed better in behavioral tests for cognition. Last, but not least, oxidative stress accompanying epileptogenesis led to an expression of a neuroinflammatory molecule, the so-called high mobility group box 1 (HMBG1). A reduced post-status epilepticus oxidative stress by the two antioxidants significantly prevented the generation of HMBG1, which could be involved in the beneficial effects of the combination [47].

7. Examples of New, As-Yet Nonapproved Compounds with Antiepileptogenic Potential

As already mentioned above, rapamycin is an antagonist of the mTOR complex 1 pathway, so a question arises whether a blockade of two mTOR pathways may offer a more efficient inhibition of epileptogenesis. Towards this aim, a 1,3,5-triazine derivative (PQR620) was evaluated in mice surviving status epilepticus induced by intrahippocampal kainate [48]. PQR620 was given for 2 weeks (at doses blocking the mTOR signaling), and the seizure evaluation began 6 weeks after the drug administration was stopped. Surprisingly, no protective effect of the pretreatment with this compound on the spontaneous seizure incidence or frequency was observed. Additionally, no desired influence on granule cell dispersion in the dentate gyrus was recorded. A significant anxiety reduction was the only behavioral response seen in pretreated mice. In the same experimental approach, another triazine derivative, PQR530 (an inhibitor of the phosphoinositide-3 kinase (PI3K)-AKT/mTOR pathway), exerted an activity similar to PQR620 [48].

Z-944, a selective and potent antagonist of the T-type calcium channel, has been examined in rats with kainate-induced status epilepticus and subsequent epileptogenesis [49]. Z944 was administered via a continuous subcutaneous infusion at 60 mg/kg daily for 4 weeks. After a 4-week interval, the animals were tested for the occurrence of spontaneous recurrent convulsions for the next 2 weeks and behavioral abnormalities (anxiety, depression and cognition). Apparently, the group receiving the calcium channel antagonist showed less intense seizure activity manifested by a reduced number of convulsions—0.8 seizures per day (vehicle-treated rats) vs. 0.01 seizures daily. Additionally, vehicle-treated rats exhibited significant deficits in spatial learning and memory tasks, as well as distinct depressive-like behavior vs. animals without status epilepticus. The pretreatment with Z944 considerably prevented the occurrence of abnormal behaviors [49].

A very intriguing hypothesis regarding the role of DNA methylation was put forward by Williams-Karnesky et al. [50], who administered adenosine at 250 ng daily (for 10 days) through silk-based polymer implants into rat brain ventricles. The administration of adenosine started 9 weeks following systemic kainate (12 mg/kg, i.p.)-induced status epilepticus. Spontaneous seizures were evaluated between 10–13 and 18–21 weeks since the induction of status epilepticus. Adenosine very potently inhibited the increase in seizure frequency per week between 10 and 13 weeks and completely blocked any further increases in this parameter when evaluated between 18 and 21 weeks. Moreover, mossy

fiber sprouting was significantly reduced when studied 12 weeks post-status epilepticus. According to the authors, the observed seizure-modifying effect was distinctly correlated with the inhibition of hippocampal DNA methylation [50].

Very promising effects were reported with the use of an antagomir (an antisense oligonucleotide) that targets microRNA-134 (ANT-134). Experiments were performed on the mice that survived intra-amygdalar kainate-induced status epilepticus. ANT-134 (30 mg/kg) was administered i.p. 2 h after the induction of status epilepticus, and EEG/spontaneous recurrent convulsions were recorded for 2 weeks and for 1 week after 1, 2 and 3 months following the status epilepticus. Evidently, spontaneous seizures were almost totally blocked by a single injection of ANT-134. Additionally, a reduced astrogliosis was found in the hippocampus [51].

Brain-derived neurotrophic factor (BDNF) is a neurotrophin involved in the modulation of synaptic plasticity and may be engaged in various functions of the central nervous system—for instance, memory processes [52]. Seizure activity is responsible for the enhanced activation of the BDNF receptor, which is tropomyosin-related kinase B (TrkB), coupled to phospholipase-C-gamma-1 [53,54]. Consequently, there is a possibility that the inhibition of TrkB signaling might be of importance in the suppression of epileptogenesis. A combined chemical–genetic approach was elaborated in order to inhibit TrkB. In wild-type mice, the enzyme is not susceptible to 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1NMPP1). However, 1NMPP1 turns into an active inhibitor after a genetic modification in the TrkB locus, which consists of the substitution of alanine for phenylalanine at residue 616. The inhibitory activity of 1NMMP1 was subsequently validated in vivo in the genetically modified mice [54]. The inhibitor was given i.p. at 16.6 μg/g 40 and 60 min after intra-amygdalar kainate (with diazepam at 10 mg/kg and lorazepam at 6 mg/kg, respectively, to terminate the status epilepticus) and then once daily. Additionally, it was available in the drinking water (at 25 μM). After 2 weeks, 1NMMP1 was withdrawn. Spontaneous seizures were measured daily in weeks 5 and 6 and behavioral testing after 8 weeks post-status epilepticus. There was a sharp reduction in the occurrence of spontaneous seizure activity in the long-term period after status epilepticus. Anxiety-like behavior was also ameliorated by the pretreatment with 1NMMP1 in genetically modified mice in the light–dark emergence test. As expected, 1NMMP1 remained ineffective in unmodified animals [54].

A similar experimental approach was used in amygdala-kindled genetically modified mice [55]. Amygdala-kindled mice (genetically modified as in the former experiment) after 6 days of a seizure-free period received an electrical stimulus inducing a seizure response (Seizure #1) and then, after 8 days, a second stimulus (Seizure #2), showing a significant progression in the seizure duration. The treatment with 1NMMP1 (16.6 μg/g) was administered i.p. after Seizure #1 every 12 h up to a total of five injections, and the mice also had access to drinking water with the TrkB inhibitor (at 25 μM) for 2 days. It turned out that, in the genetically modified mice receiving 1NMMP1, there was a clear-cut prevention of a 50% increase in the electrographic seizure duration and a 25% increase in the behavioral seizure duration, as well as a 75% increase in the duration of the ictal and postictal events. These beneficial events were not seen in the modified mice injected with the 1NMMP1 vehicle or in naïve mice administered the TrkB inhibitor itself. There is also a possibility of inhibiting TrkB signaling by the peptide pY816, uncoupling TrkB from phospholipase-C-gamma-1. Indeed, when applied after Seizure #1 at 20 mg/kg i.p. for a total of five injections in naïve amygdala-kindled mice, it very significantly prevented an increase of the seizure parameters after the induction of Seizure #2. Remarkably, carbamazepine (a conventional AED) given i.p. at 20 mg/kg every 4 h for 2 days after Seizure #1 totally failed to modify the progression of seizure activity observed at Seizure #2 [55].

8. c-Fos: A Potential Target for Antiepileptic Treatment

The stimulation of neurons by a variety of factors activates a group of immediate early genes (IEG), of which *c-fos* coding for the 37.5-kDa protein c-Fos is the one responding most rapidly [56,57]. Accordingly, epileptic seizures induced in experimental animals by electrical stimulation (electroconvulsive seizures, (ECS) [58,59]), or by a variety of chemicals (kainate, pilocarpine or pentylenetetrazol), are invariably accompanied by a rapid increase in the expression of c-Fos mRNA and/or proteins in the neurons of seizure-vulnerable brain regions (within minutes to a few hours following the induction of the first seizure) [60–66]. c-Fos activation is transient, very often receding in the latent, asymptomatic stage of epilepsy, way before the onset of recurrent seizures. Notably, in rats subjected to acute ECS, the increase of c-Fos is followed by a decrease to below the control level in the period when seizures become chronic [58]. Rapid but transient c-Fos induction has also been observed in human temporal lobe slices obtained from the surgical treatment of TLE following their epileptogenic stimulation in vitro [67].

We attempted to evaluate the so-far never analyzed relation of c-Fos expression to two characteristics of initial seizures: (i) the time lapse between the stimulus application and the onset of the first seizure and (ii) seizure intensity as measured with the Racine score. In this laboratory, each of the two parameters and their correlation with glutamatergic transmission were investigated in the lithium-pilocarpine model in young rats, until 60 min after the stimulus application in a minute timescale [68]. The study revealed considerable animal-to-animal variations with regards to parameters (i) and (ii). The variability was further accentuated in a separate group of animals treated with a seizure onset-delaying glutamatergic intervener, MSO. An analysis of the brain tissue samples derived from lithium-pilocarpine animals revealed in both the MSO-treated and nontreated group a strong negative correlation of c-Fos mRNA expression with a timelapse from the pilocarpine application to the onset of the first generalized seizure but no statistically significant correlation with the seizure intensity (Figure 1). The results suggested that, the longer the animal remains resistant to the seizure-inducing stimulus, the lesser will its c-Fos response. To express this another way, the longer the animal suffers from seizures, the higher its brain c-Fos expression.

While the above observations strongly support the status of c-Fos as an early, seizure duration-related marker of epileptogenesis, they leave open the question of the contribution of c-Fos to epileptogenesis and, thus, of its assignment to the list of therapeutic targets. Since c-Fos positively regulates several aspects of neural plasticity [68], its activation in the initial stages of epileptogenesis may be considered as a neuroprotective response. Some experimental data appear to support this view. The increase of c-Fos expression elicited by trigeminal nerve stimulation coincided with, and likely contributed to, the attenuation of pentylenetetrazol-induced seizures in rats [70]. Other than in epilepsy-prone Wistar rats, in Guyenne spiny rats (*Proechimys guyannensis*), pilocarpine-induced seizures never evolve to status epilepticus; the unusual epilepsy resistance of this species is correlated with a persistent high level of c-Fos expression after the ictal stimulus [71]. However, the transient activation of c-Fos may, in most instances, be too short-lasting to mitigate a palpable degree of progression of epileptogenesis to chronic epilepsy. A c-Fos deficit at later stages of epileptogenesis could contribute to impaired cognition and memory, the well-documented associates of advanced epilepsy [72,73].

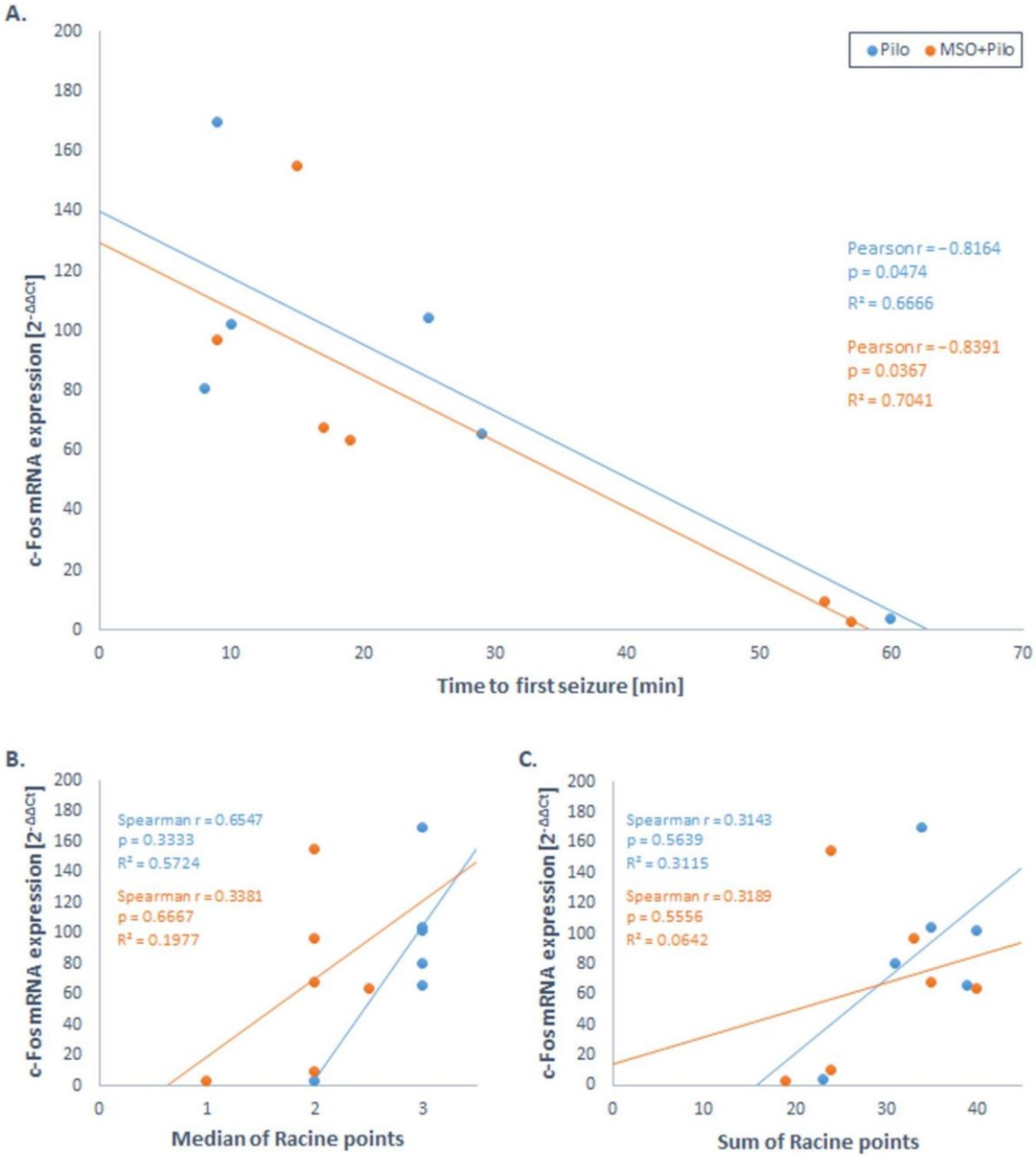

Figure 1. C-Fos mRNA expression in the hippocampus vs. time to the onset of the first generalized seizure (**A**) and the seizure intensity: median of the Racine points (**B**) and sum of the Racine points (**C**). Rats received one dose of glutamine synthetase inhibitor MSO (MSO + Pilo group, $N = 6$) or an equal volume of saline (Pilo group, $N = 6$) 2.5 h before the convulsive agent—pilocarpine. (**A**) Point 0 is the time of pilocarpine application. Assessment of the Racine score was made every 5 min up to 60 min after pilocarpine, when the animals were decapitated, and the brain tissue was dissected. A correlation coefficient was considered statistically significant at the two-tailed p-value <0.05. For a more detailed description of the model used, see Reference [68]. The total RNA was isolated from the hippocampus using a TRI reagent (Sigma). The extracted RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression was determined by Taqman Gene Expression Assays (Applied Biosystems) using 1 µL of cDNA in a reaction of 10 µL. The assay IDs were Rn02396759_m1 for rat c-Fos and Rn00667869_m1 for β-actin. The fold change in the gene expression was determined by the $2^{-\Delta\Delta Ct}$ method [69].

9. Conclusions

Analyzing the antiepileptogenic efficacy of AEDs and other agents, it is possible to delineate the most effective targets and targets playing a much lesser role in this regard. Considering AEDs, there are examples of their antiepileptogenic potential.

As regards the mechanisms of action of AEDs, they interact with the main three targets in the central nervous system: voltage-gated sodium or calcium channels, $GABA_A$ receptor-mediated inhibition and glutamate-induced excitatory events [8,13]. The $GABA_A$ receptor may, in part, appear to be a recommendable target for antiepileptogenesis, because diazepam (a positive $GABA_A$ receptor modulator) at a single but high dose of 20 mg/kg very potently inhibited spontaneous seizure activity following status epilepticus induced in rats by electrical stimulation of the amygdala [74]. Valproate, an AED with multiple mechanisms of action, is also closely associated with GABA-ergic neurotransmission in that it increases GABA turnover in brain regions responsible for seizure generation and propagation [75]. However, only some, but not all, experimental data confirmed the antiepileptogenic properties of this drug (see above). Apparently, a number of factors (a method of status epilepticus induction, administration time and dosing) may influence its final antiepileptogenic effect. After all, phenobarbital, positively modulating $GABA_A$ receptor-mediated inhibition [13], was shown to possess a seizure modifying the activity in rats after status epilepticus produced by lithium-pilocarpine [11]. However, phenobarbital was shown ineffective in animals in another model of status epilepticus, even when combined with levetiracetam [38].

A number of antioxidants have shown antiepileptogenic potential, so the question arises whether targeting the mechanism of the antioxidative defense might be of importance for the inhibition of epileptogenesis. Resveratrol was shown to exert diverse antioxidative effects—for instance, it reduces the production of free radicals and increases the activity of antioxidative enzymes: superoxide dismutase, catalase and glutathione peroxidase [41]. Certainly, its antioxidative properties are closely related to its anti-inflammatory activity, because free radicals have been found to promote inflammation [76]. Actually, resveratrol is an efficient suppressor of microglia-induced neuroinflammation and subsequent neuronal damage of inflammatory origin. Possibly, its anti-inflammatory activity may result from its direct inhibitory effect upon the synthesis of anti-inflammatory factors [41]. Sulforaphane was also effective in epileptogenesis inhibition, and its antioxidative activity is associated with Nrf2, which promotes the transcription of a number of antioxidant response genes [46]. Actually, sulforaphane has been shown to increase the expression of Nrf2 in the nucleus in vivo, and by the way, it also reduces the secretion of proinflammatory cytokines, which speaks to its direct interaction with nuclear factor kappa B [77]. N-acetylcysteine, exerting antiepileptogenic activity in combination with sulforaphane, is a direct antioxidant [78]. It is of importance that there are other examples of already approved drugs that share, among other properties, an antioxidative potential—for instance, minocycline [35]. This drug also exhibits an anti-inflammatory activity that is also shared by some antioxidative agents (resveratrol and sulforaphane). Curcumin is also a good example of an antioxidant and anti-inflammatory agents, as it can also act as an inflammasome silencer [79]. Summing up, the antiepileptogenic effects exerted by antioxidative compounds/drugs indicate that free radicals and antioxidant enzymes may become encouraging targets for antiepileptogenesis.

The anti-inflammatory drug celecoxib (an inhibitor of the cyclooxygenase 2 and HMGB1/TLR-4 pathways) has been documented to significantly reduce the remote consequences of status epilepticus in rats. These are recurrent spontaneous seizures, hippocampal neurodegeneration with aberrant neurogenesis/gliogenesis [31]. The beneficial antiepileptogenic activity of celecoxib may be interpreted in terms of targeting the inflammatory pathways.

A growing body of evidence seems to suggest that the m-TOR complex may be involved in an epilepsy-modifying effect. This may be attributed to the fact that rapamycin in some post-status epilepticus models of epileptogenesis exerted a positive activity [27,28]. However, in some experimental approaches, rapamycin was completely inactive [29,30].

Probably, these discrepancies may be partially explained by the pharmacokinetics of the drug. According to Abs et al. [80], rapamycin evidently accumulates in the rat brain following its withdrawal, with still almost 50% of the drug concentration observed during continuous rapamycin administration. Thus, it is quite possible that the drug itself may be present in the brain when spontaneous seizure activity starts. The ineffectiveness of another antagonist of two mTOR pathways, PQR620 [48], speaks rather against the involvement of these pathways in epileptogenesis inhibition.

The question arises whether the T-type calcium channel may be considered as a target for antiepileptogenesis. Indeed, the results by Casillas-Espinosa et al. [49] seemed to support such a possibility, as the potent antagonist of this channel, Z944, proved very effective in a post-status epilepticus model. Furthermore, this antagonist also very distinctly inhibited the progression of amygdala kindling in rats. Only one out of seven rats was fully kindled [81]. Interestingly, ethosuximide (also a T-type calcium channel antagonist [13]) was totally inactive in this respect—all the rats pretreated with this AED were fully kindled [81]. Perhaps the interaction mode of Z944 and ethosuximide with T-type calcium channels may vary, which can account for the completely different effects of either drug on epileptogenesis. A study by Tringham et al. [82] indicated that there were obvious differences in the mechanisms of antiseizure action of these drugs against thalamic burst firing.

A number of microRNAs may differ in their expression in patients with neurologic diseases, and a good example is microRNA-129-2-3p, which was found elevated both in cortical tissue and the plasma of patients with temporal lobe epilepsy [83]. Recently, the antagomir-induced inhibition of microRNA-129-2-3p has been documented to efficiently block the downregulation of the *gabra1* gene, which encodes receptor subunit α_1 of the $GABA_A$ receptor complex both in rat primary hippocampal neurons and in rats with kainate-induced seizure activity [84]. Additionally, the silencing of microRNA-134 in mice surviving intra-amygdalar kainate-induced status epilepticus provided distinct neuroprotection and suppressed spontaneous seizure activity recorded between weeks 3 and 4 and 7 and 8 post-status epilepticus. The suppression of spontaneous seizure activity might be, according to the authors, dependent on the reduction by antagomir-134 of the hippocampal CA_3 dendritic spine density [85]. Moreover, a clear-cut antiepileptogenic effect was shown above for the antagomir targeting microRNA-134 [51] after its peripheral administration. The systemic effectiveness of this compound was possible because of the blood/brain barrier disruption by kainate-induced status epilepticus [51].

Regarding c-Fos, it appears reasonable to propose an experimental antiepileptic therapy based on its extending persistence in the brain. Attempts towards this end could make use of one (or both) of the following two mechanisms:

Stimulus-induced c-Fos expression in the brain is prompted by an enhanced acetylation of histone H4 [86,87]. A wide spectrum histone deacetylase (HDAC) inhibitor, sodium butyrate, abrogated c-Fos accumulation in the resting brain [87] and attenuated epileptogenesis in the rat kindling model of TLE [88]. Moreover, an in vitro study revealed HDAC inhibition to be a common feature of three different AEDs: valproate, topiramate and levetiracetam [89], suggesting the possibility that the therapeutic effects of these drugs may be partly mediated by inhibition of c-Fos. Therefore, HDAC inhibitors specifically designed to target H4 may in the future become attractive AEDs. It has recently been postulated that HDAC inhibitors may alleviate epileptogenesis also by interacting with non-histone targets [90].

Elimination of c-Fos upon prolonged stimulation of pertinent brain regions in disease models other than epilepsy is associated with its increased interaction with a transcription factor ΔFosB, which is activated in a chronic but not in the early-stage post-stimulation [91,92]. If this mechanism applies to epilepsy, attempts at inactivating ΔFosB in due course may become a plausible therapeutic option as well.

As already pointed out above, DNA methylation or TrkB receptor-mediated events may be also considered as potential targets for the inhibition of epileptogenesis. Some clini-

cal studies on the inhibition of epileptogenesis were conducted in patients suffering from head traumas, however, the preventive use of AEDs failed to stop posttraumatic epilepsy (for review, [2]). Specifically, carbamazepine, phenobarbital, and phenytoin were not effective at all and valproate even tended to elevate mortality in patients with posttraumatic epilepsy. There is some hope with levetiracetam which exerted a slight, albeit statistically insignificant preventive activity in this respect, [2]. Some newer clinical data on this issue are less optimistic [93]. So far, apart from AEDs, only biperiden (an anti-parkinsonian anticholinergic drug) has been evaluated in patients after brain injury in a small phase II safety assessment, prior to a double blind, randomized, placebo-controlled trial. The initial assessment confirmed its safety in patients with brain injuries and preliminary data seem to indicate that the drug seems to reduce the incidence of post-traumatic epilepsy [94]. Interestingly, biperiden has shown clear-cut antiepileptogenic properties in post-SE (induced by pilocarpine in rats) model of recurrent spontaneous convulsions [95]. Other non-antiepileptic drugs shown above, have not been tested yet in clinical trials aimed at counteracting epileptogenesis.

All the above discussed ligands considered for use as molecular targets for antiepileptogenesis are listed in Table 1. While each of them bears a clinical antiepileptogenic potential, its practical applicability can only be verified in appropriate clinical trials. Whilst patients with brain traumas may be easily recruited for such trials, patients with presumed epileptogenesis following other initial insults would have to be checked with reliable markers for epileptogenesis. Apart from already mentioned EEG markers [10], a possibility exists that also miRNAs may become reliable markers for the process of epileptogenesis or chronic epilepsy [96] and according to the here presented data—c-Fos may be also taken into consideration in this regard.

Table 1. Anti-epileptogenic potential of various drugs and agents.

Anti-Epileptogenic Compound		Mechanism of Action [13]							Model of Epileptogenesis	Duration of Treatment	Dosage	Effect	References
		Sodium Channel Blockade	Calcium Cchannel Blockade	Potassium Channel Activation	Increase in GABA Level	Increased GABA Transmission	Inhibition of Glutamate Excitation	Other Mechanisms of Action					
Antiepileptic drugs	**Phenobarbital**	+			+	+	+	+	Lithium-pilocarpine-induced status epilepticus in rats	14 days Spontaneous seizure activity was studied for 7 days between 8 and 9 weeks after status epilepticus	15 mg/kg/ twice daily i.p.	Reduction in the number of rats with spontaneous seizure activity; the median frequency of convulsions sharply diminished from 7.5 to 1 seizure/week	[11]
	Levetiracetam		+			+		+	Pilocarpine-produced status epilepticus in mice	28 days Seizure activity was assessed in the presence of levetiracetam within four weeks after status epilepticus	500 mg/kg/ twice daily, p.o.	Reduction the number of spontaneous seizures and mortality; Present neuroprotective effect	[12]
	Eslicarbazepine	+	+						Pilocarpine-produced status epilepticus in mice	42 days	150 and 300 mg/kg/ once daily, i.p.	Reduction spontaneous seizure activity; The mossy fiber sprouting was considerably inhibited; Inhibitor of epileptogenesis	[14]
	Lamotrigine	+	+	+	+		+		Lithium-pilocarpine-induced status epilepticus in rats	7 days	10 or 20 mg/kg daily, i.p. AED given 24 hours after status epilepticus	Inhibition spontaneous seizure activity; Reduction neurodegeneration and astrogliosis in the hippocampus	[17]
	Ethosuximide		+						Lithium-pilocarpine-induced status epilepticus in rats	7 days	25 and 50 mg/kg/daily AED given i.p. 24 hours after status epilepticus	No effect	[17]

Table 1. *Cont.*

Anti-Epileptogenic Compound		Mechanism of Action [13]							Model of Epileptogenesis	Duration of Treatment	Dosage	Effect	References
		Sodium Channel Blockade	Calcium Cchannel Blockade	Potassium Channel Activation	Increase in GABA Level	Increased GABA Transmission	Inhibition of Glutamate Excitation	Other Mechanisms of Action					
Approved non-AEDs with an anti-epileptogenic potential g	Losartan			A blocker of angiotensin II type 1 receptors					Rat model of required epilepsy in which sodium deoxycholate, via a craniotomy window, was administered to the brain surface	7 days Spontaneous seizure activity was evaluated for 2 weeks Recording electrocorticographic activity for 110 days	100 mg /kg i.p. Administered 40 min following deoxycholate	Significant reduction of spontaneous convulsions, sharp reduction in the average number of convulsions	[18]
									Kainate post-status epilepticus-induced epileptogenesis in rats	4 weeks Spontaneous seizure activity was evaluated for 3 months	S.c. at 10 mg/kg 2 hours after the onset of status epilepticus and continued up to the 3rd day and then the animals were switched to losartan in drinking water	Increased the latency to the onset of seizures and provided distinct neuroprotection to CA1 hippocampal subfield, extended the latency time to the development of fully kindled seizures, neuroprotection being less expressed,in behavioral tests-considerably less deficits Some seizure parameters (threshold for after discharge induction, after discharge duration or seizure severity in fully kindled animals) were not modified	[23]
	Rapamycin			Blockade of mTOR complex 1 pathway					Kainate-induced status epilepticus in rats	21 days	6 mg/kg every 4 days	Considerable reduction in the number of spontaneous convulsions	[27]
									Electrical stimulation of the rat angular bundle	6 weeks	6 mg/kg daily for a week and then, every other day for 6 weeks following status epilepticus	Totally inhibited the occurrence of spontaneous seizure activity in 25% of animals, the remaining 75%, a significant reduction of seizure activity	[28]
									Pilocarpine-induced status epilepticus in mice	2 months	10 mg/kg	No difference in seizure frequency, Mossy fiber sprouting and dentate gyrus hypertrophy were suppressed	[29]
											3 mg/kg	Mossy fiber sprouting was reduced, hypertrophy of the dentate gyrus was decreased, generation of ectopic granule cells, loss of hilar neurons or granule cell proliferation were observed	[30]

Table 1. *Cont.*

Anti-Epileptogenic Compound		Mechanism of Action [13]							Model of Epileptogenesis	Duration of Treatment	Dosage	Effect	References
		Sodium Channel Blockade	Calcium Cchannel Blockade	Potassium Channel Activation	Increase in GABA Level	Increased GABA Transmission	Inhibition of Glutamate Excitation	Other Mechanisms of Action					
Non-antiepileptic drugs/substances	Antioxidative dietary supplements	**Celecoxib**	Blocking cyclooxygenase 2 and HMGB1/TLR-4 pathways						Lithium-pilocarpine - induced status epilepticus in rats	28 days Parameters of spontaneous convulsions (frequency and duration) observed between 28 and 42 days	20 mg/kg p.o	Parameters of spontaneous convulsions were considerably reduced, potent neuroprotection was observed in the hippocampus with aberrant neurogenesis/gliogenesis being significantly decreased	[31]
		N-acetylcysteine	Chemical structure of a reduced glutathione precursor						Systemic kainate-induced status epilepticus in rats	12 weeks	I. v. at 30 mg/kg	Increased seizure threshold to flurothyl ether, the mossy fiber sprouting was inhibited	[33]
									Brain trauma in rats induced by fluid percussion injury	5 weeks	100 mg/kg p.o.	Rats supplemented with the antioxidant did not react to the subthreshold dose of pentylenetetrazol (30 mg/kg i.p.) Which in control rats induced generalized tonic-clonic seizures The latency to the first myoclonic jerk was considerably reduced in the vehicle-treated group whilst pretreatment with N-acetylcysteine brought it back to the control value of rats without brain injury	[34]
		Minocycline	A second generation tetracycline (with anti-inflammatory and antioxidative activity)						Lithium-pilocarpine-induced status epilepticus in rats	2 weeks Spontaneous recurrent convulsions were monitored for 2 more weeks	45 mg/kg	Seizure activity was considerably suppressed in terms of its frequency, severity and duration, activation of astrocytes was not affected	[33,35]
		Resveratrol	A phytoalexin polyphenol						Intrahippocampal kainate-induced status epilepticus in rats	10 days	15 mg/kg	Considerable reduction in the number of spontaneous recurrent seizures was shown, neuroprotection in some hippocampal areas (CA1 and CA3a) and mossy fiber sprouting was distinctly inhibited	[40]
									Pentylenetetrazol-kindled convulsions in rats	10 weeks	25mg/kg, 50mg/kg and 75 mg/kg	Reduced the seizure score in pentylenetetrazol-kindled convulsions in rats, along with neuroprotection and inhibition of oxidative stress	[41]

Table 1. *Cont.*

Anti-Epileptogenic Compound		Mechanism of Action [13]							Model of Epileptogenesis	Duration of Treatment	Dosage	Effect	References
		Sodium Channel Blockade	Calcium Cchannel Blockade	Potassium Channel Activation	Increase in GABA Level	Increased GABA Transmission	Inhibition of Glutamate Excitation	Other Mechanisms of Action					
	Curcumin	Antioxidant activity, also anti-inflammatory and neuroprotective properties							Post-electrical rat model for temporal lobe epilepsy	1 week	2 µl of 2 mm curcumin solution	In vitro activity was not confirmed in hippocampal tissue	[42]
									Kainate-induced status epilepticus in rats	14 days	100 mg/kg	Did not prevent the development but reduced the severity of subsequent spontaneous convulsions and offered a significant protection against cognitive impairment	[43]
	Sulforaphane	An activator of the transcription factor (nuclear factor erythroid 2-related factor 2; Nrf2) responsible for the stimulation of various cellular defense lines through a number of cytoprotective genes							Induced status epilepticus in rats via electrical stimulation of the ventral hippocampus	7 days + 7 days Epileptogenesis progression Measured between 2 and 5 months	Sulforaphane (5 mg/kg i.p.) With N-acetylcysteine (500 mg/kg i.p.) For seven days followed by sulforaphane alone for another 7 days	Inhibited epileptogenesis progression which was reflected by the significant reduction in the frequency of spontaneous seizures evaluated 5 months after status epilepticus, Neurodegeneration was considerably less expressed and they performed better in behavioral tests for cognition	[45]
Examples of compounds with anti-epileptogenic potential PQR530 Z-944 Adenosine Antagomir	**PQR620** **PQR530**	Blockade of mTOR pathways An inhibitor of the phosphoinositide-3 kinase (PI3K)-AKT/mTOR pathway)							Status epilepticus induced by intrahippocamal kainate	2 weeks The seizure evaluation begun 6 weeks after the drug administration was stopped 4 weeks	At doses blocking the mTOR signaling	No protective effect of the pretreatment with this compound on spontaneous seizure incidence or frequency was observe, no influence on granule cell dispersion in the dentate gyrus was recorded	[46]
	Z-944	Selective and potent antagonists of T-type calcium channel							Kainate-induced status epilepticus and subsequent epileptogenesis in rats	The animals were tested for the occurrence of spontaneous recurrent convulsions for the next 2 weeks	60 mg/kg daily via continuous subcutaneous infusion	Less intense seizure activity manifested by reduced number of convulsions, significant deficits in spatial learning and memory task as well as distinct depressive-like behavior vs. Animals without status epilepticus	[47]

Table 1. *Cont.*

Anti-Epileptogenic Compound	Mechanism of Action [13]							Model of Epileptogenesis	Duration of Treatment	Dosage	Effect	References
	Sodium Channel Blockade	Calcium Cchannel Blockade	Potassium Channel Activation	Increase in GABA Level	Increased GABA Transmission	Inhibition of Glutamate Excitation	Other Mechanisms of Action					
Adenosine			Induces hypomethylation of DNA via biochemical interference with the transmethylation pathway					Systemic kainate -induced status epilepticus in rats	10 days Spontaneous seizures were evaluated between 10-13 and 18-21 weeks since the induction of status epilepticus.	250 ng daily through silk-based polymer implant into rat brain ventricles	Potently inhibited the increase in seizure frequency per week between 10-13 weeks and completely blocked the further increase in this parameter when evaluated between 18-21 weeks, mossy fiber sprouting was significantly reduced when studied 12 weeks post status epilepticus	[48]
Antagomir			An antisense oligonucleotide which targets microRNA-134 (ANT-134)					Intraamygdalar kainate-induced status epilepticus in rats	2 hours after the induction of status epilepticus	30 mg/kg, i.p.	Spontaneous seizures were almost totally blocked by a the single injection, reduced astrogliosis was found in the hippocampus	[49]
1NMMP1			1NMPP1 turns into an active inhibitor after a genetic modification in the trkb (BDNF receptor which is tropomyosin-related kinase B) locus which consists in a substitution of alanine for phenylalanine at residue 616.					Amygdalar kainate-induced status epilepticus in mice	5-6 weeks - measuring spontaneous seizures After 8 weeks post-status epilepticus - behavioral testing	16.6 μg/g ,40 and 60 min after intra-amygdalar kainate (with diazepam at 10 mg/kg and lorazepam at 6 mg/kg), respectively, to terminate status epilepticus) and then once daily. Also available in the drinking water (at 25 μm). After 2 weeks, 1NMMP1 was withdrawn.	Sharp reduction in the occurrence of spontaneous seizure activity in the long-term period after status epileptics, anxiety-like behavior was ameliorated in genetically modified mice in the light-dark emergence test, remained ineffective in unmodified animals	[52]
								Amygdala-kindled mice 6 days of seizure free period received an electrical stimulus inducing a seizure response (Seizure #1) and then, after 8 days – a second stimulus (Seizure #2)	8 days	16.6 μg/g i.p. After the seizure #1 every 12 hours up to a total of 5 injections and the mice also had access to the drinking water with the trkb inhibitor (at 25 μm) for 2 days. Applied after Seizure #1 at 20 mg/kg i.p. For a total of 5 injections in naïve amygdala-kindled mice	In genetically modified mice there was a clear cut prevention of the 50% increase in electrographic seizure duration, 25% increase in behavioral seizure duration as well as 75% increase in duration of ictal and postictal events. Beneficial events were not seen in modified mice or in naïve mice administered the TrkB inhibitor itself	[53]

Table 1. *Cont.*

Anti-Epileptogenic Compound		Mechanism of Action [13]							Model of Epileptogenesis	Duration of Treatment	Dosage	Effect	References
		Sodium Channel Blockade	Calcium Cchannel Blockade	Potassium Channel Activation	Increase in GABA Level	Increased GABA Transmission	Inhibition of Glutamate Excitation	Other Mechanisms of Action					
Combined treatments	Levetiracetam		+			+		+	Intrahippocampal kainate-induced status epilepticus in mice	5 days (latent period), starting after 6 hours from the status induction	Levetiracetam (200 mg/kg i.p.) + topiramate (30 mg/kg i.p.)	Reduction the frequency of spontaneous recurrent electroclinical seizures by 80% when compared to non-treated mice and generalized seizures (stages 4 and 5 according to a Racine scale). Reduction a number of animals with spontaneous electroclinical convulsions (some mice were completely protected); No effects on electrographic seizure activity, neurodegeneration or inflammatory reactions	[54]
	Topiramate	+	+	+	+	+	+	+					
	Levetiracetam		+			+		+	Intrahippocamal kainate-induced status epilepticus in mice	5 days (latent period), starting after 6 hours from the status induction	Phenobarbital, initiated by a bolus dose of 25 mg/kg and then 3 times daily at 15 mg/kg i.p.) + levetiracetam (200 mg/kg i.p.)	No effect on incidence or frequency of electroclinical or electrographic seizures	[54]
	Phenobarbital	+			+	+	+	+					
	Levetiracetam		+			+		+	Intrahippocamal kainate-induced status epilepticus in mice	5 days (latent period), starting after 6 hours from the status induction	Levetiracetam (60 mg/kg i.p.) + atorvastatin (3 mg/kg i.p.) + ceftriaxone (60 mg/kg i.p.)	Reduce the incidence of electroclinical seizures (by 100%) and the incidence of electrographic seizures (by 60%) as well; No effect on hippocampal neurodegeneration	[55]
	Atorvastatin	A competitive inhibitor of HMG-coa reductase [55]											
	Ceftriaxone	"reverses posttraumatic downregulation of glutamate transport in the brain" [55]											

Author Contributions: Conceptualization, S.J.C. and J.A., M.J.P., B.M., J.A. and S.J.C. contributed to the writing of this article. Table and reference preparation and editions, B.M., A.W., A.K. and M.C.-K.; writing—review and editing, S.J.C. and J.A.; funding acquisition, J.A. and visualization and execution of c-Fos experiments, M.J.P. All authors have read and agreed to the final version of the manuscript.

Funding: The authors are grateful for the financial support from the National Science Centre (NCN) of Poland (2016/23/B/NZ4/03678) (M.J.P. and J.A.), European Social Fund (ESF) (POWR.03.02.00-00-1028/17-00 (M.J.P.) and the Medical University of Lublin for the statutory grant DS/475/21 (S.J.C.).

Institutional Review Board Statement: Animal experiments described in Fig. 1. were conducted according to the guidelines of the Declaration of Helsinki and the national guidelines on animal experimentation, and were approved by the local ethical committee in Warsaw (consent no. 405/2017, 21.11.2017) in accordance with EC Directive 2010/63/EU.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: S.J.C. received financial support from Bayer, GlaxoSmithKline, Janssen, Novartis and Sanofi-Aventis for lecturing. He is also a recipient of an unrestricted grant from GlaxoSmithKline. The remaining authors have no conflicts of interest to disclose.

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Statement of contribution

To whom it may concern

Hereby, I would like to claim my contribution to the following publications:

1. M. Pawlik, M. Obara-Michlewska, M. Popek, A. M. Czarnecka, S. J. Czuczwar, J. Łuszczki, M. Kołodziej, A. Acewicz, T. Wierzba-Bobrowicz, J. Albrecht "Pretreatment with a glutamine synthetase inhibitor MSO delays the onset of initial seizures induced by pilocarpine in juvenile rats" *Brain Res.* **2021**, 1753, doi:10.1016/j.brainres.2020.147253.

I conducted most of the surgeries and *in vivo* experiments, all biochemical measurements, statistical analysis and data evaluation. I co-wrote the manuscript and responses to reviewers.

2. M. Pawlik, B. I. Aldana, L. F. Belfiori-Carrasco, M. Obara-Michlewska, M. Popek, A. M. Czarnecka, J. Albrecht "Inhibition of glutamate release, but not of glutamine recycling to glutamate, is involved in delaying the onset of initial lithium-pilocarpine-induced seizures in young rats by a non-convulsive MSO dose" *Int. J. Mol. Sci.* **2021**, 22, doi:10.3390/ijms222011127.

I conducted all *in vivo* and *ex vivo* experiments, and a part of HPLC and GC-MS measurements. I statistically evaluated all data, co-wrote the manuscript and responses to reviewers.

3. M. Pawlik, B. Miziak, A. Walczak, A. Konarzewska, M. Chrościńska-Krawczyk, J. Albrecht, S. J. Czuczwar "Selected Molecular Targets for Antiepileptogenesis". *Int. J. Mol. Sci.* **2021**, 22, 1–24, doi:https://doi.org/10.3390/ijms22189737

I executed animal experiments, c-Fos expression study, statistical analysis and co-wrote the part of the manuscript regarding c-Fos.

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I was responsible for conceptualization and supervising the research, consultation of results, writing the manuscript and responding to reviewers.

3. M. Pawlik, B. Miziak, A. Walczak, A. Konarzewska, M. Chrościńska-Krawczyk, J. Albrecht, S. J. Czuczwar "Selected Molecular Targets for Antiepileptogenesis". Int. J. Mol. Sci. 2021, 22, 1–24, doi:https://doi.org/10.3390/ijms22189737

I supervised the research on c-Fos expression and consulted the results, co-wrote and corrected the manuscript.

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I participated in the execution of animal experiments, biochemical analyzes and writing the article.

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I participated in the execution of *in vivo* and *ex vivo* experiments, and writing the article.

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I executed several EEG implantation surgeries and *in vivo* experiments, and participated in writing the manuscript.

2. M. Pawlik, B. I. Aldana, L. F. Belfiori-Carrasco, M. Obara-Michlewska, M. Popek, A. M. Czarnecka, J. Albrecht "Inhibition of glutamate release, but not of glutamine recycling to glutamate, is involved in delaying the onset of initial lithium-pilocarpine-induced seizures in young rats by a non-convulsive MSO dose" *Int. J. Mol. Sci.* **2021**, 22, doi:10.3390/ijms222011127.

I participated in the *ex vivo* experiments execution and correction of the manuscript.

I consent to the use of the above mentioned publications in the doctoral dissertation of Marek Pawlik.

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   I participated in the discussion of the obtained results and responses to the reviewers' comments.

2. M. Pawlik, B. Miziak, A. Walczak, A. Konarzewska, M. Chrościńska-Krawczyk, J. Albrecht, S. J. Czuczwar "Selected Molecular Targets for Antiepileptogenesis". *Int. J. Mol. Sci.* **2021**, 22, 1–24, doi:https://doi.org/10.3390/ijms22189737

   My responsibility was the review part of this publication so the original part (carried out by M. Pawlik) can be certainly used in his doctoral dissertation.

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I was responsible for collecting data and references used in the review part of this paper and participated in writing some parts of the review. I had nothing to do with the original part devoted to c-Fos.

I consent to the use of the above mentioned publication in the doctoral dissertation of Marek Pawlik.

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Dr. hab. Marcin Kołodziej Warsaw University of Technology Plac Politechniki 1, 00-661 Warsaw, Poland	Analysis of EEG and EMG signal
Dr. Albert Acewicz Institute of Psychiatry and Neurology Sobieskiego 9, 02-957 Warsaw, Poland	Preparation of specimens and assessment of brain tissue damage
Prof. dr. hab. Teresa Wierzba-Bobrowicz Institute of Psychiatry and Neurology Sobieskiego 9, 02-957 Warsaw, Poland	Preparation of specimens and assessment of brain tissue damage

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Warszawa, 31.03.2022 r.

Statement of contribution

To whom it may concern

Hereby, I would like to declare that in the article "M. Pawlik, B. I. Aldana, L. F. Belfiori-Carrasco, M. Obara-Michlewska, M. Popek, A. M. Czarnecka, J. Albrecht "Inhibition of glutamate release, but not of glutamine recycling to glutamate, is involved in delaying the onset of initial lithium-pilocarpine-induced seizures in young rats by a non-convulsive MSO dose" *Int. J. Mol. Sci.* **2021**, 22, doi:10.3390/ijms222011127." the participation of other authors consisted in:

Name and affiliation	Contribution
Dr. Blanca Irene Aldana University of Copenhagen Jagtvej 160, 2100 Copenhagen, Denmark	Methodology of studies employing ^{13}C metabolic precursors, GC-MS and HPLC data analysis, preparation of Scheme 1, manuscript review and editing
Dr. Lautaro F. Belfiori-Carrasco Lund University Sölvegatan 19, 221 84 Lund, Sweden	GC-MS and HPLC measurements, manuscript review and editing

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