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Systematic evaluation of rationally chosen multitargeted drug combinations: a combination of low doses of levetiracetam, atorvastatin and ceftriaxone exerts antiepileptogenic effects in a mouse model of acquired epilepsy☆

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ABSTRACT

Epileptogenesis, the gradual process that leads to epilepsy after brain injury or genetic mutations, is a complex network phenomenon, involving a variety of morphological, biochemical and functional brain alterations. Although risk factors for developing epilepsy are known, there is currently no treatment available to prevent epilepsy. We recently proposed a multitargeted, network-based approach to prevent epileptogenesis by rationally combining clinically available drugs and provided first proof-of-concept that this strategy is effective. Here we evaluated eight novel rationally chosen combinations of 14 drugs with mechanisms that target different epileptogenic processes. The combinations consisted of 2-4 different drugs per combination and were administered systemically over 5 days during the latent epileptogenic period in the intrahippocampal kainate mouse model of acquired temporal lobe epilepsy, starting 6 h after kainate. Doses and dosing intervals were based on previous pharmacokinetic and tolerability studies in mice. The incidence and frequency of spontaneous electrographic and electroclinical seizures were recorded by continuous (24/7) video linked EEG monitoring done for seven days at 4 and 12 weeks post-kainate, i.e., long after termination of drug treatment. Compared to vehicle controls, the most effective drug combination consisted of low doses of levetiracetam, atorvastatin and ceftriaxone, which markedly reduced the incidence of electrographic seizures (by 60%; $p<0.05$) and electroclinical seizures (by 100%; $p<0.05$) recorded at 12 weeks after kainate. This effect was lost when higher doses of the three drugs were administered, indicating a synergistic drug-drug interaction at the low doses. The potential mechanisms underlying this interaction are discussed. We have discovered a promising novel multitargeted combination treatment for modifying the development of acquired epilepsy.

Abbreviations: AT1, angiotensin II type 1; ASD, antiseizure drug; BBB, blood-brain barrier; COX-2, cyclooxygenase 2; EEG, electroencephalogram; GCD, granule cell dispersion; GLT-1, glutamate transporter 1; HPD, hippocampal paroxysmal discharge; HVSW, high-voltage spike wave; NCS, nonconvulsive seizures; NMDA, N-methyl-D-aspartate; SE, status epilepticus; PTE, posttraumatic epilepsy; SRS, spontaneous recurrent seizures; SV2A, synaptic vesicle protein 2A; TBI, traumatic brain injury; TGFβ, transforming growth factor beta; TLE, temporal lobe epilepsy.

☆ This paper is dedicated to the late Dieter Schmidt, who significantly contributed to numerous areas of clinical and preclinical epilepsy research, including aspects of how to develop antiepileptogenic therapies

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1. Introduction

Prevention or modification of acquired epilepsy in patients after brain injury is one of the great unmet needs in neurology (Devinsky et al., 2018; Klein et al., 2020). At least 20% of all epilepsies develop due to acute brain insults such as traumatic brain injury (TBI), stroke, CNS infections or status epilepticus (SE), including prolonged febrile seizures (Klein et al., 2018). Following these injuries, there is a latency of days to years before epilepsy develops. This latency period may offer a temporal window of opportunity to intervene with treatment to prevent or modify epilepsy by interfering with the mechanisms underlying epileptogenesis (Pitkänen et al., 2015). Epileptogenesis after acute brain injury is a complex process involving a variety of different pathophysiological processes that are only partially understood (Löscher, 2020). Widely accepted processes of epileptogenesis include neuroinflammation, oxidative stress, disruption of the blood-brain barrier (BBB) with subsequent extravasation of albumin, neurodegeneration, neurogenesis, axonal remodelling and synaptic plasticity in crucial brain regions such as the hippocampus, and development of neuronal hyperexcitability, ultimately leading to the onset of spontaneous recurrent seizures (SRS) (Pitkänen et al., 2015; Klein et al., 2018; Vezzani et al., 2019). In view of the complexity of epileptogenesis, we have previously proposed that treatment with rational combinations of drugs, which engage different targets presumed to be involved in the epileptogenic network, may be a more effective strategy than treatment with single, highly specific drugs (Löscher et al., 2013). An important benefit for translation of such a network approach to patients is the repurposing of drugs that are already clinically available.

Based on this idea, we recently started to evaluate several rationally chosen drug combinations for antiepileptogenic efficacy in a widely used mouse model of temporal lobe epilepsy (TLE), the intrahippocampal kainate mouse model. Using a drug selection strategy illustrated in Fig. 1, a literature review of numerous clinically approved drugs from a wide variety of therapeutic indications identified about 20 drugs that fulfilled our selection criteria and were used to form drug combinations that interfere with different processes thought to be involved in epileptogenesis. *In silico* analysis of drug-drug-protein network interactions by the STITCH database (Szklarczyk et al., 2016) was used to aid identifying potentially synergistic drug combinations (Fig. 1). One of the selected drug combinations (levetiracetam and topiramate) was recently shown to modify the development of epilepsy when administered during the latent period following kainate in mice, whereas administration of either drug alone was ineffective (Schidlitzki et al., 2020). This proof-of-concept that network pharmacology can modify the development of epilepsy after kainate-induced SE in mice prompted us to evaluate seven other rationally chosen combinations of 14 drugs that are illustrated in Fig. S1. As shown in this figure, these drug combinations of two to four drugs were chosen due to their ability to interfere with several critical targets of the epileptogenic process. Before performing laborious experiments on antiepileptogenic efficacy, the tolerability of the drug combinations during prolonged treatment was examined in small groups of nonepileptic control mice and mice during the latent period following SE (Fig. 1). All drug combinations were well tolerated at the chosen doses, except for the combination of valproate, losartan, and memantine which induced relatively moderate adverse effects that were considered to be acceptable for the purpose of our experiments (Klee et al., 2015; Welzel et al., 2019). For the present study, an additional drug combination (H; levetiracetam and agmatine) was added that was not previously included in the tolerability testing. The reason for adding the polyamine agmatine was its beneficial activity on oxidative damage, neuroinflammation, and proapoptotic signaling, which may mediate antiepileptogenic efficacy (Neis et al., 2017). Levetiracetam was included in most combinations due to its multitargeted mechanisms of action (Rogawski et al., 2016) and preclinical as well as clinical evidence of disease-modifying activity in acquired epilepsies (Kaminski et al., 2014; Klein et al., 2020).

For the purpose of this study, the term "antiepileptogenic" was defined as follows, using the definitions proposed by Pitkänen (2010), Schmidt (2012) and Pitkänen and Engel Jr. (2014). "Antiepileptogenic" describes treatments that prevent, stop, or reverse the development or

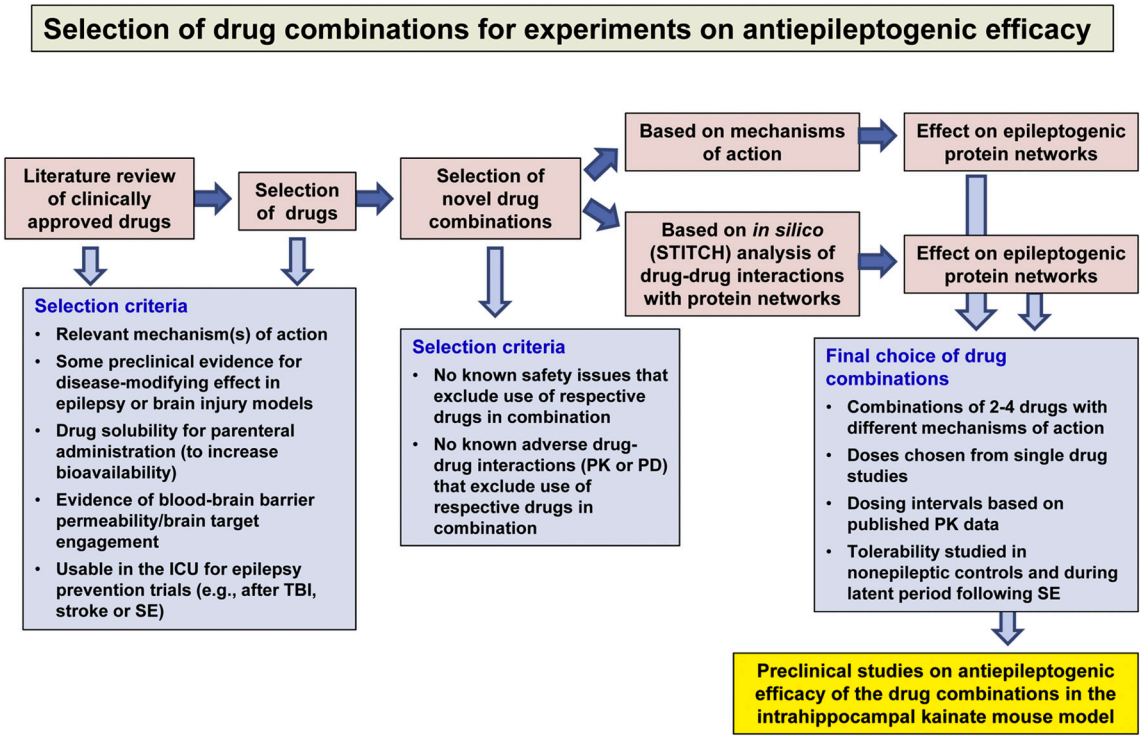

Fig. 1. Flow chart illustrating the selection process that led to the drug selections and subsequent combinations included in the present study. The STITCH database was used for *in silico* analyses of drug combinations as described recently (Schidlitzki et al., 2020).

ameliorate the epileptic condition, if given after the onset of an epileptogenic insult. According to Pitkänen and Engel Jr. (2014), antiepileptogenesis is one component of disease or syndrome modification; the other component is comorbidity modification, which was not examined here.

2. Materials and methods

2.1. Animals

Outbred male NMRI mice, which are used as a general-purpose stock in many fields of research including pharmacology (Chia et al., 2005), were obtained from Charles River (Sulzfeld, Germany) at the age of seven weeks (body weight 35-40 g). Following arrival, mice were habituated to the laboratories for at least one week. One drug combination and vehicle experiment were performed at a time, so for the seven drug combinations (eight experiments) shown in Fig. S1 and Table 1, eight separate batches of mice were used over a period of 1.5 years.

Due to hierarchical fights, all male mice were single housed and kept separately from female mice. All animals were housed under controlled conditions (ambient temperature 22-24°C, humidity 30-50%, lights on from 6:00 am to 6:00 pm). Food (Altromin 1324 standard diet) and water were freely available. Experiments were performed according to the EU council directive 2010/63/EU and the German Law on Animal Protection ("Tierschutzgesetz"). Ethical approval for the study was granted by an ethical committee (according to §15 of the Tierschutzgesetz) and the government agency (Lower Saxony State Office for Consumer Protection and Food Safety) responsible for approval of animal experiments in Lower Saxony. All efforts were made to minimize both the suffering and the number of animals. All animal experiments of this study are reported in accordance with ARRIVE guidelines (Kilkenny et al., 2010). In total, 213 mice were used (22 animals for tolerability studies for levetiracetam and agmatine (data not shown), 10 naive animals for histology, 30 animals for kainate and EEG electrode localization verfication, and 151 kainate-treated animals).

2.2. Intrahippocampal kainate mouse model

In this model, a SE is induced by unilateral injection of kainate into the CA1 of the dorsal hippocampus (Suzuki et al., 1995; Bouilleret et al., 1999). For this purpose, mice were anesthetized with chloral hydrate (500 mg/kg i.p. in 10 ml/kg saline initially, then 0.05 ml i.p. to prolong anesthesia if needed). Kainate monohydrate (Sigma-Aldrich (Steinheim, Germany) was freshly diluted (0.21 µg in 50 nl saline) and stereotaxically injected into the right CA1 of the dorsal hippocampus as described previously (Twele et al., 2016a, 2016b; Schidlitzki et al., 2017). Stereotaxic coordinates were based on the brain atlas of Paxinos and Franklin (2012), and confirmed in previous experiments using NMRI mice (Twele et al., 2016a, 2016b; Schidlitzki et al., 2017). They were verified before the beginning of and during experiments in the different batches of mice used during the experiments. Using the stereotaxic coordinates, anteroposterior -2.1, laterolateral -1.6, and dorsoventral -1.7 mm from bregma, kainate was slowly injected over 60 seconds with a 0.5 µl Hamilton® microsyringe (SAGE Europe Ltd, Milton Keynes, UK). After kainate injection, the syringe needle remained in situ for an additional two minutes to limit reflux along the injection track. For EEG recordings in the antiepileptogenic experiments, animals were immediately implanted with bipolar electrodes using the same coordinates aimed at the site of kainate injection in the ipsilateral CA1. The electrode consisted of two twisted Teflon-coated 0.2-mm-diameter stainless-steel wires separated by 0.5 mm at the tip. A screw, placed above the left parietal cortex, served as the reference electrode. Two additional skull screws, superglue, and dental acrylic cement (described below) were used to anchor the head assembly. For each experiment, the aim was to have 16-18 mice for video/EEG recording (8-9 drug-treated and 8-9 vehicle-treated animals). In order to reach this aim, up to 24 mice

Table 1
Drug combinations, vehicles, routes of administration, injection volumes, and doses used for drug efficacy testing. Based on pharmacokinetics of drugs in mice (see Klee et al., 2015, and Welzel et al., 2019), all drugs were administered 3 times daily over 5 days at the indicated doses, except for fingolimod, which was administered once daily over 5 days. Selection of doses was based on the literature shown. Before the efficacy experiments, the tolerability of all drug combinations was evaluated at the doses shown in naïve mice and mouse models of epilepsy (Klee et al., 2015; Welzel et al., 2019; present study). Abbreviations: i.p.=intraperitoneally, s.c.=subcutaneously, DMSO=dimethyl sulfoxide, PBS=phosphate-buffered saline.

	Drug cocktail	Respective vehicles	**References for selection of drugs and dosages**
A	(1) Levetiracetam (200 mg/kg i.p.) + (2) Gabapentin (200 mg/kg i.p.), (3) Topiramate (30 mg/kg i.p.)	(1) Aqua ad injectabilia i.p. (3 ml/kg)* (2) Aqua ad injectabilia i.p. (3 ml/kg)* (3) 0.9% NaCl i.p. (5 mg/kg)	(1) Klein et al. (2020) (2) Cilio et al. (2001); Klein et al. (2020) (3) Klein et al. (2020)
B	(1) Levetiracetam (200 mg/kg i.p.), (2) α-Tocopherol (250 mg/kg s.c.)	(1) Aqua ad injectabilia i.p., (3 ml/kg) (2) 10% Ethanol absolute + 90% Miglyol® 812 s.c. (3 ml/kg)	(1) Klein et al. (2020) (2) Ambrogini et al. (2014); Betti et al. (2011)
C	(1) Levetiracetam (200 mg/kg i.p.) + (2) Deferoxamine (40 mg/kg i.p.), (3) Gabapentin (200 mg/kg i.p.) + (4) Fingolimod (1 mg/kg i.p.)	(1) Aqua ad injectabilia i.p.* (3 ml/kg) (2) Aqua ad injectabilia i.p.* (3 ml/kg) (3) Aqua ad injectabilia i.p.* (3 ml/kg) (4) Aqua ad injectabilia i.p.* (3 ml/kg)	(1) Klein et al. (2020) (2) Panter et al. (1992); Gusakov et al. (1993); Liu et al. (2011) (3) Cilio et al. (2001); Klein et al. (2020) (4) Gao et al. (2012); Pitsch et al., 2019
D	(1) Levetiracetam (200 mg/kg i.p.), (2) Atorvastatin (10 mg/kg i.p.), (3) Ceftriaxone (200 mg/kg s.c.)	(1) Aqua ad injectabilia i.p. (3 ml/kg) (2) 4% DMSO + 10% Solutol® HS 15 + 86% PBS i.p. (5 ml/kg) (3) Aqua ad injectabilia s.c. (3 ml/kg)	(1) Klein et al. (2020) (2) Lee et al. (2008); Piermartiri et al. (2009); Piermartiri et al. (2010); Klein et al. (2020) (3) Goodrich et al. (2013); Klein et al. (2020)
E	(1) Levetiracetam (60 mg/kg i.p.), (2) Atorvastatin (3 mg/kg i.p.), (3) Ceftriaxone (60 mg/kg s.c.)	(1) Aqua ad injectabilia i.p. (3 ml/kg) (2) 4% DMSO + 10% Solutol® HS 15 + 86% PBS i.p. (5 mg/kg) (3) Aqua ad injectabilia s.c. (3 ml/kg)	Reduced doses (compared to D) because of effects obtained with D (see text)
F	(1) Levetiracetam (200 mg/kg i.p.), (2) Parecoxib (1 mg/kg i.p.), (3) Anakinra (100 mg/kg s.c.)	(1) Aqua ad injectabilia i.p. (3 ml/kg) (2) NaCl i.p. (3 ml/kg) (3) Aqua ad injectabilia s.c. (3 ml/kg)	(1) Klein et al. (2020) (2) Polascheck et al. (2010); Noé et al. (2013) (3) Kwon et al. (2013); Noé et al. (2013)
G	(1) Valproate (200 mg/kg i.p.), (2) Losartan (50 mg/kg s.c.), (3) Memantine (5 mg/kg i.p.)	(1) NaCl i.p. (3 ml/kg) (2) Aqua ad injectabilia s.c. (3 ml/kg) (3) Aqua ad injectabilia i.p. (3 ml/kg)	(1) Löscher and Brandt (2010) (2) Bar-Klein et al. (2014); Klein et al. (2020) (3) Klee et al. (2015); Zenki et al. (2018)
H	(1) Levetiracetam (200 mg/kg i.p.) (2) Agmatine (100 mg/kg i.p.)	(1) Aqua ad injectabilia i.p. (5 ml/kg) (2) NaCl i.p. (5 ml/kg)	(1) Klein et al. (2020) (2) Neis et al. (2017)

* Two drugs (#1 and #2 in drug combinations A and C and #3 and #4 in drug combination C) were dissolved together in the same vehicle (aqua ad injectabilia)

were included in each experiment to compensate for any losses during or after surgery.

Due to a relatively high loss of head electrode assemblies during the subsequent weeks after kainate injection in previous experiments (Schidlitzki et al., 2017), we compared Paladur® dental acrylic cement (Kulzer GmbH, Hanau, Germany) with Harvard® polycarboxlate cement (Harvard Dental International GmbH, Hoppegarten, Germany) for the fixation of the head assembly in preliminary experiments. For additional fixation of the base of the head assembly, these dental cements were combined with iBond® Universal (Kulzer GmbH), Surgibond® (SMI, Vith, Belgium), or superglue (Pattex® Ultra Gel, Henkel, Düsseldorf, Germany). Construction of the head assembly with Paladur® dental cement additionally fixated by superglue remained the most stable and durable head assembly in male NMRI mice and was therefore used for all subsequent experiments.

During all surgical procedures and for about one hour thereafter, mice were kept on a warming pad to avoid hypothermia. Directly after surgery mice were visually or video/EEG monitored to verify the development of SE by kainate. As previously described (Riban et al., 2002; Twele et al., 2016a, 2016b), the limbic SE induced by kainate was characterized by immobility, head nodding, circling, and intermittent generalized convulsive seizures; in the ipsilateral hippocampal EEG, SE was characterized by continuous activity of spikes or spike-and-waves and polyspikes. No obvious differences in this kainate-induced acute activity were observed between the treatment groups. All mice received 0.5 ml Sterofundin® VG-5 subcutaneously and pellet pap twice a day for at least seven days after surgery to compensate for fluid and nutrient deficits secondary to surgery and SE induction.

To ensure principles of animal welfare, animals were scored twice daily for two weeks after SE induction for pain, distress, and discomfort using welfare score sheets for humane endpoints (Stokes, 2002; Fentener van Vlissingen et al., 2015; Lidster et al., 2016). Using a distress scoring system (Morton and Griffiths, 1985; Lloyd and Wolfensohn, 1999), distress was rated from 0 (normal) to 3 (severe) based on food-/water intake and body weight, movement and body posture, and grooming and fur (Table S1). Mice with a score of 1 received no special treatment. Mice with a score 2 received daily treatment of 0.5 ml Sterofundin® VG-5 subcutaneously and pellet pap twice a day. The same treatment was implemented for mice that reached score 3. If score 3 persisted for more than three days, the mouse was euthanized and removed from the experiment. As all drug combinations had been evaluated for tolerability in previous experiments (Klee et al., 2015; Welzel et al., 2019), this only happened, unexpectedly, with one of the drug combinations (see below).

2.3. Drug treatment following status epilepticus in the intrahippocampal kainate model

In the present study, seven drug combinations with two to four drugs from different mechanistic categories were compared in eight experiments (A-H) (Table 1; Fig. S1). Except for levetiracetam and agmatine, the tolerability of these drug combinations had previously been tested in naïve NMRI mice and NMRI mice during the latent period following SE (Klee et al., 2015; Welzel et al., 2019). In these previous experiments, the tolerability was assessed using a modified Irwin screen, a rotarod test, rectal measurement of body temperature, and measurement of body weight, which were repeatedly performed over the course of four days. Despite animals after brain injury (e.g. post-SE) often exhibit increased adverse effects in response to drug administration (Löscher, 2016), all drug combinations were sufficiently tolerated in mice during the latent period, except for combination G (valproate, losartan and memantine), which induced moderate hypoactivity, ataxia and reduced motor coordination (Klee et al., 2015). For combination H (levetiracetam and agmatine), which we had not evaluated for tolerability before, preliminary tolerability experiments were performed in the same way as for the other drug combinations, indicating excellent tolerability in mice during the latent period after SE (data not shown). With one exception (combination C), we decided to limit the number of drugs in the combination to a maximum of three, as previously described for network pharmacology (Hopkins, 2008; Ainsworth, 2011). Preliminary tolerability/toxicity experiments with more than three drugs in one combination resulted in serious adverse effects and mortality in rats (K. Töllner, unpublished data).

In the present study, we tested the following eight combinations of 14 drugs:

A) Levetiracetam + gabapentin + topiramate
B) Levetiracetam + α-tocopherol
C) Levetiracetam + deferoxamine + gabapentin + fingolimod
D) Levetiracetam + atorvastatin + ceftriaxone
E) Levetiracetam + atorvastatin + ceftriaxone (reduced doses)
F) Levetiracetam + parecoxib + anakinra
G) Valproate + losartan + memantine
H) Levetiracetam + agmatine.

As treatment group D (levetiracetam, atorvastatin and ceftriaxone) did not exert any antiepileptogenic effects but rather exhibited pro-epileptogenic activity (see Results), this drug combination was tested with reduced doses (30% of doses used initially) in an additional anti-epileptogenesis study (treatment group E). Doses of all drugs are shown in Table 1 and were selected from previous preclinical rodent experiments with these drugs as indicated in the table. Levetiracetam was included in most drug combinations because of preliminary evidence of disease-modifying efficacy in clinical studies (Klein et al., 2020).

We previously developed solubility protocols for the 13 clinically approved drugs for parenteral (i.p. or s.c.) administration in mice (Klee et al., 2015; Welzel et al., 2019). The selected drugs, drug doses, and respective vehicles chosen for drug solutions are shown in Table 1. In the case of drugs that were used as salts, all doses (in mg/kg body weight) refer to the free acid or base forms of the respective drugs. Drug absorption following parenteral administration of drug suspensions is highly variable and lower compared to administration of drug solutions in mice (Löscher et al., 1990), which is why all drugs were administered as solutions except α-tocopherol, which was emulsified in Miglyol® 812 (90%) and ethanol (10%). Levetiracetam and deferoxamine, levetiracetam and gabapentin, and gabapentin and fingolimod were mixed in the same aqueous solution shortly before injection to reduce the number of injections over the period of treatment.

All drugs were prepared freshly once a day, except ceftriaxone, which was prepared freshly twice a day due to the limited stability of the solution. For drug combinations with three to four drugs in one combination, the injection volume was 3 ml/kg for all substances except topiramate and atorvastatin, which were not soluble below an injection volume of 5 ml/kg. All injection volumes were kept as low as possible to avoid total injection volumes of over 10-12 ml/kg in mice. Details of drug formulations and sources for drugs and drug vehicles have been reported previously (Klee et al., 2015; Welzel et al., 2019).

The study design for testing drug combinations for antiepileptogenic efficacy is shown in Fig. 2. Mice were treated with the drug combination or respective vehicle three times a day over five days (except fingolimod, which was administered once a day), starting six hours after intrahippocampal injection of kainate. Six hours was chosen to avoid the drug treatment potentially interfering with the kainate-induced SE development (Twele et al., 2016b; Schidlitzki et al., 2017). The duration of treatment with the drug combinations evaluated here was restricted to five days, because the latent period in the intrahippocampal kainate model in male NMRI mice is five to seven days (Twele et al., 2016b), after which spontaneous electrographic and electroclinical seizures develop. Thus, as shown previously (Schidlitzki et al., 2020), treatment

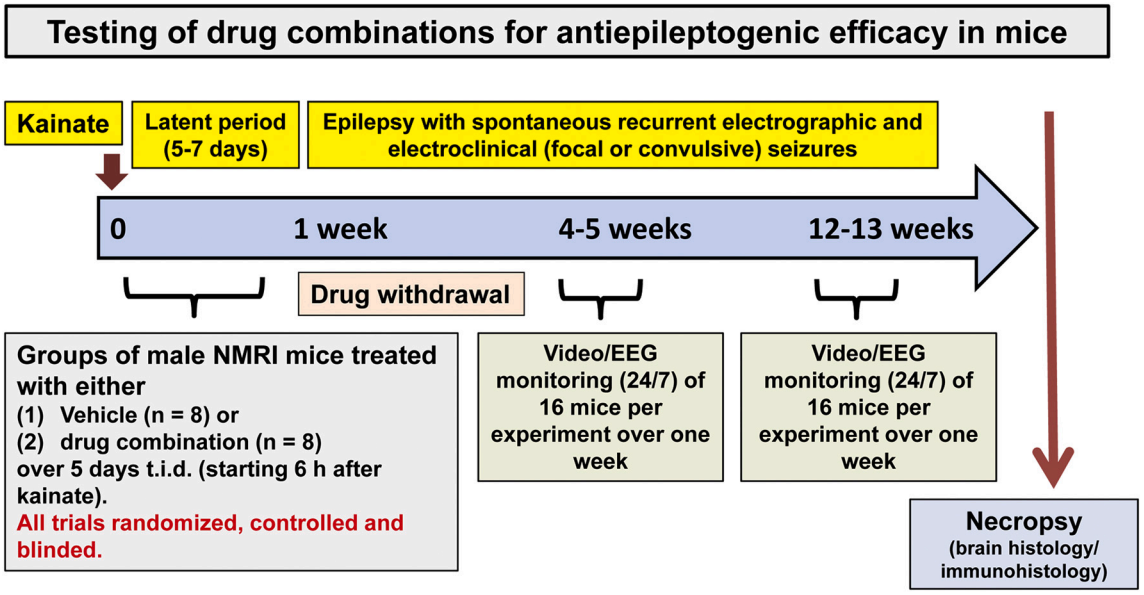

Fig. 2. Schematic illustration of the protocol used for the mouse experiments performed in this study. In all experiments, mice were randomly assigned to the drug and vehicle groups and experiments were performed in a blinded fashion. Each drug experiment was performed together with a vehicle experiment. To avoid any carry-over effects of drugs on spontaneous seizures, a sufficiently long withdrawal period (>three weeks) was included between termination of treatment and onset of seizure monitoring.

for five days should be sufficient to interfere with epileptogenesis.

The routes of administration, doses, respective vehicles, and injection volumes used for antiepileptogenesis studies are shown in Table 1. After SE, animals were randomly assigned to treatment and vehicle groups. For subsequent video/EEG monitoring and analyses, all experiments were performed in a blinded fashion, so that it was not clear which mice received drugs or the respective vehicles.

Rather than using historical controls, we decided to perform an age- and batch-matched vehicle group together with each drug-treated group to exclude the possibility that kainate injection in a particular batch of mice was less effective in inducing epilepsy than in other batches of animals. NMRI mice are outbred; thus genetic alterations that affect study outcomes can occur over time (Löscher et al., 2017). Furthermore, seasonal variation in seizure susceptibility and epilepsy development may form a bias when using historical controls (Löscher et al., 2017).

To avoid false positive (or negative) drug efficacy data, we used the following inclusion criteria: (1) The vehicle control group (n = 8-9) of each drug experiment should exhibit electrographic and/or electroclinical SRS in at least 70% of mice of this group, and (2) the treated mice should not exhibit any serious adverse effects. Based on these criteria, three of the eight groups shown in Table 1 had to be excluded from final analysis. Groups F and H were excluded because of too low SRS incidence in vehicle controls and group G because of unexpected toxicity. The toxicity of the combination of valproate, losartan and memantine, which resulted in mortalities or euthanasia of animals according to the criteria described above, had not been observed in our previous tolerability studies (Klee et al., 2015). However, in these previous experiments, this drug combination was only administered twice a day over three days compared to the three times daily dosing over five days used here, which is the most likely explanation for the observed toxicity.

As shown in Table S2, within some of the vehicle- and drug-treated groups that were included in the analysis, a few mice had to be excluded, because of insufficient accuracy of the localization of the kainate injection and electrode (see Histology below). Furthermore, a few mice lost their electrode head assembly during the three months of the experiment or had EEGs that exhibited too many artifacts to allow reliable EEG analysis of seizures. In addition, two mice (one vehicle-treated and one drug-treated animal in group E) died during generalized convulsive seizures.

2.4. Video/EEG monitoring

At 4 and 12 weeks post-SE, mice were continuously (24 h/day) video/EEG monitored for seven days (Fig. 2) to compare the occurrence of spontaneous electrographic and electroclinical seizures in vehicle- and drug-treated groups (Twele et al., 2016b). For EEG-recordings, mice were connected via a flexible cable to a system consisting of one-channel bioamplifiers (ADInstruments Ltd., Sydney, Australia) and analog-digital converters (PowerLab 4/35 PL3504/P, ADInstruments). By this system, a maximum of 16-18 mice could be monitored in parallel. Because we wanted to avoid the use of historical controls, this limited the size per group (vehicle and treated) to 8-9. The data from these mice were recorded (sampling rate 200 Hz, time constant 0.1 seconds, low pass filter of 60 Hz, 50 Hz notch filter) and analyzed with LabChart 8 for Windows (ADInstruments). The EEG recording was directly linked to simultaneous digital video-recordings of four mice per system using four infrared board cameras (Sony, Tokio, Japan) for four mice merged by one video quad processor (Monacor International GmbH & Co. KG, Bremen, Germany). For video/EEG monitoring, mice were housed singly in clear plexiglass cages (20 cm x 18 cm x 28 cm). For monitoring during the dark phase, infrared lighting was mounted above the cages.

As shown in Fig. 2, mice were video/EEG monitored in the chronic period (for one week each at 4 and 12 weeks post-SE) to compare the occurrence of spontaneous seizures in vehicle and drug-treated groups. For evaluation of effects on the development of chronic epilepsy, all electrographic and electroclinical seizures occurring after SE and in the chronic epileptic phase were analyzed visually.

After the latent period following intrahippocampal kainate injection, mice develop different types of paroxsmal EEG events and epileptic SRS (Riban et al., 2002; Maroso et al., 2011; Twele et al., 2016a, 2016b; Schidlitzki et al., 2017) as described in the following. The most frequent paroxysmal EEG events recorded by intrahippocampal electrodes in this model are high-voltage spike waves (HVSWs; Fig. 3B), which are characterized by high amplitude sharp waves ≥3 times the EEG baseline with a frequency of at least 2 Hz (spikes per second), a duration of at least five seconds, and an inter-event interval of at least three seconds (Twele et al., 2016a, 2016b). The inter-event interval is characterized by the

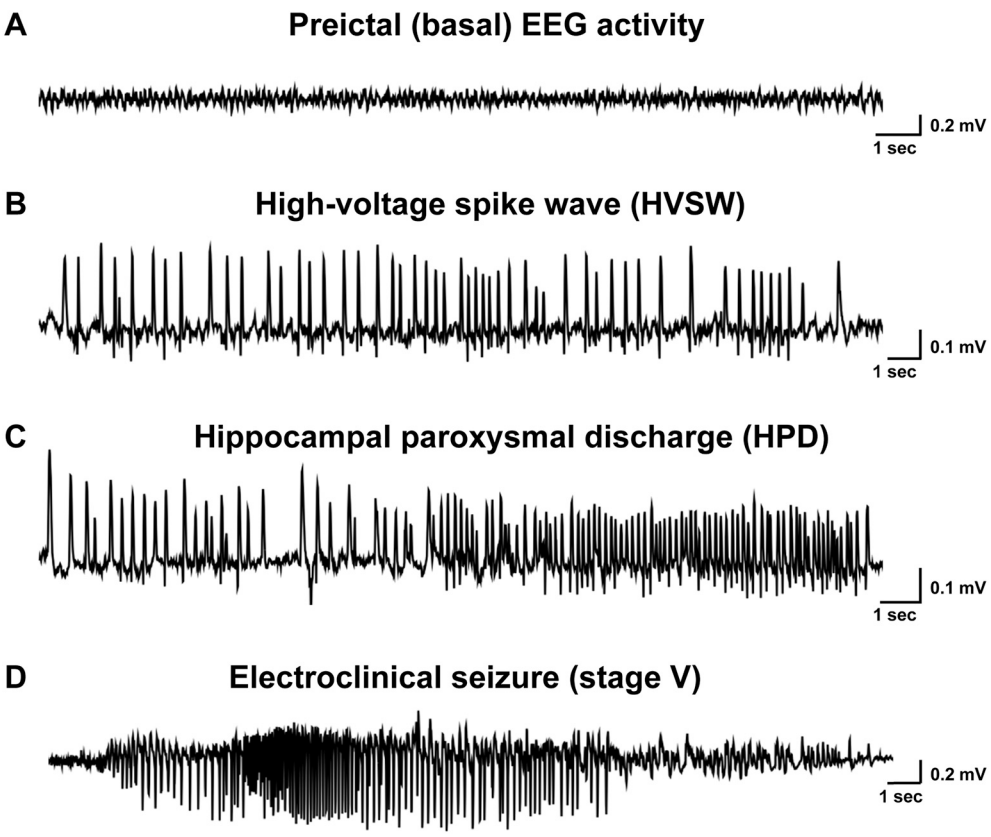

Fig. 3. Typical spontaneous paroxysmal EEG events recorded via a depth electrode in the ipsilateral CA1 in the intrahippocampal kainate mouse model. (A) Preictal (basal) EEG activity, showing typical theta oscillations (5–9 Hz). (B) A typical high-voltage sharp wave (HVSW) discharge, recorded in an epileptic mouse; such HVSWs start after the latent period (five to seven days) following kainate. (C) A typical hippocampal paroxysmal discharge (HPD), recorded in an epileptic mouse; such HPDs start after about 10-14 days following kainate and are considered electrographic seizures. (D) A generalized convulsive electroclinical seizure (Racine stage V), recorded in an epileptic mouse in the chronic phase of epilepsy.

occurrence of either no epileptic EEG activity, isolated spikes, or spike trains with an amplitude of less than three times the baseline. Spikes or spikes trains <3 times the baseline are considered interictal activity. HVSWs can show evolution in frequency or pattern, but can also be regular. They occur without any obvious behavioral alterations or motor correlates.

The second most frequent paroxysmal EEG event are hippocampal paroxysmal discharges (HPDs; Fig. 3C), which can only be detected at the kainate injection site of the ipsilateral hippocampus (Riban et al., 2002; Maroso et al., 2011). HPDs are often longer (over 20 seconds) than typical HVSWs and always show evolution in morphology and frequency. As shown in Fig. 3C, HPDs typically start with large amplitude HVSWs, followed by a train of lower-amplitude spikes (≥2 times the baseline) with at least five seconds of increased frequency (≥5 Hz). HPDs also have an inter-event interval of at least three seconds, in which either no epileptic EEG activity, isolated spikes, or spike trains with an amplitude of less than two times baseline are observed (also considered as interictal activity). In our hands, HPDs occur without any obvious behavioral alterations or motor correlates and are therefore considered electrographic seizures (Twele et al., 2016a), whereas the interpretation of HVSWs is ambivalent as described in the Discussion.

For comparison of the frequency of HVSWs and HPDs in vehicle- and drug-treated mice, electrographic seizures were counted visually in the EEG during the one-week video/EEG monitoring periods at 4 and 12 weeks post-SE. Four 30-min periods (typically at 6:00 am and 12:00, 6:00, and 11:00 pm) were selected and analyzed for days one, four, and seven of the respective video/EEG monitored weeks for calculation of the average number of electrographic seizures occurring per hour.

In addition to highly frequent HVSWs and HPDs, male NMRI mice develop less frequent focal and generalized electroclinical (convulsive) seizures (Twele et al., 2016b), which occur several times per week. Focal and generalized convulsive electroclinical seizures are characterized by a high spike frequency and amplitude, and a typical postictal depression of the EEG baseline (Fig. 3D). For comparison of the frequency of electroclinical seizures in vehicle- and drug-treated mice, seizures were counted manually in the video/EEG recordings of the seven days of continuous (24/7) recordings at 4 and 12 weeks post-SE. Based on the video recordings, the electroclinical seizures were rated for severity using the following modified scale by Racine (1972): stage 1, behavioral arrest with minor facial clonus (stereotypical sniffing, tremor of tactile hair); stage 2, severe facial clonus (head nodding, mouth or facial movements); stage 3, unilateral forelimb clonus; stage 4, bilateral forelimb clonus with rearing; stage 5, generalized tonic-clonic seizure with loss of righting reflexes. Stage I-III seizures were considered as focal and stage IV and V seizures as generalized convulsive seizures. Furthermore, the average duration of electroclinical seizures was determined and compared between the vehicle- and drug-treated groups. As an additional parameter for the severity of the disease, the seizure load was calculated based on the severity (summation of number of electroclinical seizures multiplied by seizure stage) or the duration of electroclinical seizures (cumulative seizure duration) as described recently (Schidlitzki et al., 2020).

2.5. Histology

For histological analysis, all mice of the diverse treatment groups were anesthetized with chloral hydrate (720 mg/kg i.p. in 10 ml) after the last video/EEG recording (13-14 weeks after intrahippocampal kainate injection; see Fig. 2) and transcardially perfused with 0.01 M phosphate-buffered saline followed by 4% paraformaldehyde. The brains were removed after one hour, postfixed in 10% sucrose solution (4% paraformaldehyde) for 24 hours, and then transferred to 30% sucrose solution (saline). 1 mg/ml of thymol was added to the sucrose solution if the brains were stored for a longer period of time. As previously described (Bröer et al., 2016), four series of coronal brain sections (40 μm) were prepared using a cryomicrotome and subsequently stained with cresyl violet (containing thionin). Naive age-matched groups of mice were used as controls. The correct localization of the kainate

injection and EEG electrode in the hippocampus was verified in each mouse.

For determining neurodegeneration in the hippocampus, five to six thionin-stained brain sections (at -1.56 to -2.18 mm AP from bregma) were semi-quantitatively scored using a scoring system described by Gröticke et al. (2008). The left and right hippocampi were scanned in a quasi-random fashion and scores were noted for each of the subregions of the hippocampal formation (CA1, CA2, CA3a, Ca3c, and hilus): score 0 = no obvious damage; score 1 = abnormal appearance of the structure without clear evidence of visible neuronal loss; score 2 = moderate neurodegeneration (lesions involving 20–50% of neurons); score 3 = severe neurodegeneration (lesions involving over 50% of neurons). Furthermore, the extent of the granule cell dispersion (GCD) in the dentate gyrus was visually assessed with a score system: score 0 = no GCD, score 1 = mild GCD, score 2 = moderate GCD, score 3 = severe GCD.

2.6. Study design and data analysis

In all experiments, mice were randomly assigned to the drug and vehicle groups and experiments were performed in a blinded fashion. For the antiepileptogenesis studies, the sample size was restricted to 8-9 vehicle controls and 8-9 drug-treated mice due to the video/EEG monitoring spaces available. Individual vehicle control experiments were performed in parallel to each drug experiment instead of using historical controls to minimize the bias of batch-to-batch and seasonal differences in animal responsiveness to the convulsant and seasonal effects on data (Löscher et al., 2017). Based on a sample size of eight mice per group and the typical seizure frequency in this model determined by us previously (Schidlitzki et al., 2017; Schidlitzki et al., 2020), the statistical power to determine a significant treatment effect on seizure frequency was calculated at 0.81 (at alpha $\leq$0.05) before beginning the studies. The software G*Power 3.1 was used for post hoc power analysis to compute the achieved power of the experiments and to calculate the estimated group size to achieve a power $\geq$0.8.

All antiepileptogenesis experiments were analyzed separately and were also compared with a pooled vehicle group to facilitate inter-treatment comparisons; the seizure incidence of the five control groups did not differ significantly (see Results). Depending on whether data was normally distributed, either parametric or nonparametric tests were used for statistical evaluation. For pairwise comparisons or for intragroup comparisons, either the Student's t-test or the Mann-Whitney U-test were used. For comparison of several groups, and depending on data distribution, either the ANOVA F-test, followed post hoc by Dunnett's multiple comparisons test, or the Kruskal-Wallis test, followed post hoc by Dunn's multiple comparisons test, were used. For analysis of body weights over the first two weeks post-SE, a two-way ANOVA followed post hoc by Sidak's multiple comparisons test were used for intergroup comparisons. For comparison of seizure incidences in a 2 x 2 table, Barnard's unconditional test (Barnard, 1947) was used, because this test preserves the significance level and generally is more powerful than Fisher's exact test for moderate to small sample sizes (Lydersen et al., 2009). Before the various statistical analyses, few outliers were detected and removed by Grubb's outlier test, using a significance level (alpha) of 0.05. Except for Barnard's unconditional test, which was performed by SciStatCalc version 1.5 (http://scistatcalc.blogspot.com/2013/11/barnards-test-calculator.html) and verified by R (version 4), all statistical analyses were performed with the Prism 8 software from GraphPad (La Jolla, CA, USA). All tests were used two-sided and a P $\leq$ 0.05 was considered significant.

3. Results

3.1. Incidence, frequency, severity and duration of spontaneous seizures in vehicle controls

As described in the Methods section, five of the eight experiments with different drug combinations and respective vehicle controls met the criteria for inclusion and were analyzed for antiepileptogenic efficacy. Based on the two one-week long periods of continuous (24/7) video-EEG monitoring at 4 and 12 weeks following kainate, for the five individual vehicle control groups (n = 39), 81% of the animals developed electrographic seizures and 74% electroclinical seizures. All mice with electrographic seizures (HPDs) exhibited also HVSWs. Incidence of SRS did not differ at 4 vs. 12 weeks after kainate. The average (± SEM) frequency of paroxysmal electrographic events (HVSWs and HPDs) in vehicle controls was 10.1 ± 1.6 seizures/h at 4 weeks and 11.8 ± 1.9 seizures/h at 12 weeks following kainate. When the electrographic events were subclassified into HVSWs (Fig. 2B) and HPDs (Fig. 2C) the average frequency of HVSWs in vehicle controls was 6.4 ± 1.1 HVSWs/h and 5.4 ± 1.4 HVSWs/h respectively for the 4 and 12 week timepoints, while the average frequency of HPDs in vehicle controls was 3.7 ± 0.9 HPDs/h at 4 weeks and 6.3 ± 1.2 HPDs/h at 12 weeks following kainate. The frequency of HPDs was significantly higher (P = 0.05) at 12 vs. 4 weeks, indicating the progression of epilepsy with time.

For electroclinical seizures the average frequency for vehicle controls was 3.1 ± 0.6 seizures/week at 4 weeks and 6.8 ± 1.8 seizures/week at 12 weeks following kainate, which was not significantly different (P = 0.1485). We also differentiated the frequency of electroclinical seizures according to seizure type. Average frequency of focal (stage I-III) seizures in vehicle controls was 0.4 ± 0.1 seizures/week at 4 weeks and 0.4 ± 0.2 seizures/week at 12 weeks following kainate. In contrast the average frequency of generalized convulsive (stage IV-V) seizures in vehicle controls was 2.8 ± 0.6 seizures/week at 4 weeks and 6.6 ± 1.8 seizures/week at 12 weeks following kainate (P = 0.0661). Vehicle-treated epileptic mice exhibited significantly more generalized convulsive than focal seizures (P<0.0001 at both time points). Average severity of electroclinical seizures was 4.4 ± 0.19 (score) at 4 weeks and 4.7 ± 0.1 at 12 weeks. Average duration of electroclinical seizures was 30.5 ± 1.8 sec at 4 weeks and 38.1 ± 1.8 sec at 12 weeks following kainate, which was significantly different (P = 0.0042), substantiating progression of epilepsy over the duration of the experiment.

3.2. Tolerability of drug treatments

Except drug combination G (valproate, losartan and memantine; see Methods), all drug combinations were well tolerated when administered during the latent period following kainate, corroborating our previous tolerability experiments (Klee et al., 2015; Welzel et al., 2019). This is illustrated by the lack of drug effects on body weight illustrated in Fig. S2. Body weight significantly decreased by about 10-15% following kainate in both vehicle controls and drug-treated mice. The only significant inter-group difference was observed for combination B (levetiracetam and α-tocopherol) at one day after kainate when weight loss in the drug-treated group was significantly less marked than in vehicle controls (Fig. S2B). In most mice, body weight returned to pre-kainate levels within two weeks following kainate surgery.

3.3. Antiepileptogenic efficacy of drug combinations in the intrahippocampal kainate mouse model

To allow a better comparison of effects across the different drug combinations, these are shown together with pooled vehicle controls in Figs. 4-6 and Fig. 10; cohort-specific data are shown in Figs. S3-S7. Seizure incidence or frequency did not significantly differ across individual vehicle control groups. As described in Methods, one drug combination (levetiracetam, atorvastatin, ceftriaxone) was evaluated at two

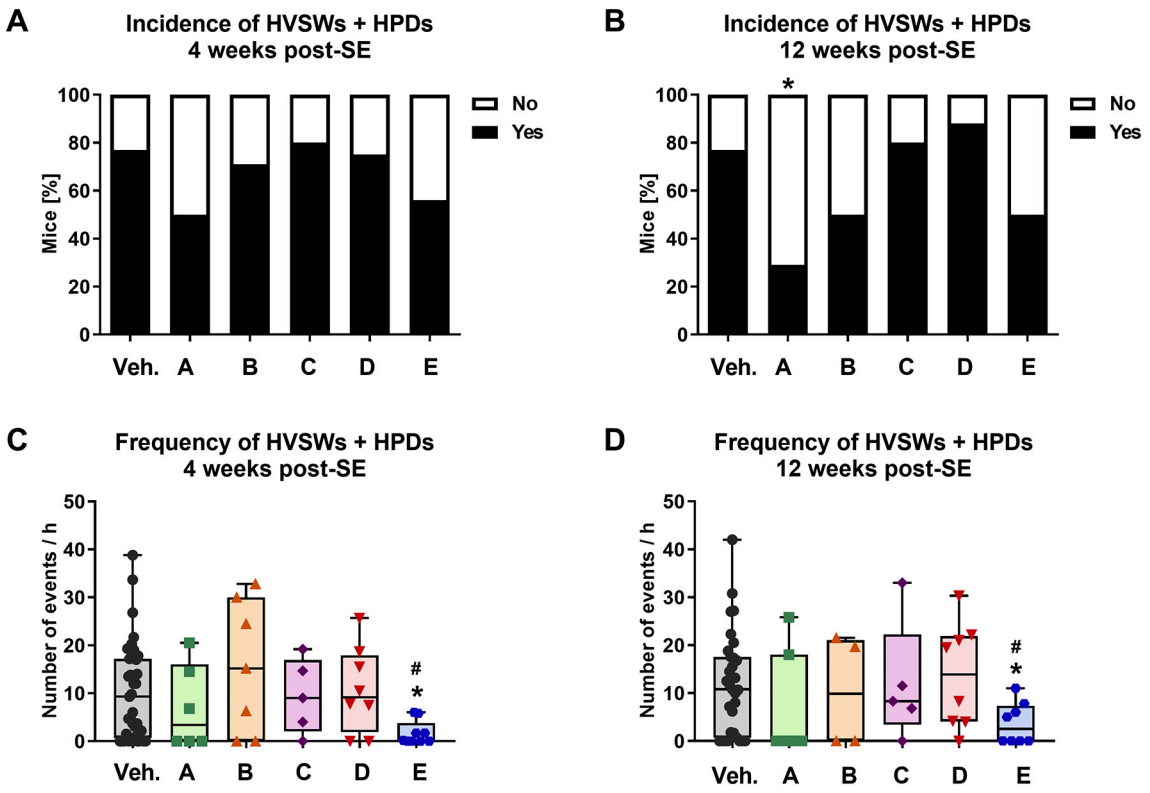

Fig. 4. Effect of treatment with drug combinations during the latent period following kainate on incidence and frequency of spontaneously recurrent paroxysmal electrographic events (HVSWs and HPDs) determined at 4-5 and 12-13 weeks after kainate. Data were calculated from the sum of HVSWs and HPDs; see Figs. 5 and 6 for individual data. Data in A and B (event incidence) are illustrated as percentage of mice within each group exhibiting spontaneous electrographic events within each one-week recording period, whereas data in C and D (event frequency) are illustrated as number of electrographic events per hour and shown as boxplots with whiskers from minimal to maximal values; the horizontal line in the boxes represents the median value; in addition, individual data are shown. The data shown for vehicle (n = 39 at 4 weeks and 31 at 12 weeks after kainate) are from the five individual control groups of the five drug combination experiments illustrated here. Statistical comparison of data in individual vehicle groups did not indicate any significant inter-group differences. Sample size of the drug-treated groups is n=5-9 at 4 weeks and n=4-8 at 12 weeks after kainate (some mice lost their head EEG electrode assembly during the course of the experiment). Significant differences to vehicle controls are indicated by asterisk (*P<0.05; **P<0.01), while significant differences between groups D and E are indicated by the hash sign (P<0.05). Combination A = levetiracetam, gabapentin and topiramate; combination B = levetiracetam, and α-tocopherol; combination C = levetiracetam, deferoxamine, gabapentin and fingolimod; combination D = levetiracetam, atorvastatin and ceftriaxone; combination E = reduced doses of levetiracetam, atorvastatin and ceftriaxone. Doses of drugs and dosing intervals are shown in Table 1.

dose levels, after the experiment with the initial doses (combination D; Table 1) indicated pro- rather than antiepileptogenic effects (see below). Thus, for combination E, doses of levetiracetam, atorvastatin, and ceftriaxone were reduced by 70% (Table 1).

As shown in Fig. 2, spontaneous seizures were recorded long after withdrawal from drug treatment, thus excluding any direct drug effects on SRS. As shown in Fig. 4B, the incidence of paroxysmal electrographic events (HVSWs and HPDs) was significantly reduced only by combination A (levetiracetam, gabapentin, topiramate) at 12 weeks after kainate. The frequency of electrographic events was significantly decreased by combination E (reduced doses of levetiracetam, atorvastatin, ceftriaxone) at both 4 and 12 weeks after kainate (Fig. 4C, D). Furthermore, the frequency of electrographic events following treatment with combination E was significantly lower than the frequency following treatment with combination D (high doses of levetiracetam, atorvastatin, ceftriaxone), demonstrating the significant effect achieved by lowering the doses of this drug combination.

When paroxysmal electrographic events were differentiated into HVSWs and HPDs, again only combination A reduced the incidence of both HVSWs (Fig. 5) and HPDs (Fig. 6) at 12 weeks following kainate. The frequency of HVSWs was significantly reduced by combination E at 4 but not 12 weeks after kainate (Fig. 5C, D). Combination E also decreased the incidence and frequency of HPDs at both 4 and 12 weeks after kainate (Fig. 6), whereas none of the other drug combinations significantly reduced the frequency of electrographic seizures.

The incidence of electroclinical seizures was significantly reduced by combination E at 12 weeks after kainate (Fig. 7B). Indeed, during video-EEG monitoring at 12 weeks after kainate, none of the mice exhibited any electroclinical seizures following treatment with this drug combination. As a consequence, also seizure frequency was significantly reduced (Fig. 7D). In contrast, treatment with combination D (high doses of levetiracetam, atorvastatin, ceftriaxone) tended to increase seizure frequency at 12 weeks after kainate (Fig. 7D), which was the reasoning for reducing the doses of this drug combination in group E. None of the drug combinations, including combination E, significantly decreased the incidence or frequency of electroclinical seizures at 4 weeks after kainate (Fig. 7A, C). At 12 weeks, combination E was the only drug combination that exerted significant effects on incidence and frequency of electroclinical seizures (Fig. 7B, D).

Typically, there was an overlap in the occurrence of electrographic and electroclinical seizures in epileptic mice. Indeed, as shown in Fig. 8 and Fig. S8, the majority of mice showing epileptic seizures had both electroclinical and electrographic seizures at 4 weeks and 12 weeks post-SE. Furthermore, all mice with electrographic seizures exhibited both HVSWs and HPDs. Overall, 82% and 81% of the vehicle control mice had SRS at 4 or 12 weeks after kainate, respectively. None of the treatments exerted significant effects on seizure incidence at 4 weeks after kainate (Fig. 8A, Fig. S8A; Table S3), while treatment A and E exerted significant effects on seizure incidence at 12 weeks following kainate (Fig. 8B, Fig. S8B; Table S3). Treatment A significantly decreased the number of

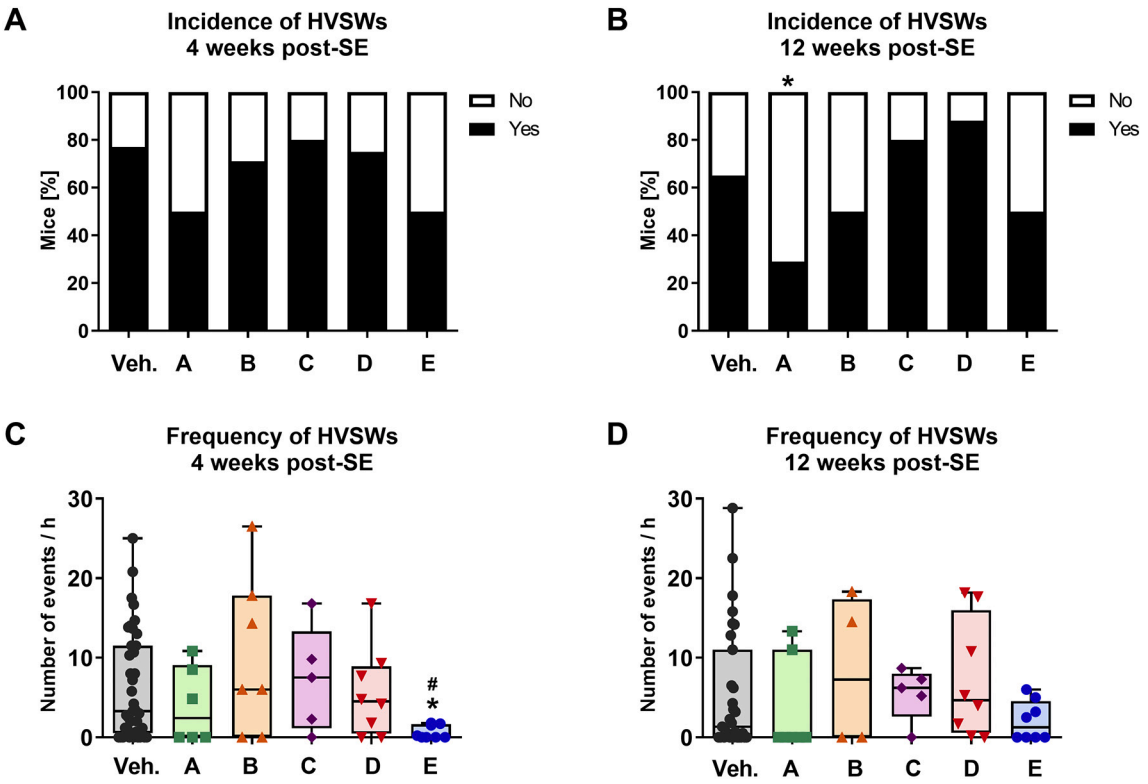

Fig. 5. Effect of treatment with drug combinations during the latent period following kainate on incidence and frequency of HVSWs determined 4-5 and 12-13 weeks after kainate. See Fig. 4 legend for details.

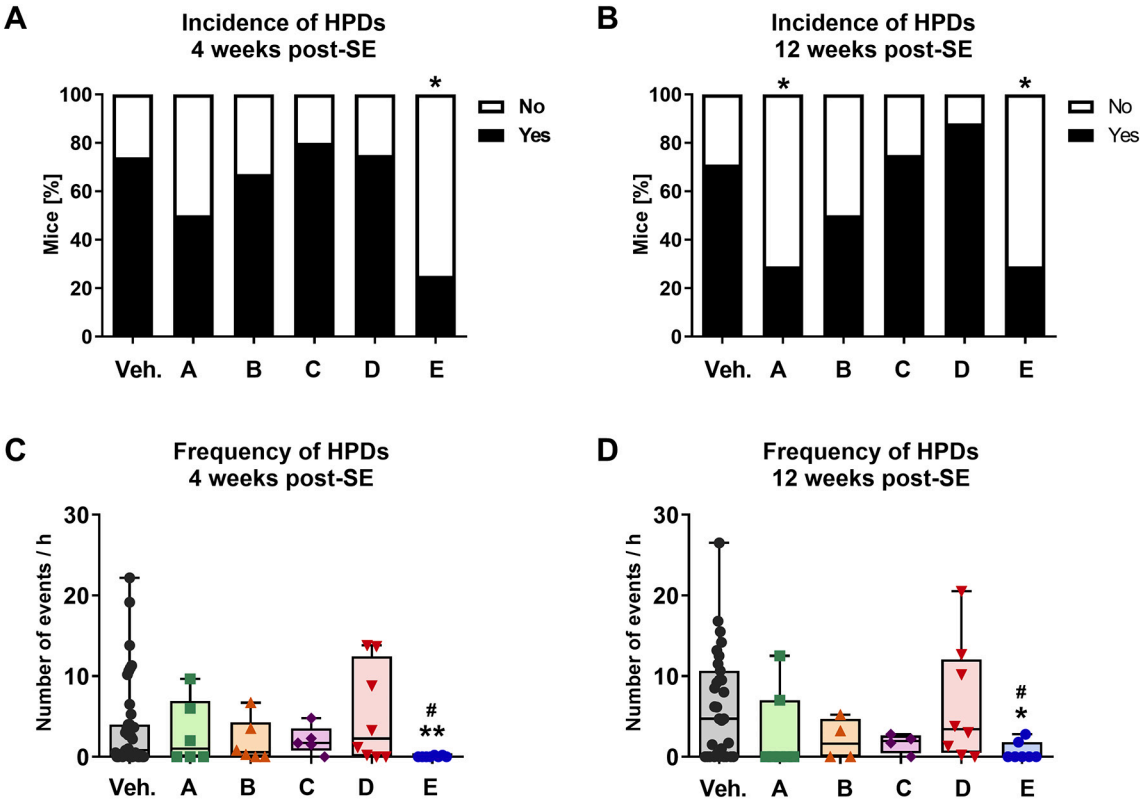

Fig. 6. Effect of treatment with drug combinations during the latent period following kainate on incidence and frequency of HPDs determined 4-5 and 12-13 weeks after kainate. See Fig. 4 legend for details.

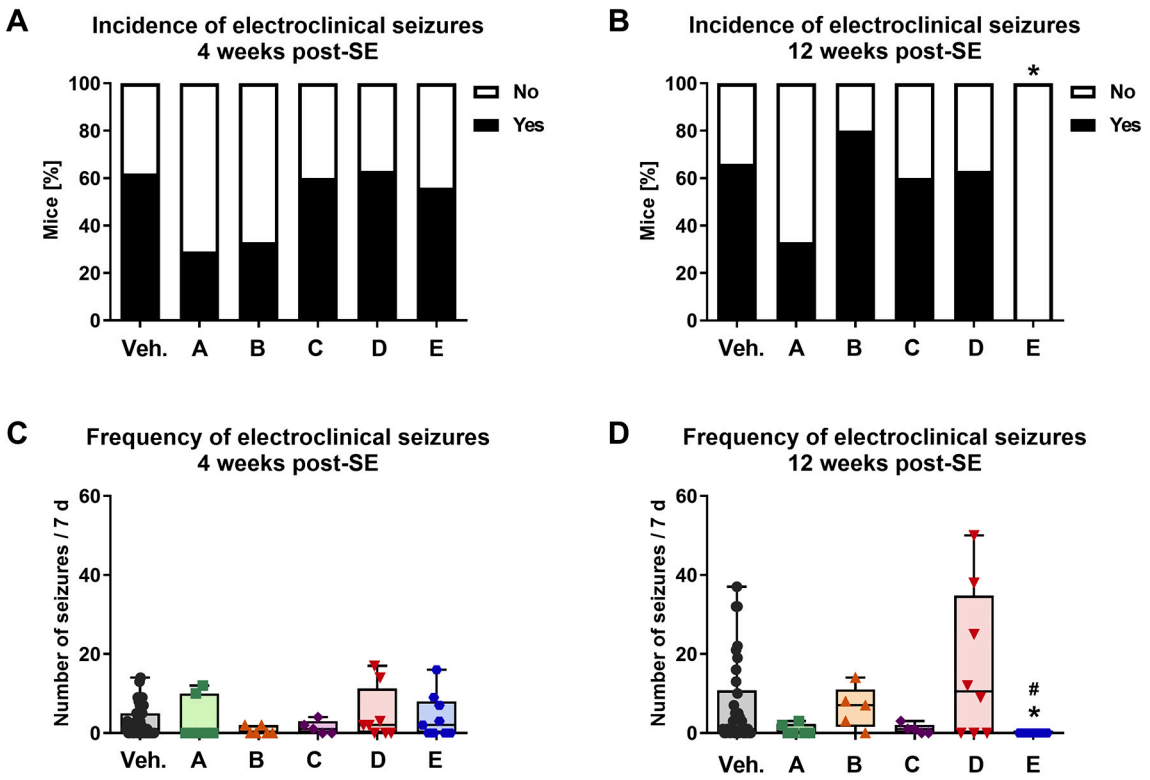

Fig. 7. Effect of treatment with drug combinations during the latent period following kainate on incidence and frequency of electroclinical seizures determined 4-5 and 12-13 weeks after kainate. Sample size of the drug-treated groups is n=5-9 at 4 weeks and n=5-8 at 12 weeks after kainate. See Fig. 4 legend for further details.

mice with any type of seizures, while treatment E significantly decreased the number of mice with any type of seizures and the number of mice with electrographic and electroclinical seizures. Furthermore, all mice treated with combination E (reduced doses of levetiracetam, atorvastatin and ceftriaxone) that only had electrographic seizures at 12 weeks, had both electroclinical and electrographic seizures at 4 weeks (Fig. 8).

The duration of electroclinical seizures was not reduced by any treatment, but an increased seizure duration was determined for combination C, suggesting a pro-epileptogenic effect (Fig. S9D). Similarly, average seizure severity was not reduced by any treatment (Fig. S9A, B), except that no seizures were recorded for combination E at 12 weeks (Fig. S9A, B). The seizure load was only significantly decreased by combination E (Fig. S10).

In addition to combination E, some of the other drug combinations (A and C) tended to decrease frequency of HPDs (Fig. 6D) or electroclinical seizures (Fig. 7D) at 12 weeks after kainate. However, none of these effects were statistically significant.

3.4. Comparison of monotherapy vs. two or three drugs combinations

To determine whether a three drugs combination has better efficacy than two or one drug, we took combination A (levetiracetam, topiramate and gabapentin) as an example. First, as shown in Fig. 8 and Table S3, this combination significantly decreased seizure incidence at 12 weeks after kainate and, second, we previously tested levetiracetam, topiramate and the combination of the two drugs in the same model and with the same doses as in the present study (Schidlitzki et al., 2020), so we could use these data for the comparison. As shown in Fig. 9A, levetiracetam alone exerted no effect on the incidence of electroclinical seizures, while topiramate alone reduced seizure incidence by 25% vs. individual vehicle controls. The combination of levetiracetam and topiramate reduced seizure incidence by 36.4%. The most marked effect (46% reduction in seizure incidence) was obtained with the triple combination, indicating that the three drugs combination has better efficacy than two or one drug. As shown in Fig. 9B, the effect of the triple combination vs. double combination or monotherapy was even more marked for incidence of electrographic seizures, clearly indicating a synergistic effect of the triple combination.

3.5. Hippocampal neurodegeneration after kainate

Consistent with previous reports (Bouilleret et al., 1999), kainate induced marked neurodegeneration and granule cell dispersion in the ipsilateral hippocampus, whereas no obvious changes were observed in the contralateral hippocampus (Fig. 10). In the ipsilateral hippocampus, severe neuronal loss was seen in the CA1 and CA3 layers and the dentate hilus (Figs. 8 and 9) as compared to the contralateral hemisphere or naïve controls. When the extent of neuronal loss and granule cell dispersion was scored (as described in Methods), none of the drug treatments significantly reduced neurodegeneration or granule cell dispersion compared to vehicle controls (Fig. 11). However, except for combination B, granule cell dispersion of drug treated groups did not significantly differ from naïve controls, which was due to large inter-individual variation of granule cell dispersion in drug treated mice (Fig. 11D).

4. Discussion

The 14 drugs chosen for the present study are clinically used for diverse therapeutic indications, but, based on their mechanisms of action, have previously been suggested to be interesting candidates for antiepileptogenic therapy (Löscher and Brandt, 2010; Löscher, 2020; Löscher, 2020; Klein et al., 2020). Levetiracetam, gabapentin, valproate, and topiramate are antiseizure (antiepileptic) drugs (ASDs) which, however, are also used for other indications, including neuropathic pain (gabapentin), bipolar disorder (valproate), and migraine (valproate, topiramate), and have shown antiepileptogenic potential in preclinical studies (Löscher and Brandt, 2010; Klein et al., 2020). Deferoxamine is

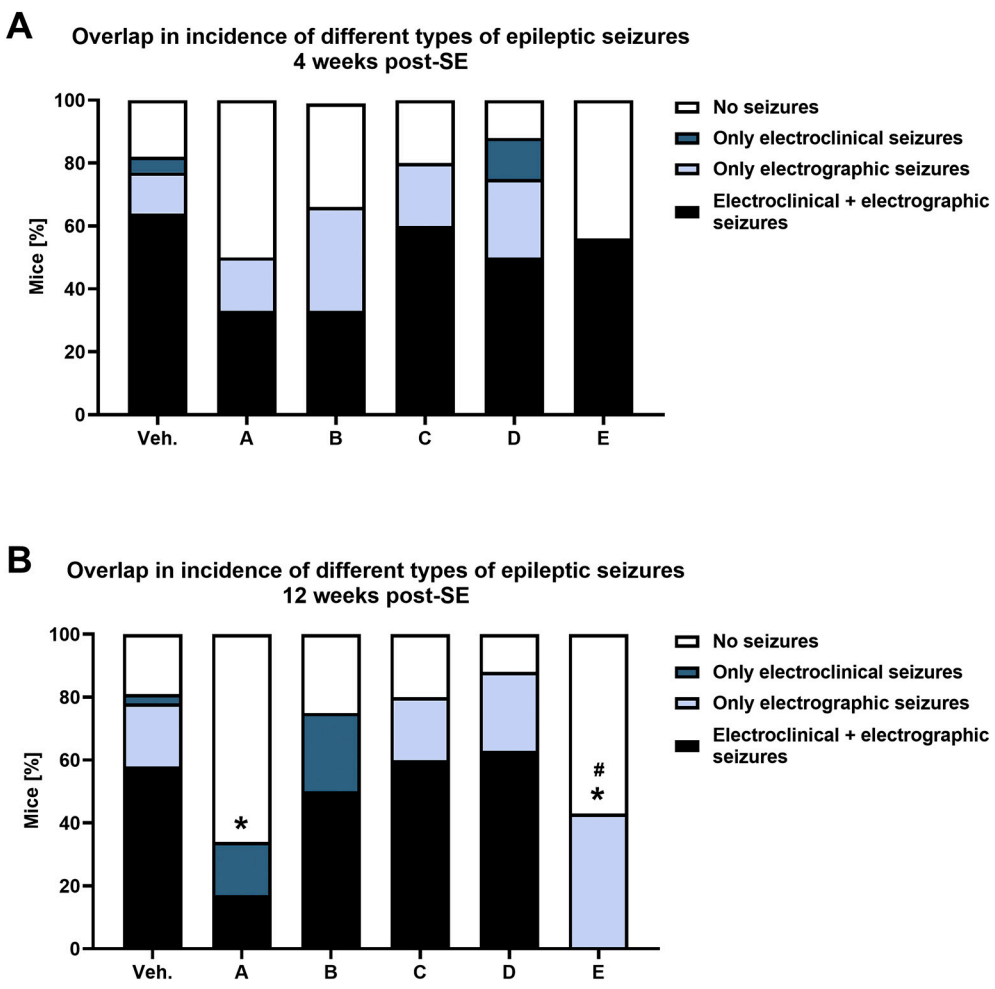

Fig. 8. Overlap in the occurrence of electrographic and electroclinical seizures in vehicle and treatment groups at 4-5 weeks (A) and 12-13 weeks (B) after kainate. The data shown for vehicle (n = 39 at 4 weeks and 31 at 12 weeks after kainate) are from the five individual control groups of the five drug combination experiments illustrated here. All mice with electrographic seizures (HPDs) exerted also HVSWs. Significant differences to vehicle controls in the number of mice without seizures is indicated by asterisk (*P<0.05), while significant differences in the number of mice with electroclinical and electrographic seizures is indicated by the hash sign ($^{\#}$P = 0.0053).

an iron chelator that is used for therapy of aluminum and iron intoxication, but was also shown to be effective in preventing the formation of free radicals and lipid peroxidation and to exert antiinflammatory and neuroprotective efficacy (Hall et al., 2010). α-Tocopherol, the most lipophilic and active form of vitamin E, exerts antioxidant properties by acting as a free radical scavenger and thereby protects cell membranes against lipid peroxidation, which is relevant for interfering with epileptogenesis (Mori et al., 2004; Ambrogini et al., 2018). Fingolimod, an immunotherapeutic drug targeting the sphingosine-1-phosphate receptor, is a widely used medication for relapsing-remitting multiple sclerosis; its antiinflammatory and antioxidant effects are likely to explain its disease-modifying effects in models of epileptogenesis (Klein et al., 2020). Similarly, atorvastatin, a competitive inhibitor of HMG-CoA reductase that is primarily used for the treatment of dyslipidemia, exerts antiinflammatory and free radical quenching effects that may mediate antiepileptogenic efficacy (Scicchitano et al., 2015; Klein et al., 2020). The antiinflammatory drug parecoxib acts by inhibiting the prostaglandin-synthesizing enzyme cyclooxygenase 2 (COX-2) and has been reported to exert disease-modifying activity in a model of acquired epilepsy (Polascheck et al., 2010). Similar effects were reported for the antiinflammatory drug anakinra, an antagonist of interleukin 1 receptors (Klein et al., 2020; Terrone et al., 2020). Losartan, *an angiotensin* II type 1 (AT1) receptor antagonist used in the treatment of hypertension, diabetic nephropathy, and congestive heart failure, exerts both neuroprotective and antiepileptogenic effects that are thought to be mediated by inhibition of albumin-induced transforming growth factor beta (TGFβ) signaling (Friedman et al., 2014; Klein et al., 2020; Löscher and Friedman, 2020). The glutamate N-methyl-D-aspartate (NMDA) receptor subtype antagonist memantine is used for treatment of dementia; like other NMDA receptor antagonists it exerts neuroprotective activity and interferes with the effects of glutamate during epileptogenesis (Löscher and Brandt, 2010). The β-lactam antibiotic ceftriaxone reverses posttraumatic downregulation of glutamate transport in the brain and enhances glutamate clearance in the acute and subacute periods after trauma, when glutamate toxicity is likely first to occur, which is highly relevant for epileptogenesis (Yimer et al., 2019; Klein et al., 2020). The only compound that is not clinically approved is agmatine, which is being studied for several indications such as cardioprotection, diabetes, impaired renal function, neuroprotection (stroke, severe CNS injuries, epilepsy, glaucoma, and neuropathic pain), and psychiatric conditions (depression, anxiety, schizophrenia, and cognition). We included it because of its beneficial activity on oxidative damage, neuroinflammation, and proapoptotic signaling, which is likely to mediate antiepileptogenic efficacy (Neis et al., 2017). The specific combinations of these 14 drugs were chosen "rationally" to combine drugs with different potentially antiepileptogenic mechanism of action in order to produce multitargeted mechanistic drug combinations (Fig. S1).

As described in the Introduction, levetiracetam was included in most combinations due to its multitargeted mechanisms of action (Rogawski et al., 2016) and preclinical as well as clinical evidence of disease-modifying activity in acquired epilepsies (Kaminski et al., 2014; Klein et al., 2020). Clear indication of a disease-modifying effect of levetiracetam was reported for the amygdala kindling model of TLE, in which the effects of the drug on kindling acquisition persisted long after its withdrawal (Löscher et al., 1998; Stratton et al., 2003), while data from post-SE models are inconsistent. In a rat model in which SRS develop

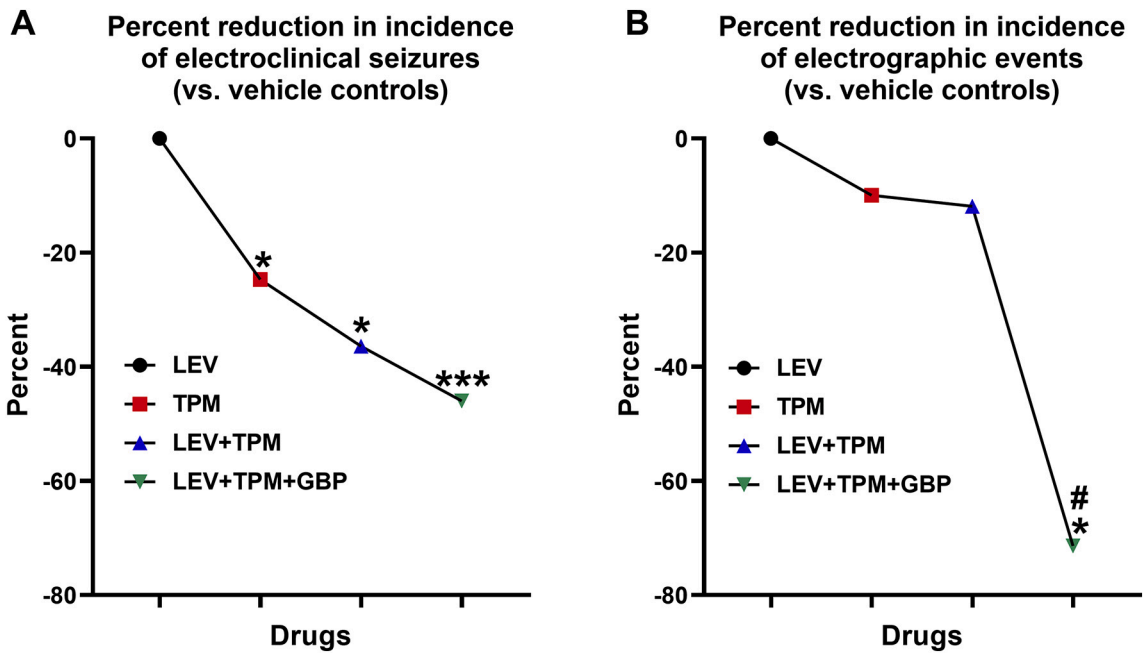

Fig. 9. Efficacy comparison of combination A (levetiracetam, topiramate, gabapentin) with monotherapy (levetiracetam or topiramate) and double combination (levetiracetam and topiramate) in the intrahippocampal kainate mouse model of TLE. Efficacy is shown as percent reduction of incidence of electroclinical seizures (A) or paroxysmal electrographic events (B) vs. individual vehicle control groups, recorded at 12-13 weeks after kainate. Sample size was 11 (levetiracetam alone), 12 (topiramate alone), 11 (levetiracetam and topiramate) and 7 (levetiracetam, topiramate, gabapentin). Size of individual vehicle groups was 8, 13, 14, and 5, respectively. Significant differences to seizure incidence after treatment with levetiracetam alone is indicated by asterisks (*P<0.05; ***P<0.001), whereas significant difference between the triple vs. double combination is indicated by the hash sign ($^{\#}P = 0.0315$). Data for monotherapies and the double combination were taken from the experiments of a recent study (Schidlitzki et al., 2020); doses and onset and duration of treatment were the same as those used for combination A, thus allowing direct comparison of efficacies.

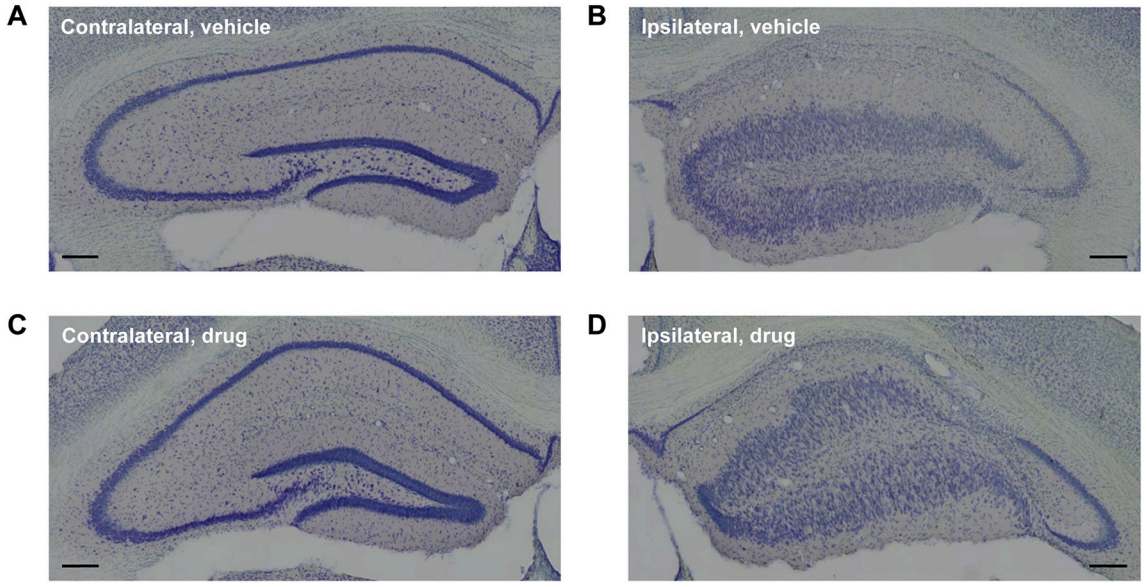

Fig. 10. Representative photomicrographs illustrating neurodegeneration and granule cell dispersion in the ipsilateral (right) vs. contralateral (left) hippocampus of epileptic mice. Thionin-stained coronal hippocampal sections of the contralateral (A, C) and the ipsilateral (B, D) hippocampus at -1.90 mm from bregma are shown. Mice were treated with either vehicle (A, B) or the drug combination levetiracetam, gabapentin and topiramate (C, D) and transcardially perfused 13-14 weeks after kainate. Severe neurodegeneration was observed in the ipsilateral CA1, CA3, and dentate hilus (B, D); furthermore marked granule cell dispersion was observed in the ipsilateral dentate gyrus (B, D). Scale bar = 200 μm.

after electrically induced SE, treatment with levetiracetam during the latent period did not exert any antiepileptogenic or neuroprotective effects (Brandt et al., 2007). In the mouse pilocarpine model of post-SE TLE, treatment with levetiracetam after SE reduced incidence and severity of seizures, BBB disruption, and hippocampal damage (Itoh et al., 2015). In the rat kainate model of post-SE TLE, levetiracetam treatment after SE significantly decreased the mean duration but not frequency of spontaneous electrographic EEG seizures, indicating a disease-modifying effect (Sugaya et al., 2010). Furthermore, marked disease-modifying effects of levetiracetam were observed in different

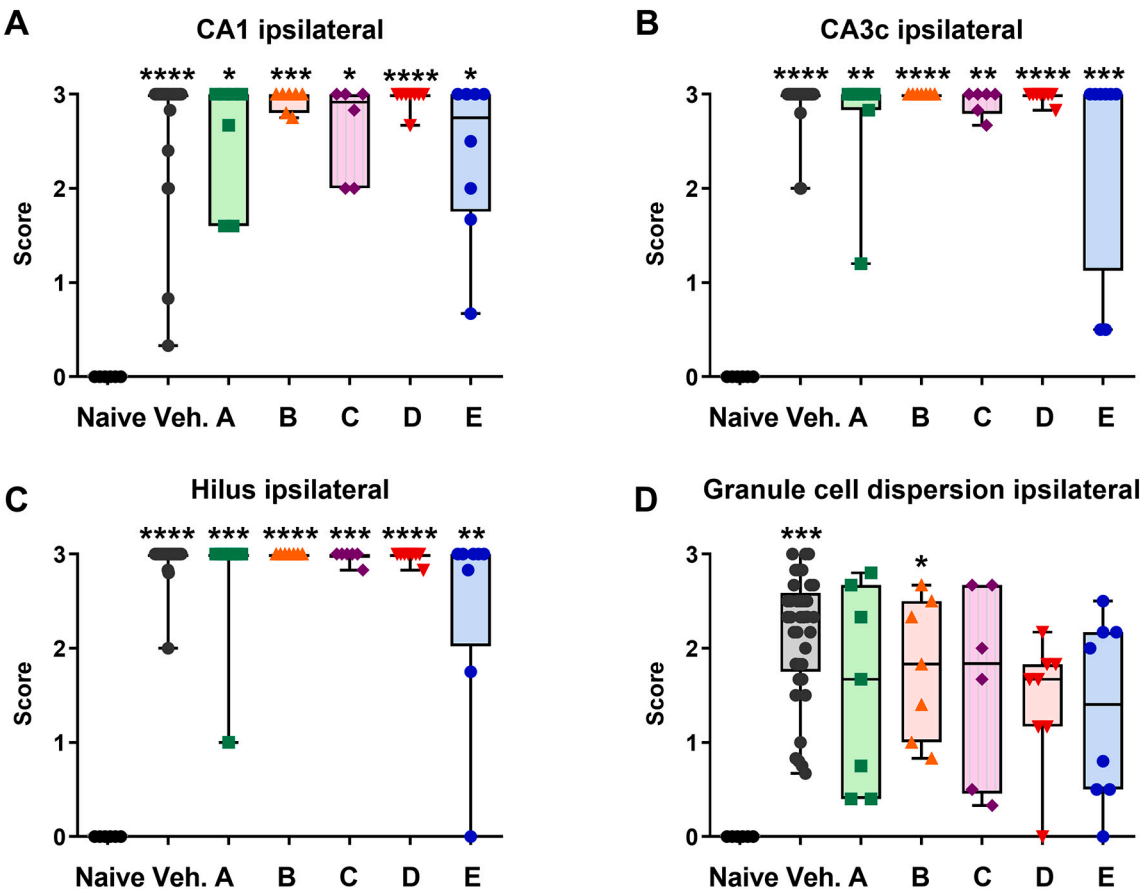

Fig. 11. Treatment with drug combinations after status epilepticus (SE) does not significantly reduce the neurodegeneration or granule cell dispersion in the ipsilateral hippocampal formation. Mice were perfused 13-14 weeks after kainate. In addition to vehicle- and drug-treated mice, naive control mice are shown. Severity scores for neurodegeneration and granule cell dispersion are shown as boxplots with whiskers from minimal to maximal values; the horizontal line in the boxes represents the median value; in addition, individual data are shown. Sample size was 6 (naïve), 39 (vehicle), 7 (combination A), 7 (B), 6 (C), 8 (D), and 8 (E) mice, respectively. Significant differences to naive controls are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). None of the drug-treated groups significantly differed from vehicle controls.

TBI models (Chen et al., 2016; Browning et al., 2016; Caudle et al., 2016; Chen et al., 2018), which has been critically discussed in detail recently (Klein et al., 2020; Löscher, 2020). However, in apparent contrast to these previous studies, treatment with levetiracetam alone did not significantly modify epileptogenesis in the intrahippocampal kainate mouse model (Schidlitzki et al., 2020). Thus, overall it is questionable whether levetiracetam alone exerts any true antiepileptogenic effect.

Several previous studies indicated that epilepsy is difficult to prevent or modify in the intrahippocampal kainate model, including studies with glutamate receptor antagonists (Twele et al., 2015; Schidlitzki et al., 2017), mTOR antagonists (Shima et al., 2015; Gericke et al., 2020), an inhibitor of adenosine kinase (Sandau et al., 2019), genetically engineered cells (Ali et al., 2017), and genetic manipulation of urokinase-type plasminogen activator receptor (Ndode-Ekane and Pitkänen, 2013) or BDNF-mediated TrkB signaling (Heinrich et al., 2011). This may be related to the double-hit insult produced in this model by the traumatic insult caused by surgical implantation of the EEG electrode into the hippocampus and the intrahippocampal injection of the excitotoxic kainate (Brackhan et al., 2018). Similar to mesial TLE in patients, this double-hit insult produces marked BBB disruption, neuroinflammation, and neurodegeneration in the ipsilateral hippocampus and associated areas as reported previously (Riban et al., 2002; Pernot et al., 2011; Zattoni et al., 2011; Bitsika et al., 2016; Brackhan et al., 2018). Thus, to our knowledge, combination E described in the present study exerted the most pronounced antiepileptogenic effect reported as yet in this model.

Unfortunately, not all drug combinations examined in the present study could be included in the final analysis, as the incidence of epilepsy was too low in two of the vehicle groups (of combinations F and H) and one drug combination (G) was too toxic. The toxicity of combination G (valproate, losartan, memantine) could be decreased by reducing the dose of memantine during the course of the experiment, but sample size was already too low to allow any meaningful analysis of data. Recent preclinical data indicate that memantine and losartan protect the integrity of the BBB when administered in combination (https://www.israel21c.org/novel-combination-therapy-treats-neurological-disorders/). Thus, we plan to repeat experiments on this combination with lower doses of memantine. Also, combinations F and H need to be re-evaluated in another batch of mice.

Of the five combinations with eight drugs that could be analyzed for antiepileptogenic efficacy, a combination of low doses of levetiracetam, atorvastatin and ceftriaxone (combination E) was more effective to prevent and modify epileptogenesis than several combinations evaluated in the present and previous studies (Table S3). As shown in Table S3, the combination of reduced doses of levetiracetam, atorvastatin and ceftriaxone (combination E) markedly decreased the incidence of electrographic seizures (HPDs) both at 4 and 12 weeks after kainate and, more importantly, the incidence of electroclinical seizures at 12 weeks after kainate. Furthermore, it significantly decreased the frequency of electrographic seizures. Such pronounced effects on both electrographic and electroclinical seizures were not observed with any other drug combination, including our previously reported

combinations (Schidlitzki et al., 2017; Schidlitzki et al., 2020).

When examining the mechanisms of action of levetiracetam, ceftriaxone and atorvastatin in more detail, a unique and likely synergistic combination of mechanisms evolves (Table S4), which may explain the striking antiepileptogenic effects of this combination. For all three drugs, antiinflammatory, anti-oxidative and neuroprotective effects have been reported, but additional mechanisms, e.g., modulation of presynaptic neurotransmitter release via SV2A (levetiracetam), postsynaptic effects at GABA and glutamate receptors (levetiracetam), reduction of BBB leakage (levetiracetam), and anti-excitotoxic effects by altering astroxytic glutamate receptors (ceftriaxone) or cell cholesterol homeostasis (atorvastatin) are drug specific and likely add to the synergistic efficacy of the combination. Thus, it is unlikely that the efficacy of this drug combination is due to drug interactions at a single target. More likely, it may be due to the multitargeted (network pharmacological) action of the three drugs.

Interestingly, the antiepileptogenic efficacy was only observed after decreasing the doses of the initially tested combination (D) by 70%, which would indicate dose-specific synergistic drug-drug interaction as typically observed in network pharmacology (Ainsworth, 2011). The idea of decreasing the doses of combination E was based on the observation that the initially tested combination D with the high doses of levetiracetam, atorvastatin and ceftriaxone, which were based on the literature (Table 1), tended to exert pro-epileptogenic effects (see e.g. Fig. S9D). Cephalosporins are known to have proconvulsant activity and may precipitate seizures at high doses (Sander and Perucca, 2003), whereas such activity is not known for levetiracetam or atorvastatin. The 70% dose reduction was chosen because in case of synergistic pharmacodynamic drug-drug interactions between three drugs, each drug should be effective at one third of its dose (or lower) compared to the doses of the drugs used alone (Niu et al., 2019), which is in line with the principles of network pharmacology (Ainsworth, 2011).

Remarkably, although combination E reduced the incidence of HPDs at both 4 and 12 weeks, incidence of electroclinical seizures was only reduced at 12 weeks, which could indicate that this drug combination exerted a lasting inhibitory effect on the progression from focal electrographic seizures to clinical seizures. An alternative explanation would be that the frequency of electroclinical seizures was reduced so markedly that no seizures could be recorded during the one-week EEG recording period at 12 weeks after kainate. In theory, combination E could also have induced regression or remission of the disease. This would need to be confirmed with longer continuous video-EEG monitoring.

Of the three drugs in combination E, we have so far only tested levetiracetam alone in the intrahippocampal kainate mouse model (Schidlitzki et al., 2020). At 200 mg/kg t.i.d., this drug exerted no significant antiepileptogenic effects. We cannot exclude that ceftriaxone or atorvastatin would exert significant antiepileptogenic effects when administered alone, although we consider this unlikely, particularly at the low doses used in combination E. To our knowledge, neither ceftriaxone nor atorvastatin have been previously evaluated in the intrahippocampal kainate mouse model. In the only preclinical test of ceftriaxone's antiepileptogenic capacity, treatment with 200 mg/kg/d for one week starting 30 minutes after TBI (lateral fluid percussion injury) in rats restored astrocytic glutamate transporter 1 (GLT-1) expression in the lesioned cortex to near normal levels, reduced posttraumatic astroglial activation seven days after TBI by 43%, and reduced seizure frequency 12 weeks after injury from 151 seizures/24 hours to 47, and seizure duration by 19% (Goodrich et al., 2013). For atorvastatin (10 mg/kg), disease-modifying effects were reported for the pilocarpine rat model of TLE (Oliveira et al., 2018), whereas no effects were observed in an electrically induced SE model of TLE in rats (van Vliet et al., 2011).

Importantly, for two of the three drugs in combination E, some clinical evidence of antiepileptogenic or disease-modificating effects exists (Klein et al., 2020). Three clinical studies, including a large study on post-stroke epilepsy, suggest possible antiepileptogenic effects of statins such as atorvastatin (Pugh et al., 2009; Etminan et al., 2010; Guo et al., 2015). Similarly, two human studies indicated an antiepileptogenic effect of levetiracetam (Jehi et al., 2012; Klein et al., 2012), although the effect of levetiracetam in the pilot study of Klein et al. (2012) on development of posttraumatic epilepsy (PTE) was suggestive rather than statistically significant in that 20% (8/40) of the untreated patients developed PTE vs. 10.9 % (5/46) of the treated patients ($P = 0.18$).

Similar to our previous antiepileptogenesis experiments in the intrahippocampal kainate mouse model (Schidlitzki et al., 2017; Schidlitzki et al., 2020), the duration of treatment after kainate was restricted to five days, as this corresponds to the latent period before onset of SRS in male NMRI mice in this model (Twele et al., 2016b). We cannot exclude that longer treatment would have been more effective, yet for an antiepileptogenic effect, treatment during the latent period should be sufficient. As in our previous studies, treatment was started six hours after intrahippocampal injection of kainate to avoid the possibility that the drug treatment interfered with the kainate-induced SE development and thus to minimize any initial insult-modifying effect. As shown recently (Schidlitzki et al., 2020), the excitotoxic kainate itself, the kainate-induced SE or both damage hippocampal neurons within six hours, before the onset of drug treatment, which explains that treatments starting six hours after kainate do not prevent neurodegeneration in this model. Thus, in order to target the neurodegenerative consequences of kainate, treatment should start as early as possible after kainate. This, however, could result in initial insult modification rather than an antiepileptogenic effect (Löscher and Brandt, 2010; Galanopoulou et al., 2012).

The present findings and previous studies (e.g., (Brandt et al., 2004; Brandt et al., 2003; Schidlitzki et al., 2020) indicate that neuroprotection may not be necessary for prevention or reduction of SRS in models of acquired epilepsy, at least when using SE as the initial brain insult. Interestingly, in a recent study in which a combination of the AMPA receptor antagonist NBQX and the NMDA receptor antagonist ifenprodil was administered during the latent period, starting six hours after kainate, reduced granule cell dispersion, less neuronal degeneration in the dentate hilus and less electroclinical seizures were observed two weeks following kainate, but these effects were lost at subsequent weeks (Schidlitzki et al., 2017). Such early neuroprotective effects were not observed for any of the drug combinations evaluated here, when neurodegeneration was assessed one week after intrahippocampal kainate injection (Welzel et al., 2019).

We have recently reported that a combination of levetiracetam and topiramate modifies the development of epilepsy when administered during the latent period following kainate in mice (Schidlitzki et al., 2020). Adding gabapentin to levetiracetam and topiramate (combination A) was much more effective to reduce the incidence of paroxysmal EEG events (HVSWs and HPDs) than the double combination, which exerted no significant effects on the development of such events (Schidlitzki et al., 2020). Also, as shown in Fig. 9, the triple combination was more effective to reduce the incidence of electroclinical seizures. Furthermore, the analysis of overlap in the occurrence of electrographic and electroclinical seizures in vehicle and treatment groups (Fig. 8) showed that combination A (levetiracetam, topiramate, gabapentin) was quite effective in reducing the incidence of mice that exhibited both types of seizures. However, as shown in Table S3, combination E (low dose levetiracetam, atorvastatin and ceftriaxone) was clearly more efficient in preventing or modifying epilepsy than either combination A or the combination of levetiracetam and topiramate. The other drug combinations, (levetiracetam and α-tocopherol, levetiracetam, deferoxamine, gabapentin, fingolimod) were ineffective. Thus, although *in silico* bioinformatic approaches and database mining for drug repurposing are useful tools for predicting drug combinations for potential clinical uses (Sun et al., 2016), they cannot replace *in vivo* experiments in adequate preclinical models.

Repurposing (or repositioning) of approved drugs has recently gained new momentum for rapid identification and development of new therapeutics for diseases that lack effective drug treatment (Nosengo, 2016; Sun et al., 2016). A recent report lists 118 repurposed drug products for 203 new CNS indications prior to January 2016; 102 approved and 101 in development (Clout et al., 2019). Drug repurposing holds the potential to bring medications with known safety profiles to new patient populations. Drug combinations of two or more compounds with different mechanisms of action increase successful drug repositioning (Sun et al., 2016). The use of drugs in combination can produce a synergistic effect if each of the drugs impacts a different target or signaling pathway that results in reduction of required drug doses for each individual drug. Over the past decades, multitargeted and combinatorial therapies achieved considerable therapeutic efficacy by modulating the activities of the targets in complex diseases such as HIV-1 infection, cancer, asthma and diabetes mellitus (Muhammad et al., 2018). In neurology, several repurposed drugs, including statins, and their combinations are currently being investigated as potential disease-modifying treatments for Parkinson's disease (Athauda and Foltynie, 2018). Furthermore, clinical trials on combinations of repurposed drugs, including losartan and atorvastatin or memantine and donepezil, are currently being performed in Alzheimer's disease (Cha et al., 2018; Ihara and Saito, 2020). In clinical epilepsy, drug repurposing has become an important strategy in the treatment of patients with therapies targeted to their specific pathophysiology (Demarest and Brooks-Kayal, 2018). One important example is the use of the ASD vigabatrin for prevention or modification of epilepsy in patients with tuberous sclerosis complex (Jozwiak et al., 2020).

As outlined in the Introduction, the strategy (illustrated in Fig. 1) that we used to identify novel antiepileptogenic combinations of repurposed drugs consisted of (1) selection of drugs (approved for other therapeutic indications) based on their mechanism of action and previous evidence of disease-modifying activity in epilepsy models; (2) forming diverse potentially synergistic combinations with these drugs, both based on mechanism of action and *in silico* (STITCH) analyses; and (3) systematic evaluation of these novel combinations of repurposed drugs in a battery of mouse experiments for tolerability and antiepileptogenic efficacy, taking pharmacokinetic aspects into account. In the ~6 years since we started, this strategy identified three interesting combinations, i.e., levetiracetam and topiramate (Schidlitzki et al., 2020) and the two efficacious combinations (A and E) presented here. Several other combinations, including a combination of ifenprodil and NBQX (Schidlitzki et al., 2017), a combination of levetiracetam and phenobarbital (Schidlitzki et al., 2020) and most of the combinations evaluated here were found to be either ineffective or too toxic. Thus, these data emphasize that - for identifying antiepileptogenic drug combinations - a systematic approach is needed and one preferably that can prioritize drugs and combinations that will likely bring about positive results. However, several aspects are not addressed by our approach, including age, sex, different preclinical models of different types of epilepsy, and the impact of different doses (except for combination E) and onset and duration of treatment. These aspects need to be addressed in the future for the most efficacious combination(s) identified by our strategy.

One limitation of the present study is the relatively small group size for each drug combination, resulting in relatively low statistical power to identify significant effects on incidence of SRS, whereas the power to determine significant effects on seizure frequency was sufficiently high. Rather than using historical controls, as often done in preclinical trials on antiepileptogenic treatments, we used a batch-matched vehicle control group with each drug-treated group. This reduced the size of the treatment group, as the maximal capacity of our video-EEG monitoring system limits monitoring to 16-18 animals at a time. However, if historical controls had been used, we would have falsely interpreted combinations F and H as having a high antiepileptogenic efficacy. With batch-matched controls, both vehicle and drug groups of these experiments exhibited a low incidence of epilepsy.

A second limitation is that the relatively low frequency of electroclinical seizures (on average 6.8 per week) and the short one-week period of continuous video-EEG monitoring at 12-13 weeks after kainate may lead to chance effects of treatments. However, transient treatment with combination E shortly after kainate also significantly reduced the frequency of the much more frequent electrographic seizures at both 4-5 and 12-13 weeks after kainate, which makes a random observation unlikely.

A third potential limitation is that we cannot exclude that the antiepileptogenic effects of combination E were just due to delaying the epileptogenic process, because epileptogenesis was still progressing at 12 weeks after kainate. However, the main effect of combination E was on incidence and frequency of electroclinical seizures, which did not significantly differ in vehicle controls at 12 vs. 4 weeks post-kainate. Thus, we believe that the effects of combination E represent "true" antiepileptogenic activity.

A fourth limitation is that, except for levetiracetam (Schidlitzki et al., 2020), the drugs of the efficacious combination E were not tested alone or in combinations of only two of the three drugs in combination E. Thus, at present this is a limitation that undermines extrapolation of which is the key mechanism for combination E and the very few other combinations that showed some effects and also limits the option of reducing the number of drugs to absolute necessary. Thus, an important next step will be to examine this aspect in more detail. However, as shown in Fig. 9, we performed an efficacy comparison of combination A (levetiracetam, topiramate, gabapentin) with monotherapy (levetiracetam or topiramate) and double combination (levetiracetam and topiramate), which indicated the highest efficacy for the triple combination. A similar analysis is planned for combination E.

Fifth, we did not perform pharmacokinetic analyses of potential drug-drug interactions. We recently showed that such interactions do not affect a combination of levetiracetam and topiramate (Schidlitzki et al., 2020), and similar pharmacokinetic experiments are also planned for combination E of the present study. However, using the Drug Interactions Checker (https://www.drugs.com/drug_interactions.html), no pharmacokinetic interactions between levetiracetam, ceftriaxone and atorvastatin were found. Indeed, the three drugs used in combination E have a very favorable pharmacokinetic profile, with no known drug-drug interaction, which would add to their attraction for human translation. All three medications are in very common use and therefore frequently used together in the clinic, e.g., levetiracetam with atorvastatin (epilepsy and hyperlipidemia), levetiracetam and ceftriaxone (seizures/epilepsy and meningitis) and ceftriaxone and atorvastatin (bacterial infections and hyperlipidemia).

Combination E (reduced doses) should be retested with larger group sizes to confirm the data, as done recently for the combination of levetiracetam and topiramate (Schidlitzki et al., 2020). Our aim in this study was to test as many rationally chosen and tolerable drug combinations as possible. However, as these experiments are very labor-intensive, small groups are better suited for initial screening. To our knowledge, the marked antiepileptogenic effect we saw with combination E here has not been reported before for any other treatment in the intrahippocampal kainate mouse model or other rodent models of acquired epilepsy. We plan to evaluate the antiepileptogenic efficacy of combination E in a rodent model of TBI-induced epilepsy. A positive outcome would facilitate translation of this combination to the clinic.

The intrahippocampal kainate mouse model used in the present study is widely used as a model of mesial TLE to study antiseizure or antiepileptogenic effects of novel treatments (Guillemain et al., 2012; Löscher, 2016; Duveau and Roucard, 2017; Sandau et al., 2019; Löscher, 2020). The high frequency of paroxysmal focal electrographic events (HPDs and HVSWs) recorded from the hippocampal kainate focus is an advantage both for studies on antiseizure drugs and for studies on prevention or modification of epilepsy. HPD- or HVSW-like EEG patterns do not occur in sham-treated nonepileptic mice (Twele et al., 2017). We

have previously compared the characteristics of HPDs and HVSWs in the intrahippocampal kainate model of TLE with those of nonconvulsive seizures (NCS) in humans with TLE and found many similarities (Twele et al., 2016a)(see also more detailed discussion below). However, the main difference to epilepsy patients is certainly the high frequency of the HPDs and HVSWs in the kainate mouse model, which may be a consequence of the direct infusion of kainate into the hipocampus and the resulting chronic alterations in hippocampal structure and functionality (Duveau and Roucard, 2017). In addition to the frequent focal electrographic seizures, we also quantified the less frequent electroclinical seizures, which are commonly used in other epilepsy models as outcome measure (Löscher, 2020). Interestingly, as shown in Fig. 8, the long-term consequences of short-term treatment with combination E on electrographic and electroclinical seizures differed, which could either indicate that the mechanisms underlying electrographic and electroclinical seizures are different or, as discussed above, that this drug combination reduced the progression from focal electrographic seizures to clinical seizures or even reversed the development of the epileptic condition.

One may question whether HVSWs are electrographic (or nonconvulsive) seizures or rather an interictal pattern as initially suggested by Riban et al. (2002). The latter group argued that HVSWs are dissociated from seizures but they may initiate HPDs, whereas HPDs are focal NCS resembling hypersynchronous high-voltage spikes observed in sclerotic hippocampus of patients with TLE, particularly when this structure is the focus of epileptic activity. Indeed, HVSWs have a relatively low SW frequency ($\geq$2 Hz) and are often monomorphic which, however, does not argue against a definition as NCS, as for instance classical spike-wave discharges of absence can be $\geq$2 Hz and sometimes relatively monomorphic but are considered seizures. Indeed, in subsequent studies by other groups, both HVSWs and HPDs were considered epileptic seizures (e.g., Maroso et al., 2011).

We recently compared the characteristics of clinical NCS (recorded with depth electrodes in the intensive care unit; cf., Sinha and Hirsch, 2014) with those of HVSWs, HVSWs fulfilled several of the features of NCS (Twele et al., 2016a). As discussed in this previous study, even though HVSWs often lack any clear evolution, this does not argue against the possibility that they represent electrographic seizures or NCS. Monomorphic focal EEG seizures are not uncommon in patients with different types of epilepsy (Ikeda et al., 2009; Nickels et al., 2012; Butler et al., 2013) and have also been described in other animal models of acquired epilepsy, such as the perinatal hypoxia model of epilepsy (Rakhade et al., 2011; Lippman-Bell et al., 2013). Furthermore, the fact that HVSWs can be suppressed by rapidly acting ASDs such as diazepam (Klein et al., 2015; Twele et al., 2016a) would be consistent with a NCS definition (Sinha and Hirsch, 2014).

However, because the issue on whether HVSWs are interictal or ictal phenomena cannot be resolved yet, it is best to use the term ictal/interictal pattern or ictal/interictal continuum for these paroxysmal EEG events. Continuous EEG monitoring is becoming increasingly used in neurologic and non-neurologic intensive care units, allowing to detect and define electrographic seizures in critically ill patients (Rubinos et al., 2018). In patients with acute brain injury, EEG findings that are highly associated with seizures but do not qualify as definitive seizures by strict criteria are considered to lie on the ictal-interictal continuum (Singla et al., 2020). These findings, which encompass periodic and rhythmic patterns, are common in such patients and may lead to secondary brain injury, thus warranting treatment. The present data on HVSWs may be useful clinically as effective treatments for these conditions and patterns are needed (Rubinos et al., 2018).

For determination of drug effects, HPDs and HVSWs are often counted together (e.g., Maroso et al., 2011; Duveau et al., 2016). This was avoided in the present study, but both events were calculated and illustrated separately. Importantly, as illustrated in Table S3, when only HPDs would have been used as NCS (or electrographic seizures), the outcome of our experiments would have been the same.

As pointed out above, we plan to examine the most effective treatment (combination E) in other models of acquired epilepsy. If combination E is effective in a TBI model of posttraumatic epilepsy, we will perform pharmacokinetic studies and determine plasma levels. Clinical dosing in a subsequent human PTE prevention study would be derived from targeted plasma levels, which can be used for allometric scaling and dose conversion between animals and humans (Nair and Jacob, 2016). The well known and relatively benign human clinical tolerability, safety and pharmacokinetic profiles of levetiracetam, ceftriaxone and atorvastatin may be of advantage in potential human translation of prevention of epilepsy after acute brain injury, for instance of post-traumatic epilepsy.

In conclusion, combination of low doses of three repurposable drugs (levetiracetam, atorvastatin, ceftriaxone) from different therapeutic areas with different antiepileptogenic mechanisms of action, exhibited potent antiepileptogenic effects in a mouse model of acquired TLE. These three drugs affect various epileptogenesis-related targets, thus providing a novel network pharmacology approach for epilepsy prevention or modification following brain injury. As predicted for network pharmacology (Löscher et al., 2013), this novel drug combination may be a promising strategy for epilepsy prevention in humans. The data presented here represent first exploratory studies that need further documentation with increased sample sizes and pharmacokinetics and different doses of the drugs.

Declaration of Competing Interest

Drs Klein and Löscher are co-founders as well as CEO and CSO, respectively, of PrevEp, Inc. (Bethesda, MD, USA). PrevEp did not fund this research and played no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. All other authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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