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Long term chemogenetic suppression of spontaneous seizures in a mouse model for temporal lobe epilepsy

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Summary

Objective

More than one third of patients with Temporal Lobe Epilepsy (TLE) continue to have seizures despite treatment with anti-epileptic drugs and many experience severe drug related side effects, illustrating the need for novel therapies. Selective expression of inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) allows cell-type specific reduction of neuronal excitability. In this study we evaluated the effect of chemogenetic suppression of excitatory pyramidal and granule cell neurons of the sclerotic hippocampus in the intrahippocampal mouse model (IHKA) for temporal lobe epilepsy.

Methods

IHKA mice were injected with an adeno-associated viral vector carrying the genes for an inhibitory DREADD hM4Di in the sclerotic hippocampus or control vector. Next, animals were treated systemically with different single doses of CNO (1, 3, 10 mg/kg) and clozapine (0.03 and 0.1 mg/kg) and the effect on spontaneous hippocampal seizures, hippocampal EEG power, fast ripples (FRs) and behavior in the open field test was evaluated. Lastly, animals received prolonged treatment with clozapine for three days and the effect on seizures was monitored.

Results

Treatment with both CNO and clozapine resulted in a robust suppression of hippocampal seizures for at least 15 hours only in DREADD-expressing animals. Moreover, total EEG power and the number of FRs significantly reduced. CNO and/or clozapine had no effects on interictal hippocampal EEG, seizures or locomotion/anxiety in the open field test in non-DREADD epileptic IHKA mice. Repeated clozapine treatment every eight hours for three days resulted in almost complete seizure suppression in DREADD animals.

Significance

This study shows the potency of chemogenetics to robustly and sustainably suppress spontaneous epileptic seizures and pave the way for an epilepsy therapy in which a systemically administered exogenous drug selectively modulates specific cell types in a seizure network, leading to a potent seizure suppression devoid of the typical drug related side effects.

Key words

DREADD, hM4Di, clozapine, Hippocampus, Intrahippocampal Kainic Acid

Key points box

- Single dosing of CNO & clozapine to activate an inhibitory DREADD in excitatory hippocampal neurons suppresses seizures for at least 15 hours in the IHKA mouse model
- Selective inhibition of excitatory hippocampal neurons reduces total EEG power and number of pathological hippocampal fast ripples in sclerotic hippocampus
- Chemogenetic inhibition of sclerotic hippocampus has no overt side-effects on locomotion and anxiety in the open field test in non-DREADD animals
- Repeated administration of subclinical clozapine doses resulted in an almost complete and sustained seizure suppression for three days

Introduction

Epilepsy is one of the most common neurological disorders, characterized by spontaneously recurring epileptic seizures. More than one third of patients continue to have seizures despite best available treatment with anti-epileptic drugs (AED) and many patients suffer from unacceptable drug related side effects[1]. Therefore, research towards new means to suppress seizures is very important.

Recent technological advances allow modulation of local neuronal populations with unprecedented cell specificity using light-activated ion channels (optogenetics) or Designer Receptors Exclusively Activated by Designer Drugs (DREADDs, chemogenetics).

Optogenetic trials have demonstrated the possibility to interrupt ongoing seizures upon local light application. With this approach however seizures still occur but they are quickly aborted[2–4]. Current translational downsides of optogenetics are the requirement for implantation of optic fibers, the limited penetrance of light in brain tissue and the need for a very sensitive closed loop system to trigger light application upon detection of an epileptic seizure.

Chemogenetics involve the selective expression of modified receptors, such as DREADDs, in specific cell types and regions of the brain, allowing selective modulation of neuronal populations using systemically applied drugs[5]. A selective promotor system carried by a locally injected recombinant adeno-associated viral (rAAV) vector achieves region- and cell-type specific

expression of DREADDs. The most commonly used DREADDs are modified human muscarinic receptors engineered to be insensitive to the native ligand acetylcholine but activated by drugs such as clozapine and its inert breakdown product Clozapine-N-Oxide (CNO). Depending on the type of DREADD, neurons can be selectively activated or inhibited. hM3Dq is a DREADD coupled to Gq signaling thus activating neurons, in contrast to hM4Di which induces Gi signaling leading to neuronal and synaptic silencing[5–8]. Several preclinical studies demonstrated potent effects of DREADD-based treatments in epilepsy models. Seizure activity could be suppressed both by hM3Dq mediated activation of inhibitory interneurons[9,10] as well as hM4Di mediated inhibition of excitatory neurons both in vitro[10,11] and in vivo[9,12–14]. In kainic acid and pilocarpine rodent models for temporal lobe epilepsy (TLE) chemogenetic suppression of excitatory neurons successfully lead to a suppression of spontaneous seizures[9,13].

In this study we applied chemogenetics in the intrahippocampal kainic acid mouse (IHKA) model for drug refractory TLE. We selectively targeted Ca^{2+}/calmodulin-dependent kinase II alpha (CamKIIα) expressing pyramidal and granule cells in the sclerotic hippocampus by selective expression of hM4Di receptors. Both CNO- and clozapine-induced silencing of these neurons reduced total power of hippocampal EEG, decreased the number of fast ripples (FRs) and suppressed hippocampal seizure activity. Moreover, in non-DREADD animals no effects on seizures, interictal EEG or side-effects on locomotion and anxiety in the open field test were observed. These results support a crucial role for excitatory hippocampal neurons in the generation of FRs and spontaneous seizures and provide an approach for the development of a novel epilepsy therapy, devoid of the typical drug related side effects.

Methods

Animals

Male C57BL/6 Jola Hsd mice were obtained from Envigo (Horst). All animals were housed at controlled temperature (21-22°C) and relative humidity (40-60%) conditions with a fixed 12-hour light/dark cycle (light on between 6AM–6PM) and food and water ad libitum. The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University (ECD16/31). Treatment and care were in compliance with the ARRIVE guidelines. All animals were 8 weeks old at the start of the experiments.

Intrahippocampal kainic acid (KA) injection

Mice (n=108) were anesthetized with isoflurane and their head was immobilized in the stereotactic frame. KA (200 ng in 50 nl saline, R&D systems) was infused unilaterally in the dorsal hippocampus (coordinates relative to bregma -2.0 mm anterioposterior (AP), +1.5 mm mediolateral (ML), -1.8 mm dorsoventral (DV)). After waking up from anesthesia mice developed a status epilepticus as consequence of the KA injection.

Intrahippocampal electrode implantation and adeno-associated viral vector (rAAV) injection

At least two weeks after KA injection all mice were again anesthetized with isoflurane for implantation of EEG electrodes. A bipolar recording electrode (200 µm tip separation) was implanted at the site of KA injection as well as an epidural recording electrode over ipsilateral parietal cortex. In addition, an epidural ground/reference electrode over ipsilateral frontal cortex and 3 anchor screws were placed. A subset of animals (n=26) were implanted with a cannula above the hippocampus (-0.1 mm DV) which allowed to record hippocampal EEG before injection of rAAV and to evaluate the effect of DREADD-expression on seizures. The other IHKA mice (n=82) were injected with rAAV at the same time of electrode implantation (rAAV2/7-CamKIIα-hM4Di-mCherry, 500 nl, 2.7E+13 genome-copies/ml, DREADD group or rAAV2/7-CamKIIα-mCherry, 500 nl, 2.1E+13 genome-copies/ml, non-DREADD group).

EEG recording

Two weeks after electrode implantation mice were connected to the EEG setup. This setup consisted of a headstage with a unity gain preamplifier, a 6-channel cable, commutator and an

amplifier (512x). Signals were digitized at 2kHz with a data acquisition card and stored on a PC for off-line analysis. Electrographic seizures were defined as a repetitive pattern (>2 Hz) of complex, high amplitude EEG spikes that lasted for a minimum of 7 seconds and are separated by at least 7 seconds from a previous seizure (representative example shown in Figure 2). Mice with clear electrographic hippocampal seizures were selected to evaluate effects of chemogenetic treatment on seizures and interictal EEG. Unfortunately, only in 32/108 mice clear hippocampal seizures could be recorded. Four of these 32 mice were implanted with a cannula. In total 21 mice were in the DREADD group (4 implanted with cannula, 17 without cannula) versus 11 mice in the non-DREADD group). Next, all animals were treated with different doses of CNO (1 mg/ml, 0.3% DMSO in saline) and clozapine (0.01 mg/ml, 3% DMSO in saline) between 4-8 hours after start of the light-on cycle (an overview of treatments in supplementary).

Effect on interictal EEG

Five seizure-free (interictal) one-minute EEG segments were randomly selected from two one-hour epochs, the first starting 24 hours before and the second 30 minutes after injecting CNO (10 mg/kg in n=21, 0.3% DMSO in saline). We evaluated the effect of chemogenetic inhibition on hippocampal EEG power and FRs in respectively 6/21 and 8/21 DREADD mice and 6/11 non-DREADD animals. FRs were detected in the hippocampal EEG electrodes after bandpass filtering between the signal 200-500 Hz. FRs were defined as transient oscillatory events consisting of at least 4 oscillations with amplitudes larger than 4 standard deviations of the ongoing signal. For power spectral analysis the differential EEG signal obtained from the two hippocampal electrodes was low-pass filtered (0.5 Hz, first-order Butterworth), segmented into 2-second sweeps with 50% overlap and windowed (Blackman-Harris) where after the FFT delivered the power spectrum. For each animal a mean was constructed from 80 realizations. Between animal variation was so small that mean spectra of the population could be constructed to compare before and after CNO treatment. Effects on FRs and EEG spectral power were evaluated in a two-way ANOVA.

Effect on spontaneous hippocampal seizures

Per hour the fraction of time spent in seizures (FTS) was calculated. To evaluate effects of viral vector injection or DREADD expression on seizure activity FTS was compared before and three weeks after injection with rAAV in four mice (see higher) using a paired t-test. DREADD

animals received single intraperitoneal injections of 1, 3 and 10 mg/kg CNO and 0.03 and 0.1 mg/kg clozapine. In non-DREADD animals the highest doses of CNO and clozapine were administered (an overview of treatments in supplementary). Effects were compared to a baseline FTS value recorded during four hours the days before treatments. First the effect of the highest CNO and clozapine doses were compared between DREADD and non-DREADD animals using the MIXED procedure in IBM SPSS Statistics[15]. Random effects models were constructed with FTS as dependent variable, subject ID as random factor, group, time and group by time interaction as fixed factors. To compare different doses similarly a random effects model was constructed with FTS as dependent variable, subject ID as random factor, dose, time and dose by time as fixed factors. The autoregressive moving average model was the used covariance structure in both models.

Sustained treatment with 0.1 mg/kg clozapine, every eight hours for three days, was evaluated by comparing averaged FTS/hour calculated for eight-hour post-injection epochs with the FST/hour during an eight-hour baseline period. Again, a random effect model was constructed with averaged FTS values as dependent variable, subject ID as random factor, dose and treatments as fixed factors and the first-order ante-dependence covariance structure was selected. Bonferroni correction was used in all post hoc tests.

Effect on locomotion and anxiety-related behavior

A subset of non-DREADD mice (n=7) were used to test whether the highest doses of DREADD agonists used to suppress seizures in DREADD animals, had effects on locomotion and anxiety in the open field test (OFT). Mice were habituated to the arena (60x60x35 cm, 5x5 squares) for 10 minutes on three days during their active period. On the fourth day, one hour before the test, 10 mg/kg CNO, 0.1 mg/kg clozapine or vehicle was administered, and animals were tested in the field for 10 minutes. A cross-over design was used, and testing days were separated by one habituation day. For automatic, unbiased behavioral analysis of video-files commercial software (Ethovision XT 11.5, Noldus) was used. The fraction of time an animal spent in the center of the field (inner 9 squares) was analyzed during the first 5 minutes of the test to evaluate effects on anxiety. Distance travelled was evaluated during 10 minutes in the field. Effects were compared in a one-way ANOVA test.

Immunohistochemistry

Mice were transcardially perfused with phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde/0.1M phosphate buffer solution. Brains were cryoprotected with 30% sucrose, snap-frozen in isopentane and coronal sections were made using a microstat (Leica). Sections were washed twice and kept for 30 and 60 minutes in 0.5% and 1% H_2O_2. After two PBS wash steps sections were permeabilized and blocked in 0.2% Trition-X-100/0.4% Fish Skin Gelatin (=blocking buffer) after which they were transferred into the primary antibody solution (rabbit anti-RFP, 1:2000, Rockland 600-401-379) and kept overnight. Next, sections were washed twice in blocking buffer followed by 1h in secondary antibody (goat anti-rabbit Alexafluor 594, 1:1000, abcam ab150088). After two PBS washing steps, a nuclear DAPI staining was performed (1 µg/ml, Sigma-Aldrich) and slices were mounted on glass slides. Pictures taken with a fluorescent microscope were stitched using the Fiji stitching tool[16].

Data Analysis

All data are presented as mean ± standard error of the mean (SEM). A P-value<0.05 was assumed for rejection of the null hypothesis.

Results

To evaluate whether hM4Di expression in excitatory hippocampal neurons has constitutive effects on seizure activity in IHKA mice the fraction of time in seizures (FTS), determined during four hours spread over one day, was compared before and three weeks after injection of rAAV2/7-CamKIIα-hM4Di-mCherry in four mice. Expression of hM4Di, without activation by its ligand, has no effect on spontaneous seizure activity as FST values before and after rAAV injection were respectively 16±4% and 16±3%. However, activation of hM4Di receptors by their agonists CNO or clozapine had clear effects on hippocampal EEG and epileptic activity.

Effects on interictal EEG

Two interictal EEG features were analyzed: (1) FRs and (2) changes in spectral power. The occurrence rate of FR events 30-90 minutes after CNO injection was decreased (5±1 FRs/minute) compared to one day before (11±3 FRs/minute, P=0.02, n=6, Figure 1). In non-DREADD mice CNO injection had no effect (Post-treatment 13±2 FRs/minute versus pre-treatment 11±3 FRs/minute, P=0.99, n=6). Power spectral analysis revealed a reduction of the local hippocampal EEG power to 21±2% of baseline (P=0.048, n=6, figure 2E-G), while in non-DREADD animals CNO treatment had no effect on hippocampal EEG power (141±21% of baseline, P=0.35, n=6).

Effects on spontaneous hippocampal seizures

Chemogenetic inhibition of excitatory hippocampal neurons led to a strong and robust suppression of spontaneous epileptic seizures. Figure 2 shows a representative EEG example 20 minutes before and 20-40 minutes after injecting CNO in two representative DREADD and two non-DREADD animals. In DREADD animals 10mg/kg CNO (n=13) reduced FTS from 15±2% to 8±1% during the 23 hours monitoring period after treatment (Figure 3A). In non-DREADD control animals CNO had no effect (Baseline 15±2%, post-treatment 16±1%, n=11). The first hours after CNO administration the FTS in DREADD animals was close to zero and maximally different from non-DREADD animals that were not affected by CNO. It took at least 15 hours before the FTS in DREADD animals was no longer different from baseline. DREADD and non-DREADD animals differed significantly up till 19 hours after treatment (P<0.001).

Seizure-suppressing effects of 1, 3 and 10 mg/kg CNO were compared in 10, 6 and 13 animals respectively (Figure 3B). Seizure suppression lasted for several hours for all doses (FTS

was different from baseline for at least 6, 14 and 15 hours respectively). Treatment with 1 mg/kg CNO reduced the FTS from 16±2% to 12±2%. This effect was significantly lower compared to the FTS reduction after injection of 3 mg/kg CNO (from 16±3% to 7±2%, P<0.001) and 10mg/kg CNO (from 15±2% to 8±1%, P<0.001). Seizure suppression was similar after treatment with 3 and 10 mg/kg CNO (P=0.83).

Next, we evaluated whether similar seizure suppressing effects could be obtained by a very low dose of clozapine, the mother compound of CNO. Recent studies report that CNO does not cross the blood-brain barrier (BBB) but is metabolized to clozapine (1:100 conversion rate) which does pass the BBB and has high affinity for the DREADDs[17]. We thus expected an equally potent DREADD-mediated seizure suppression after injection of 0.1 mg/kg clozapine. Indeed, treatment of DREADD animals reduced the FTS from 14±2% to 5±1% (Figure 3C, n=10). In non-DREADD animals the FTS was not affected (n=4, baseline FTS of 21±3%, post-treatment FTS of 20±1%). The time course of suppression by clozapine was very similar to after CNO. FTS values were maximally reduced the first hours and were statistically different from baseline up to 16 hours after treatment. DREADD and non-DREADD groups were significantly different from each other up to 20 hours after treatment (P<0.001). Injection with 0.03 mg/kg clozapine resulted in a smaller reduction of FTS from 15±2% to 9±1% (versus 14±2% to 5±1% in case 0.1 mg/kg clozapine) and the effects lasted only 9 hours compared to 16 hours.

Side-Effects of CNO and clozapine in non-DREADD mice

Non-DREADD mediated behavioral effects of clozapine and CNO doses, used to activate hM4Di, were evaluated in non-DREADD IHKA mice using the OFT. The distance travelled (DT) and percentage of time spent in center in the open field (TC) were not different between treatments (vehicle, CNO, clozapine; DT P=0.75, TC P=0.67, Figure 4). We therefore conclude that neither CNO, nor clozapine induce measurable off-target side effects on the spontaneous locomotion or anxiety of IHKA mice in this test.

Repeated clozapine treatment

To evaluate the potency of this chemogenetic technique to sustainably suppress epileptic seizures, animals were treated repeatedly with 0.1 and 0.03 mg/kg clozapine every eight hours for three days (n=5 and n=4, Figure 5). This resulted in an almost complete suppression of epileptic

activity during the entire treatment period. FTS values following all nine injections were significantly different from baseline ($P<0.05$), independent of the used dose ($P=0.12$). Although not reaching significance FTS values during 17-24 hours after the last treatment reached considerably higher values than baseline ($P=0.09$), suggesting a state of rebound hyperexcitability after termination of this prolonged chemogenetic seizure suppression.

DREADD expression

Histological analysis revealed both KA-induced damage to the right hippocampus as well as hM4Di-expression (Figure 6). DAPI-based nuclear staining revealed a clear loss of pyramidal neurons in the hippocampal CA1/CA3 region as well as a dispersion of the granule cell layer of dentate gyrus, typically seen in the sclerotic hippocampus of IHKA mice. Expression of mCherry reporter, coupled to hM4Di, is mainly localized in the sclerotic hippocampus. Positive mCherry staining is also present in other brain regions including the contralateral hippocampus, cortical areas, thalamic and hypothalamic nuclei. This pattern is indicative for 1) spread of viral vector outside the hippocampus during the injection procedure (e.g. mCherry expression in cell bodies of cortical neurons aside the injection tract), 2) anterograde transport of hM4Di in axons of transduced hippocampal (and neocortical) neurons projecting to other brain regions (e.g. positive fibers in reticular thalamic nucleus, retrosplenial cortex, etc.) and 3) axonal uptake of viral vector and retrograde transport to cell bodies of afferent neurons (e.g. positive cell bodies of mossy cells in contraleral hilus).

Discussion

This preclinical proof of concept study demonstrated potent suppression of spontaneous seizures and epileptiform FRs in the hippocampus upon chemogenetic suppression of excitatory hippocampal neurons. A chemogenetic approach has previously been used to suppress spontaneous seizures in the IHKA mouse model for TLE[9]. In this study chemogenetic inhibition of CamKIIα principal neurons as well as activation of parvalbumin interneurons reduced the number of generalized seizures during an eight hour monitoring period after 1 mg/kg CNO administration[9]. We now show that using this approach also focal hippocampal seizures are suppressed for an extended period of several hours, and that increasing the dose leads to a more effective seizure suppression.

The seizure-suppressing effect in our study was dependent on the activation of the inhibitory DREADD hM4Di, since expression of the DREADD alone had no effect on hippocampal seizures. Moreover, DREADD ligands did not modulate epileptic activity in non-DREADD animals. Seizure suppressing effects could be obtained with two different hM4Di ligands: CNO or clozapine. Although the designer receptors were initially designed to be sensitive to the inert molecule CNO, it recently became clear that in vivo effects of CNO are mediated by its the back-conversion to clozapine, which crosses the blood brain barrier and activates DREADD receptors[17,18]. Therefore novel ligands, such as compound 21, perlapine, JHU37160/152, and clozapine have been suggested for direct DREADD activation[5,19]. We now confirm that low doses of clozapine can effectively be used as hM4Di activators in vivo. Clozapine is already used in clinical practice but at doses that are typically 100–1000 fold higher than those sufficient to activate DREADDs. The calculated human equivalent for the 0.1 mg/kg highest clozapine dosage used in this study, is 0.49 mg/kg whereas standard dosage for schizophrenic patients amounts to 300-450 mg/kg per day[20]. However, off target effects may still occur given the interactions of clozapine with a broad range of receptors, including dopaminergic, adrenergic, serotonergic, histaminergic and muscarinergic receptors. Studies report effects of clozapine on anxiety-related behavior (both anxiolytic and anxiogenic) and locomotion (sedative effects) at doses of 0.05 mg/kg and higher in wild-type rats[21,22] and 0.5 mg/kg and higher in wild-type mice[23–25]. In this study such effects could not be demonstrated in the OFT after a single administration of 0.1 mg/kg clozapine or 10 mg/kg CNO to non-DREADD IHKA mice. Since clozapine has been used in clinical practice for decades, repurposing this drug to be used at subclinical doses for the activation of DREADDs should be feasible in order to apply this chemogenetic therapy to patients. However, in future effects chemogenetic inhibition on hippocampal function should also be investigated both in normal and epileptic animals. Wang et al.[9] showed memory impairment of epileptic animals compared to normal controls but most importantly chemogenetic suppression did not further reduce memory function. In view of clinical translation, hippocampal functionality and epilepsy comorbidities should be evaluated during chronic chemogenetic treatment

Differences in efficiency between lower (1 mg/kg CNO, 0.03mg/kg clozapine) and higher (3 & 10 mg/kg CNO and 0.1mg/kg clozapine) agonist doses were observed indicating that dose fine-tuning might be necessary in some applications of chemogenetics. Repeated treatments with clozapine suppressed epileptic activity almost completely suppressed for three days, indicating the

potency of this technique to sustainably suppress seizures. Following this prolonged seizure suppression, a rebound effect was observed after withdrawal. Similar effects are known to occur in the hippocampus upon recovery from inhibition, and should be taken into account when translating this approach to the clinic[26]. However the observed seizure suppressing effect is stronger than what can be achieved using standard AEDs in this animal model, given that only some AEDs lead to mild seizure suppression, for a short time span only in a subset of animals (leading to responders versus non-responders)[27,28].

Besides the strong seizure suppressing effects, chemogenetic treatment also influenced the interictal hippocampal EEG and FRs. Fast Ripples (200-500Hz) are only observed in the dentate gyrus in the context of epilepsy pathology and they are thought to be important during ictogenesis. In this experiment recording electrodes are located in the KA lesion where only dentate gyrus seems to be remaining, so the majority of recorded FRs are most likely pathological. A suppression of these epilepsy biomarkers could thus correspond to a suppression of the underlying ictogenic activity[29,30].

Unfortunately, in our study clear spontaneous seizures could only be recorded in a low fraction of IHKA mice. Many mice displayed severe hippocampal sclerosis, probably due to the three invasive procedures (KA and rAAV injections and electrode implantation). In some animals recording electrodes were not located in hippocampal tissue but in the enlarged lateral ventricle instead. In further studies, electrodes will be positioned in hippocampal regions outside of the KA lesion to increase the yield of animals where epileptic seizures can be detected.

We observed effects of CNO and clozapine up to 16 hours following injection, although reported half-lives of CNO and clozapine are much shorter (<1 hour for CNO[31] and about 2 hours for clozapine[32]). This is in line with previous observations that DREADD-mediated effects outlast the presence of its agonists which is possibly due to the long-term modification in downstream second messenger systems[31,33]. Activation of the hM4Di receptor by its agonist initiates a Gi-coupled signaling cascade that suppresses neuronal activity via two main mechanisms: (1) hyperpolarization induced by the activation of G-protein coupled inward rectifying potassium channels and (2) directly or indirectly a reduction of neurotransmitter release[5–8].

Using a CamKIIα0.4 promotor we targeted excitatory neurons with a high specificity[34,35]. In hippocampus the pyramidal neurons of the hippocampus proper and the granule cells and mossy cells of the dentate gyrus express CamKIIα[34–36]. Taking into account that many pyramidal cells

and mossy cells are lost during epileptogenesis following IHKA injection, the majority of silenced neurons are most likely dentate granule cells[37,38], although we cannot exclude the possibility that a small amount of GABA-ergic neurons were silenced as well[34,35]. Dentate granule cells have been identified before as a potential therapeutic target in epilepsy as chemogenetic inhibition of newborn dentate granule cells effectively suppressed seizure activity in a pilocarpine mouse model for TLE[13].

Our results thus indicate that the targeted neurons are essential in the seizure generation process in the IHKA model for TLE. Expression of the mCherry fusion protein was visible in the entire sclerotic hippocampus. As expected some expression of the mCherry-tag was observed in the contralateral hippocampus and surrounding cortex and thalamus, possibly due to mCherry-expressing axons originating from the injected hippocampus, retrograde transport of viral particles that transduced passing fibers or ectopic leakage of vector solution during the injection procedure[39]. In future we aim to optimize this and adjust rAAV serotype in order to limit the number of non-hippocampal transduced cells, limiting DREADD expression to the epileptogenic region. However, the extension of DREADD expression in excitatory neurons strongly connected to the sclerotic hippocampus could also possibly be beneficial to seizure suppression, leading to inhibition of an even larger part of the seizure network. It is indeed known that chemogenetic suppression of brain regions other than the epileptic focus can be effective in reducing the spread of epileptic activity, thus contributing to seizure reduction[14,40].

We conclude that both CNO- and clozapine-mediated chemogenetic suppression of CamKIIα expressing neurons in the sclerotic hippocampus represent very potent tools to block spontaneous seizures and FRs in the IHKA mouse model for TLE and indicate the essential role of these neurons in epilepsy pathology. This provides an approach for the development of an epilepsy therapy in which a systemically administered drug is made to very selectively modulate specific cell types inside the seizure network without affecting other cells in the brain or body, resulting in potent suppression of epileptic seizures with minimal side effects.

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Legends

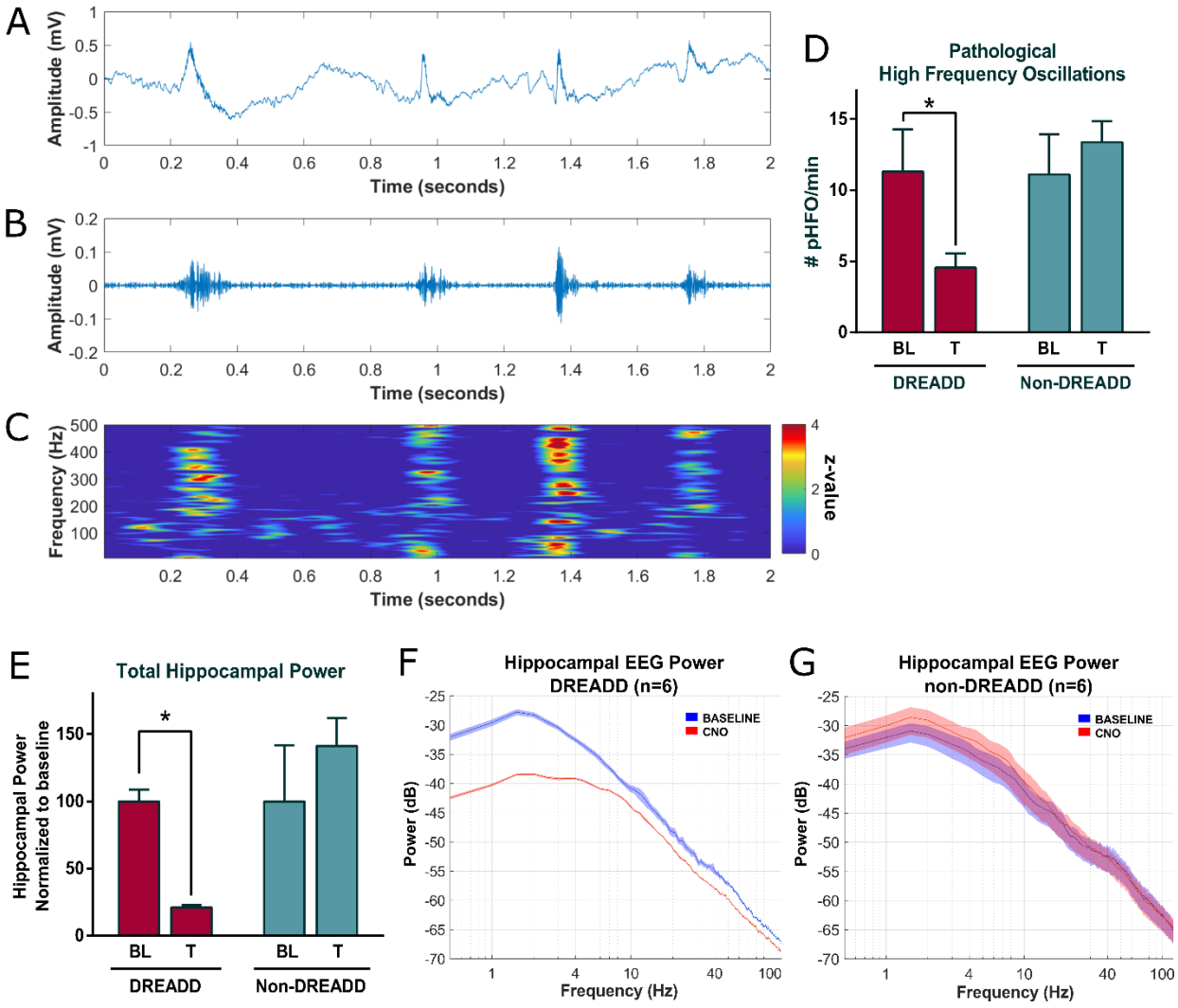

Figure 1 Chemogenetic inhibition of hippocampal excitatory neurons decreases the number of FRs and decreases the interictal hippocampal EEG power. **(A-C)** Baseline dentate gyrus EEG trace of an epileptic mouse containing FRs. **(A)** Unfiltered **(B)** Bandpass filtered between 200 and 500 Hz **(C)** time-frequency plot **(D)** The number of FRs decreases significantly after chemogenetic treatment of DREADD animals (n=8), this had no effect in non-DREADD animals (n=6). **(E)** The total power decreases after treatment with CNO only in DREADD-expressing animals **(F)** The power decrease is visible in all frequency bands in DREADD animals (n = 6) **(G)** In non-DREADD controls CNO had no effect on hippocampal power (n = 6), data are shown as mean ± SEM, * indicates P<0.05, BL = Baseline T = between 30-90minutes following CNO treatment

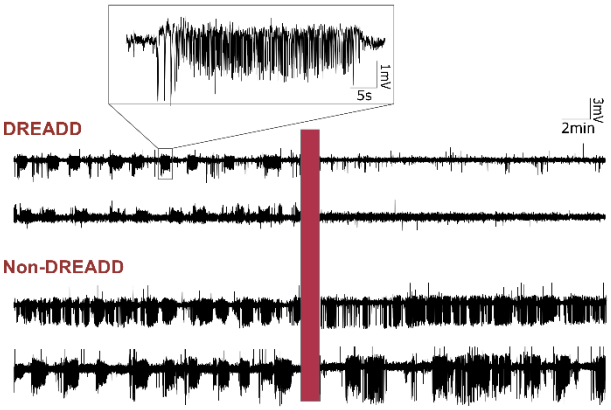

Figure 2 Effect of hM4Di receptors activation on hippocampal EEG and epileptic seizures. Administration of a DREADD-agonist (10mg/kg CNO) has a robust influence on hippocampal EEG of DREADD-mice. Twenty-minute EEG segments collected during the hour before (left) and after (right) agonist administration in two representative DREADD and two non-DREADD animals are shown. (Red bar = interval of about 20min during which CNO was injected)

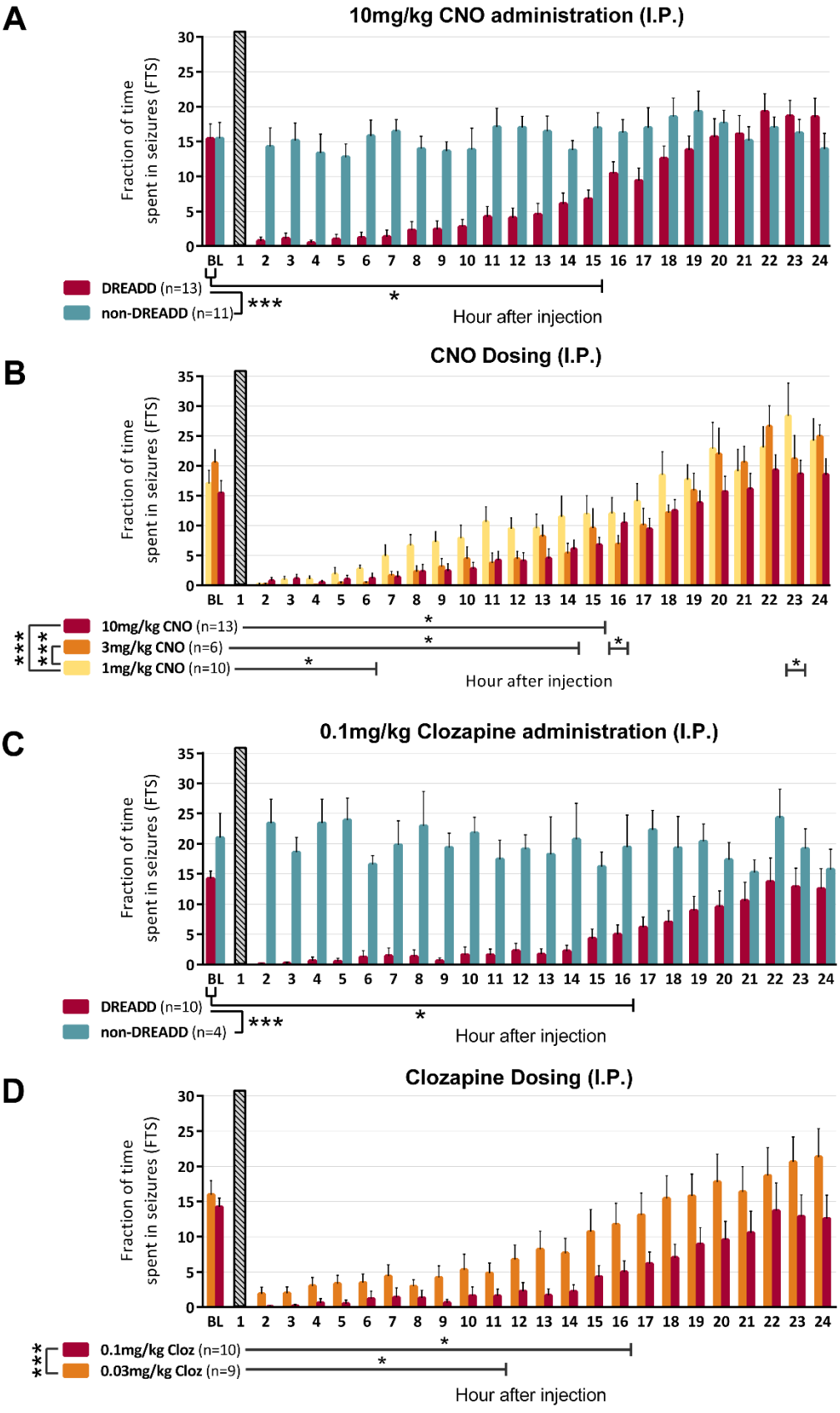
A
10mg/kg CNO administration (I.P.)
Fraction of time spent in seizures (FTS)
BL 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
DREADD (n=13)
non-DREADD (n=11)
***
*
Hour after injection
B
CNO Dosing (I.P.)
Fraction of time spent in seizures (FTS)
10mg/kg CNO (n=13)
3mg/kg CNO (n=6)
1mg/kg CNO (n=10)
***
***
***
*
*
*
*
Hour after injection
C
0.1mg/kg Clozapine administration (I.P.)
Fraction of time spent in seizures (FTS)
DREADD (n=10)
non-DREADD (n=4)
***
*
Hour after injection
D
Clozapine Dosing (I.P.)
Fraction of time spent in seizures (FTS)
0.1mg/kg Cloz (n=10)
0.03mg/kg Cloz (n=9)
**
**
*
*
Hour after injection

Figure 3 Chemogenetic inhibition of hippocampal excitatory neurons suppresses spontaneous hippocampal epileptic seizures. (A) Administration of 10 mg/kg CNO suppressed epileptic seizures of DREADD animals for at least 15 hours **(B)** Comparison of three different doses of CNO (1, 3 & 10 mg/kg) **(C)** Similar seizure-suppressing effects were obtained by administration of 0.1 mg/kg clozapine (**D)** Comparison of two different doses of Clozapine (0.1 & 0.03 mg/kg). Data are shown as mean ± SEM, * indicates P < 0.05, FTS = Fraction of Time spent in Seizures, grey bars indicate one-hour gaps during which the animals were disconnected form the EEG setup for intraperitoneal injections of CNO or clozapine

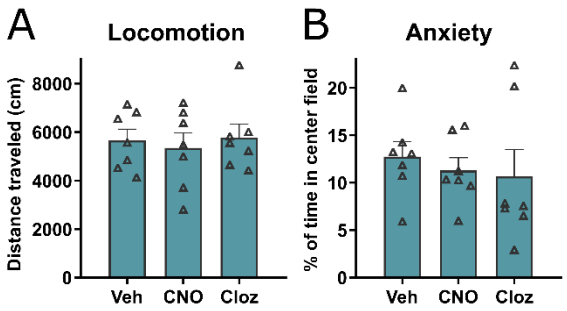

Figure 4: Effect of DREADD agonists in non-DREADD animals on behavior in the open field test. Chemogenetic inhibition of hippocampal excitatory neurons had no effect on **(A)** locomotion (distance travelled) and **(B)** anxiety (time spent in center of field) in the open field test both in non-DREADD control animals (n = 7), data are shown as mean ± SEM, every animal is represented by a mark, Veh = vehicle, Cloz = clozapine

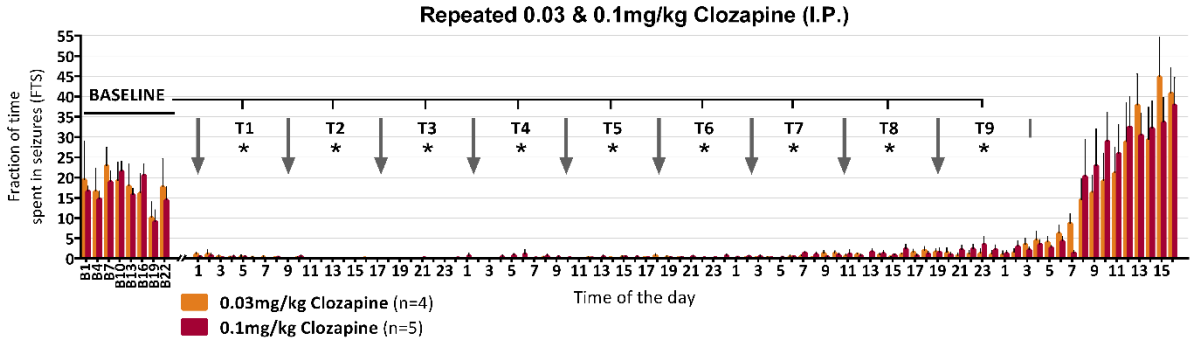

Figure 5: Repeated clozapine administration every eight hours sustainably suppresses spontaneous seizures for three days. Repeated treatments with both 0.03 and 0.1mg/kg sustainably suppress the fraction of time an animal spent in seizures (FTS) following all nine treatments (T1-9, every arrow indicates a clozapine injection), baseline measurements are indicated as B followed by the hour during which baseline was recorded on the day prior to treatment. Data are shown as mean ± SEM, * indicates P<0.05

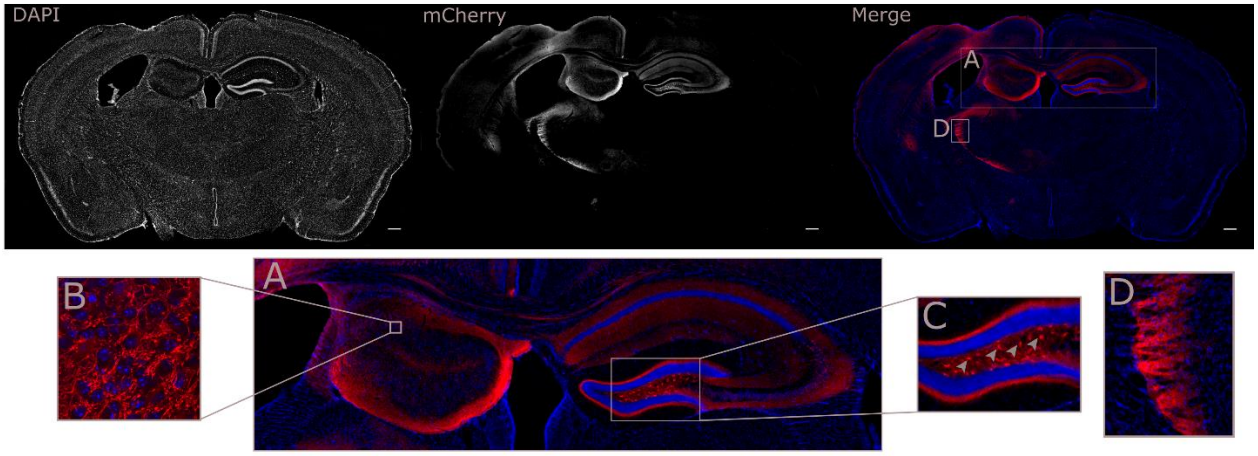

Figure 6 Immunofluorescent staining of the hippocampus Upper panel: DAPI, middle panel: mCherry, lower panel: merge). Boxes in the right image indicates locations of the zooms visualized below. Typical alterations induced by intrahippocampal kainic acid (neuronal loss, hippocampal sclerosis, granule cell dispersion) as well as the location of the mCherry tag coupled to the hM4Di designer receptor are visualized. Fluorescence is located mainly in sclerotic hippocampus **(A-B)** but mCherry positive cells and fibers are observed in surrounding regions (cortex and underlying thalamus) and regions connected to the transduced hippocampus, such as the contralateral hippocampus contains positive fibers as well as positive cells (**A-C**, positive cells, which are most likely mossy cells, are indicated with an arrow), reticular thalamic nucleus which contains positive fibers (**D**) and midline thalamic nuclei contains some mCherry positive cells) Scale bar = 500µm

Supplementary table I: Overview over treatment schedules for IHKA mice with clear seizures included in our study. EEG before rAAV indicates in which animals EEG was recorded prior to rAAV injection. Repaeted indicates administrations every eight hours for three days.

		CNO			Clozapine			
ID	EEG before rAAV	1 mg/kg	3 mg/kg	10 mg/kg	0.1 mg/kg	0.03 mg/kg	Repeated 0.1 mg/kg	Repeated 0.03 mg/kg
DREADD								
MS 1		X		X				
MS 2		X		X				
MS 3		X		X				
MS 4		X		X				
MS 5		X	X	X				
MS 6		X	X	X				
MS 7		X	X	X				
MS 8		X	X	X				
MS 9		X	X	X				
MS 10		X	X					
MS 11	X			X	X	X		
MS 12	X			X	X	X		
MS 13	X			X	X	X		
MS 14	X			X	X	X		
MS 15					X	X	X	X
MS 16					X	X		
MS 17					X	X	X	
MS 18					X	X	X	X
MS 19					X	X		
MS 20					X		X	X
MS 21							X	X
non-DREADD								
MS 22				X				
MS 23				X				
MS 24				X				
MS 25				X				
MS 26				X				
MS 27				X				
MS 28				X	X			
MS 29				X				
MS 30				X	X			
MS 31				X	X			
MS 32				X	X			
Total								
DREADD	n=4	n=10	n=6	n=13	n=10	n=9	n=5	n=4
non-DREADD				n=11	n=4			