Chemical–genetic attenuation of focal neocortical seizures

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Focal epilepsy is commonly pharmacoresistant, and resective surgery is often contraindicated by proximity to eloquent cortex. Many patients have no effective treatment options. Gene therapy allows cell-type specific inhibition of neuronal excitability, but on-demand seizure suppression has only been achieved with optogenetics, which requires invasive light delivery. Here we test a combined chemical–genetic approach to achieve localized suppression of neuronal excitability in a seizure focus, using viral expression of the modified muscarinic receptor hM4Di. hM4Di has no effect in the absence of its selective, normally inactive and orally bioavailable agonist clozapine-N-oxide (CNO). Systemic administration of CNO suppresses focal seizures evoked by two different chemoconvulsants, pilocarpine and picrotoxin. CNO also has a robust anti-seizure effect in a chronic model of focal neocortical epilepsy. Chemical–genetic seizure attenuation holds promise as a novel approach to treat intractable focal epilepsy while minimizing disruption of normal circuit function in untransduced brain regions or in the absence of the specific ligand.

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Epilepsy affects up to 1% of the population and is resistant to drug therapy in at least 20% of cases. Epilepsy can be focal (arising from a specific brain area) or generalized (arising from both hemispheres). People with focal-onset epilepsy are especially prone to pharmacoresistance. The epileptogenic zone in such cases is frequently restricted to a small region that can often be localized with imaging and electrophysiological techniques. However, surgical removal of the seizure focus can successfully treat only about 5% of pharmacoresistant patients, and is often inappropriate in focal neocortical epilepsy because of proximity to eloquent cortex.

Gene therapy targeted to the epileptogenic zone has been shown to be effective in rodent models of epilepsy including focal neocortical epilepsy. However, viral delivery of transgenes that alter excitability permanently may impair essential function of circuits near the seizure focus. An attractive strategy would be to suppress circuit excitability ‘on demand’ upon detection of a seizure. Recently, progress in optogenetic seizure suppression in rodents has shown that this is, in principle, feasible. One of the main hindrances to clinical translation is the need to deliver light of the appropriate wavelength, intensity and duration to the region of transduced neurons. This necessitates the implantation of optical devices and suffers from the strong attenuation of light in brain tissue.

We report an alternative gene therapy approach to achieve targeted and temporally limited suppression of neuronal excitability that relies on systemic delivery of a small molecule inhibitor. Specificity is achieved by regional and cell-type specific expression of a designer receptor exclusively activated by a designer drug (DREADD). Neurons transduced with a DREADD are in principle unaffected in the absence of the selective ligand, and only affected when the ligand is present, thereby avoiding permanent alteration of their properties. We chose the engineered inhibitory G protein-coupled human muscarinic receptor hM4Di, which has been made sensitive to the orally bioavailable and normally inert metabolite of clozapine, clozapine-N-oxide (CNO). Importantly, hM4Di is relatively insensitive to acetylcholine, the endogenous agonist of the parent receptor. hM4Di activation leads to the opening of G-protein gated inwardly rectifying potassium channels, resulting in membrane hyperpolarization and neuronal inhibition.

Results

Chemical–genetic silencing of pilocarpine-induced seizures.

To test the ability of the DREADD to modify seizure activity, we injected an adeno-associated virus (AAV) encoding hM4Di under the CamKIIα promoter (AAV5-CaMKIIα-HA-hM4D(Gi)-IRES-Citrine) into the forelimb area of primary motor cortex (M1) of rats weighing 263–325 g under isoflurane anaesthesia. At the same time we implanted both a Teflon cannula guide above the injection site to allow administration of chemoconvulsants and a subcutaneous transmitter (Open Source Instruments Inc.) with the active lead overlying M1 for wireless electroencephalography (EEG) recording. The transmitter samples the EEG at 512 Hz continuously for several weeks. Expression of hM4Di (Fig. 1a,b) had no detectable effect on behaviour or limb use (Fig. 1c), and was confirmed by fluorescence microscopy for all rats sacrificed after 4–20 weeks.

We first examined seizures acutely evoked by chemoconvulsant injection into layer 5 of the motor cortex 17–52 days after hM4Di AAV injection. Pilocarpine (5 M, 200–900 nl) injected via the implanted cannula guide (1.6–2.0 mm from skull surface) elicited large-amplitude spike-wave deflections at a frequency between 0.5 and 2 Hz, starting within 5 min of injection and lasting between 45 and 90 min (Fig. 2a,b). Spike-wave complexes either had a single negative peak (‘simple’ spike-waves, SS) or featured one or more shoulders (polyspike-waves or ‘complex’ spike-waves, CS; Fig. 2b). They were interspersed with runs of intermediate frequency (IF) discharges (5–12 Hz) lasting 0.2–12 s, which typically had a smaller amplitude (Fig. 2b). IF discharges correlated with the occurrence of more severe motor seizures. Motor seizures ranged from brief twitching of the contralateral limb, the head or the body (score 1), repetitive head, limb or body shaking (score 2), to rearing, retrograde locomotion and generalized convulsions lasting several seconds (score 3) (Fig. 2c). We therefore used the EEG power in an overlapping frequency band (4–14 Hz) as a surrogate marker to assess the anti-seize effect of hM4Di activation.

We randomly interleaved experiments on alternate days on which either CNO (1 mg kg$^{-1}$ in dimethyl sulfoxide (DMSO)/saline vehicle) or vehicle alone, was administered by intraperitoneal injection immediately after focal neocortical...
pilocarpine infusion. In CNO trials, both electrographic and motor convulsions were substantially reduced (n = 6 rats). Repeated measures analysis of variance (ANOVA) for the first six 10 min intervals post vehicle/CNO injection revealed a significant decrease in the mean frequency of negative deflections in the EEG, the mean 4–14 Hz power, and the number of IF runs that correlate with severe motor seizures (Fig. 2c,d; P < 0.05). The seizure activity was on average larger in the vehicle trial than in the matched CNO trial for each rat and every 10 min interval (Fig. 2e,f).

**Chemical–genetic silencing of picrotoxin-induced acute seizures.** CNO thus profoundly suppressed pilocarpine-triggered seizures. However, the interpretation of this anti-seizure effect is potentially confounded by an overlap of downstream signalling pathways.

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**Figure 2 | Chemical–genetic silencing of pilocarpine-induced seizures.** (a) Morlet-wavelet EEG spectra from a rat administered intracortical pilocarpine (time 0), with either intraperitoneal vehicle (top) or CNO (bottom). (b) EEG segment from (a, top), showing simple spikes (SS), complex spikes (CS) and runs of intermediate frequency (IF) activity (expanded below). (c) Behavioural seizures correlated with EEG. SS activity was associated with no motor seizures (severity score 0) or twitching of limb, head or body (score 1), while IF was associated with repetitive head or body shaking (score 2) or rearing, retrograde locomotion and generalized convulsions (3). Six hundred consecutive 4-s-intervals per rat were assessed in three rats (numbered 1–3). (d) Detection of Intermediate Frequency (IF)-activity by Fourier transformation: Fourier transform (right) of EEG segments in one rat showing either SS (black) or IF (red) activity (two 5-s periods shown to the left) induced by pilocarpine. The IF Fourier transform shows a peak around 7 Hz (and a harmonic at 14 Hz). (e) Temporal evolution of spike frequency (left), 4–14 Hz power (middle), and number of IF runs (right), in vehicle (red) and CNO (blue) trials (consecutive 10-min-intervals before (-10–0 min) and after pilocarpine and vehicle/CNO injection). N = 6 rats (10 pairs of trials, averaged within rat where repeated, data are shown as mean ± s.e.m.). (f) Same data as in e, but plotted as cumulative electrographic seizure metrics (frequency, power, number of IF events), comparing vehicle versus CNO for each animal (indicated by colour). Symbols indicate consecutive cumulative metrics at 10-min-intervals. The 45-degree line (grey) indicates equivalence of CNO and vehicle.
cascades of pilocarpine acting on muscarinic receptors and CNO acting on hM4D. We therefore tested a second chemoconvulsant, the GABA<sub>A</sub> receptor blocker, picrotoxin. Picrotoxin injection into the primary motor cortex (10 mM, 100–600 nl) also elicited electrographic and motor seizures. These were similar in overall duration, composition of spike-wave and IF complexes, and behavioural correlates (Fig. 3a–d) to seizures caused by pilocarpine. Among minor differences, electrographic bursting switched on and off more abruptly and individual polyspike-wave complexes lasted longer (Fig. 3b). When CNO was administered by intraperitoneal injection immediately after focal picrotoxin the electrographic discharges were again attenuated (Fig. 3e; n = 5 rats; repeated measures ANOVA, P < 0.05). This was especially marked for IF activity (Fig. 3e,f), which correlated with more severe motor seizures (Fig. 3c,d).

We asked whether off-target effects of CNO independent of hM4D could account for its anti-seizure effect. We conducted control experiments in both, rats injected with an analogous virus expressing the optogenetic actuator ArchT instead of hM4D as well as rats, which had no virus injected. Animals underwent the same experimental protocol as described above, using local intracortical injection of either pilocarpine (six virus-injected and

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**Figure 3 | Chemical–genetic silencing of picrotoxin-induced seizures.** (a) Morlet-wavelet power spectra of EEG in an animal injected with picrotoxin at time 0 (1 mm below pia, 10 mM, 300 nl), together with intraperitoneal vehicle (top) or 1 mg ml<sup>−1</sup> CNO in vehicle (bottom) on consecutive days. (b) EEG activity. Bottom, expanded sections from times indicated (2-s-duraiton) showing SS, CS and IF activity. (c) Motor seizures were more severe in association with IF activity than with SS activity, and intermediate with CS activity (severity scale as in Fig. 2c). Six hundred consecutive 4-s-intervals per rat were assessed in three rats (numbered 1–3). (d) Fourier transform (right) of 5-s-traces displayed (left, middle) containing SS (black) and IF (4–14 Hz, peak around 11.5 Hz; red) activity induced by picrotoxin. (e) Temporal evolution of spike frequency (left), 4–14 Hz power (middle), and number of IF runs (right), in vehicle (red) and CNO (blue) trials (consecutive 10-min-intervals before (− 10–0 min) and after picrotoxin and vehicle/CNO injection). N = 5 rats (12 pairs of trials, averaged within rat where repeated, mean ± s.e.m.). (f) Same data as in e, but plotted as cumulative electrographic seizure metrics for each animal as in Fig. 2e.
We observed no significant reduction of seizure activity in CNO compared with vehicle trials in any of the three measures of seizure severity in any of the control groups (repeated measures ANOVA, $P > 0.05$).

Finally, we asked if chemical–genetic inhibition of focal seizure activity would result in a significant reduction of behavioural seizures. We focused on the picrotoxin model, where the anti-seizure effect of CNO/hM4Di was less pronounced, to establish a conservative benchmark. Rats ($n = 7$) underwent a similar

![Figure 4](image-url)
treatment as before, but the virus was left to express for 3 months before the first experiment was conducted to achieve high expression levels. Seizures of the most severe class (score 3, see above and Methods) were counted by an experimenter who was blind to the identity of the injected compound (vehicle versus CNO, 4 mg kg\(^{-1}\)). The number of severe behavioural seizures was reduced in CNO trials relative to vehicle trials in all subjects (\(P = 0.012\), paired \(t\)-test), with an average decrease of 39.5 ± 8.7% (mean ± s.e.m., Fig. 5a) or 19.3 seizure episodes (Fig. 5c). No effect was observed in control animals, which were not injected with virus (\(n = 6\); \(P = 0.510\), paired \(t\)-Test, Fig. 5b).

**Chemical–genetic silencing of focal neocortical epilepsy.** hM4D\(_i\) activation with CNO is thus effective in two chemogenetic silencing of focal neocortical epilepsy schemes: first, activation leads to the opening of G-protein gated inwardly rectifying potassium channels, resulting in membrane hyperpolarization\(^{11}\). Second, synaptic transmission from excitatory cortical synapses might be directly inhibited, as has been shown for the native muscarinic receptor M4, which is expressed presynaptically\(^{21,22}\). While the former hyperpolarizing effect has been repeatedly documented for the modified receptor hM4D\(_i\), it is unclear if its potential for synaptic silencing may also contribute to the inhibitory effect we observe. We injected the hM4D\(_i\)-expressing AAV into the CA3 subfield of the hippocampus in three rats under isoflurane anesthesia at 4 weeks of age, and prepared acute hippocampal slices 10–11 weeks later. A field excitatory postsynaptic potential (fEPSP) was evoked in stratum radiatum of CA1 by extracellular stimulation. Bath perfusion of CNO (10 \(\mu\)M) reversibly decreased the initial slope of the fEPSP without affecting the fibre volley, consistent with a decrease in glutamate release (\(n = 9\) slices; Fig. 7). Although the depression was modest, this experiment underestimates the effect of hM4D\(_i\), activation on individual synapses because not all stimulated axons were supplied by transduced neurons. The inhibition of synaptic transmission likely contributes to the overall antiepileptic effect of hM4D\(_i\).

**Discussion**

This study shows that chemical–genetics offers the prospect of attenuating seizures on demand. We demonstrate the

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Figure 5 | Chemical–genetic silencing of picrotoxin-induced motor seizures. (a,b) Pair-wise comparison of the number of episodes with severe motor seizures (class 3, as rated for Fig. 3c, see Methods) between vehicle and CNO trials in hM4D\(_i\)-transfected (\(a, n = 7\)) and untransfected (\(b, n = 6\)) rats. Two pairs of trials were conducted per animal and the counts averaged within each animal. Orange bar indicates the decrease (\%, right axis), where significant (paired \(t\)-test; \(P < 0.05\)). (c) The absolute difference in the number of severe motor seizures for hM4D\(_i\)-transfected (black) and untransfected rats (grey; error bars indicate s.e.m.; asterisk indicates statistical significance, \(P = 0.031\), one-tailed unpaired \(t\)-test).

Figure 6 | Chemical–genetic silencing of focal neocortical epilepsy. (a) Sample EEG traces from a tetanus toxin injected animal, showing background activity (1) and four types of epileptiform activity: ‘long events of low amplitude’ (2), ‘short events of high amplitude’ (3), ‘long event of high amplitude’ (4), and ‘high amplitude plus intermittent spikes’ (5) (see ref. 7). (b–d) Pair-wise comparison of the frequency of epileptiform events (b), coastline (c) and high-frequency power (d) between vehicle and CNO trials. \(N = 6\) rats; 15 pairs of trials, averaged within animal where repeated. Orange bars indicate the decrease (\%, right axis), where significant (Wilcoxon test; \(P < 0.05\)).

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hM4D\(_i\) inhibits synaptic transmission. While the optogenetic approach of on-demand silencing of epilepsy relies on direct hyperpolarization via outward currents, the more indirect, chemogenetic inhibition via hM4D\(_i\) may recruit at least two strategies of neuronal inhibition: first, activation leads to the opening of G-protein gated inwardly rectifying potassium channels, resulting in membrane hyperpolarization\(^{11}\). Second, synaptic transmission from excitatory cortical synapses might be directly inhibited, as has been shown for the native muscarinic receptor M4, which is expressed presynaptically\(^{21,22}\). While the former hyperpolarizing effect has been repeatedly documented for the modified receptor hM4D\(_i\), it is unclear if its potential for synaptic silencing may also contribute to the inhibitory effect we observe. We injected the hM4D\(_i\)-expressing AAV into the CA3 subfield of the hippocampus in three rats under isoflurane anesthesia at 4 weeks of age, and prepared acute hippocampal slices 10–11 weeks later. A field excitatory postsynaptic potential (fEPSP) was evoked in stratum radiatum of CA1 by extracellular stimulation. Bath perfusion of CNO (10 \(\mu\)M) reversibly decreased the initial slope of the fEPSP without affecting the fibre volley, consistent with a decrease in glutamate release (\(n = 9\) slices; Fig. 7). Although the depression was modest, this experiment underestimates the effect of hM4D\(_i\), activation on individual synapses because not all stimulated axons were supplied by transduced neurons. The inhibition of synaptic transmission likely contributes to the overall antiepileptic effect of hM4D\(_i\).
Figure 7 | Chemical–genetic inhibition of synaptic transmission. (a) Experimental configuration; a stimulation electrode was placed in stratum radiatum to activate Schaffer collateral fibres expressing HA-hM4D(Gi)-mCitrine. The resulting fEPSP, evoked every 30 s, was recorded further medial in CA1 with an extracellular electrode. (b) Sample traces showing a stimulation artifact, fibre volley and subsequent fEPSP under baseline condition (black), during CNO wash-in (10 μM; red) and after washout (blue). (c) Average normalized slope of evoked fEPSPs (black) and average normalized amplitude of evoked fibre volleys over time before (6 min), during (10 min) and after (6 min) CNO (10 μM) across slices (n = 9). Slices were only included if mCitrine fluorescence was visible in CA3 and CA1. Error bars show s.e.m.

Assessment of motor coordination. Rats were either injected with AAV5-CamkIIz-HA-hM4D(Gi)-IRES-mCitrine unilaterally into the right-motor cortex (as described above) or underwent sham surgery (cut and suturing of skin). After 25–32 days, rats were placed on an elevated metal grid for 3 min. Two observers, blind to whether the rat had undergone sham surgery or hM4D(Gi)-injection, counted left and right as the whole foot falling through the space between painted metal wires that made up the grid, spaced 4 cm apart. Four hours later, all rats were injected with CNO (1 mg kg\(^{-1}\)) and the behavioural assessment repeated 25–33 min post-injection. Typically, the number of foot faults was lower on the second assessment as a result of training and habituation. Two months later, the same rats were assessed in a similar manner, but with the reverse order of treatment: the first trial was conducted 24–37 min prior injection. Data from the two CNO and the two non-injection trials were each averaged for analysis.

Brain slice experiments. AAV5-CamkIIz-HA-hM4D(Gi)-IRES-mCitrine virus (1 μl) was injected into the CA3 subfield of the dorsal hippocampus of 4-week-old male Sprague–Dawley rats at 3.6 mm lateral (right), 2.8 mm posterior and 2.9 mm ventral from bregma at 100 nl min\(^{-1}\) (ref. 26). Ten to eleven weeks later, the animals were transcardially perfused with a room temperature solution containing patients with drug-resistant epilepsy have seizures that are preceded by premonitory auras, or cluster at predictable times (for example, catamennial epilepsy), and hence might benefit from such chemical–genetic silencing. CNO’s bioavailability implies that it could be administered buccally or intranasally. For even faster on-demand administration, subcutaneous puffs, as used to deliver insulin\(^{1,6}\), could, in principle be used in people with epilepsy. Recent evidence that seizures can be predicted by automated EEG analysis\(^{22}\) offers the prospect of a closed-loop device.

A further potential application of chemical–genetics to epilepsy is to test the hypothesis that continued alteration of neuronal excitability for a fixed period might ‘reset’ epileptogenic circuits in some circumstances, bringing about a persistent reduction in seizures that outlasts the administration of the ligand. The reversibility, together with the regional and cell-type specificity of chemical–genetics, distinguishes this approach from available small molecules or gene therapies based on expression of ion channels or other signalling molecules.

In conclusion, we have shown that chemical–genetics is a promising approach to achieve region- and cell-type specific attenuation of neuronal excitability to suppress seizures. The pathway to translation is likely to be more direct than for optogenetics.

Methods

AAV of serotype 5 containing a CamkIIz-HA-hM4D(Gi)-IRES-mCitrine cassette provided by Dr Bryan Roth (University of North Carolina, UNC) was obtained from UNC Vector Core at a concentration of 8 × 10\(^{12}\) infectious units (IU) per ml. For control experiments (Fig. 4) a similar virus was injected expressing the optogenetic silencer ArchT instead of the chemical–genetic silencer hM4D, (AAV5-CamkIIz-ArchT-GFP).

Stereotactic surgery. Animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986, and approved by the local ethics committee. Male Sprague–Dawley rats (6–12 weeks old, 263–325 g) were anaesthetized using isoflurane and placed in a stereotaxic frame (Kopf, CA, USA). hM4D(Gi)-expressing AAV5-virus (1.5 μl) was injected at 100 nl min\(^{-1}\) into layer 5 of the forelimb area of right primary motor cortex (coordinates, 2.2–2.4 mm lateral and 1.0 mm anterior of bregma at a depth of 1.0 mm from pia; in some rats half of the volume each was deposited at 1.1 and at 0.7 mm from pia). An EEG transmitter (A3019D, Open Source Instruments) was implanted subcutaneously with a subdural intracranial recording electrode positioned above the injection site. A reference electrode was implanted in the contralateral skull. For sequential injections of chemovolcanists a Teflon cannula guide (C313GT/SP, PlasticsOne) was implanted above the injection site. For chronic epilepsy experiments, 12–16 ng of tetanus toxin (gift of Dr G. Schiavo) was injected together with hM4D(Gi)-expressing AAV5 virus in a final volume of 1.6–1.8 μl. Animals were housed separately in Faraday cages and EEG was recorded continuously for up to 8 weeks post surgery. Animal numbers per cohort were chosen to allow for detection of a therapeutic effect, while avoiding unnecessary procedures given their severity level.

Assessment of motor coordination. Rats were either injected with AAV5-CamkIIz-HA-hM4D(Gi)-IRES-mCitrine unilaterally into the right-motor cortex (as described above) or underwent sham surgery (cut and suturing of skin). After 25–32 days, rats were placed on an elevated metal grid for 3 min. Two observers, blind to whether the rat had undergone sham surgery or hM4D(Gi)-injection, counted left and right as the whole foot falling through the space between painted metal wires that made up the grid, spaced 4 cm apart. Four hours later, all rats were injected with CNO (1 mg kg\(^{-1}\)) and the behavioural assessment repeated 25–33 min post-injection. Typically, the number of foot faults was lower on the second assessment as a result of training and habituation. Two months later, the same rats were assessed in a similar manner, but with the reverse order of treatment: the first trial was conducted 24–37 min prior injection of CNO, while the second trial was conducted ~5.5 h later without prior injection. Data from the two CNO and the two non-injection trials were each averaged for analysis.

Brain slice experiments. AAV5-CamkIIz-HA-hM4D(Gi)-IRES-mCitrine virus (1 μl) was injected into the CA3 subfield of the dorsal hippocampus of 4-week-old male Sprague–Dawley rats at 3.6 mm lateral (right), 2.8 mm posterior and 2.9 mm ventral from bregma at 100 nl min\(^{-1}\) (ref. 26). Ten to eleven weeks later, the animals were transcardially perfused with a room temperature solution containing...
trials was randomized. Vehicle (1% DMSO/saline) or CNO (1 mg kg

Behavioural seizure analysis
Morlet-wavelet power spectra.

epochs were also exported into Labview (National Instruments) to compute
determined as the sum of the absolute difference between successive points. EEG

fluorescence and immunohistochemical analysis

Statistical analysis. Paired t-tests or Wilcoxon tests, or unpaired t-tests were

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Author contributions

D.K. performed in vivo experiments. E.N. performed in vitro experiments. D.M.K., S.S. and E.N. assisted with behavioural experiments requiring blind assessors. D.K. and D.M.K. designed the study and wrote the manuscript. D.K., D.M.K., E.N. and M.C.W. analyzed data. S.S., M.C.W. and D.M.K. provided rodent telemetry facilities. All authors revised the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: D.K., S.S., M.C.W. and D.M.K. have applied for a patent relating to the use of DREADDs in the treatment of epilepsy (Intellectual Property Office, United Kingdom, filing number GB1404470.5). E.N. declares no competing financial interests.

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