Selective silencing of hippocampal parvalbumin interneurons induces development of recurrent spontaneous limbic seizures in mice

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Abbreviated title: Parvalbumin interneurons in epileptogenesis

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Abstract: 247 words
Introduction: 547 words
Discussion: 1308 words
Abstract

Temporal lobe epilepsy (TLE) is the most frequent form of focal epilepsies and generally associated with malfunctioning of the hippocampal formation. Recently, a preferential loss of parvalbumin (PV) neurons has been observed in the subiculum of TLE patients and in animal models of TLE. To demonstrate a possible causative role of defunct PV neurons in the generation of TLE, we permanently inhibited GABA release selectively from PV neurons of the ventral subiculum by injecting a viral vector expressing tetanus toxin light chain in male mice. Subsequently, mice were subjected to telemetric EEG recording and video monitoring. Eighty-eight percent of the mice presented clusters of spike-wave discharges (C-SWD, 40.0 ± 9.07 per month) and 64% showed spontaneous recurrent seizures (SRS, 5.3 ± 0.83 per month). Mice injected with a control vector neither presented C-SWD nor SRS. No neurodegeneration was observed due to vector injection or SRS. Interestingly, mice that presented only C-SWD but no SRS, developed SRS upon injection of a sub-convulsive dose of pentylenetetrazole after 6 weeks. The initial frequency of SRS declined by about 30% after 5 weeks. In contrast to permanent silencing of PV neurons, transient inhibition of GABA release from PV neurons through the designer receptor hM4Di selectively expressed in PV containing neurons transiently reduced the seizure-threshold of the mice but induced neither acute nor recurrent seizures. Our data demonstrate a critical role for perisomatic inhibition mediated by PV-containing interneurons suggesting that their sustained silencing could be causally involved in the development of TLE.

Key words:
epilepsy; parvalbumin; subiculum; hippocampus; basket cell; feed-forward inhibition; epileptogenesis;
SIGNIFICANCE STATEMENT. Development of temporal lobe epilepsy (TLE) generally takes years after an initial insult during which maladaptation of hippocampal circuitries takes place. In human TLE and in animal models of TLE parvalbumin neurons are selectively lost in the subiculum, the major output area of the hippocampus. The present experiments demonstrate that specific and sustained inhibition of GABA release from parvalbumin expressing interneurons (mostly basket cells) in sector CA1/subiculum is sufficient to induce hyper-excitability and spontaneous recurrent seizures in mice. Like in patients with non-lesional TLE, these mice developed epilepsy without signs of neurodegeneration. The experiments highlight the importance of the potent inhibitory action mediated by parvalbumin cells in the hippocampus and identify a potential mechanism in the development of TLE.
Introduction

Temporal lobe epilepsy (TLE) is the most frequent form among focal epilepsies. It is characterized by spontaneous recurrent seizures (SRS) arising from limbic brain structures and is often associated with Ammon’s horn sclerosis (Spielmeyer, 1927; Honavar and Meldrum, 1997), and consequently with memory deficits and emotional disturbances. TLE may be provoked by an initial insult like prolonged febrile seizures, head injury or by sustained seizures. It often takes years until TLE becomes manifest (Bragin, et al., 2000). During the preceding "silent phase", neuronal circuitries are altered to permit recurrent hyper-synchronization and hyper-excitability displayed as epileptic seizures (Paz and Huguenard, 2015). The underlying cellular and molecular mechanisms mediating this transition from a normal to an epileptic brain are still poorly understood.

For instance, Cossart et al. (2001) proposed that a deficit in dendritic feedback inhibition, presumably due to a loss in oriens-lacunosum moleculare (O-LM) neurons, reduces the seizure-threshold whereas augmented somatic feed-forward inhibition limits epileptiform activity (Cossart et al., 2001; Dinocourt et al., 2003). Feed-forward inhibition is exerted particularly by fast-spiking parvalbumin (PV)-containing basket cells that establish perisomatic synapses upon pyramidal cells of the hippocampus and subiculum and decisively control their excitatory output (Szabo et al., 2010; Gulyas and Freund, 2015).

Thus, a sudden loss of perisomatic inhibition in the hippocampus leads to uninhibited massive bursting of pyramidal cells and the occurrence of fast ripples (Alexander et al., 2016; Salami et al., 2014) and may give rise to the formation of an epileptic focus (Gulyas and Freund, 2015).

Interestingly, PV-containing interneurons are preferentially lost in the subiculum of TLE patients even without Ammon’s horn sclerosis (Andrioli et al., 2007). Also in animal models of TLE, PV-containing interneurons of the subiculum selectively degenerate after an initial status epilepticus (Dinocourt et al., 2003; Knopp et al., 2008; Drexel et al., 2011).
Thus, a loss or impairment of PV-containing basket cells could be directly connected to the subsequent development of TLE.

To test this hypothesis, we now permanently inhibited GABA release selectively from PV interneurons of the ventral subiculum of mice and subjected them to continuous telemetric EEG and video monitoring. For silencing locally PV-containing neurons, we injected an adeno-associated viral vector (AAV) expressing tetanus toxin light chain (TeLC; fused with a green fluorescence protein (GFP) tag) with its reading frame inverted in a flip-excision (FLEX) cassette (termed AAV-TeLC) into the ventral subiculum of transgenic mice expressing Cre-recombinase under the PV promoter (PV-cre mice). Cre-recombinase then inverts the TeLC construct selectively in PV-expressing neurons, allowing permanent transgene expression (Murray et al., 2011). TeLC cleaves vesicle associated membrane protein 2 (VAMP2, synaptobrevin), thereby permanently inhibiting GABA release (Schiavo et al., 1992). Control mice were injected with a corresponding GFP vector (AAV-GFP).

AAV-TeLC injected mice developed clusters of spike-wave discharges (C-SWD) that were often followed by spontaneous recurrent seizures (SRS). Furthermore, to test whether also transient inhibition of GABA release would be sufficient to induce C-SWD and SRS we took advantage of the DREADD (designer receptors exclusively activated by designer drugs) technology (Roth, 2016). We expressed hM4Di receptors specifically in PV-containing interneurons and subsequently inhibited GABA release from these neurons transiently by injection of clozapine N-oxide (CNO), selectively inhibiting neurons containing the DREADD receptors. In contrast to permanent silencing of PV/GABA neurons with TeLC their transient inhibition did not induce C-SWD or SRS.

**Materials and Methods**

*Mice.* Animal experiments were conducted according to national guidelines and European Community laws and were approved by the Committee for Animal Protection of the Austrian Ministry of Science. *Parvalbumin (PV)-cre* transgenic mice (*Pvalbtm1(cre)Arbr*).
RRID:IMSR_JAX:017320) were originally purchased from Jackson Laboratories (Farmington, CT, USA) through Charles River Germany (Sulzfeld, Germany) and then maintained on a C57BL/6N background. These mice in adulthood express Cre-recombinase under the PV promoter. C57BL/6N wild type (WT) mice were obtained from Charles River (Sulzfeld, Germany). The mice were housed in groups of 3-5 in single ventilated cages under standard laboratory conditions (12/12h light/dark cycle, light turns on at 06:30 AM) and had access to food and water ad libitum. For the experiments, 10 to 14 weeks old male heterozygous mice ($Pvalbtm1(cre)Arbr^{+/-}$) were used, which exhibited a similar number of PV-expressing interneurons in the subiculum as WT C57BL/6N mice (not shown).

Preparation of vectors. The AAV1/2 vectors contained the genes for TeLC fused with a GFP tag or GFP alone, respectively with its reading frame inverted in a flip-excision (FLEX) cassette (AAV-TeLC and AAV-GFP, respectively). Thus, AAV-mediated transgene expression is limited to PV-expressing cells at the injection site. The FLEX cassette and the AAV2-based vector backbones on a cytomegalovirus virus (CMV) enhancer/chicken $\beta$-actin promoter were constructed (Murray et al., 2011). Vectors pseudotyped for AAV1 were produced and highly purified as previously described (Mietzsch et al., 2014). Briefly, human embryonic kidney HEK-293 cells were co-transfected with rAAV plasmids pAM-FLEX-TeLC or pAM-FLEX-GFP and pDP1rs helper plasmids by calcium phosphate co-precipitation. Benzonase-treated, cleared cell lysates were HPLC-purified on AVB columns with successive dialysis. AAV titers were determined by qPCR as genomic particles/ml (gp/ml). Titers were $2.4 \times 10^{10}$ gp/ml for pAM-FLEX-TeLC, and $1.4 \times 10^{11}$ gp/ml for pAM-FLEX-GFP.

AAV-FLEX-hM4Di vector. For experiments using DREADD technology (designer receptors exclusively activated by designer drugs), the recombinant AAV2-hSyn-FLEX-hM4Di-mCherry plasmid deposited by Bryan Roth, University of North Carolina at Chapel Hill.
Hill, USA (Krashes et al., 2011) was obtained from Addgene, Cambridge, MA, USA. It was packaged into AAV2 capsids with a final titer of $2.7 \times 10^{12}$ gp/ml and was injected 1:4 diluted ($6.7 \times 10^{11}$ gp/ml).

Surgery. Stereotaxic surgeries were performed as described in detail previously (Jagirdar et al., 2015). Heterozygous male $PV$-$cre$ transgenic mice (age 10-14 weeks) were treated with the analgesic drug carprofen (5 mg/kg, sc; Rimadyl, Pfizer, USA) 60 min prior to surgery. We anesthetized the mice with 150 mg/kg i.p. ketamine (Ketasol, stock solution 50 mg/ml, Ogris Pharma Vertriebs-GmbH, Wels, Austria) and maintained anesthesia by applying 1-4% sevoflurane (Sevorane, Abbott, Vienna, Austria) through a veterinary anesthesia mask using oxygen (400 ml/min) as a carrier gas. We placed the mice into a stereotaxic frame (David-Kopf Instruments, Tujunga, CA, USA) and opened the skin above the skull. A telemetric EEG transmitter (TA10EA-F20, Data Sciences International, Arden Hills, USA) was inserted into a subcutaneous pocket at the right abdominal wall and the electrode wires were pulled through the subcutaneous channel formed from the pocket to the skull, and the pocket was carefully sutured. Bilateral holes were drilled for AAV-vector injection and insertion of a depth-electrode (coordinates from bregma in mm: posterior, 3.8; lateral, 3.5) and for the epidural reference electrode (2 mm posterior, 1.6 mm lateral).

We injected the AAV vectors, AAV-$TeLC$ or AAV-$GFP$ (for controls) unilaterally into the left ventral subiculum (in mm: 3.8 posterior, 3.5 left, 3.5 ventral). AAV-$TeLC$ or AAV-$GFP$ injections were done in mice of the same litters on the same days. For telemetric EEG recordings we implanted a tungsten depth electrode (Cat. no 577100; Science Products GmbH, Hofheim, Germany) into the left ventral subiculum (in mm: 3.8 posterior, 3.5 left, 3.0 ventral) and, as reference electrode, attached a stainless steel screw (M1*2, Hummer und Rieß GmbH, Nürnberg, Germany) to the skull in an epidural position (2.0 mm posterior, 1.6 mm right). In some mice, an epidural electrode was set above the ipsilateral dorsal hippocampus instead of the depth electrode. Electrodes were fixed to the skull.
using dental cement (Paladur, Heraeus Kulzer, Henry Schein, Innsbruck, Austria). After surgery and during EEG recording mice were single housed.

EEG recordings and video monitoring. EEGs were recorded continuously using a telemetry system (Dataquest A.R.T. Gold 4.33 Acquisition, Data Sciences International, Arden Hills, USA). For behavioral analysis, continuous video recordings were performed using Axis 221 network cameras (Axis communications AB, Lund, Sweden) and infrared illumination (Conrad Electronics GmbH, Wels, Austria) during darkness. EEGs were recorded at a sampling rate of 1,000 Hz without a priori filter cut-off and saved on external hard disk drives.

EEG traces of local field potentials were visually inspected by two independent observers using the Dataquest A.R.T. Gold 4.33 Analysis software (Data Sciences International, Arden Hills, USA). We defined seizures as EEG segments with continuous high frequency activity with an amplitude of at least two times the baseline amplitude, a duration of at least 10 seconds, and the presence of a post-ictal depression (EEG-signal below baseline amplitude). C-SWD were defined as series of at least 5 high amplitude (at least 2 times baseline amplitude) spike-wave discharges (SWD) not more than 60 s apart. We used corresponding synchronized video recordings to analyze behavioral correlates to EEG seizures. At the end of the experiment (6 to 8 weeks after the initial vector injection), mice were deeply anesthetized (killed) with thiopental (150 mg/kg), perfused with 4% paraformaldehyde and their brains dissected out for immunohistochemistry.

Evaluation of motor seizures. All seizures defined by EEG were also investigated for a possible behavioral correlate by evaluating the synchronized video recordings. Seizure rating was done in accordance to that of kainic acid-injected rats (Sperk et al., 1983): Stage 1, staring, arrest, chewing; stage 2, unilateral or bilateral tonic movements/seizure; stage 3, rearing without falling; stage 4, rearing with falling, limbic seizures.

Determination of seizure threshold using a threshold dose of pentylenetetrazole (PTZ). PTZ (30 mg/kg in saline) was injected intraperitoneally. This dose was established in a
pilot experiment (data not shown). Only one out of 13 AAV-GFP injected mice and no WT (n = 5) revealed an acute convulsion. Immediately after PTZ-injection, the transmitter-implanted mice were placed into cages for continuous video- and EEG-recordings.

Transient silencing of PV-neurons using the DREADD system. To achieve specific and reversible chemogenetic silencing of PV-expressing interneurons in the subiculum, we used DREADD technology. Twelve heterozygous male PV-cre mice were injected with an AAV-hM4Di vector (1.0 μl) expressing the inhibitory hM4Di receptor (together with a red fluorescence protein, RFP tag) into the left ventral subiculum using the same coordinates and protocol as for AAV-TeLC injections. Epidural EEG-electrodes were set above the ipsilateral hippocampus (2.0 mm posterior and 1.6 mm lateral) and fixed to the skull using dental cement. Fifteen days later, seven mice were injected i. p. with the selective hM4Di agonist clozapine-N-oxide (CNO; Tocris Bioscience, Bristol, United Kingdom; 10 mg/kg, i.p., dissolved in sterile 0.9% NaCl at a concentration of 1.0 mg/ml) and EEGs were inspected for the occurrence of C-SWD or seizures. AAV-hM4Di vector injected controls (n = 5) were injected with saline instead of CNO.

To prove the efficacy of DREADD treatment in vivo we investigated the effect of transient silencing of PV-interneurons on the seizure threshold. For this, another 12 AAV-hM4Di vector injected mice were injected with CNO (10 mg/kg, i.p.; n = 6) or saline (n = 6) after 15 days, and 45 min later with a threshold dose of the GABA_A-receptor antagonist PTZ (30 mg/kg; i.p.). The interval between CNO and PTZ injections has been chosen based on previous experiments showing a maximal behavioral read-out due to inhibition of PV-expressing interneurons in the nucleus accumbens already 30 min after CNO application (Wirtshafter and Stratford, 2016). We then monitored acute seizures and C-SWD by telemetric EEG recording for 24 hrs.

Slice preparation and patch-clamp electrophysiology of evoked inhibitory postsynaptic currents (eIPSCs). To verify a loss of inhibitory input to pyramidal neurons of the...
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subiculum, we applied whole-cell voltage-clamp recordings in acute brain slices also
containing the subiculum 2-3 weeks after vector injection. Mice (AAV-TeLC: n = 6; AAV-
GFP: n = 5) were anesthetized with isoflurane (Baxter, USA), decapitated, and their
brains were rapidly removed and placed in ice-cold oxygenated (95% O₂/5% CO₂) artificial
cerebrospinal fluid containing (aCSF; in mM): 126 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 26
NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose. Horizontal hippocampal slices at 300 μm
thickness were cut on a vibratome (VT1200, Leica Microsystems, Germany) and
recovered in aCSF (32-34°C) for 1 h prior to recordings.

For recording eIPSCs, recording pipettes with a final tip resistance of 2-5 MΩ, prepared
using a micropipette puller (P-1000 Sutter Instruments, Novato, CA, USA) contained (in
mM): 135.0 CsCl, 10.0 CsOH-HEPES, 0.2 CsOH-EGTA, 2.0 Mg-ATP, 0.3 Na₃-GTP, 8.0
NaCl and 5.0 N-ethyl-lidocaine chloride (Abcam; pH of 7.2–7.4; osmolarity of 295–305
mOsm; Murray et al., 2011). Subicular pyramidal neurons were visually identified based
upon their anatomical location and pyramidal morphology using an upright microscope
(BX51, Olympus Deutschland GmbH, Hamburg, Germany) equipped with a 40x water-
immersion objective, infrared light with differential interference contrast and a digital
camera. eIPSCs were recorded at a holding potential of -70 mV in response to electrical
stimulation (10-35 μA; set to the minimum current required to evoke IPSCs with maximal
amplitude) delivered using a concentric, bipolar platinum/iridium electrode with a 2-3 μm
diameter (Microprobes, Gaithersburg, MD, USA) connected to a constant current
stimulator (Digitimer, Hertfordshire, UK). Brain slices were constantly perfused with
artificial cerebrospinal fluid (32-34°C) containing glutamate AMPA and NMDA receptor
agonists, 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 100 μM DL-2-amino-5-
phosphonopentanoic acid (DL-AP5; Abcam, UK), respectively, and 1 μM WIN 55,212-2
mesylate (Tocris, Bristol, UK) was dissolved in aCSF immediately prior to recordings.
Data were filtered at 2.9 kHz and sampled at 10 kHz with an EPC10 patch-clamp amplifier
and analyzed using PatchMaster and FitMaster software (HEKA Electronic, Lambrecht,
Germany).
Patch-clamp electrophysiology of miniature inhibitory and excitatory postsynaptic currents (mIPSCs and mEPSCs). To record mIPSCs and mEPSCs, we used 3-6 month old male mice (n = 21) and a protective recovery method for slice preparation (Zhao et al., 2011). Briefly, mice were deeply anesthetized and perfused with 40 ml ice-cold pre-oxygenated (95% O₂/5% CO₂) solution containing (in mM): 93 N-methyl-D-glucamin (NMDG-HCl), 30 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 20 HEPES-NaOH, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl₂, 8 MgSO₄ and 25 glucose, pH = 7.4).

Next, brains were rapidly extracted and immersed in the same solution. Subsequently, 300 μm-thick horizontal hippocampal slices were cut on a vibratome (VT1200S, Leica) and transferred to a recovery chamber filled with the same solution (32°C) for 12 min. Thereafter, slices were kept (for at least 60 min prior to the recordings) in a solution containing (in mM): 90 NaCl, 26 NaHCO₃, 3 KCl, 1.2 NaH₂PO₄, 20 HEPES-NaOH, 5 Na-ascorbate, 3 Na-pyruvate, 1.5 CaCl₂, 2 MgSO₄, 0.5 L-Glutathione and 25 glucose, pH = 7.4).

For voltage-clamp recordings of mIPSCs, pipettes with a resistance of 3-4.5 MΩ contained (in mM): 120 K-gluconate, 6 KCl, 10 HEPES-KOH, 5 EGTA, 4 ATP-Mg, 0.3 GTP (pH was adjusted to 7.3 with KOH). For mEPSCs recordings the pipette solution contained (in mM): 135 KCl, 1 MgCl₂, 10 HEPES-KOH, 1 EGTA, 2 Mg-ATP, 0.3 GTP (pH = 7.4). To record mIPSCs and mEPSCs, pyramidal neurons were voltage-clamped at -70 mV in aCSF. The amplitude and frequency of mEPSs and mIPC were analyzed from a 100 s trace recorded 5 minutes after the application of tetrodotoxin (TTX, 1 μM) and 50 μM bicuculline for mEPSCs recordings, or 25 μM NBQX+ 50 μM D-AP5 for mIPSCs recordings. Electrophysiological data were analyzed using Clampfit 10.0 (Molecular Devices), MiniAnalysis Program (Synaptosoft) and SigmaPlot (Systat Software Inc.).

Immunohistochemistry. These studies were performed after terminating recordings (6 to 8 weeks after vector injection) on free-floating, 4% paraformaldehyde-fixed, 30 μm thick
horizontal sections using indirect peroxidase labeling, or by immunofluorescence as described previously (Sperk et al., 2012; Wood et al., 2016). The following antisera were used for immunohistochemistry: monoclonal rat anti-GFP (1:2,000; 04404-84, RRID:AB_10013361, Nacalai Tesque Inc., CA, USA through Gerbu, Heidelberg, Germany), rabbit anti-ΔFosB (1:2,000; sc-48, RRID:AB_631515, Santa Cruz Biotechnology Inc., Heidelberg, Germany), rabbit anti-PV (1:15,000; PV 25, RRID:AB_10000344, Swant, Switzerland), rabbit anti-dynorphin (a gift by Dr. Philippe Ciofi, INSERM, Bordeaux, France) (Sperk et al., 2012), rabbit anti-somatostatin, 1:1,000 (Sperk and Widmann, 1985), rabbit anti-GABA (1:1,000; A2052, RRID:AB_477652, Sigma Aldrich, Vienna, Austria), and rabbit anti-RFP (red fluorescence protein; 1:1,000; RRID:AB_2209751, Rockland 600-401-379, Sanova Pharma, Vienna, Austria). The antibodies were characterized by the supplier or were validated by us in previous experiments by immunocytochemistry (Sperk and Widmann, 1985; Sperk et al., 2012; Wood et al., 2016). In brief, horizontal sections were incubated free-floating with 10% normal goat or horse serum (Biomedica, Vienna, Austria) in Tris-HCl buffered saline (TBS; 50 mM, pH 7.2) containing 0.4% Triton X-100 (TBS-Triton) for 90 min, followed by incubation with the respective primary antisera (room temperature, 16 h), followed by washing with TBS-Triton. Primary antibodies bound to the respective antigens were then visualized by incubation with horseradish peroxidase (HRP)-coupled secondary antibodies reacting host specific for the primary antiserum (1:250, goat anti-rabbit P0448; RRID:AB_2617138, Dako, Vienna, Austria; 1:500, donkey anti-rabbit, 711035152, RRID:AB_2617138, Dako, Vienna, Austria; 1:500, donkey anti-rat secondary antibodies, 712035153, RRID:AB_2340639, Jackson ImmunoResearch, Suffolk, UK) at room temperature for 150 min. After washing with TBS, HRP bound to the secondary antibodies was revealed with 0.05% diaminobenzidine tetrahydrochloride dihydrate (DAB, Fluka, Sigma-Aldrich Handels GmbH, Vienna, Austria) and 0.005% H2O2 substrate. Sections were washed in TBS, mounted on slides, dehydrated in ethanol series and cover-slipped with Eukitt (Gröpl, Vienna, Austria).
Double immunofluorescence was performed as described (Wood et al., 2016). Two sections per animal were anatomically matched to those from the other animals (n = 29) and processed either for GABA and GFP, somatostatin and GFP, or for PV and GFP. The same antibodies as described above were used at the same concentrations (room temperature, 16 h).

For double labeling of GFP and PV, the secondary reaction was done by simultaneous incubation with a donkey anti-rat antibody coupled to Alexa Fluor 488 (1:500; Molecular Probes, Eugene, OR, USA through Thermo Fisher Scientifics, Vienna) for GFP, and with a HRP coupled goat anti-rabbit antibody (1:250; P0448, RRID:AB_2617138, Dako, Vienna, Austria) for PV at room temperature for 120 min. The HRP-coupled antibody was then further reacted with TSA-Cy3 (homemade, Lumiprobe, Hannover, Germany; 1:100 in 50 mM PBS, 0.005% H2O2) at room temperature for 5 min.

Double labeling of GABA and GFP. GFP was labeled in the same way as described above, and GABA was reacted with the HRP-coupled goat-anti-rabbit antibody (see above) at room temperature, 120 min. The HRP-coupled antibody was then incubated with TSA-AMCA (1:100 in 50 mM PBS, 0.02% H2O2; Thermo Fisher Scientifics, Vienna) at room temperature for 5 min. For concomitant labeling of somatostatin and GFP we used the HRP-coupled donkey anti-rabbit antibody (1:500; 711035152, RRID:AB_10015282, Jackson ImmunoResearch, Suffolk, UK) and the anti-rat antibody coupled to Alexa Fluor 488 (1:500; Molecular Probes, Eugene, OR, USA), respectively as secondary antibodies. The HRP-coupled donkey anti-rabbit antibody was then further reacted with TSA-Cy3 as described above.

For identifying expression sites of hM4Di in PV-containing interneurons after AAV-hM4Di injection, double labeling for the RFP tag of the hM4Di vector and for PV was performed also using the primary antibodies described above. As secondary antibodies biotinylated donkey anti-mouse (1:200; Vectastain PK 4002, RRID:AB_2336811, Scabo-Scandic, Vienna, Austria) and HRP coupled goat anti-rabbit antibodies (1:250; P0448,
For double labeling of caspase 3 and GFP, we incubated three sections obtained at different levels of the ventral hippocampus (approximately 240 μm apart) from 4 AAV-TeLC injected mice with a rabbit anti-caspase 3 antibody (1:500; #9661, RRID:AB_2341188, Cell Signaling Technology, Frankfurt, Germany) together with the monoclonal rat anti-GFP antibody (1:2,000; 04404-84, RRID:AB_10013361, Nacalai Tesque Inc., CA, USA) at room temperature for 16 h. For detection of the caspase 3 antibody we used a biotinylated goat anti-rabbit antibody (1:200; Vectastain PK 4001, RRID:AB_2336810, Scabo-Scandic, Vienna, Austria; room temperature, 120 min), which was then reacted with streptavidin (1:100; Dylight streptavidin 649 SA 5649, RRID:AB_2336421, Vector Labs, Szabo Scandic, Vienna, Austria; room temperature for 120 min) and goat anti-rabbit antibodies with TSA-Cy3 (homemade, Lumiprobe, Hannover, Germany; 1:100 in 50 mM PBS, 0.005% H2O2; room temperature for 5 min).

For double labeling of caspase 3 and GFP, we incubated three sections obtained at different levels of the ventral hippocampus (approximately 240 μm apart) from 4 AAV-TeLC injected mice with a rabbit anti-caspase 3 antibody (1:500; #9661, RRID:AB_2341188, Cell Signaling Technology, Frankfurt, Germany) together with the monoclonal rat anti-GFP antibody (1:2,000; 04404-84, RRID:AB_10013361, Nacalai Tesque Inc., CA, USA) at room temperature for 16 h. For detection of the caspase 3 antibody we used a biotinylated goat anti-rabbit antibody (1:200; Vectastain PK 4001, RRID:AB_2336810, Scabo-Scandic, Vienna, Austria; room temperature, 120 min), which was then reacted with streptavidin (1:100; Dylight streptavidin 649 SA 5649, RRID:AB_2336421, Vector Labs, Szabo Scandic, Vienna, Austria; room temperature for 120 min). For detecting the GFP antibody, an Alexa Fluor 488-coupled antibody was used (1:500; RRID:AB_221477, Molecular Probes, Eugene, OR, USA) concomitantly with the biotinylated goat anti-rabbit antibody at room temperature for 120 min. We used hippocampal sections from mice unilaterally injected with kainic acid (350 pmol/70 nl; Jagirdar et al., 2015) as positive controls for identifying caspase 3-positive degenerating neurons (not shown).

All double immunofluorescence labeled sections were mounted on fluorescence-free glass slides and covered in 86% glycerol and 2.5% DABCO (D27802, Sigma Aldrich, Vienna, Austria) and analyzed by confocal microscopy.

Semi-quantitative analysis of immunohistochemical data.
To determine the number of PV-labeled neurons in the subiculum of wild-type mice (n = 5) and of heterozygous PV-cre mice (n = 5), cell numbers in the intermediate to ventral subiculum were counted on 30 μm thick horizontal sections reacted for PV (2 matched sections per mouse) at 40x primary magnification. Values obtained from left and right hemispheres and from different sections were averaged and expressed as neurons per region (3,473 neurons were analyzed).

For counting neurons co-expressing TeLC/GFP together with GABA, PV, or somatostatin at the site of AAV vector injection, microphotographs of 30 μm thick horizontal double immunofluorescence-labeled sections (29 mice, 2 sections per mouse) were taken at 20x primary magnification using a fluorescence microscope (Zeiss Imager.M1, Carl Zeiss GmbH, Jena, Germany). Images were imported into NIH ImageJ 1.51d (NIH, Bethesda, MD, USA) and photographs of the individual channels were displayed side by side. The area evaluated was defined by the region containing GFP-positive neurons (directly affected by the vector injection). Using the "Cell Counter" plugin, the numbers of single-labeled and double-labeled cells were determined. In total, 10,590 labeled neurons (GFP: 2,724; GABA: 4,966; PV: 1,114; somatostatin: 1,786) were analyzed for identifying co-labeling with other markers.

To identify neurons stimulated during spontaneous seizures we labeled sections for ΔFosB accumulating in activated neurons (Morris et al., 2000). To determine numbers of intensely labeled ΔFosB expressing neurons, 8-bit microphotographs of individual subregions of the in the hippocampal formation were taken from Ni-DAB-stained 30 μm thick horizontal immune-labeled sections (2 sections per mouse, 34 mice in total) at 10x magnification and constant illumination. Microphotographs were imported into NIH ImageJ, unspecific background (measured in the alveus or fimbria) was measured and subtracted from the pixels’ gray values, and a common threshold was set for all images to obtain binary images displaying black neurons on white background. The ImageJ program function "Analyze Particles" was used to determine the numbers of intensely labeled...
neurons in the outlined regions of interest (parameters: size 5 - 25 μm, circularity 0.3 - 1).

Due to the dense packing of granule cells in the dentate gyrus, these neurons were counted manually. The area of each outlined region was measured and cell numbers per mm² were then calculated. Values from both hemispheres and individual sections were averaged.

Statistical analyses. All statistical analyses were performed using GraphPad Prism statistical software (v.5.0f, GraphPad Inc., La Jolla, USA). Fisher’s exact test was used for analysis of the effect of low-dose PTZ in controls (AAV-GFP) and AAV-TeLC-injected mice. Kruskal-Wallis test with Dunn’s multiple comparison post-hoc test was used for comparing multiple groups with one control group (evaluation of ΔFosB-positive neurons). Two-way repeated measures ANOVA was used to analyze electrophysiological data and unpaired Student’s t-test was used for analyzing mean differences of two groups. A P-value < 0.05 was considered as statistically significant.

Results

TeLC expression is selective for PV interneurons

AAV-TeLC injection resulted in TeLC expression in 61.4 ± 2.43% of PV-containing interneurons at the injection site in the subiculum (total number of neurons counted: 1,114 PV cells; Figs. 1A, C, G-L). Variable expression of TeLC was also detected in the sector CA1, presubiculum, and parasubiculum, and occasionally in parts of the entorhinal cortex and dentate gyrus (Fig. 1A). Double immunofluorescence labeling for GFP (the tag for TeLC) was evaluated in 25 mice (total number of GFP positive neurons: 2,724) and revealed 95.7 ± 0.68%, 83.2 ± 1.27%, and 23.7 ± 1.61% co-labeling with GABA (Fig. 1D-F), PV (Fig. 1G-L) or somatostatin (not shown), respectively (Fig. 1M), indicating a high specificity of transfection/viral transduction for the PV-containing subpopulations of GABA-
ergic basket and axo-axonic cells. About 15 to 30% of PV-expressing neurons contain also somatostatin (Jinno and Kosaka, 2000; Klausberger and Somogyi, 2008) (Fig. 1M), which is supportive of the quantitative data ratio we obtained above. These neurons represent a subpopulation of O-LM and bistratified cells (Fig. 1O) co-expressing PV; they were therefore also affected by the TeLC vector. In reverse, the majority of PV expressing neurons expressed TeLC (60%), whereas only minor populations of GABA and of somatostatin-immunoreactive neurons expressed TeLC (around 18% and 15%, respectively; Fig. 1N). This again underlines the specificity of our experimental manipulation using the TeLC vector. Considering that majorities of GABA and of somatostatin neurons do not contain PV, their lack of labeling confirms specificity of vector delivery.

**Electrophysiology**

To verify a loss in perisomatic inhibition of pyramidal cells by TeLC injection, we used whole-cell voltage-clamp recordings in slices of the subiculum 2-3 weeks after vector injection. We observed only an insignificant reduction in evoked inhibitory postsynaptic currents (eIPSCs) but no change in either miniature IPSCs (mIPSCs) or EPSCs (mEPSCs) (Fig. 2). However, when we applied the CB1 receptor agonist WIN 55,212-2 to suppress GABA release from non-PV, CCK-containing basket cells we observed, consistent with a previous report on CA1 neurons (Murray et al., 2011), that the amplitude of the WIN 55,212-2 insensitive component of the eIPSC (representing inhibition mediated by PV-containing basket cells) was significantly reduced in the subiculum of AAV-TeLC-injected mice compared with AAV-GFP injected controls (Fig. 2A-2E; treatment: $F(1/20) = 4.72, P < 0.05$; time: $F(8/160) = 7.74, P < 0.01$; interaction: $F(8/160) = 0.82, P > 0.05$).

Taken together, our experiments show a significant reduction in eIPSC caused by silencing of PV neurons, while the inhibitory tone of other interneurons (notably that of CCK containing basket cells) is not affected. Importantly, in addition neither mEPSCs nor mIPSCs are altered after vector injection.
Development of recurrent clusters of spike-wave-discharges (C-SWD) and of SRS after AAV-TeLC injection

We then performed telemetric EEG recordings for up to 57 days in freely moving mice injected with AAV-TeLC (n = 25) or AAV-GFP (n = 9) into the subiculum (Fig. 3). At day 16, 22 of the 25 mice (88%) exhibited unprovoked C-SWD (Figs. 3B, D). In average these C-SWD consisted of 42.1 ± 3.14 SWD (Fig. 3G) and probably reflect a pre-ictal state or sub-threshold epileptic activity (Table 1). Among these mice, 16 (representing 64% of all AAV-TeLC injected mice or 72.7% of mice with C-SWD) developed SRS (1 to 19, median: 5.5 SRS per 30 days; Figs. 3E, F, H). SRS developed in parallel with C-SWD although 6 mice did not present SRS by day 42 after AAV-TeLC injection in spite of showing C-SWD (Fig. 3B). As summarized in Table 2, these SRS were mostly (89.5% of 102 total SRS recorded) preceded by pre-ictal C-SWD (Fig. 3E), had a mean duration of 24.6 ± 0.62 s, and were followed by post-ictal depression of the EEG (Fig. 3F, H). None of the AAV-GFP injected mice exhibited C-SWD or SRS during the 6 to 8 weeks recording period (Fig. 3C). Three mice (12%) developed neither C-SWD nor SRS (Fig. 3B).

Importantly, all SRS recorded by EEG were accompanied by motor seizures. Video recordings revealed that 93% of EEG seizures could be evaluated also for the animals’ behavior. Among these, 100% of mice revealed generalized limbic motor seizures with rearing (stage 3 to 4).

Figs. 3 I and J show the frequencies of C-SWD and of SRS during the 6-week period after AAV-TeLC injection, respectively. Although the occurrence of both C-SWD and SRS varied between animals, it was relatively equal, on average amounting to 1.5 seizures and 15 C-SWD per animal and week. Notably, the frequency of C-SWD and SRS tended to decline by weeks 5 and 6.

Neither AAV-TeLC injection nor recurrent seizures caused neurodegeneration
After EEG monitoring (6 to 7 weeks after vector injection), the mice were killed and their brains processed by immunohistochemistry for PV, GABA, somatostatin, caspase 3, \( \Delta \text{FosB} \), or by Nissl stain. Importantly, cell counts at the injection site revealed neither a loss in PV-containing neurons (expressing TeLC) nor in GABA-immunoreactive neurons in \textbf{AAV-TeLC} injected mice that had presented only C-SWD or C-SWD and SRS compared to un-injected controls or \textbf{AAV-GFP} injected mice (Figs. 4A, B). Since immunohistochemistry was performed after concluding EEG monitoring (6 to 8 weeks after vector injection; see Table 1). Only a few caspase 3-positive neurons were seen in close vicinity to the needle tract 3 and 10 days after \textbf{AAV-TeLC} or \textbf{AAV-GFP} injection (Fig. 4F) indicating minimal neurodegeneration due to the injection procedure per se. Furthermore, neither changes in Nissl staining (Figs. 4C, D) nor mossy fiber sprouting, an indicator for seizure-induced loss of mossy cells in the dentate hilus (Houser et al., 1990; Pirker et al., 2001) (Fig. 4E), were observed in any of the \textbf{AAV-TeLC} injected mice. Taken together, these data indicate that neither \textbf{AAV-TeLC} injections per se nor chronic silencing or SRS caused damage of the transfected PV neurons, in spite of inhibiting release of GABA.

To determine the anatomical sites of seizure propagation we investigated expression of the transcription factor \( \Delta \text{FosB} \) after EEG monitoring. \( \Delta \text{FosB} \) accumulates in neurons activated by seizure activity (Morris et al., 2000). Whereas \( \Delta \text{FosB} \) was only barely expressed in controls and in mice that exhibited only C-SWD (Fig. 5A and D), it prominently labeled neurons in the hippocampus, subiculum, entorhinal and perirhinal cortices after frequent SRS (Fig. 5B, C and E to J) corroborating a role of these temporal lobe areas for seizure propagation.

\textbf{Seizure-threshold is decreased also in mice exhibiting C-SWD only}
We then hypothesized that mice presenting C-SWD but no SRS may already have a lowered seizure-threshold and thus be prone to epileptic seizures. To test this, we injected six mice that had been seizure-free for six weeks (in spite of presenting C-SWD: 1-27 per 6 weeks, mean $11.5 \pm 4.87$, $n = 69$), with a threshold dose of the GABA$_A$ receptor antagonist PTZ (30 mg/kg, i.p.). This treatment provoked acute tonic-clonic seizures in all TeLC vector-injected mice, but only in one of seven AAV-GFP injected controls and in none of 5 WT mice ($P = 0.0047$; Fig. 6A-C). The EEG of the mice was monitored for additional 14 days. Importantly, during this time all mice revealed recurrent C-SWD (not shown) and SRS ($0.8 \pm 0.25$ per mouse and week; Fig. 6A).

The seizure-threshold is decreased already shortly after silencing of PV containing interneurons

From these experiments the question arose whether the decrease in seizure-threshold was a consequence of silencing PV-containing interneurons or was induced (like in kindling) by repeated C-SWD experienced over a prolonged period of time. We therefore challenged another group of six mice (still seizure-free and only $2.3 \pm 0.84$ C-SWD/mouse/10 days) with the threshold dose of PTZ already 10 days after TeLC vector injection (at this interval the vector had already resulted in full expression of TeLC). All mice responded with acute and thereafter with recurrent seizures (mean $9.0 \pm 3.55$ per 30 days, $n = 6$; Figs. 6D). This demonstrates that inhibition of GABA release from PV-containing interneurons of the subiculum/CA1 *per se* is sufficient to lower the seizure-threshold and induces a pre-ictal state also in mice that exposed (only a few) C-SWD.

Transient inhibition of PV-expressing interneurons is not sufficient to induce acute or spontaneous seizures

At this point, we asked whether already transient inhibition of GABA release from PV-containing interneurons would be sufficient to induce C-SWD and SRS. We therefore
injected *PV-cre* mice into the ventral subiculum with an AAV vector expressing hM4Di receptors (AAV-hM4Di), which are exclusively activated by designer drugs (DREADD) (Roth, 2016). This treatment resulted in specific expression of the inhibitory hM4Di-DREADD in PV-neurons (Fig. 7A-E). Fifteen days later, we temporarily inhibited virally-transduced PV-containing interneurons by injecting the mice with CNO. Neither hM4Di expression in PV-neurons *per se* nor transient silencing of PV neurons at the injection site for as short as 1 to 2 hrs induced C-SWD or SRS during the subsequent 5 days (n = 7; Fig. 7F). This confirmed that only sustained but not transient inhibition of GABA release from PV-containing neurons is capable to induce seizures/epilepsy.

To verify that GABA release was indeed transiently inhibited after injecting CNO in the transgenic mice expressing hM4Di, we injected another group of mice fifteen days after AAV-hM4Di injection, with CNO (n = 6) or with saline (n = 6) and 45 min later with a threshold dose of PTZ (30 mg/kg), and recorded their EEGs for additional 24 hrs. Now, all 6 mice injected with CNO and PTZ presented C-SWD (4.2 ± 1.14 per 24 hrs) and 2 of 6 mice showed acute convulsions after PTZ (Fig. 7G). In contrast, none of the AAV-hM4Di mice injected with saline (instead of CNO) revealed C-SWD or seizures upon PTZ injection. This experiment documented that CNO indeed had transiently impaired GABA release. Thus, inhibition of PV neurons in the ventral subiculum/CA1 by CNO may transiently lower seizure-threshold, but is *per se* not sufficient to induce acute convulsions.

**Discussion**

Our present experiments build up on previous findings reporting selective losses in PV-containing interneurons in the subiculum of TLE patients and in animal models of TLE (Andrioli et al., 2007; Knopp et al., 2008; Drexel et al., 2011). We now directly interfered with the activity of PV-containing basket cells, a central component of the hippocampal circuitry mediating feed-forward inhibition upon pyramidal cells. We demonstrate that
anatomically restricted inhibition of GABA release from PV-containing interneurons in the subiculum, notably without signs of neurodegeneration, induces recurrent C-SWD that progress to SRS in about 73% of the mice. Recurrent C-SWD reflect a permanent decrease in seizure-threshold since already a threshold dose of the GABA\(_A\) receptor antagonist PTZ precipitates acute convulsions followed by SRS (epilepsy). Importantly, in contrast to permanent inhibition of GABA release from PV-containing neurons (by expressing TeLC), only transient silencing (for about 1 to 2 hrs) of the same neurons using DREADD (Wirtshafter and Stratford, 2016; Roth, 2016) was not sufficient to induce SRS, clearly documenting that only prolonged silencing of PV/GABA neurons results in SRS or a pre-ictal state in which recurrent seizure activity can be provoked.

AAV-TeLC injections into the subiculum selectively targeted PV-containing interneurons as revealed by double-label immunohistochemistry. The injections were not exclusively restricted to the subiculum, but often affected also some PV-containing interneurons in sector CA1 and the pre- and parasubiculum or, in a few cases, the dentate gyrus. Although the subiculum was affected in all experiments we therefore cannot exclude a contribution of PV neurons silenced to some extent also in other hippocampal subfields. The slight variations in targeting individual areas of the hippocampus were due to the anatomical situation injecting into the ventral hippocampus. We have chosen the ventral hippocampus as target area because of the much larger presentation of the subiculum in the temporal part of the hippocampus and the presumed higher relevance of the ventral hippocampus for epileptogenesis (Becker et al., 1997).

As expected, TeLC expression was found in several classes of interneurons of the subiculum known to contain PV (Fig. 1O). The most prominent population comprises PV-containing basket cells forming perisomatic synapses on pyramidal cells (Lee et al., 2014). However, also axo-axonic cells targeting the axon initial segment of pyramidal cells and somatostatin-containing O-LM cells and bistratified neurons, both impinging on pyramidal cell dendrites (Klausberger and Somogyi, 2008), contain PV and were therefore
affected by the AAV-TeLC injections. The major populations of neurons expressing TeLC, however, were basket and axo-axonic cells, and only about 15% of the somatostatin-positive O-LM and bistratified cells expressed TeLC.

Fast-spiking PV-containing basket cells exert extremely potent, but presumably transient feed-forward inhibition upon the perisomatic region of pyramidal cells, whereas O-LM and bistratified cells mediate enduring feedback inhibition on pyramidal cell dendrites (Freund and Katona, 2007; Gulyas and Freund, 2015). On the other hand, axo-axonic cells may also exert an excitatory function by a locally reversed chloride gradient at their synapses (Szabadics et al., 2006).

PV containing interneurons clearly have a prominent role in protecting the brain from initiation of spontaneous seizures. Thus, onset of epileptic activity rapidly induces an inhibitory barrage by activation of fast-spiking PV-containing basket cells, but not of somatostatin containing O-LM cells (Cammarota et al., 2013; Lee et al., 2014; Toyoda et al., 2015). In the pilocarpine model of TLE PV interneurons, but not O-LM cells, become activated in all hippocampal subfields (most prevalently in the subiculum) already several hrs before the onset of spontaneous seizures (Toyoda et al., 2015). A transient drop in their activity may eventually allow seizure initiation (Toyoda et al., 2015). Our present experiments are in line with these findings by showing that focal silencing of PV neurons unilaterally in the ventral subiculum was sufficient to induce lasting SRS in the absence of cellular signs of neurodegeneration.

In spite of the significant effects of vector injections on the animals' behavior, neither overall evoked nor miniature IPSCs or EPSCs were affected in subicular pyramidal cells of slices of AAV-TeLC injected mice. This is rather surprising but consistent with the previous data by Murray et al (2011). There is no clear explanation for this. However, In the presence of the cannabinoid receptor 1 (CB1) agonist WIN 55,212-2, selectively
suppressing GABA release from cholecystokinin (CCK)/CB1-containing basket cells (Takács et al., 2015), we observed a significant reduction of the amplitude of eIPSCs (by 33%). This reduction was almost twice as high as in AAV-GFP injected mice (59%) reflecting the component of CCK/CB1-containing basket cells. These experiments indicate that both types of basket cells exert a primary inhibitory action upon pyramidal cells of CA1 and the subiculum (Takács et al., 2015). Silencing PV-containing interneurons, however, seems to affect only the inhibitory tone exerted by these neurons, but is already sufficient to induce SRS in the mice.

It is important to note that only irreversible silencing of PV neurons by TeLC expression, but not a transient inhibition (for about 1 to 2 hrs) through the DREADD system results in C-SWD or SRS. However, when transient inhibition of GABA release from PV cells of the ventral subiculum was coinciding with systemic application of a threshold dose of PTZ, C-SWD or even acute seizures developed rapidly. Thus, PV neurons in the subiculum are crucial protectors during excitatory challenge and limit the spread of epileptic activity, while they may be transiently dispensable under basal conditions.

The frequency of SRS is modest (around one to two per week), but still beyond the range of seizure frequency in most TLE patients. It was striking, however, that the frequency of SRS appeared to decline after several weeks. The underlying mechanisms are unclear. One may consider a variety of mechanisms responsible such a fading of seizure frequency reflecting re-adaptation of the disturbed neuronal circuitry. One such mechanism may include sprouting of inhibitory neurons. Thus, sprouting of somatostatin terminals (presumably O-LM cells) terminating in the outer molecular layers of the dentate gyrus and of the subiculum has been observed in the kainic acid model of TLE (Drexel et al., 2012; Peng et al., 2013), in kindling (Botterill et al., 2017) and in TLE patients (Mathern et al., 1995; Fürtinger et al., 2001). Other mechanisms may include a reduction of cannabinoid signaling affecting GABA release from CCK basket cells, or a compensatory increase in the activity of PV neurons not affected by the AAV-TeLC.
injection (e.g. contralateral to the injection site). We observed such a compensatory
activation of PV neurons (as indicated by increased expression of PV mRNA) in surviving
PV-interneurons of the subiculum of kainic acid treated rats (Drexel et al., 2011). SRS
could also cause a rapid increase in the expression of neuroprotective neuropeptides
mostly inhibiting glutamate release as observed in human TLE and in respective models
recently (Mathern et al., 1995; Patrylo et al., 1999; Furtinger et al., 2001; Pirker et al.,
2001; Vezzani and Sperk, 2004; Drexel et al., 2012).
Thus, Ledri et al. (2014) recently used selective optogenetic activation of somatostatin-
and PV-containing interneurons to investigate their effect on epileptiform activity in
hippocampal slices. They demonstrated that activation of either dendritic (mediated by
somatostatin neurons) or perisomatic inhibition (mediated by PV-containing basket cells)
suppressed epileptiform activity, although global activation of all GABA neurons was even
more effective indicating important roles of both mechanisms in maintaining physiological
transmission (Ledri et al., 2014). We are currently investigating such mechanisms that
may be involved in the late reduction in the frequency of SRS.
Taken together, our experiments demonstrate a pivotal role of PV-containing basket cells
for controlling the excitability of pyramidal neurons in the subiculum. We provide
experimental evidence that selective permanent inhibition of PV-containing interneurons
may reduce perisomatic feed-forward inhibition in vivo resulting in a decrease in seizure-
threshold with the development of C-SWD and SRS (i.e. epilepsy) without signs of
neurodegeneration. In contrast, transient inhibition of PV-containing basket cells requires
an additional excitatory challenge for evoking C-SWD or seizures.
References


Figure Legends

Figure 1. AAV-TeLC induces selective expression of TeLC in PV-containing interneurons in the subiculum of PV-cre mice.

(A) Distribution of TeLC (immunoreactivity for the GFP tag) after unilateral injection of AAV-TeLC into the subiculum of PV-cre mice and after EEG monitoring for up to 8 weeks. The overall distribution of PV-containing interneurons and fibers in the subiculum of C57BL/6N wild type (WT) mice (B) is similar to the distribution of TeLC expressing neurons in AAV-TeLC injected mice (C). Representative images for WT (B; n = 5) and AAV-TeLC injected mice (A, C; n = 25) For regional distribution of TeLC expression in individual animals see Table 1.

(D-L) TeLC is specifically expressed in PV-containing interneurons: (D-F) Only a subpopulation of GABA neurons (blue) expressed TeLC (green; white arrows in F indicate double labeled neurons). The majority of GABA containing neurons did not express TeLC (red arrows in F). (G-L) The majority of PV-containing neurons were positive for TeLC/GFP at the site of AAV-TeLC injection, shown at high (G-I) and low magnification (J-L; white arrows indicate some of the double labeled cells).

(M, N) Cell counts of double labeled cells (in total 2,724 GFP-positive neurons were evaluated in 25 mice): (M) More than 95% of TeLC expressing cells contain GABA, 80% PV and about 20% also somatostatin (interneurons containing somatostatin and PV (Jinno and Kosaka, 2000)). (N) In reverse, about 60% of PV neurons express TeLC at the injection site, whereas less than 20% of GABA-expressing neurons and about 15% of SOM-expressing interneurons are TeLC/GFP positive.

(O) Scheme of PV containing interneurons in the subiculum: The major population comprises PV containing basket cells forming perisomatic synapses on pyramidal cells (PC). Minor populations are axo-axonic cells forming synapses on PC axon initial segments and subpopulations of somatostatin-containing O-LM cells and bistratified cells mediating feedback inhibition through PC dendrites.
Abbreviations in (A) and (B): DG, dentate gyrus; PaS, parasubiculum; PrS, presubiculum; Sub, subiculum. In O: oml, outer molecular layer; iml, inner molecular layer; pcl, pyramidal cell layer. The dashed line in (B) indicates the border between the subiculum and sector CA1. Scale bar in (A): 600 μm; scale bar in (C; for B, C): 300 μm; scale bar in (I; for D-I): 25 μm; scale bar in (L; for J-L): 100 μm.
Figure 2. TeLC-expression in PV-containing interneurons attenuates inhibitory input at synapses on pyramidal neurons of the subiculum.

To quantify the loss of inhibition, 2-3 weeks after AAV-TeLC or AAV-GFP injection (time when C-SWD had become manifest), whole-cell voltage-clamp recordings from pyramidal neurons of the subiculum were obtained and electrically evoked inhibitory postsynaptic currents (eIPSCs) were recorded in the presence of glutamate receptor antagonists (DNQX, 10 μM and DL-AP5, 100 μM). In the subiculum, eIPSCs originate from various types of local interneurons, including cholecystokinin (CCK)- and PV-containing neurons.

To partially isolate the eIPSC component from PV-containing neurons, the cannabinoid receptor 1 (CB1) agonist WIN 55,212-2 (1 μM) was bath applied to selectively suppress GABA release from CCK-containing neurons (known to express the CB1 receptor), as previously described for pyramidal neurons of sector CA1 (Glickfeld et al., 2008; Murray et al., 2011).

(A, B) The baseline maximal amplitude of eIPSCs and the stimulation intensity required to evoke maximal responses was not different between groups (\(P = 0.286\) and 0.651, respectively; t-test). Two-way repeated measures ANOVA revealed that the amplitude of eIPSCs in the presence of WIN 55,212-2 was significantly reduced in AAV-TeLC-compared to AAV-GFP-injected mice (C; Treatment: \(F_{(1/20)} = 9.83, P = 0.0052\); Time: \(F_{(8/160)} = 5.97, P < 0.0001\)). Importantly, the WIN 55,212-2 insensitive component of the eIPSC was significantly larger in mice injected with AAV-GFP compared to AAV-TeLC-injected mice (D; treatment: \(F_{(1/20)} = 4.72, P = 0.0419\); Time: \(F_{(8/160)} = 7.74, P < 0.0001\)). Specifically, WIN 55,212-2 reduced the amplitude of eIPSCs by 33% in AAV-GFP injected mice, consistent with previous reports (Glickfeld et al., 2008), while mice injected with AAV-TeLC exhibited a 59% reduction in the amplitude of eIPSCs (E). Thus, TeLC expression in PV-containing neurons results in a significant reduction of inhibitory input to subicular pyramidal neurons (n = 10 cells from 6 AAV-TeLC-injected mice and 12 cells from 5 AAV-GFP-injected mice). Values are given as means ± S.E.M.
(F) The representative traces of the mIPSC recordings of subiculum pyramidal neurons from AAV-GFP (black) and AAV-TeLC-injected (gray) mice. (G) Visualization of subiculum pyramidal neurons (magenta color) after patch-clamping (arrows) with solution containing biocytin. PV+ neurons in the subiculum are visible in green because of stereotaxic injection of Cre-driven AAV-GFP construct. (H-I) Analysis of mEPSCs (H) and mIPSCs (I) amplitude (H,I, left) and frequency (H,I, right) in subiculum pyramidal neurons from Pvalb<sup>tm1(cre)Arbr</sup> mice injected with AAV-GFP (black bars, n = 10 and 9 cells for mEPSC and mIPSC recordings, respectively) and AAV-TeLC (red bars, n = 11 and 10 cells for mEPSC and mIPSC recordings, respectively). Data are not significantly different between 2 experimental groups.
Figure 3. Permanent silencing of PV-neurons by unilateral AAV-TeLC injection into the ventral subiculum of PV-cre mice results in spontaneous recurrent seizures (SRS).

(A) Scheme of electrode positions and anatomical site of vector injection. (B) Cumulative presentation of C-SWD and of SRS. Eighty-eight percent of mice developed C-SWD by day 16, and 64% of mice presented at least one SRS by day 28.

(C-E) Representative 8 min EEG-traces obtained in mice 18 d after AAV-GFP (C) or AAV-TeLC (D-E) injection. Whereas AAV-GFP injected mice showed an unchanged EEG, AAV-TeLC injected mice presented either C-SWD only (D) or C-SWD and SRS (16 of 25 mice, E). The intermittent C-SWD lasted about 5 min and were detected in 22 of 25 AAV-TeLC-injected mice (D). About 90% of SRS occurred immediately after pre-ictal C-SWDs (E). (F) Magnitude spectrum (0-80 Hz, logarithmic power scale) of the EEG-recording in (E). Note the profound and long-lasting post-seizure depression of EEG amplitude in all frequency bands.

(G, H) High resolution EEG-traces of a pre-ictal single SWD (G, duration about 300 ms, marked as G in panel E) and (H) of a spontaneous tonic-clonic seizure (marked as H in panel E). (E, F, H) EEG seizures lasted about 25 seconds and were always accompanied by generalized tonic-clonic motor seizures with loss of posture (stage 3 to 4). (I, J) Mean C-SWD (± SEM) (I) and mean SRS (± SEM) presented per week (J). Note the reduction in C-SWD and seizure frequencies after 5 to 6 weeks.
Figure 4. AAV-TeLC injection or related seizures did not induce signs of neurodegeneration.

(A, B) AAV-TeLC did not affect cell numbers of PV neurons at the injection site. Numbers (mean ± S.E.M.) of PV- and GABA-positive neurons were neither reduced in the subiculum of mice that had experienced C-SWD only (n = 6) nor in mice presenting SRS after AAV-TeLC injection (n = 16). Cell counts were done 6 to 8 weeks after AAV-TeLC injection (and monitoring). Numbers are shown for the injected subiculum; they did not differ from those in the contralateral subiculum (data not shown). Controls: Uninjected mice (n = 6); data were not different from AAV-GFP injected mice (n = 9; data not shown).

(C, D) Representative Nissl stains in brain slices at the level of the ventral hippocampus of (C) mice with SRS after AAV-TeLC injection (n = 16), and (D) after AAV-GFP injection (seizure free; n = 9).

(E) To test for possible mossy fiber sprouting as a consequence of loss in highly vulnerable mossy cells, we labeled hippocampal mossy fibers by immunohistochemistry for dynorphin. Although mossy fibers were strongly positive for dynorphin, no immunoreactivity was observed in the inner molecular layer of the dentate gyrus, which would be indicative for sprouted mossy fibers (red arrow in E) in AAV-TeLC injected mice that had experienced SRS for 6 to 8 weeks. The black arrow (E) indicates the area of the granule cell layer that is also devoid of dynorphin-immunoreactivity (representative for 16 mice).

(F) Double labeling for the apoptosis marker caspase 3 (red cells, white arrows) and for TeLC/GFP (green cells, blue arrows) at the injection site of AAV-TeLC, 10 days after injection. Labeling was performed in three horizontal sections obtained at different dorso-ventral levels (about 240 µm apart from each other) from 4 mice killed 3 or 10 days after AAV-TeLC injection. Zero to maximally five apoptotic cells per section were detected close to the needle tract. Note the intact GFP-positive cells expressing TeLC but not caspase 3 in close vicinity to presumably apoptotic cells. As positive controls we investigated caspase 3 expression in the dorsal hippocampus of mice injected locally with...
kainic acid two and 10 days before (Jagirdar et al., 2015) and observed caspase 3-
positive cells at both intervals (data not shown).

Scale bars: in (D) for (C, D): 500 μm; in (E): 200 μm; in (F): 20 μm.
Figure 5. Expression of ΔFosB in the hippocampal formation after spontaneous seizures induced by AAV-TeLC injection.

To identify neurons stimulated during spontaneous seizures, sections of the ventral hippocampus were obtained after EEG monitoring (up to 8 weeks after AAV-TeLC or AAV-GFP injection) and labeled for ΔFosB-immunoreactivity, a cellular marker accumulating in activated neurons (Morris et al., 2000).

(A, D) Only very faint ΔFosB labeling was detected in some neurons of the hippocampal formation (A) including the subiculum (D; higher magnification) in AAV-GFP injected mice (no EEG alterations) and in AAV-TeLC injected mice with C-SWD but no SRS (not shown).

(B) ΔFosB was clearly expressed in neurons of mice that experienced 1 to 6 SRS and (C, E, F) high numbers of ΔFosB-positive neurons were present throughout the hippocampal formation and the deep and superficial layers of the entorhinal and perirhinal cortices of mice that had experienced between 9 and 19 SRS after AAV-TeLC; (E) higher magnification of the subiculum of these mice.

(C, F) Note the extremely strong expression of ΔFosB in the granule cell layer of these mice, sometimes particularly concentrated in the inner and outer portions of the granule cell layer (F).

(G-J) Semi-quantitative estimates of ΔFosB-positive cells using the ImageJ program were done in blinded fashion and are depicted in (G) sector CA3, (H) granule cells of the dentate gyrus, (I) the subiculum, and (J) layers V to VI of the perirhinal cortex after injection of AAV-GFP (GFP; n = 10) or AAV-TeLC (TeLC). AAV-TeLC-injected mice had either experienced at most C-SWD (TeLC sw; n = 9), or 1 to 6 (TeLC rs+; n = 10), or more frequent (9 to 19) SRS (TeLC rs++, n = 5). Scale bars: in (C) for (A-C), 500 μm; in (F) for (D, E, F), 100 μm. Similar increases in numbers of ΔFosB-positive neurons were also obtained for sector CA1, the superficial layers of the perirhinal cortex (PRC), and the deep and superficial entorhinal cortices (not shown).
Statistics: Kruskal-Wallis test with Dunn's multiple comparison post hoc test (all groups compared to controls; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. AAV-GFP group). Values are given as means ± S.E.M. Abbreviations: CA1 and CA3, hippocampal sectors CA1 and CA3; DG, dentate gyrus; EC, entorhinal cortex; PRC, perirhinal cortex; Sub, subiculum.
Figure 6. The seizure-threshold is reduced in mice exposing C-SWD but not SRS after AAV-TeLC.

Seizure-threshold was tested with a threshold dose of pentylenetetrazole (PTZ; 30 mg/kg, i.p.) in PV-cre mice that did not develop SRS after AAV-TeLC.

(A) PTZ induces acute (red bar) and then recurrent seizures and C-SWD (not shown) in mice that were seizure-free for 6 weeks after AAV-TeLC. Bars represent mean seizures (± S.E.M.) per day. The red bar depicts acute PTZ induced seizures the gray bars show SRS.

(B) Representative EEG traces after PTZ in mice initially injected with AAV-GFP (upper trace) and AAV-TeLC (lower trace), respectively.

(C) After PTZ, seizures were observed in all AAV-TeLC injected mice (n = 6) but only in one of 7 AAV-GFP injected controls.

(D) Injection of a threshold dose of PTZ in still seizure-free mice (n = 6) already 10 d after AAV-TeLC provoked one acute seizure (red bar) and subsequently SRS (gray bars) and C-SWD (not shown), but not in AAV-GFP injected controls (not shown).
Figure 7. Only transient inhibition of PV neurons in the subiculum

(A) Distribution of hM4Di/RFP expressed after AAV-hM4Di injection. (B) hM4Di is found in neurons and dendrites at the injection site. (C-E) At the injection site the majority of PV neurons was also labeled for red fluorescence protein (RFP; tag for hM4Di).

(F) PV-cre mice injected with AAV-hM4Di (n = 7) were treated after 12 days with saline (not indicated) and after 15 days with CNO (10 mg/kg, i.p.). Neither treatment resulted in acute or spontaneous seizures (EEG recordings for 5 days after CNO). (G) To investigate whether CNO treatment was efficient we injected additional 12 mice with CNO or saline on day 15 after AAV-hM4Di and, 45 min later, with a threshold dose of PTZ (30 mg/kg, i.p.). EEGs were monitored for further 24 hrs. All CNO and PTZ-injected mice (n = 6) showed acute C-SWD (4.2 ± 1.14 per 24 hrs) and 2 mice acute seizures (1 and 3 seizures, respectively) during the initial 2 hrs indicating that GABA-ergic transmission is reduced after CNO (right bars). Six AAV-hM4Di injected mice (n = 6) treated concomitantly with saline (instead of CNO) and later with PTZ revealed neither C-SWD nor seizures (left bars). Abbreviations in (A): CA1 and CA3, hippocampal sectors CA1 and CA3; DG, dentate gyrus; EC, entorhinal cortex; Sub, subiculum. Scale bar in (A): 500 μm; Scale bar in (E, for B-E): 25 μm.
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<th>PrS</th>
<th>PaS</th>
<th>DG</th>
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**Group 2: AAV-FLEX-TeLC injected, with seizures**

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**Group 3: AAV-FLEX-GFP injected**

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**Group 4: AAV-FLEX-TeLC injected and 10 days later with PTZ**

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**Table 1.**

Summary of mice injected with AAV-TeLC or with AAV-GFP, the anatomical distribution of TeLC expression, and the total number of seizures observed.
Injections were done unilaterally into the ventral subiculum and mice were subjected to telemetric EEG and video monitoring. **Group 1** (# 1 - 9): Mice that revealed no spontaneous seizures, but (except # 1 - 3) clusters of spontaneous spike-wave discharges (C-SWD). Six of these mice (# 4 - 9) that had experienced C-SWD but were seizure-free at day 42 (see Fig. 3) were then injected with a threshold dose of PTZ resulting in acute and then recurrent spontaneous seizures (Fig. 6). **Group 2** (# 10-25): Sixteen of 25 AAV-TeLC injected mice (# 10 - 25) revealed spontaneous recurrent seizures and C-SWD. **Group 3** (# 26-34): None of nine AAV-GFP injected mice exhibited recurrent seizures and were considered as controls. The fact that these mice also did not present C-SWD argues against an unspecific effect of vector injection. **Group 4** (# 35 - 40): These mice were injected with AAV-TeLC and after 10 days with PTZ. They were seizure-free after AAV-TeLC but exhibited acute and then spontaneous recurrent C-SWD and seizures after PTZ. Mouse 37 died from a status epilepticus (se) on day 34.

Expression of GFP (tag for AAV-TeLC) was most prominent in the subiculum/sector CA1, often extending to the pre- and parasubiculum (PrS, PaS) and, in some instances to the entorhinal cortex (EC) or to the dentate gyrus (DG).
Table 2. Characteristics of spontaneous recurrent seizures and clusters of spike-wave discharges (C-SWD)

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<td>Incidence</td>
<td>64% of mice presented at least 1 spontaneous seizure during the first 6 weeks after virus-injection</td>
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<td>Onset of spontaneous seizures</td>
<td>9.5 ± 1.86 days after AAV-injection</td>
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<td>50% of mice with seizures presented first seizure before day 6</td>
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<td>Frequency of seizures</td>
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<td>Mean seizure duration</td>
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<td>Pre-ictal C-SWD</td>
<td>89.5% of spontaneous seizures were preceded by C-SWD</td>
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<td><strong>Clusters of spike-wave discharges (C-SWD)</strong></td>
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<td>Incidence</td>
<td>88% of mice</td>
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<td>50% of mice with seizures presented first C-SWD before day 5</td>
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<td>Number of SWD per C-SWD</td>
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<td>Frequency of SWD within C-SWD</td>
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<td>Mean duration of C-SWD</td>
<td>294 ± 13.7 seconds</td>
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Data are presented as mean ± SEM