Silencing of Hippocampal Somatostatin Interneurons Induces Recurrent Spontaneous Limbic Seizures in Mice

Meinrad Drexel, Sadegh Rahimi and Günther Sperk
Department of Pharmacology, Medical University Innsbruck, 6020 Innsbruck, Austria

Abstract—The hippocampus proper and the subiculum contain two major populations of somatostatin (SST)-containing interneurons, oriens-lacunosum moleculare (O-LM) cells projecting from the stratum oriens to the stratum lacunosum moleculare and bistratified cells with their cell bodies close to the pyramidal cell layer and axons terminating in the strata radiatum and oriens. Both types of interneurons innervate pyramidal cell dendrites and exert prominent feedback inhibition. We now investigated whether impairing this type of feed-forward inhibition by selectively inhibiting GABA release from SST expressing interneurons in hippocampal sector CA1 and subiculum may be sufficient to induce spontaneous recurrent seizures. We injected transgenic mice expressing Cre-recombinase on the SST promoter unilaterally into the ventral CA1 sector and subiculum with an adeno-associated viral (AAV) vector expressing tetanus toxin light chain (TeLC) with its reading frame inverted in a flip-excision (FLEX) cassette. This treatment resulted in specific expression of TeLC and silencing of SST-containing interneurons. We continuously monitored the EEG and behavior of the mice for six weeks. Nine out of eleven mice within 10 days developed series of pre- or interictal spikes (IS, 21.4 ± 6.83 per week) and four mice exposed recurrent spontaneous seizures (SRS, 1.5 ± 0.29 per week). All 23 SRS observed were preceded by IS series. Our data demonstrate a critical role of feed-forward inhibition mediated by SST-containing interneurons suggesting that their sustained malfunctioning can be causatively involved in the development of TLE.

Key words: feed-forward inhibition, O-LM cells, bistratified cells, preictal spikes, epileptogenesis.

INTRODUCTION

Temporal lobe epilepsy (TLE) is the most frequent form of focal epilepsies. It is characterized by spontaneous recurrent seizures (SRS) arising from limbic brain structures and is often associated with hippocampal sclerosis, and consequently with memory deficits and emotional disturbances. TLE may be provoked by an initial insult like head injury, sustained seizures or prolonged febrile seizures in early childhood. It often takes years until the syndrome becomes manifest. During this “silent phase” neuronal circuitries, most likely in the hippocampal formation, are altered and lead to recurrent hyper-synchronization, hyper-excitability, and seizures (Paz and Huguenard, 2015). The cellular and molecular mechanisms underlying this process are still poorly understood although it is believed that a malfunctioning of GABA-ergic neurons in the hippocampus may importantly contribute to the transition from a normal to an epileptic brain (Cossart et al., 2001; Paz and Huguenard, 2015).

The hippocampal circuitry is based on the so-called tri-synaptic pathway consisting of a sequence of excitatory projections reaching from the entorhinal cortex to the dentate gyrus, from there to sector CA3 of the Ammon’s horn, CA3 to CA1 and from CA1 to the subiculum, the main output region of the hippocampus. These excitatory projections are under tight control of inhibitory interneurons. More than 25 subtypes of interneurons have been characterized in the hippocampus so far (Freund and Buzsáki, 1996; Klausberger and Somogyi, 2008). Among these, two groups of interneurons are especially important for the functioning of the hippocampal circuitry: (1). Basket cells and axo-axonic cells with their cell bodies at the pyramidal cell layer and axons making their inhibitory contacts with pyramidal neurons close to their cell bodies; these neurons exert primarily feed-forward inhibition. They separate into several subpopulations characterized by their content of the calcium-binding protein parvalbumin (PV) or the neuropeptides cholecystokinin (CCK) or vasoactive intestinal peptide (VIP). (2) The other important populations are so called O-LM (oriens-lacunosum moleculare) cells projecting from the stratum oriens to the stratum
lacunomus molecular and bistratified cells targeting pyramidal cell dendrites in the strata radiatum and oriens. Both interneuron types exert potent feedback inhibition upon pyramidal cell dendrites. O-LM and bistratified cells contain the neuropeptide somatostatin (SST) as co-transmitter to GABA. In addition, SST is also expressed by GABA-ergic neurons back projecting from the hippocampus to the septum (Freund and Buzsáki, 1996; Jinno and Kosaka, 2002) and interneurons co-expressing PV and SST have been reported in the presubiculum of SST-cre mice (Nassar et al., 2015).

Malfunctioning of PV-containing basket cells as well as of SST-containing O-LM and bistratified cells has been associated with the development of epilepsy. Thus, TLE patients, even without signs of Ammon’s horn sclerosis, show a selective loss of PV-containing basket cells in the subiculum (Andrioli et al., 2007), and horn sclerosis, show a selective loss of PV-containing.

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Furthermore, silencing of PV-containing basket cells and axo-axonic cells of the subiculum results in recurrent series of pre- or interictal spikes (IS) and spontaneously recurrent seizures (Drexel et al., 2017). These data indicate an important role of PV neurons in the manifestation of TLE and also underline a particular role of the subiculum in the development of TLE.

One of the hallmark characteristics of animal models of TLE is a loss of SST neurons in the hilus of the dentate gyrus (Sloviter, 1987). On the other hand, surviving SST- and NPY-expressing interneurons become markedly activated (Gruber et al., 1994) and can even sprout in the molecular layer of the dentate gyrus (Mathern et al., 1995; Peng et al., 2013) and to the outer molecular layer of the subiculum (Drexel et al., 2012). And, a deficit in dendritic feed-back inhibition in sector CA1, presumably caused by a loss in SST-containing O-LM neurons, decreases the animals’ seizure-threshold (Cossart et al., 2001; Dinocourt et al., 2003).

To further substantiate the hypothesis that impaired feed-back inhibition mediated by SST-containing interneurons may be causative for the development of TLE, we now permanently inhibited GABA release selectively from SST neurons of sector CA1 and the subiculum in mice and assessed continuous telemetric EEG and video monitoring for six weeks. We injected an adeno-associated virus vector (AAV1/2) containing either the gene for tetanus toxin light chain (TeLC) fused with a GFP tag or GFP alone, with the reading frames inverted in a flip-excision (FLEX) cassette (AAV-TeLC and AAV-GFP; respectively) and a cytomegalovirus virus (CMV) enhancer/chicken β-actin promoter were prepared as described previously (Murray et al., 2011; Drexel et al., 2017). AAV titers were determined by qPCR as genomic particles per ml (gp/ml).

Surgery and vector injection

In total 26 mice were used for the experiments. Vector injection and EEG monitoring were done as described in detail previously (Drexel et al., 2017). Briefly, heterozygous male SST-cre transgenic mice (age 10–14 weeks) were treated with the analgesic drug carprofen (5 mg/kg, s.c.; Rimady, Pfizer) 60 min before surgery. They were anesthetized with 150 mg/kg, i.p. ketamine (stock solution 50 mg/ml; Ketasol, Ogris Pharma Vertriebs-GmbH, Wels, Austria) and anesthesia was maintained by applying 1–3% sevoflurane (3% for induction, 1–1.5% for maintenance; Sevorane, Abbott, IL, USA) through a veterinary anesthesia mask using oxygen
(400 ml/min) as a carrier gas. Mice were placed into a stereotaxic frame (David Kopf Instruments) and the skin above the sculls was opened. We then placed a telemetry EEG transmitter (ETA-F10, Data Sciences International, St. Paul, MN, USA) into a subcutaneous pocket at the left abdominal wall, and the two connected electrode wires were pulled through the subcutaneous channel (between the pocket and exposed skull); the pocket was carefully sutured afterwards. For the AAV vector injection, insertion of the tungsten depth electrode, and reference electrode we drilled three holes. We implanted a tungsten depth electrode (Cat. no 577100; Science Products GmbH, Hofheim, Germany) into the left ventral subiculum (3.8 mm posterior, 3.5 mm lateral, 3.0 mm ventral; Fig. 3A) for later EEG recordings 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 lateral; Fig. 3A). In some mice, we placed an epidural electrode above the ipsilateral dorsal hippocampus instead of the depth electrode. Electrodes were fixed to the skull using dental cement (Paladur, Heraeus Kulzer supplied by Henry Schein, Innsbruck, Austria). We then injected 1.5 µL of AAV-TeLC or AAV-GFP at a rate of 0.1 µl/min unilaterally into the transition zone of the left ventral subiculum and sector CA1 (3.8 mm posterior, 3.5 mm lateral, 3.5 mm ventral). AAV-TeLC or AAV-GFP injections were done on the same days in mice of the same litters. Thereafter the mice were single housed.

We performed two experiments, differing in the titer of the injected AAV-vectors:

**Pilot experiment** (using a higher vector dose). We injected the SST-cre mice with AAV-TeLC (also expressing the GFP tag) at a titer of 2.4 × 10^{10} gp/mL (n = 7) or with AAV-GFP (tier 2.8 × 10^{10} gp/mL, n = 4). Subsequent immunohistochemistry revealed some TeLC expression in pyramidal cells (see Results, Fig. 1). We therefore used lower concentrations of the vectors in the main experiment resulting in almost exclusive expression of the vectors in SST containing cells (Fig. 2).

**Main experiment** (low vector dose). Transgenic mice were injected with AAV-TeLC at a titer of 1.2 × 10^{10} gp/mL (n = 11) or with AAV-GFP (n = 5; tier 1.4 × 10^{10} gp/mL). EEG recordings were performed for 42 days and the mice were perfused with PFA afterwards and their brains processed for immunohistochemistry.

**EEG recordings and video monitoring.** Telemetric EEG recordings were performed as described previously (Drexel et al., 2017). In brief, telemetry implants (Physiotel transmitter ETA-F10, weighing 1.6 g, 1.1 cm^3, battery lifetime 2 months, Data Science international, DSI, St. Paul, MN, USA) were applied, allowing the continuous measurement of EEG, physical activity, and temperature. EEGs were recorded at a sampling rate of 1000 Hz with no a priori filter cutoff. Two independent observers visually inspected the EEG traces of local field potentials using the Ponemah v6.0.x analysis software (Data Sciences International). Seizures were defined as EEG segments with continuous activity with an amplitude of at least two standard deviations of pre-seizure baseline amplitude, a duration of at least 10 s, and the presence of a postictal depression (Fig. 3E, F).

**Evaluation of motor seizures.** Synchronized video recordings were used to analyze behavioral correlates to EEG seizures. For this purpose, continuous video recordings, conducted via Axis 221 network cameras (Axis Communications, Lund, Sweden) and infrared illumination (Conrad Elektronik, Hirschau, Germany) during darkness were performed. Seizure severity was evaluated according to staging used for kainic acid-induced seizures in rats (Sperk et al., 1983), in which stages 1 to 4 are defined as staring or chewing (1), unilateral or bilateral tonic movements/seizure (2), rearing without falling (3), and rearing with falling/limbic seizures (4), respectively.

**Paraformaldehyde fixation of the brains.** After terminating EEG recordings (6 weeks after vector injection), the mice were deeply anesthetized with a lethal dose of thiopental (Sandoz, Austria) and subsequently perfused via the left ventricle with 25 ml ice-cold 50 mM phosphate-buffered saline (PBS) followed by 100 ml 4% paraformaldehyde (PFA). The brains were then removed from the sculls and placed in 4% PFA/PBS for 90 min and afterwards transferred to 20% sucrose/PBS and kept there overnight. The brains were then snap-frozen in −70 °C isopentane (Merck, Darmstadt, Germany, 3 min) and kept in sealed vials at −70 °C until they were cut in a cryostat-microtome (Carl Zeiss AG, Vienna, Austria). Microtome sections (40 µm) were kept at cold room temperature (4 °C) until they were processed.

**Immunohistochemistry**

Immunohistochemistry was performed on free-floating, 4% PFA-fixed, 40-µm-thick horizontal sections applying indirect peroxidase labeling or immunofluorescence as described previously (Wood et al., 2016; Drexel et al., 2017). The following primary antisera were used: monoclonal rat anti-GFP (1:2000; catalog #04404-84, Nacalai Tesque (through GERBU Biotechnik, Heidelberg, Germany)), rabbit anti-SST (1:1000; (Sperk and Widmann, 1985)) and a monoclonal mouse antibody (1:5000; MAB 377, clone A60; Chemi-Con, Millipore, Vienna, Austria) for neuronal nuclear protein (NeuN).

The antibodies were characterized by the supplier and/or validated by us previously (Sperk and Widmann, 1985; Marksteiner and Sperk, 1988; Wood et al., 2016). We incubated horizontal sections containing the ventral hippocampus free floating with 10% normal goat or horse serum (Biomedica) 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 (at room temperature for 16 h), followed by washing with TBS-Triton. Primary antibodies bound to the respective antigens were then tagged by incubation with horseradish peroxidase (HRP)-coupled secondary antibodies, donkey anti-rabbit (1:150; catalog #711035152, Jackson ImmunoResearch, Ely, UK) for
SST and NeuN or donkey anti-rat (1:500; catalog #712035153, Jackson ImmunoResearch; RRID: AB_2340639) for GFP at room temperature for 150 min. After washing with TBS, HRP bound to the secondary antibodies was revealed with 0.05% diaminobenzidine (DAB; Fluka and Sigma-Aldrich Handels GmbH, Vienna, Austria) and 0.005% H$_2$O$_2$. After washing the sections in TBS, they were mounted on slides, dehydrated in ethanol series, and cover-slipped with Eukitt (Gröpl, Vienna, Austria).

Double-labeling by immunofluorescence was performed as described previously (Wood et al. 2016). Two sections per animal were matched to anatomically same sections from the other animals ($n = 5$ for AAV-GFP and $n = 11$ for AAV-TeLC) and processed for SST and GFP. The same antibodies as described above were used at the same concentrations (at room temperature for 16 h). The secondary reaction was performed by simultaneous incubation with a donkey anti-rat antibody coupled to Alexa Fluor 488 (1:500; Thermo Fisher Scientific, Vienna, Austria) for GFP and with an HRP-coupled goat anti-rabbit antibody (1:250; catalog #P0448, Dako; RRID: AB_2617138) for SST at room temperature for 120 min. The HRP-coupled antibody was then further reacted with TSA-Cy3 (homemade; Lumiprobe; 1:100 in 50 mM PBS and 0.005% H$_2$O$_2$) at room temperature for 5 min. The sections were then mounted on slides and covered in 86% glycerol, 2.5% DABCO (D27802, Sigma Aldrich, Vienna, Austria) and analyzed by confocal microscopy.

Cell counts

For determining the distribution of GFP labeled interneurons, we counted their numbers (at 40-fold magnification) in the different hippocampal subfields of the same 40 µm thick horizontal sections (obtained close to the injection site) after reacting the sections for GFP immunoreactivity (2 matched sections per mouse). Values obtained for each region were averaged and expressed as GFP-positive neurons per total number of GFP-positive neurons within the entire section.
Proportion of GFP positive pyramidal cells. NeuN immunoreactive pyramidal cells were determined in the same way in the pyramidal cell layers of CA1 and in the subiculum. The total numbers of NeuN labelled cells were 332 and 739 per region, respectively. Assuming that 90% of the cells represented pyramidal neurons we corrected the numbers respectively. GFP-positive pyramidal cells and GFP-positive dendrites of pyramidal cells were counted in the same area. GFP positive pyramidal cells were well identified by their shape and/or attached dendrites. The proportion of GFP expressing pyramidal cells/pyramidal cell dendrites of total number of (NeuN-positive) pyramidal cells was calculated.

Number of interneurons co-expressing of TeLC/GFP and SST. For estimating numbers of interneurons co-expressing TeLC/GFP and SST at the site of AAV vector injection, microphotographs of 40 μm thick horizontal double immunofluorescence-labeled sections were taken at 20-fold magnification using a fluorescence microscope (Zeiss Imager.M1, Carl Zeiss GmbH, Jena, Germany). Images were imported to NIH ImageJ (NIH, Bethesda, MD, USA) and photographs of the individual channels were displayed next to each other. Using the “Cell Counter” plugin, the numbers of single-labeled and double-labeled cells were determined.

Statistics
A paired Student’s t-test was used for comparing summative SRS and IS exposed by the mice during the interval 20 to 28 with those between 30 to 38 days after AAV-TeLC injection.

RESULTS
The mice recovered well after vector injection and EEG-transmitter implantation. They had no weight loss, exposed normal motor behavior already after 24 h and their surgical wounds were entirely healed after four to
five days. They were single-housed and their EEGs and seizure spells were recorded by telemetric recording and video EEG for 6 weeks. We then verified the correct localization of the vector injections and the specificity of its expression for SST containing interneurons in all mice by immunohistochemistry/immunofluorescence.

**Specificity of vector injection**

In initial pilot experiments, we investigated the specificity of the site of vector injections and their specificity in targeting SST neurons in three SST-cre mice that were euthanized already after five days. We used an AAV-TeLC vector concentration of $2.4 \times 10^{10}$ gp/mL. At this concentration of the vector, we observed, however, GFP-immunoreactivity (IR) – besides its abundant expression in interneurons – also in some pyramidal neurons (Fig. 1B). This indicated unspecific activation of the SST promoter in some pyramidal cells causing expression of TeLC. For our subsequent experiments we therefore decided to use lower concentrations of the AAV-TeLC ($1.2 \times 10^{10}$ gp/mL) and AAV-GFP ($1.4 \times 10^{10}$ gp/mL, in control mice). Using these lower vector concentrations for the AAV-TeLC or AAV-GFP injections, we observed GFP-IR primarily in hippocampal interneurons of the mice. Under these conditions GFP-IR was rarely present in pyramidal cells or pyramidal cell dendrites (Fig. 2C). For obtaining an estimate for the percentage of pyramidal cells silenced by TeLC expression, we counted GFP-IR pyramidal cells and pyramidal cell dendrites and calculated their proportion of total pyramidal neurons estimated in the same section after labelling for NeuN-IR. We observed a portion of $1.79 \pm 0.567\%$ and $0.36 \pm 0.112\%$ GFP positive pyramidal cells (including pyramidal cell dendrites; mean ± SEM, $n = 11$) from the total numbers of (NeuN labeled) pyramidal cells in the same area of sector CA1 and the subiculum, respectively. The vast majority of GFP-IR cells, however, were...
interneurons. As can be also seen in Fig. 1A, GFP-IR interneurons were primarily located in the subiculum (mean % ± SEM of total labeled cells per section: 33.3 ± 0.58%), sector CA1 (26.3 ± 0.4%) and entorhinal cortex (18.7 ± 0.63%) and were less abundant in the temporal (10.2 ± 0.24%) and perirhinal (6.78 ± 0.13%) cortices; they were rarely observed in the presubiculum (1.39 ± 0.054%) and the dentate gyrus (1.13 ± 0.06%). At the injection site, about 74% of the GFP expressing interneurons also contained SST-IR and 66% of the SST-IR cells expressed GFP.

Development of recurrent series of pre- or interictal spikes (IS) and of spontaneous recurrent seizures (SRS) after AAV-TeLC injection

After injection of AAV-TeLC or AAV-GFP, the SST-cre mice were subjected to continuous telemetric EEG recording and video monitoring for 42 days. Similar as described for PV-cre mice, we detected typical recurring events. Early after AAV-TeLC injection we observed series of spikes (Fig. 3B, E) identical to those previously observed in PV-cre mice after intrahippocampal AAV-TeLC injection (Drexel et al., 2017). We originally had termed them “spike-wave-discharges”. We now changed this terminology and named them “preictal or interictal spikes” (IS) according to their electrophysiological characteristics. IS series represented series of at least 5 high amplitude spikes (at least 2 times baseline amplitude) spikes that are not more than 60 s apart and occur interictally or preictally to spontaneous recurrent seizures (SRS) or without accompanying seizures. Less frequently, we observed spontaneous recurrent seizures (SRS) as indicated by 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 with an EEG-signal below baseline amplitude (Fig. 3E, F) (See Table 1).

The time patterns of the occurrence of IS and SRS were highly variable during the six weeks observation period (days 1–42 after AAV-TeLC) and are shown individually for all mice in Fig. 4. The characteristics of IS and of SRS are summarized in Table 2. All AAV-TeLC vector injected mice (n = 11), but none of the mice treated with the control vector (AAV-GFP; n = 5) presented series of IS (Table 2, Fig. 3D, E). First series of IS were observed already two days after AAV-TeLC injection and after 17 days, ten mice had shown at least one IS series (Figs. 4, 5A) with a mean onset of 10.2 ± 1.94 days. One mouse had rare separated IS (# 6382). Between days 7 and 24 after AAV-TeLC injection, four out of the 11 mice also developed SRS (Fig. 4). SRS were always preceded by at least one series of IS (Fig. 4). The average interval between the last IS and the subsequent SRS was 8.8 ± 0.92 s. The four mice with SRS had 803 series of IS; among these, 23 were directly followed by seizures. Seizures identified by EEG were always accompanied by behavioral seizures (rating 3 or 4) observed in the video recordings (not shown). As shown in Fig. 5C, the maximum frequency of IS series (10.5 ± 4.53 IS per mouse per two days; n = 11) was reached after 12–17 days and that of SRS after 20–25 days (0.4 ± 0.24 SRS per mouse per two days; n = 11).

Interestingly, the frequency of IS seemed to decline after day 30 (Fig. 5A). To investigate whether this suspicion was significant, we compared both the total numbers of IS (n = 10) and of SRS (n = 3; excluding mouse # 8974 that evaded monitoring on day 29) during days 20–28 (mean ± SEM, IS: 29.4 ± 9.73, SRS: 3.33 ± 0.88) and days 30–38 (IS: 28.4 ± 6.86; SRS: 1.0 ± 1.0) by paired Student’s t-test. Neither the apparent decline between IS series nor that of SRS (Fig. 5A, B) was statistically significant (P = 0.678 and 0.230, respectively). Mice injected with the AAV-GFP vector (n = 5) developed neither SRS and nor IS series (see Fig. 3D). Extremely rare IS-like events likely represented electrode artefacts (not shown).

Table 1. Summary of mice injected with the low titer of AAV-TeLC or with AAV-GFP (controls) and occurrence of series of preictal spikes (IS) and spontaneous recurrent seizures (SRS)

| Mouse # | AAV- | IS | Days to first IS | Number of IS | SRS +/- | Days to first SRS | Number of SRS | Day of last SRS | EEG until day |
|---------|------|----|-----------------|--------------|---------|-----------------|---------------|----------------|--------------|
| 6376    | TeLC | +  | 12              | 93           | -       | -               | -             | -              | 42           |
| 6377    | TeLC | +  | 14              | 28           | -       | -               | -             | -              | 42           |
| 6378    | TeLC | +  | 12              | 15           | -       | -               | -             | -              | 42           |
| 6379    | TeLC | +  | 15              | 53           | -       | -               | -             | -              | 42           |
| 6380    | TeLC | +  | 3               | 145          | +       | 11              | 8             | 29             | 42           |
| 6382    | TeLC | +  | 17              | 11           | -       | -               | -             | -              | 42           |
| 6383    | TeLC | +  | 14              | 62           | -       | -               | -             | -              | 42           |
| 6389    | TeLC | +  | 3               | 476          | +       | 7               | 6             | 26             | 42           |
| 8975    | TeLC | +  | 8               | 160          | +       | 20              | 6             | 40             | 42           |
| 8974    | TeLC | +  | 10              | 22           | +       | 26              | 3             | 29             | 29           |
| 6391    | TeLC | +  | 2               | 234          | -       | -               | -             | -              | 42           |
| 6375    | GFP  | -  | -               | -            | -       | -               | -             | -              | 42           |
| 6381    | GFP  | -  | -               | -            | -       | -               | -             | -              | 42           |
| 6384    | GFP  | -  | -               | -            | -       | -               | -             | -              | 35           |
| 6386    | GFP  | -  | -               | -            | -       | -               | -             | -              | 42           |
| 6387    | GFP  | -  | -               | -            | -       | -               | -             | -              | 42           |
We are presenting evidence that selective silencing of GABA/SST-containing interneurons of the ventral hippocampus induces recurrent IS and SRS. We used injection of AAV-TeLC into transgenic mice expressing Cre-recombinase on the SST promoter resulting in selective expression of TeLC in SST-containing O-LM and bistratified cells of the ventral hippocampal sector CA1 and the subiculum. The toxin cleaves vesicle-associated membrane protein 2 (VAMP2, synaptobrevin) and thereby permanently inhibits GABA release selectively in SST neurons (Schiavo et al., 1992). We recently demonstrated that the AAV vector injections do not induce neurodegeneration when injected in PV-cre mice (Drexel et al., 2017) and vector expressing neurons are well recovered also after six weeks (Drexel et al., 2017, and present experiments). Interestingly, however, using the same AAV vector concentrations as in our previous experiments for silencing PV neurons in PV-cre mice, we observed (in contrast to the previous experiments) expression of the GFP-tag also to some extent in pyramidal cells. This indicates that the SST-cre mice

**Table 2.** Characteristics of spontaneous recurrent seizures and series of inter- and preictal spikes (IS)

| Series of preictal spikes (IS)                                                                 |
|---------------------------------------------------------------------------------------------|
| • Incidence of IS: 100% of mice                                                            |
| • Nine out of eleven mice developed repeated series of IS, two mice had rare and more separated IS |
| • Onset of IS series: 10.2 ± 1.94 days                                                      |
| • Number of IS: 65.4 ± 14.19 per month/mouse                                               |
| • Duration of spike series: 352.5 ± 56.13 s                                                |
| • No of spikes per series: 46.9 ± 7.2                                                      |
| • Frequency of spikes in series: 0.135 ± 0.0029 Hz                                         |
| • Interspike interval: 7.74 ± 0.229 s                                                       |
| • Interval from last spike to SRS: 8.8 ± 0.92 s                                             |
| • Four mice with seizures had 803 series of IS, 23 series of IS were directly followed by seizures |

**Spontaneous recurrent seizures**

- Incidence: 36% of mice presented at least 1 spontaneous seizure during the first 6 weeks after virus-injection
- Onset of spontaneous seizures: 16.0 ± 4.30 days after AAV-injection
- Frequency of seizures: 1.2 ± 0.56 seizures per month
- Mean seizure duration: 14.6 ± 0.90 seconds
- Pre-ictal spikes: 100% of spontaneous seizures were preceded by series of IS

Data are presented as mean ± SEM.
are genetically not as tightly controlled over the expres-
sion of the viral transgene as the previously used PV-
cre mice (Drexel et al., 2017). By lowering the concentra-
tion of the injected vector we were, however, able to avoid
almost entirely TeLC expression in pyramidal cells and to
achieve rather selective expression of the transgenes
(TeLC and GFP) in SST interneurons (O-LM and bistrat-
bified cells). Extensive co-labelling of GFP-IR with SST-
IR indicates silencing of SST interneurons and thus inhibi-
tion of GABA release from these cells. The resulting
impaired feedback inhibition at pyramidal cell dendrites
of the stratum lacunosum moleculare of CA1 and the
outer molecular layer of the subiculum is causative for
subsequent series of IS and for SRS. Importantly, our
injections and thus expression of TeLC were unilateral
and restricted to a small area of the ventral hippocampus.
Whereas SRS detected in the EEG were always accompa-
nied by generalized motor seizures, IS series had no obvious correlate in motor behavior. The role of IS for the development of epilepsy is not fully understood. IS may produce conditions that are permissive for seizure activity (de Curtis and Avanzini, 2001; Staley and Dudek, 2006). It is important to note that mice exposing IS series but not SRS after silencing PV containing basket cells in the subiculum instantly developed seizures and subsequent SRS upon application of a low subconvulsive dose of pentylenetetrazole indicating a markedly reduced seizure threshold in IS series present-
ing mice (Drexel et al., 2017).

SST-containing O-LM cells constitute about 92% of the inhibitory synapses at the pyramidal cell dendrites in CA1 emphasizing their important role in modulating pyramidal cell activity. Cossart et al. demonstrated the importance of a loss in inhibition of pyramidal cell dendrites for seizure generation in pilocarpine treated rats although somatic inhibition was initially increased (Cossart et al., 2001; Dinocourt et al., 2003). We now show that silencing GABA release from SST-expressing O-LM and bistratified cells (without neurodegeneration) results in development of IS series and SRSs. Using the same experimental approach, we previously inhibited GABA release selectively from PV containing neurons in the subiculum (Drexel et al., 2017). PV is preferentially expressed in basket cells and axo-axonic cells, and only to a minor extent in bistratified cells. Basket cells and axo-axonic cells target pyramidal cells at perisomatic sites or at the axon initial segments, respectively. In contrast to O-LM and bistratified cells, PV cells exert potent feedforward inhibition on pyramidal cells. Silencing these neu-
rons also resulted in a similar picture of recurrent IS series and SRSs indicating a crucial role for feed-back inhi-
bition in controlling pyramidal cell excitability and prevent-
ing seizures. We used the same conditions for both types of experiments, in particular only a single focal and unilat-
eral injection of the viral vector into a defined part of the ventral hippocampus. This site-restricted application may explain why the frequency of SRS was only limited in both experiments. Silencing of PV-containing cells

Fig. 5. Characterization of development of series of preictal spikes (IS) and of spontaneous recurrent seizures (SRS). (A, B) mean numbers of IS (± SEM) (A) and mean numbers of SRS (± SEM) (B) presented per mouse and per two days. Due to the fact that recording had to be stopped for one mouse (loss of electrodes) on day 29, the numbers of mice were 11 (day 1 to day 29) and 10 (day 30 to day 42) for IS (A) and four (day 1 to day
29) and three (day 30 to day 41) for SRS (B), respectively. Note that frequencies of IS appeared to decline to some extent between days 25 and 40. Comparing the respective data by paired Student’s t-test we, however, found no significant difference (see Results section). Mice injected with the AAV-GFP vector (n = 5) had no SRS and no or extremely rare IS-like events (no series of IS) that likely represented electrode artefacts (see also Fig. 3D). (C) Cumulative presentation of IS and of SRS. All mice had developed at least one IS by day 18, and 36% of mice presented at least one SRS by day 27.
(inhibiting feed-forward inhibition) resulted in more than twice as many mice exposing SRS than inhibiting feedback inhibition mediated by SST neurons (88% vs 36%), comparing the present data with the previous ones (Drexel et al., 2017). Also, the average frequency of seizures from week one to week six was somewhat lower and SRS were about 10 s shorter (14.6 ± 0.90 vs. 24.6 ± 0.62 s) after inhibiting GABA release from SST neurons. On the other hand, the frequency of IS was about two times higher after silencing SST neurons (21.4 ± 6.83 vs. 11.7 ± 2.07).

In principle, both experimental approaches almost equally reduce the seizure threshold in the mice. After silencing PV neurons, the animals, however, go more readily from a more pre-ictal state (reflected by IS series) to fully epileptic state with SRS. Somatic inhibition through PV containing basket and axo-axonic cells is activated just before the seizure onset and represents a major source of inhibitory barrages opposing ictal discharge propagation (Cammarota et al., 2013; Toyoda et al., 2015). This early activation of PV neurons in the pre-ictal period is, however, followed by a significant activation of SST neurons during the late pre-ictal state, which can persist throughout the ictal events (Cossart et al., 2001; Parrish et al., 2019). Bursting of both sets of interneurons is intensive with rates up to 300 Hz (Parrish et al., 2019). Thus, impairing feed-forward or feed-back inhibition by injection of TeLC vector either in PV-cre or SST-cre mice similarly antagonizes the corresponding inhibitory barrages and leads to the development of SRS and epilepsy.

In this context it is interesting to note that mice, which are deficient in the homeobox transcription factor DLX1 show early post-embryonal degeneration of SST- and calretinin-containing neurons in the cortex and hippocampus and consequently develop generalized electrographic seizures (Cobos et al., 2005). In contrast to our present experiment, in which we silenced hippocampal SST neurons in adult mice and observed spontaneous limbic seizures already after one to two weeks, seizures in DLX1-deficient mice develop only about 4 weeks after degeneration of SST neurons. It is likely that functioning basket and axo-axonic cells not affected by the DLX1 knock out may initially protect the mice from seizure generation.

Whereas our experiments show that silencing of SST cells induces SRS, it is long known that severe seizures can result in a loss of SST neurons in the hilus of the dentate gyrus and in the sector CA1 (Sloviter, 1987; Sperk et al., 1992; Dinocourt et al., 2003; Kobayashi and Buckmaster, 2003). After a kainic acid-induced status epilepticus in rats, SST neurons are also lost in the subiculum, presubiculum and in layers V/VI of the entorhinal cortex (Drexel et al., 2012). Thus, seizures may induce a loss in SST neurons, but on the other hand impairing neurotransmission in SST interneurons (even without neurodegeneration) results in seizures.

The loss in feedback inhibition after the degeneration of O-LM cells in the kainic acid model of TLE is also accompanied by “repair mechanisms”. Surviving SST-positive cells respond with pronounced over-expression of SST and glutamate decarboxylase (GAD) mRNAs and IR in the cortex, the dentate gyrus and the subiculum reflecting markedly increased neuronal activity of these neurons (Marksteiner and Sperk, 1988; Feldblum et al., 1990; Drexel et al., 2012). Furthermore, SST and vesicular GABA transporter (VGAT)-IR accumulate in the dentate molecular layer and in the outer molecular layer of the subiculum indicating axonal sprouting of respective SST/GABA neurons (Davenport et al., 1990; Mathern et al., 1995; Drexel et al., 2012). Interestingly, sprouted axon terminals in the molecular layers of the subiculum and of the dentate gyrus may originate from O-LM cells (Peng et al., 2013). Both mechanisms may contribute to a compensation in GABA transmission impaired by the loss in O-LM cells. But overexpression of the co-transmitter SST may also contribute to endogenous anti-epileptic activity. Thus, SST by itself exerts modest anticonvulsive actions through its SST4 and SST2 receptors (Moneta et al., 2002; Qu et al., 2008).

Ledi et al. (2014) recently used selective optogenetic activation of SST- and PV-containing interneurons to investigate their influence on epileptiform activity in hippocampal slices. They demonstrated that activation of dendritic (mediated by SST neurons) as well as of 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 (Ledi et al., 2014).

Taken together, our present experiments demonstrate a pivotal role of SST-containing interneurons (O-LM and/or bistratified cells) for controlling the excitability of hippocampal pyramidal neurons. We provide experimental evidence that selective permanent inhibition of SST-containing interneurons may reduce feed-back inhibition of pyramidal cells in vivo resulting in a decrease in seizure-threshold with development of recurrent IS series and SRS (i.e. epilepsy) without signs of neurodegeneration.

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