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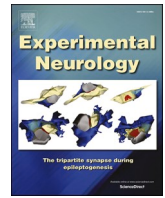
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Research paper

Collagen VI: Role in synaptic transmission and seizure-related excitability[☆]

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ABSTRACT

Collagen VI (Col-VI) is an extracellular matrix protein primarily known for its bridging role in connective tissues that has been suggested to play a neuroprotective role. In the present study we report increased mRNA and protein expression of Col-VI in the hippocampus and cortex at a late stage of epileptogenesis in a post-*status epilepticus* (SE) model of epilepsy and in brain tissue from patients with epilepsy. We further present a novel finding that exposure of mouse hippocampal slices to Col-VI augments paired-pulse facilitation in Schaffer collateral-CA1 excitatory synapses indicating decreased release probability of glutamate. In line with this finding, lack of Col-VI expression in the knock-out mice show paired-pulse depression in these synapses, suggesting increased release probability of glutamate. In addition, we observed dynamic changes in Col-VI blood plasma levels in rats after Kainate-induced SE, and increased levels of Col-VI mRNA and protein in autopsy or post-mortem brain of humans suffering from epilepsy. Thus, our data indicate that elevated levels of ColVI following seizures leads to attenuated glutamatergic transmission, ultimately resulting in less overall network excitability. Presumably, increased Col-VI may act as part of endogenous compensatory mechanism against enhanced excitability during epileptogenic processes in the hippocampus, and could be further investigated as a potential functional biomarker of epileptogenesis, and/or a novel target for therapeutic intervention.

1. Introduction

Extracellular matrix (ECM) proteins modulate several characteristics of synaptic transmission and plasticity in the central nervous system (CNS). Increasing evidence supports the notion that the ECM plays a dual role as a promoter of structural and functional plasticity and as a degradable stabilizer of neural microcircuits, both processes being key elements for mental health (Dityatev et al., 2010). Collagen VI (Col-VI) belongs to the category of ECM proteins and is primarily known for its bridging role in connective tissues (Kielty et al., 1991). Col-VI is a form of collagen that is composed of three different alpha-chains ($\alpha 1$, $\alpha 2$ and $\alpha 3$) that, in order to create flexible networks in skeletal muscles and

other organs, interlink collagen types I, II, IV, proteoglycans, hyalurons and cells, thus forming a microfilamentous network (Bonaldo et al., 1990; Christensen et al., 2012; Keene et al., 1988; Kuo et al., 1997; Specks et al., 1992). In addition, three recently discovered collagen VI chains, which bear a resemblance to the $\alpha 3$ chain, were identified and designated as $\alpha 4$, $\alpha 5$, and $\alpha 6$. Each of these newly recognized chains is associated with a unique gene (COL6A4, COL6A5, and COL6A6) (Fitzgerald et al., 2008; Gara et al., 2008). These chains can substitute $\alpha 3$ chain forming $\alpha 1\alpha 3\alpha 4$, $\alpha 1\alpha 2\alpha 5$ or $\alpha 1\alpha 2\alpha 6$ heterotrimers, but in humans $\alpha 4$ is not functional due to COL6A4 gene disruption (Gara et al., 2008).

Col-VI is also expressed in meninges (Kuo et al., 1997; Sievers et al., 1994) as well as in the CNS and peripheral nervous system (Hirvonen

[☆] We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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et al., 1996; Palumbo et al., 2002), both during development and in adulthood (Cheng et al., 2009; Marvulli et al., 1996). Although the role of Col-VI has been mainly related to its unique supramolecular assembly in the connective tissue, several recent data support a notion of its protective role in the CNS. For example, a neuroprotective role has been suggested by increased apoptosis due to toxic effect of A β -peptides in Col-VIa1 $-/-$ mouse neuronal cultures (Cheng et al., 2009). In line with this observation, the addition of Col-VI rescued UV-induced apoptosis and counteracted dendritic shrinkage through the Akt and JNK pathways in mouse primary hippocampal neurons (Cheng et al., 2011). Interestingly, the Col-VI mRNA and protein expression are increased in the CNS of subjects affected by Alzheimer's disease (AD), as well as in transgenic mice bearing AD-related mutations (Cheng et al., 2009). Mutation in the COLVIA2 gene has recently been reported in a consanguineous family with progressive myoclonus epilepsy, suggesting a potential causative link between mutated Col-VI and epilepsy (Karkheiran et al., 2013), which is a common comorbidity of AD (Noebels, 2011; Palop and Mucke, 2009; Vossel et al., 2013).

We hypothesized that Col-VI might influence synaptic transmission and/or plasticity. Notably, it has been proposed that the Col-VI α 3 chain has the capacity to interact with presynaptic cannabinoid 1 receptors (CB1R), thereby hindering the suppression of excessive glutamate release that is typically mediated through endocannabinoid binding to CB1Rs (Lam et al., 2022). This interaction suggests a novel modulatory mechanism where Col-VI could directly impact synaptic efficacy and neurotransmitter dynamics through a specific interplay with cannabinoid CB1R signaling pathways (Lam et al., 2022) and could mediate the antiepileptic effects of Col-VI. In the present study, we show that excitatory glutamatergic transmission is diminished, likely through presynaptic mechanisms, following the treatment of hippocampal slices with exogenously applied collagen VI (Col-VI). Furthermore, we observed an enhanced excitatory neurotransmission, presumably due to elevated glutamate release, in slices derived from collagen VI knockout (Col-VI KO) mice. Additionally, our findings reveal an upregulation of collagen VI (Col-VI) expression in the hippocampus of animals subjected to the post-SE model of chronic epilepsy (Reddy and Kuruba, 2013), indicating that heightened Col-VI levels could play a role in modulating neuronal hyperexcitability during the later phases of epileptogenesis. This observation is further corroborated by dynamic fluctuations in the blood levels of Col-VI in rats, alongside elevated mRNA and Col-VI protein expression detected in human epileptic brain biopsies as well as in post-mortem samples. These results align with the animal model data, reinforcing the potential of Col-VI as a significant modulator in the pathophysiology of epilepsy.

2. Material and methods

2.1. Ethical approval

All animal experiments were conducted according to international guidelines on the use of experimental animals, as well as the Swedish Animal Welfare Agency guidelines, and were approved by the local Ethical Committee for Experimental.

Animals and performed in accordance with the guidelines of the European Community Council Directives 2010/63/EU. For the in vivo part of the study, ethical permits were obtained from Lund/Malmö Ethics Committee M205-12/M166-14 and M204-12 and M403-12 for the in vitro part. Human brain tissue was handled according to declaration of Helsinki, and the local ethical committee approvals at Amsterdam and Bonn Universities, respectively. The study is reported in accordance with ARRIVE guidelines.

2.2. Animals

A total of 76 young Sprague-Dawley male rats, 3 weeks old at arrival (Charles River); 14 C57Bl6 wild type mice (breeding) and 10 Col-VI

knock-out (Col-VI $-/-$) mice, 3–4 months old (C57/Bl6 background, a contribution from Prof. Paolo Bonaldo, Padova University and previously described in (Bonaldo et al., 1998) were used for this study. To maintain consistency with previous research and minimize variability, only male animals were used in this study. This decision was made to avoid the potential confounding effects of the estrous cycle in female animals, which can influence synaptic transmission and seizure susceptibility. We recognize the importance of including both sexes in epilepsy research and plan to include both male and female animals in future studies to account for potential sex differences in epilepsy-related outcomes (Christian et al., 2020).

2.3. Slice preparation

Animals (both rats and mice) were briefly anesthetized with isoflurane and decapitated. The head was quickly immersed in ice-cold sucrose-based cutting solution, constantly oxygenated with carbogen (95% O₂ and 5% CO₂), and containing the following (in mM): sucrose 75, NaCl 67, NaHCO₃ 26, glucose 25, KCl 2.5, NaH₂PO₄ 1.25, CaCl₂ 0.5, and MgCl₂ 7 (pH 7.4, osmolarity 305–310 mOsm). After quickly removing the brain, the cerebellum was discarded and the hemispheres were divided. Horizontal slices (300 μ m thickness) containing the hippocampus and entorhinal cortex were cut on a vibratome (VT1200S, Leica Microsystems) in cutting solution maintained at 3–4 °C. Individual slices were transferred to a first incubation chamber containing cutting solution, constantly oxygenated with carbogen, and maintained at 32 °C in a water bath for 20 min. After the first incubation, slices were transferred to artificial cerebrospinal fluid (aCSF) constantly oxygenated with carbogen, and containing the following (in mM): NaCl 119, NaHCO₃ 26, glucose 11, KCl 2.5, NaH₂PO₄ 1.25, CaCl₂ and MgSO₄ 1.3 (pH 7.4, osmolarity 295–305 mOsm). The second incubation was performed at room temperature for 40 min.

2.4. Col-VI treatment of acute hippocampal slices

Rat hippocampal slices were transferred in holding chambers (multiwell plates, which were coated with silicone to prevent Col-VI from adhering to the walls) and incubated at a concentration of 40 nM for 6 h under continuous oxygenation either in aCSF (composition as reported above; osmolarity 295–305 mOsm; pH 7.4), referred to as control solution from here after, or aCSF containing human purified Col-VI (Fitzgerald Industries International, Acton, MA, USA). (Fig. 1A; osmolarity 295–305 mOsm; pH 7.4, at this pH Col-VI has a fibrillar structure (Cheng et al., 2009)). The concentration of Col-VI was chosen based on previous studies demonstrating effective modulation of e.g. A β neurotoxicity in cultured neurons (Cheng et al., 2009). The 6-h duration was selected to ensure sufficient time for Col-VI to exert its effects, based on our previous experience with other proteins (Asztely et al., 2000). The stability of Col-VI was expected to be maintained throughout the incubation period according to the manufacturer's protocol. After the incubation, slices were transferred to the recording chamber, constantly perfused (2–3 ml per min) with oxygenated aCSF and maintained at 31–33°C. Field excitatory postsynaptic potentials (fEPSPs) were then recorded as described below. For the experiments conducted on Col-VI $-/-$ mice, slices were prepared as described above and transferred to the recording chamber immediately after 40 min recovery in aCSF at room temperature.

2.4.1. Electrophysiology

After transferring the slices to the recording chamber, they were held in place with a nylon mesh, and a bipolar stimulation electrode and a pipette filled with aCSF.

(tip resistance of 0.8–3 M Ω) were placed in *stratum radiatum* of CA1, approximately.

300–500 μ m apart. Recording pipettes were pulled from borosilicate glass with a Flaming-Brown horizontal puller (P-97, Sutter Instruments).

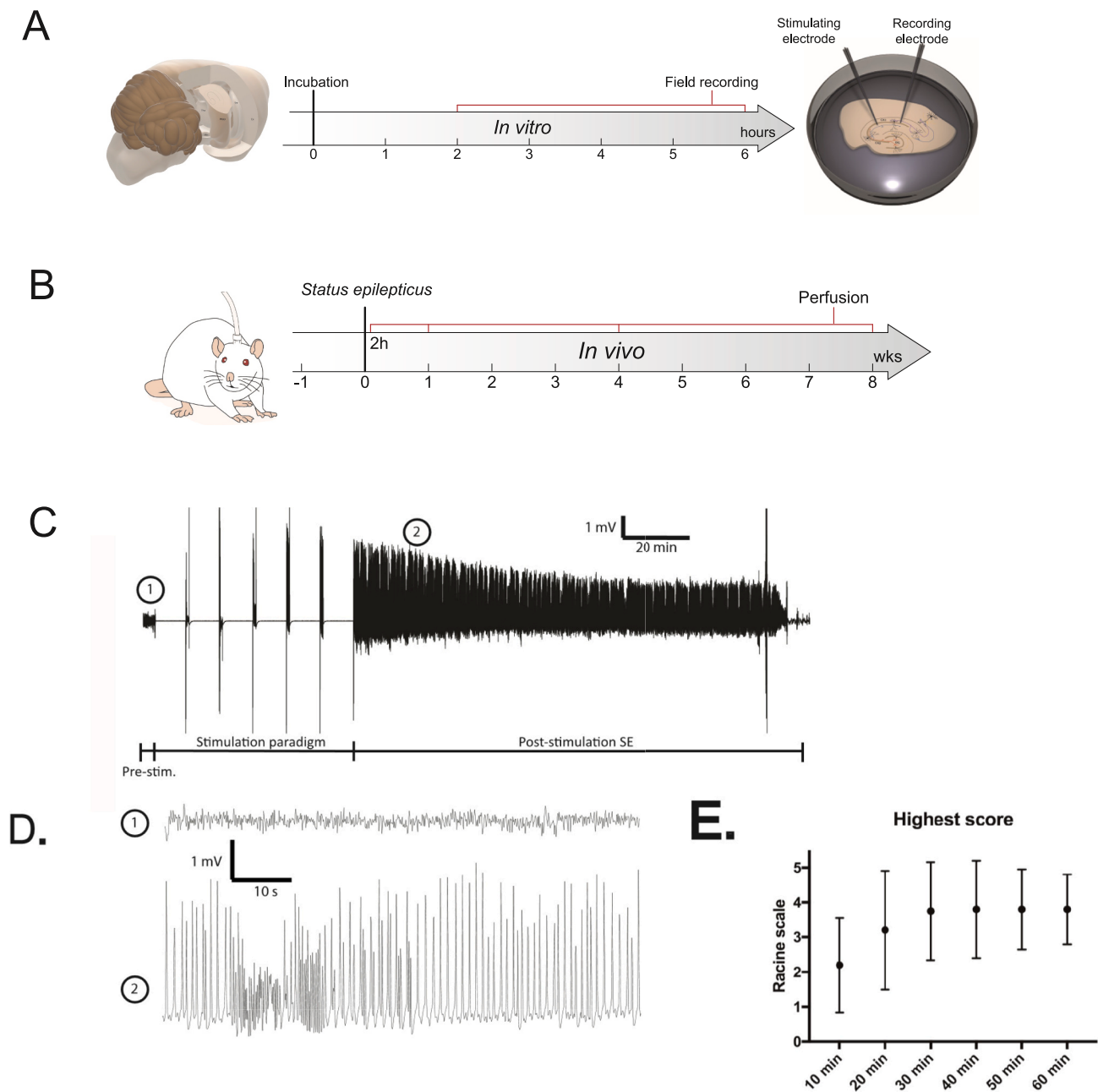


Fig. 1. Schematic illustration of the experimental design and overview of SE features. **A**, Acute hippocampal slices were incubated with Col-VI for 6 h. Field recordings were performed in CA1 region after exposing the acute hippocampal slices to Col-VI. **B**, SE was induced 1 week after implanting the electrodes and animals were allowed to survive for 2 h, 1, 4 or 8 weeks. At these time points, the brains were taken for in situ hybridization or immunohistochemistry. **C**, Electrically induced SE. The stimulation threshold was set during the first phase of the experiment (Pre-stim protocol), then followed by 6 epochs of stimulation (Stimulation paradigm) and finally 2 h of continuous monitoring (Post-stimulation SE). **D**, Two episodes of the EEG trace are enlarged: 1. Normal EEG before any stimulation, 2. Hyper-synchronous spiking during post-stimulation SE. **E**, Seizure severity is gradually increased during the stimulation. A plateau at an average Racine score of 3.8 is reached after 30 min of stimulation.

fEPSPs were then induced by electrical stimulation, and recorded using the protocols previously described by Sørensen et al. (Sorensen et al., 2009). Briefly, electrical stimulation of Schaffer collaterals induced synaptic activation of CA1 pyramidal neurons recorded as fEPSPs. Only slices exhibiting fEPSP amplitudes over 1 mV, and exceeding presynaptic fiber volleys (PSFV, a measure of axon fiber activation) at least 3 times (a commonly used criteria to verify good quality of slices), were included in the analysis. Increasing stimulation strength was used to estimate so-called input-output relationship of fEPSPs by plotting the PSFV amplitudes (mV) against the initial slope (mV/ms) of the corresponding fEPSP. The initial slope was measured between the initial

descending point and the point before the fEPSP curve was disrupted by population spike, usually lasting for 1.5–2 ms. The stimulation strength was then normalized across the slices to 30–40% of maximal fEPSP. The stability of the fEPSPs were monitored for 5–10 min (at 0.05 Hz) before paired-pulse stimulations were delivered at interstimulus intervals (ISI) of 25, 50, 100 and 200 ms. In a set of experiments, fEPSPs and respective paired-pulse ratios (facilitation-depression; PPF, PPD), were recorded during continuous application of 100 mM picrotoxin (PTX) to block gamma-aminobutyric acid A (GABAA) receptors. It is widely accepted that the high PPF is indicative of relatively low release probability of neurotransmitters in synapses, while low PPF and PPD is commonly

observed in synapses with high release probability (see, e.g., (Cho and Askwith, 2008; Debanne et al., 1996; Di Castro et al., 2016)).

2.5. Epilepsy models

2.5.1. Animals

A total of 52 male Sprague Dawley rats (200–230 g, Charles River Germany), individually housed with ad libitum access to food and water, were used. The animals were sacrificed by overdose of pentobarbital at 2 h, 1 week, 4 weeks and 8 weeks after SE induction (Fig. 1B). For each time point, 8 animals were implanted with electrodes, 4 animals were electrically stimulated to induce SE, while 4 animals served as sham and were only connected to stimulation cable without applying stimulation current. Another 4 animals were not implanted with electrodes and served as naïve controls. Additional 4 animals were stimulated for immunohistochemical analysis together with 4 naïve animals, and were analyzed at 4 weeks timepoints. Finally, in order to verify that Col-VI protein expression changes depend on seizure events and not electrode implantation, 4 animals received intrahippocampal kainate (KA) injections for induction of SE and another 4 were used as control animals. These animals were analyzed at chronic epilepsy phase about 1 year after KA.

2.5.2. Electrode implantations

Animals were anesthetized using inhalable isoflurane mixture (2.5%; Baxter.

Chemical AB) and the skull was subsequently fixed in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). Through drill holes made in the skull, a bipolar stainless steel stimulating/recording electrode (PlasticsOne, Roanoke, VA) was implanted in the right ventral hippocampus (coordinates from bregma, midline, dura: AP -4.8 mm, ML -5.2 mm, DV -6.3 mm) and a reference electrode was placed between the skull and the cheek muscle. The electrode contacts were secured to a connector pedestal (PlasticsOne, Roanoke, VA), which was cemented onto the skull. All animals were allowed to recover for 1 week before initiation of electrical stimulations.

2.5.3. Status epilepticus: electrical stimulation-induced

The individual threshold for afterdischarge induction was determined for each animal 30 min before the initiation of the SE stimulations. Step-wise stimulations of 50 Hz, 1 ms square-wave pulses for 1 s with 10 μ A increments (MacLab system; AD Instruments, Bella Vista, Australia) were applied until an epileptic afterdischarge longer than 5 s was detected on the electroencephalogram (EEG). SE was induced by continuous supra-threshold stimulation (50 Hz, 1 ms square-wave pulses for 10 s) for 1 h and with an interruption every 10 min, during which 1 min EEG recording was acquired. Averaging the highest seizure score of each 10 min stimulation epoch revealed a gradual increase in seizure severity during the electrical stimulation. The seizure severity reached a plateau after 30 min of stimulation and during the last epoch the average max score was 3.8 ± 1.0 ($n = 20$, Fig. 1C, D). Every minute during the stimulation and during the self-sustained phase of SE the behavioral seizure severity was scored according to the Racine scale: stage 0, no behavioral changes; stage 1, facial twitches; stage 2, chewing and head nodding; stage 3, unilateral forelimb clonus; stage 4, rearing, body jerks, bilateral forelimb clonus; stage 5, imbalance and falling (Fig. 1E). After the stimulation, the EEG was continuously recorded during the self-sustained phase of SE for 2 h. After 2 h SE, the animal was given an intraperitoneal injection of sodium pentobarbital (40 mg/kg) to terminate the SE. The EEG was recorded until all epileptic activity completely stopped. After 4 weeks, 4 of the stimulated and 4 naïve rats were perfused and the brains were removed for subsequent cutting and staining.

2.5.4. Status epilepticus: Kainate-induced

Kainate injections for induction of SE and subsequent self-sustained

chronic seizures were performed in rats like previously described in (Ledri et al., 2016), where 0.4 μ g/ 0.4 μ l of kainic acid (Ascent scientific, Cambridge, UK) dissolved in PBS was injected into the medial part of the right hippocampus, with the following coordinates: (reference from bregma, midline, dura): AP -5.3 mm, ML -4.5 mm, DV -3.2 mm. As soon as the animals started to wake up from the anesthetics (isoflurane), behavioral convulsions were observed. SE was defined as continuous motor seizures for at least 10 min within 3 h after kainate injection. After 1 year, the animals were transcardially perfused with saline and subsequently with 4% paraformaldehyde before the brain was collected. Analysis of the optical density was performed as described in section for optical density measurements and analyzed as described in section for statistical analysis.

2.5.5. In situ hybridization

At each time point (2 h, 1 week, 4 weeks and 8 weeks, please see Fig. 1B), animals that were subjected to SE stimulations were sacrificed under halothane anesthesia, by using a guillotine. The brain was immediately removed while submerged in frozen oxygenated artificial cerebral spinal fluid (119 mM NaCl, 2.5 mM KCl, 1.3 MgSO₄, 2.5 mM CaCl₂, 26 NaHCO₃, 1.0 NaH₂PO₄). Subsequently, the brain was put into a zip-lock bag before being frozen in dry ice. All brains were stored in the -80 °C freezer until further processing. In situ hybridization was performed as described earlier, with minor modifications (Christensen et al., 2006; Woldbye et al., 2005).

Brains previously frozen on dry ice were cut into sections that were thaw-mounted onto Superfrost glass slides and stored at -80 °C until further processing. The slides were subsequently defrosted for 10 min at room temperature and fixed in 4% paraformaldehyde for 5 min followed by 2 \times 5 min wash in 1 \times PBS. Sections were then acetylated (0.9% NaCl, 0.25% acetic anhydride, 1.4% triethanolamine) for 10 min and dehydrated in series of 70% ethanol, 96% ethanol, and 99.9% ethanol before they were treated by chloroform for 5 min. Slides were subsequently washed again in 99.9% and 96% ethanol and left to dry. In situ hybridization was performed using the following synthetic antisense oligonucleotide DNA probe targeting rat Col-VI mRNA: 5'-TGA AGG GAG AAA AGG GGA GCC GTG GAG AGA AGG GTT CCA GAG GAC-3'. The oligoprobe was labeled at the 3'-end with [33P]dATP (3000 Ci/mmol; NEG312H250UC; PerkinElmer, DK) using terminal deoxynucleotidyl transferase (Roche Diagnostics, Mannheim, Germany). The labeled probe was added to a specific activity of 1×10^6 cpm/100 ml to the hybridization buffer (50% formamide (v/v), 20% 20 \times SSC (v/v), 10% dextran sulfate (w/v), 2% 50 \times Denhardt's solution (v/v), 0.5% yeast t-RNA (w/v), 5% DNA salmon sperm (v/v)), and 10 μ M dithiothreitol. After adding a volume of 120 μ l hybridization mixture to each slide, the slides were covered with a coverglass and left at 37 °C in humidity boxes overnight. At room temperature, the slides were subsequently briefly rinsed in 1 \times SSC, washed for 60 min in 1 \times SSC at 60 °C, and dehydrated in ethanol. Finally, the slides were air-dried and exposed together with 14C-microscales to 33P-sensitive Kodak BioMax MR films (Amersham Biosciences, DK) for 2–4 weeks and developed in Kodak GBX developer.

For the semiquantitative determination of mRNA levels, the films were scanned (Epson, DK) and optical densities (Bq/g) based on calibration curves were obtained with the 14C-microscales and computer-assisted image analysis (Scion Image). Optical densities were measured by manual delineation bilaterally in at least three adjacent sections per animal over the dorsal hippocampal dentate gyrus granular layer (DG), CA1 pyramidal layer and neocortex (S1BF) (Paxinos, 2006) (Fig. 4B top panel). The values obtained from the right- and left-side of the brain values were averaged and used to calculate the mean for each animal. Background measurements immediately adjacent to each brain section were subtracted from each measurement before calculations. A person blinded to the treatment of the animals performed all measurements. We confirmed the specificity of our oligoprobe by showing that an alternative antisense oligoprobe complementary to another part of

the Col-VI mRNA sequence (5'-ATG TGC TCC TGC TGT GAG TGC ACA TGT GGA CCC ATT GAC ATC CT-3') resulted in a similar labeling pattern. In control experiments, addition of corresponding unlabeled antisense probes competitively blocked labeling of slices by the oligoprobes.

2.5.6. Immunohistochemistry

2.5.6.1. Human brain tissue. Surgical and post-mortem brain tissue included in this study was obtained from the archives of the Amsterdam University Medical Centers. Hippocampal samples were obtained from 6 patients undergoing surgery for intractable epilepsy and diagnosed with temporal lobe epilepsy with hippocampal sclerosis (TLE-HS). Autoptic brain tissue was obtained from 5 patients who died after SE as well as from 6 age-matched controls, without a history of seizures or other neurological diseases. All autopsies were performed within 24 h after death. Tissue was obtained and used in accordance with the Declaration of Helsinki and the Amsterdam UMC Research Code provided by the Medical Ethics Committee. All cases were reviewed independently by two neuropathologists.

Human brain tissue was fixed in 10% buffered formalin and embedded in paraffin.

Paraffin embedded tissue was sectioned at 6 μm , mounted on pre-coated glass slides (Star Frost, Waldemar Knittel, Braunschweig, Germany) and processed for immunohistochemical staining. Sections were deparaffinated in xylene, rinsed in ethanol (100%, 95%, 70%) and incubated for 20 min in 0.3% hydrogen peroxide diluted in methanol to block endogenous peroxidase activity. Antigen retrieval was performed using a pressure cooker in 0.01 M sodium citrate buffer (pH 6.0) at 121 °C for 10 min. Sections were washed with phosphate buffered saline (PBS; 0.1 M, pH 7.4) and incubated overnight with primary antibody (rabbit anti-collagen VI, ab6588, 1:2000, Abcam, Cambridge, UK) in antibody diluent (VWR International, Radnor, PA, USA) at 4 °C. Thereafter, sections were washed in PBS and then stained with a polymer-based horse radish peroxidase immunohistochemistry detection kit (Brightvision plus kit, ImmunoLogic, Duiven, the Netherlands) according to the manufacturer's instructions. After washing in PBS, sections were stained using 3,3'-diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in the presence of 0.015% H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6). The reaction was stopped by washing in distilled water. Sections were counterstained with Haematoxylin-Mayer solution (Klinipath, Breda, the Netherlands), washed with tap water, dehydrated in alcohol and xylene and coverslipped using Pertex (VWR International, Radnor, PA, USA).

2.5.6.2. Rat brain tissue. After transcardial perfusion with 4% paraformaldehyde, the brains were post-fixed in the same solution overnight, at 4 °C. The following day brains were transferred to 20% sucrose until sinking and then cut to 30 μm slices on a microtome. Slices were rinsed in.

KPBS, quenched and then blocked by 5% goat serum in 0.25% Triton X-100 in KPBS.

Sections were incubated overnight in 1:250 dilution rabbit anti-Col-VI antibody (Abcam, Cambridge, UK) in blocking solution. The validation of the primary antibody for Col-VI was provided by Abcam by means of western blots and using Col-VI^{-/-} animals (see: <http://www.abcam.com/rab27a-antibody-ab55667.html>).

Some slices were incubated without any primary antibody. Finally, slices were incubated in biotinylated goat-anti-rabbit secondary antibody (BA1000; 1:200; Vector.

Laboratories, Burlingame, CA) in 2% normal goat serum. An amplification system.

(Vectastain ABC KIT, Vector Laboratories) was used and was further visualized by 3-3'-diaminobenzidine (DAB). Images of the stained brain slices were acquired using an Olympus microscope.

Optical densities were measured on Col-VI immunohistochemical images acquired from slices of stimulated, non-stimulated electrode implanted and naïve animals by using the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Three equally large circular areas (100 μm in diameter) were measured in four consecutive slices bilaterally in the dentate hilus, CA1 and CA3 areas. Values were corrected by subtraction of background optical density.

2.5.7. mRNA expression analyses in human TLE

Human hippocampal biopsy tissue from TLE patients who underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center were selected. Clinical characteristics of the patients have been described in detail before (van Loo et al., 2015). All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Bonn Medical Center. Informed written consent was obtained from all patients. 78 patients with hippocampal sclerosis (HS) were included and 35 patients with lesion-associated TLE, i.e. in which hippocampi lack the damage pattern of HS and seizures are explained by lesions including low-grade neoplasms and/or focal dysplasia in vicinity of the hippocampal formation. In all selected patients, presurgical evaluation revealed that seizures originated in the mesial temporal lobe (Kral et al., 2002).

The mRNA analyses for COL6A1, CIL6A3 and COL6A5 were carried out as described previously (Pernhorst et al., 2013). Briefly, total RNA from biopsies representing all hippocampal subfields was isolated using All Prep DNA/RNA Mini Kit.

(Qiagen) according to the manufacturer's protocol. cRNA was produced using the.

Illumina Total Prep-96 RNA Amplification Kit (Life Technologies Corporation, Darmstadt) and 750 ng of synthesized cRNA was used for hybridization on Human HT-12 v3 Expression BeadChips with Illumina Direct Hybridization Assay Kit (Illumina, San Diego, CA) according to standard procedures. Data for COL6A1, COL6A3 and COL6A5 were extracted and analyzed by Illumina's GenomeStudio Gene Expression Module and normalized using Illumina BeadStudio software suite by quantile normalization with background subtraction.

2.5.8. Blood sampling in rats

A cohort of 32 rats were injected by intrahippocampal KA to induce SE by intrahippocampal kainate, as described above, with the purpose of longitudinal analysis of Col-VI concentration in plasma. Blood was sampled from the tail vein under isoflurane anesthesia before and after SE induction at timepoints -7, 2, 7, 14, 21, 28 days and finally 3 months (sacrificial sampling from heart) after SE. The blood was collected in RNase free tubes which were washed with DEPC water and autoclaved before use. 0.5 M EDTA was added to the tubes shortly before blood collection (10 μl EDTA / 1 ml blood). The sampled blood was stored on ice for a maximum of 2 h and then centrifuged at 1300g at RT to separate plasma. 200 μl from the upper phase was stored in -80 °C. To confirm the occurrence of SRS, the animals were implanted with electrodes one month after SE induction and monitored for seizures (see above for procedure). All animals but two exhibited SRS (data not shown). The plasma samples were analyzed with ELISA (antibodies-online, ABIN432559).

according to the instructions included in the kit.

2.6. Statistical analyses

For all the optical measurements, statistical analysis between the groups was performed using a Bonferroni-adjusted unpaired two-tailed Student's *t*-test. Differences were considered significant at $p < 0.05$. Data are presented as average \pm standard error of the mean (SEM). The investigator conducting the optical density measurements was blind to the group identity of individual animals.

Analysis of electrophysiological data was performed using Fitmaster

(HEKA elektronik) and Igor Pro (Wavemetrics). PPF (and PPD) was expressed as the ratio between the initial slope of the second and the first fEPSPs of the pair, in percentage. Statistical analysis of the data was done using unpaired Student's *t*-test, and the level of significance was set at $p < 0.05$. All data are presented as mean \pm SEM. In the figures, the level of significance is indicated with asterisks as follows: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3. Results

3.1. Effects of exogenous Col-VI on short-term synaptic plasticity in rat hippocampal slices

Although it is known that various ECM components can be involved in different forms of synaptic plasticity (De Luca and Papa, 2016; Oohashi et al., 2015), little or no data are available on the potential role of Col-VI in this process. We hypothesised that Col-VI may directly alter synaptic transmission and plasticity in the hippocampus. To test this hypothesis, we exposed rat hippocampal slices to Col-VI for 6 h (Fig. 1A) prior to electrophysiological recordings of fEPSPs generated by Schaffer collateral-CA1 cell excitatory synapses. In acute hippocampal slices pretreated with Col-VI, PPF was observed to be significantly higher compared with control slices at inter stimulus interval (ISI) of 25 ms (Fig. 2A, B, C, two-tailed unpaired *t*-test; Col-VI = 5 slices, aCSF = 5 slices, $n = 10$ animals), suggesting a decreased release probability of glutamate. This effect was much more pronounced when inhibitory transmission was blocked by application of picrotoxin (PTX) to block GABAergic inhibitory transmission (Fig. 2D; significant difference only at ISI of 25 ms; $p < 0.01$, two-tailed unpaired *t*-test; Col-VI = 10 slices, aCSF = 8 slices, $n = 14$ animals). Actual PPF % values are presented in Table 1.

Table 1
Data Corresponding to Fig. 2C-D.

ISI (ms)	Control PPF (%)	Col-VI PPF (%)	Control PPF with PTX (%)	Col-VI PPF with PTX (%)
25	113.7 \pm 2.6	127.1 \pm 1.5	154.7 \pm 17.3	287.9 \pm 36.5
50	116.7 \pm 3.3	124.7 \pm 2.1	125.0 \pm 8.7	175.9 \pm 22.7
100	113.5 \pm 3.0	119.0 \pm 2.7	119.3 \pm 2.2	122.3 \pm 1.7
200	98.2 \pm 3.4	105.3 \pm 4.7	108.1 \pm 3.0	106.4 \pm 6.8

There was also a trend towards increased PPF at ISI of 50 ms (not statistically significant).

3.2. Short-term synaptic plasticity in Col-VI knock-out mouse hippocampal slices

After observing alterations of short-term excitatory synaptic plasticity in Col-VI gain-of-function experiments, we investigated whether this might also be confirmed by loss-of-function approach using Col-VI deficient mice. First, we recorded field potentials in Schaffer collateral-CA1 cell excitatory synapses in hippocampal slices from Col-VI $-/-$ and measured PPF. No differences in PPF were observed between Col-VI $-/-$ and wild type (Col-VI $+/+$) hippocampal slices when perfused with normal aCSF (control conditions; Fig. 3 A, B, C; $p > 0.05$, one-tailed unpaired *t*-test, $n = 16$ slices, 6 Col-VI $-/-$ animals; $n = 10$ slices, 5 Col-VI $+/+$ animals). Actual PPF/PPD % values are presented in Table 2. However, when in the same slices inhibitory synaptic transmission was blocked by application of GABA receptor antagonist PTX, PPF was converted into PPD only in Col-VI $-/-$ slices at ISI of 25 ms

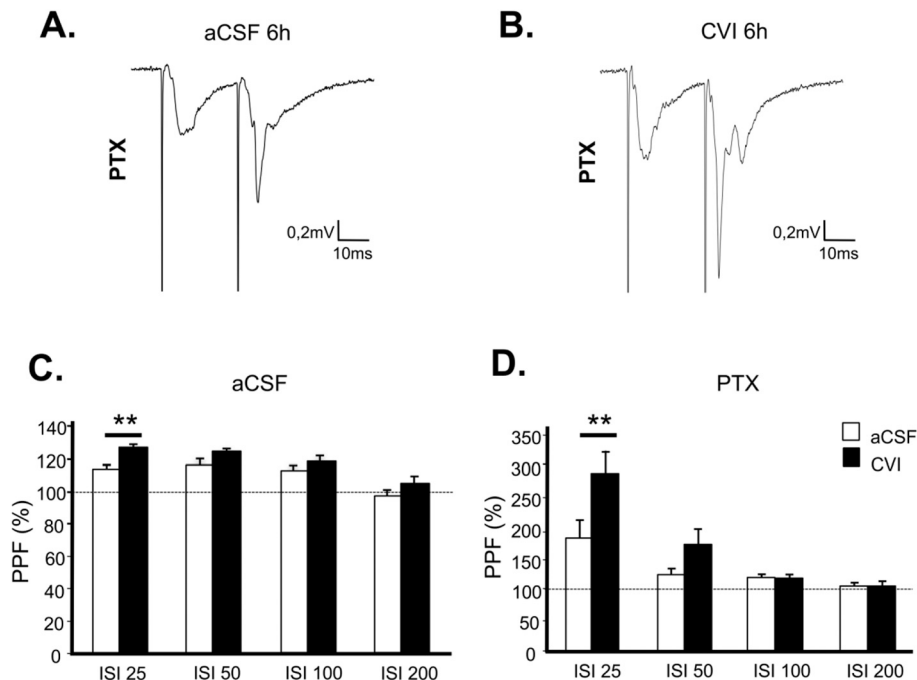


Fig. 2. Paired-pulse facilitation (PPF) of fEPSPs in Schaffer collateral-CA1 excitatory synapses is increased after exposing acute hippocampal slices to Col-VI for 6 h. **A,**

Representative trace of PPF of fEPSPs at the interstimulus interval (ISI) of 25 ms after incubating the acute hippocampal slice in aCSF for 6 h and treated with picrotoxin (PTX) before recording (control). **B,** Representative trace of PPF of fEPSPs at ISI 25 ms after exposing the acute hippocampal slice to Col-VI for 6 h and treating with PTX before recording. **C,** Bar graph representing % PPF ratio after incubating slices with Col-VI for 6 h. The plot shows an increased PPF at the ISI of 25 ms. $N = 10$ slices from 10 rats. **D,** The increase in average PPF of fEPSPs.

(measured at initial slopes) is more pronounced when blocking synaptic GABAergic inhibition with PTX. $N = 8$ control, and 10 Col-VI incubated slices from 14 rats. **: $p < 0.01$, two-tailed unpaired *t*-test. Data is presented as mean \pm SEM.

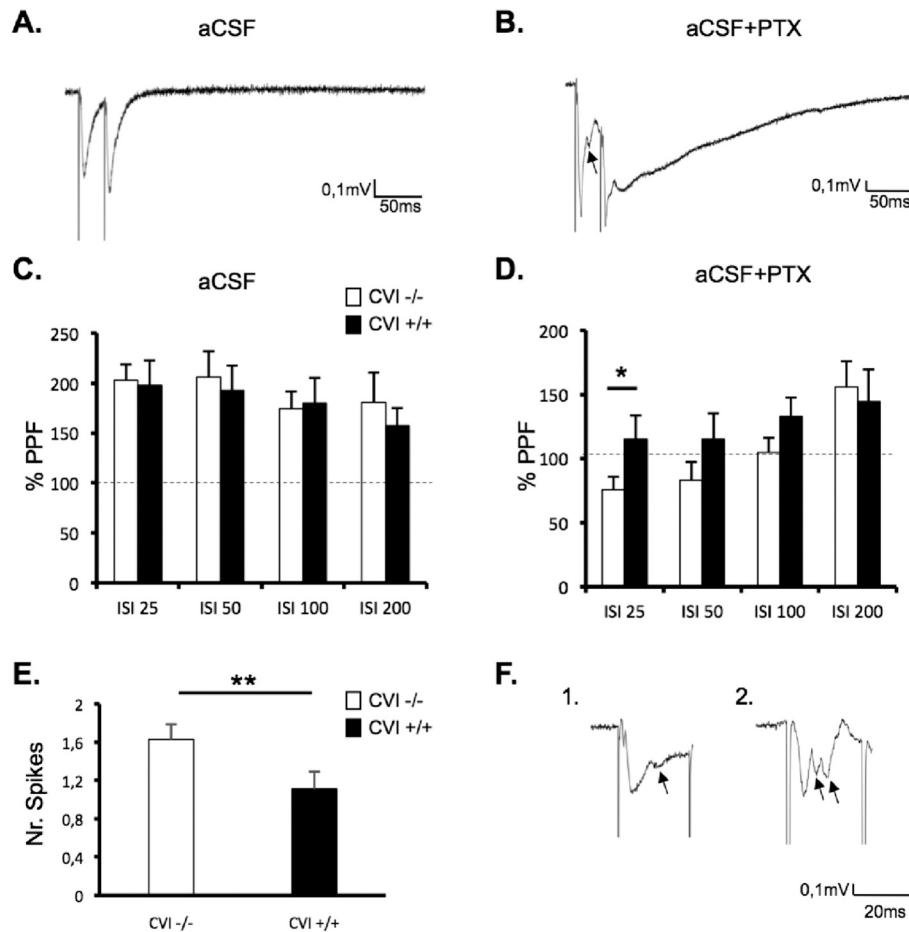


Fig. 3. Paired-pulse facilitation (PPF) of fEPSPs is lower in Schaffer collateral-CA1 excitatory synapses of acute hippocampal slices obtained from Col-VI knock out ($-/-$) mice. **A**, Representative trace of fEPSPs from Col-VI ($-/-$) mouse acute hippocampal slices in aCSF at the ISI of 25 ms. **B**, Representative trace of fEPSPs from Col-VI ($-/-$) mouse acute hippocampal slices in aCSF at the ISI of 25 ms after adding PTX to aCSF. Arrows denote deflections (population spikes) on the first evoked fEPSPs. **C**, Percentage of fEPSP PPF ratio (measured at initial slopes) at the different ISIs. No statistical difference was observed at any of the ISIs ($p < 0.05$; one-tailed unpaired t -test. Data is presented as mean \pm SEM. $n = 16$, 6 Col-VI ($-/-$) animals; $n = 10$, 5 Col-VI ($+/+$) animals). **D**, Percentage of fEPSP PPF ratio after blocking GABAergic synaptic transmission with PTX. A significant difference is observed at the 25 ms ISI (*: $p < 0.05$; one-tailed unpaired t -test. Data is presented as mean \pm SEM. $n = 12$ slicers from 6 Col-VI ($-/-$) animals; $N = 9$ slices from 5 Col-VI ($+/+$) animals). **E**, Bar graph showing the average number of events/population spikes generated by the first fEPSP of the paired-pulse stimulations, after applying PTX. Statistical differences are found between Col-VI ($-/-$) and Col-VI ($+/+$) mice (**: $p = 0.01$; one-tailed unpaired t -test. Data is presented as mean \pm SEM. $N = 59$ slices from 6 Col-VI ($-/-$), and $n = 36$ slices from 5 Col-VI ($+/+$) animals). **F**, 1 and 2 are representative traces of fEPSPs from Col-VI ($-/-$) mouse acute hippocampal slices in aCSF at the ISI of 25 ms after adding PTX. Arrows denote population spikes on evoked first fEPSPs.

Table 2
Data Corresponding to Fig. 3C-D.

ISI (ms)	WT PPF (%)	Col-VI KO PPF (%)	WT PPF with PTX (%)	Col-VI KO PPF with PTX (%)
25	197.8 \pm 24.8	203.0 \pm 15.6	115.4 \pm 18.3	75.9 \pm 10.0
50	192.9 \pm 24.6	206.0 \pm 25.7	115.4 \pm 19.7	83.1 \pm 14.3
100	180.2 \pm 25.1	174.4 \pm 17.0	132.8 \pm 14.8	104.7 \pm 11.6
200	157.7 \pm 17.6	181.0 \pm 29.4	144.3 \pm 25.4	155.7 \pm 20.1

(Fig. 3D; $p < 0.05$, one-tailed unpaired t -test. $n = 12$ –16 slices, 6 Col-VI ($-/-$) animals; $n = 9$ slices, 5 Col-VI ($+/+$) animals). Note also a trend for PPD of fEPSPs at 50 ms (not significantly different from Col-VI ($+/+$)).

These data suggest increased glutamate release probability in perforant path-CA1 cell excitatory synapses, presumably leading to increased network excitability. This notion gained further support by increased number of polysynaptic events and/or evoked population

spikes in response to the first (PS1) stimulation pulses (a measure of hippocampal excitability (Feng et al., 2011)), when GABA receptors were blocked with PTX application. The polysynaptic events and/or population spikes were counted as deflections on evoked fEPSP traces (Fig. 3B, E, F; arrows). The Col-VI ($-/-$) animals showed significantly higher average number of these events evoked by PS1 (Fig. 3E; Col-VI ($-/-$): 1.6 ± 0.1 vs Col-VI ($+/+$): 1.1 ± 0.1 ; $p = 0.01$; one-tailed unpaired t -test. $n = 59$ slices from 6 Col-VI ($-/-$) mice and $n = 36$ slices from 5 Col-VI ($+/+$) mice). It should be kept in mind that all fEPSPs were normalized across the slices to 30–40% of their maximal amplitude respectively.

Collectively, outcomes from gain- and loss-of-function experiments indicate that elevated Col-VI levels may diminish the release probability of glutamate in Schaffer collateral-CA1 excitatory synapses. Conversely, reduced levels or the absence of Col-VI in the hippocampus might enhance the release probability of glutamate, potentially correlating with decreased or increased excitability in the hippocampal circuits, respectively.

3.3. Col-VI expression post status epilepticus

Once we established that absence of Col-VI may increase, while Col-VI elevation may decrease hippocampal excitability, we hypothesised that Col-VI expression may be altered during epileptogenesis, and thereby either contribute to, or counteract this process. In support, there is increasing evidence that ECM molecules are engaged in different aspects of epileptogenesis (Dityatev and Fellin, 2008). To test this hypothesis, we electrically stimulated hippocampi of young adult rats to induce SE (Kanter-Schlifke et al., 2007; Lothman et al., 1989), which routinely triggers epileptogenesis leading to chronic epileptic condition (Lothman et al., 1990) and analyzed the expression levels of.

Col-VI mRNA in the hippocampus and in the neocortex at different time points after SE. We chose these regions since they are highly engaged in the process of epileptogenesis in SE model (Hsieh and Watanabe, 2000). In situ hybridization showed a significant increase in Col-VI mRNA expression only in the DG at 4 weeks after SE (Fig. 4A;

Sham = 846.3 ± 6.6 OD, SE = 1210.0 ± 98.2 OD; $p < 0.05$, Bonferroni-adjusted Student's *t*-test for 8 groups; 4 experimental groups at different time points, and 4 respective controls). This increase in mRNA expression was also translated into a parallel increase in protein immunoreactivity for Col-VI at the same time points, as estimated by optical density analysis of immunoreactivity in the DG (Fig. 4C; Sham = 11.5 ± 1.3 , SE = 17.0 ± 1.0 ; without primary antibody (Ab-) = 11.2 ± 2.2 ; $p < 0.01$ in the KA-injected side and $p < 0.05$ for the contralateral side (not shown); Bonferroni adjusted unpaired.

Student's *t*-test). In addition, Col-VI immunoreactivity was significantly increased in CA1 region (Fig. 4D; Sham = 17.3 ± 0.7 , SE = 25.4 ± 1.2 ; without Ab 19.5 ± 1.0 [not shown]; $p < 0.01$ for the contralateral side and $p < 0.001$ for the electrode side; Bonferroni adjusted unpaired Student's *t*-test) and significantly increased only in the ipsilateral (electrode side) CA3 area (Sham = 9.1 ± 1.0 , SE = 15.4 ± 1.5 , Ab- = 10.2 ± 2.0 , $p < 0.01$) but not for the contralateral side; (Bonferroni adjusted unpaired Student's *t*-test). As upregulation of Col-VI immunoreactivity was observed in both contralateral and ipsilateral sides DG and CA1 relative to the electrode implantation, and no upregulation was observed in the sham group, we can exclude that this response was due to the electrode implantation per se. This was further confirmed by data in chronic epileptic rats assessed 1 year after kainate-induced SE (Fig. 5), which revealed significantly increased levels of Col-VI immunoreactivity in all sub-regions of the hippocampus (CA1: without Ab 11.2 ± 1.4 , Control 12.2 ± 1.9 , KA 8.9 ± 1.4 ; CA3: without Ab 4.1 ± 2.8 , Control 3.0 ± 1.7 , KA 7.5 ± 1.4 ; DG hilus: without Ab 2.5 ± 1.4 , Control 0.0 ± 1.3 , KA 4.4 ± 1.5 ; Fig. 5), suggesting that Col-VI upregulation is a specific response to seizures and not related to possible acute damage resulting from electrode implantation. Although the resolution of the mRNA images does not allow for the precise identification of specific cell types, the increased signal is clearly located in the pyramidal layers of the hippocampus and dentate gyrus granule cells. Measurements were performed on these areas, demonstrating increased optical density. It is likely that principal neurons in the hippocampus, such as CA1 pyramidal neurons and dentate gyrus granule cells, exhibit increased levels of COL6 mRNA. Because Col-VI is released from the cells and stained by antibodies, achieving cell type-specific resolution is challenging, making attempts to determine such specificity based on immunoreactivity potentially misleading. In this context, mRNA expression provides a better indication of cell specificity.

3.4. Validating the observed changes in Col-VI levels in rat models by examination of brain tissue obtained from individuals with temporal lobe epilepsy

To investigate the validity of the elevated Col-VI expression in individuals with epilepsy, we conducted immunohistochemical and mRNA analyses on human brain tissue samples obtained either post-mortem or from surgeries of patients diagnosed with temporal lobe epilepsy. The qualitative analysis of immunoreactivity in postmortem tissue of patients who died after status epilepticus (SE; Fig. 6C, D) or in resected brain tissue from patients with temporal lobe epilepsy displaying hippocampal sclerosis.

(TLE-HS; Fig. 6E, F), showed higher levels of Col-VI both in dentate gyrus (DG) and CA1 area of the hippocampus compared to postmortem controls (control; Fig. 6A, B). The mRNA expression for COL6A1 was significantly higher in samples from TLE patients with amon horn sclerosis (AHS) compared to those from patients with lesion-associated (LA) tissue samples of the hippocampus (Fig. 6G). No such differences were observed for any other subtypes (transcript variants COL6A3 or COL6A5) (Fig. 6H), as well as in a hypothetical protein (not shown). These data indicate that elevated mRNA and protein expression are also increased in patients with epilepsy.

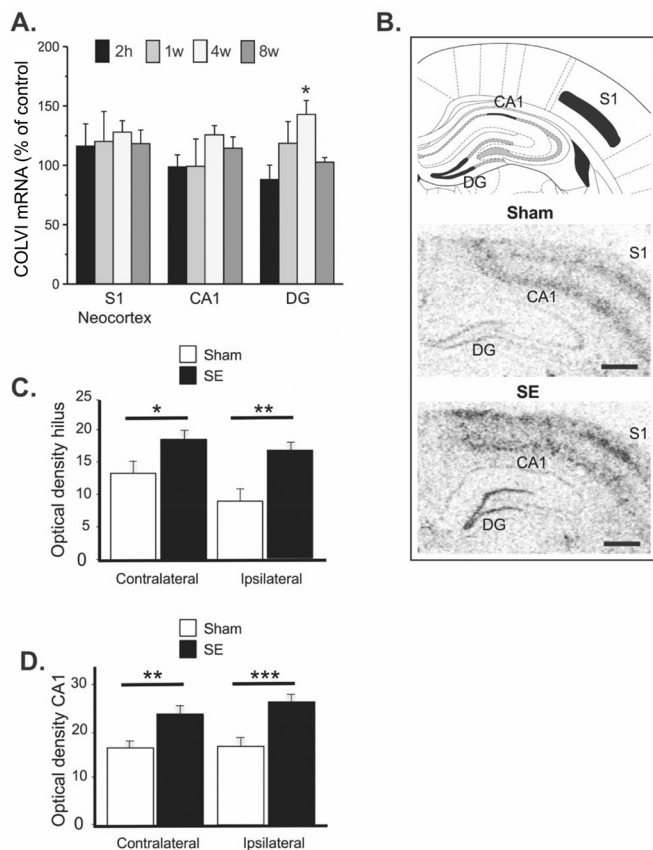


Fig. 4. COL6 mRNA and Col-VI protein expression in the rat epileptic hippocampus. A, Bar graph showing COL6 mRNA expression (arbitrary units, % of control) in primary somatosensory (S1) cortical area, and dentate gyrus (DG) granule cell layer at 2 h, 1, 4 and 8 weeks after SE induction. Statistical difference between control (Sham) and SE animals is observed in the DG at 4 weeks after SE. B, Representative images of in situ hybridization performed in the brain sections from the SE and normal sham-operated animals. Note the increase of COL6 mRNA expression in the DG (time point = 4 weeks after SE). Top panel on B shows the regions where COL6 mRNA labelling was measured (modified from (Paxinos, 2006)). Scale bars: 1 mm. C and D, Bar graphs showing Col-VI protein levels (immunoreactivity; arbitrary units) at 4 weeks after SE induction measured by optical density in the DG (C) and in CA1 region (D) of the hippocampi contralateral and ipsilateral to the electrode implantation. DG: Dentate gyrus; CA1: *Cornu ammonis* area 1. S1: primary somatosensory neocortical area. SE: status epilepticus. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; Bonferroni adjusted unpaired Student's *t*-test. Data is presented as mean \pm SEM ($n = 4$ animals per group). The SE was induced by electrical stimulation as described in details in the Methods section.

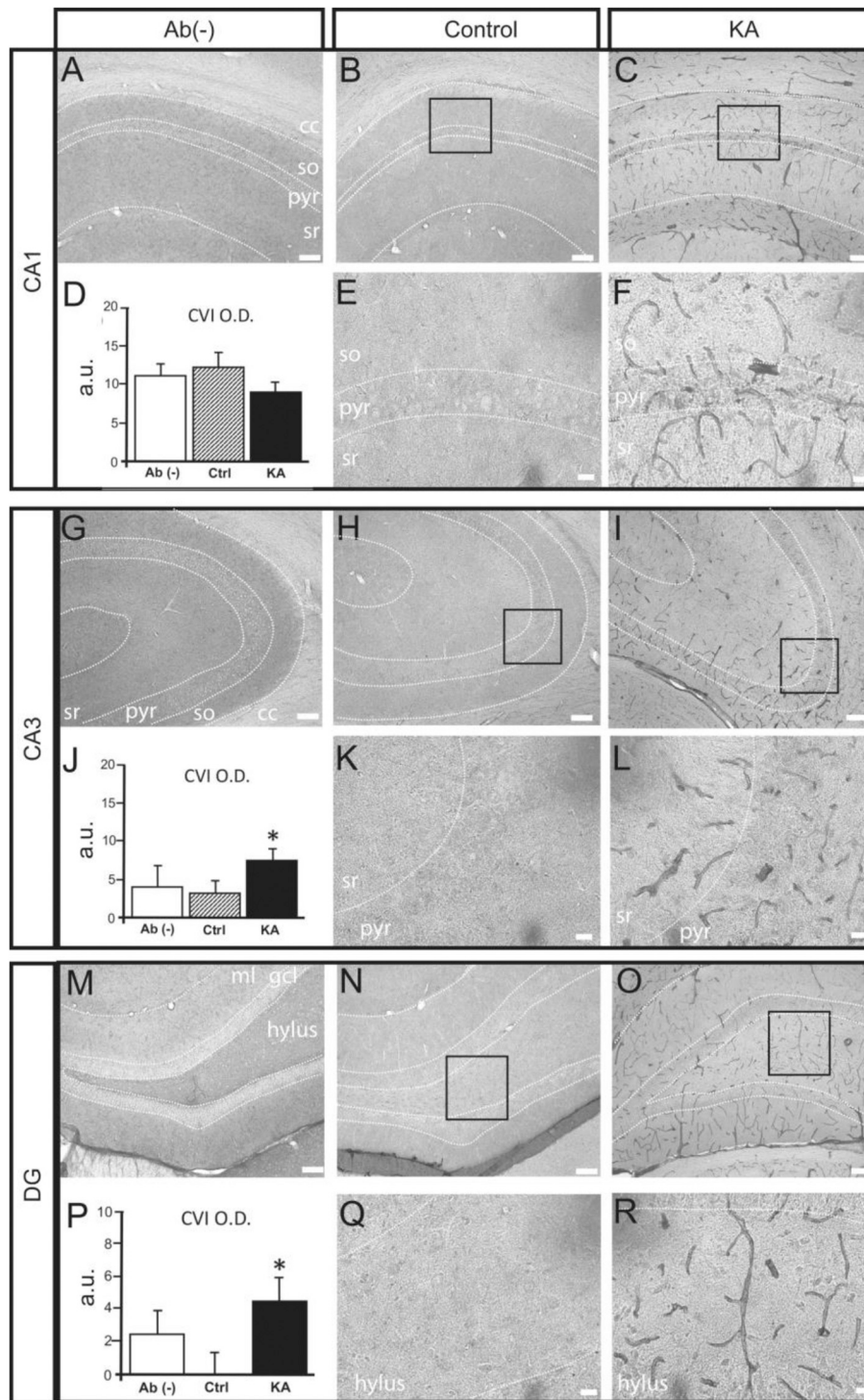


Fig. 5. Immunoreactivity to Col-VI is increased in hippocampus of chronic epileptic animals. A, G and M show representative cases for Col-VI immunohistochemistry without primary antibody in CA1, CA3 and DG, respectively. B and C, H and I, N and O show representative cases for Col-VI immunohistochemistry in CA1, CA3 and DG of the hippocampi in the control and kainate injected animals. E and F, K and L, Q and R, enlargements of respective images above. D, J and P, bar graphs showing quantifications of optical density measurements (arbitrary units, a.u.) in hippocampus slices in the CA1, CA3 and DG areas, respectively. Scale bars in O and R: 200 and 50 μ m, respectively. cx: cortex; cc: corpus callosum; so: stratum oriens; pyr: pyramidal layer; sr: stratum radiatum; slm: stratum lacunosum moleculare; ml: molecular layer; gcl: granule cell layer. SE was induced by 0.4 μ g/ 0.4 μ l of kainic acid injected into the medial part of the right hippocampus (see Methods). SE was defined as continuous motor seizures for at least 10 min within 3 h after kainate injection. After 1 year, the animals were transcardially perfused with saline and subsequently with 4% paraformaldehyde before the brain was collected.

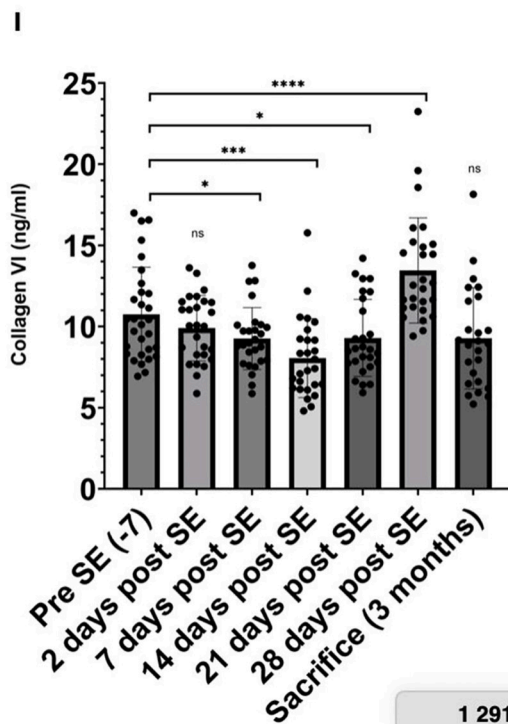
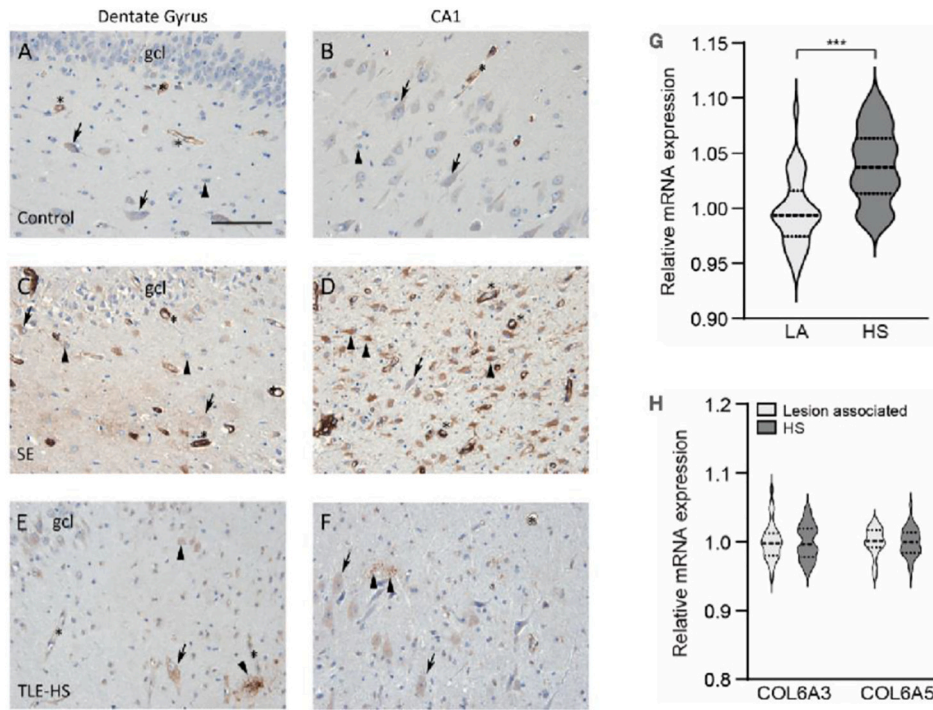
3.5. Col-VI blood levels after SE in rats

Since Col-VI is a soluble molecule and crosses BBB, we asked whether changes in the brain associated with seizure activity could also be

reflected in blood levels of Col-VI. To address this question, we collected blood samples longitudinally from same rats at different time points after intrahippocampal kainate (KA)-induced SE. The ELISA analysis of these blood samples revealed moderate but statistically significant

dynamic alterations of Col-VI blood levels after KA-induced SE (Fig. 6I). Fig. 6I-J shows the changes in Col-VI blood levels post-SE. Individual animal data points are included in the bar charts to illustrate data distribution. The analysis revealed a significant effect of time following SE on Col-VI levels ($F(3.911, 100.4) = 23.78, p < 0.0001$). Post hoc Dunnett's test indicated differences in Col-VI levels at 7, 14, 21, and 28 days post SE compared to the first pre-SE measurement. The Col-VI was decreased during early epileptogenesis period, between 7 and 21 days

post-SE, while it was increased at 28 days at which time point most of the animals have developed spontaneous recurrent seizures (SRS). No changes in blood levels were observed in chronic epileptic state at 3 months after SE. The changes are depicted in Fig. 6I as ColVI levels in ng/ml of blood, and are compared to pre-SE control values. Actual values in numbers are presented on the Fig. 6J. These data indicate that changes in Col-VI expression in the brain could also be reflected in its blood levels.



J

Time post SE	Col-VI ng/ml	n
-7 days (pre SE)	10.75±0.5311	30
2 days	9.898±0.3879	27
7 days	9.254±0.3673*	27
14 days	8.052±0.4689***	27
21 days	9.293±0.4566*	27
28 days	13.45±0.6243****	27
Sacrifice (3 months post SE)	9.273±0.6248	25

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Fig. 6. Col-VI immunoreactivity and COL6 mRNA expression are increased in the hippocampus of patients with mTLE, and Col-VI blood levels measured by ELISA are dynamically changed post SE. **A and B** Shows strong staining in blood vessels (asterisks), and moderate staining in neurons (arrows) and glia (arrowheads) in the hippocampus from post-mortem samples of healthy individual (controls; $n = 6$ individuals). **C and D**, increased Col-VI immunoreactivity observed in the hippocampus of patients who died after SE, in the DG and CA1 (respectively), in neurons (arrows), and glia (arrowheads), as well as in blood vessels (asterisks). This increase was most evident in the CA1 area. **E and F**, hippocampal slices from brain tissue resected from patients with drug-resistant temporal lobe epilepsy with hippocampal sclerosis (mTLE-HS). Note moderate increase of Col-VI immunoreactivity observed in the DG and CA1 areas in neurons (arrows), as well as in glial cells (arrowheads), while the in blood vessels' immunoreactivity was similar to the controls (asterisks) ($n = 5$ individuals). **G and H**, Violin plots showing the relative expression levels of COL6A1 (**A**), and two of its family members, i.e. COL6A3 and COL6A5 (**B**) in hippocampal biopsy tissue of patients with TLE-HS versus hippocampi from patients with lesion-associated (LA)-TLE (i.e. seizures originating outside hippocampus; controls) (LA: $n = 35$; HS: $n = 78$; t -test: $***p \leq 0.001$, with β -actin as reference gene).

Blood was taken from tail vein longitudinally from same rats at various time points. **I**, Graph demonstrates significant reduction of Col-VI levels in blood plasma, between 7 and 21 days post-SE (during late epileptogenesis phase), while about 4 weeks post-SE, the levels were significantly higher as compared to pre-SE control. Three months post-SE, the levels of Col-VI blood plasma were back to control values. **J**, Actual values in ng/mL are presented as means \pm SE. One-Way ANOVA with repeated measures, * - **** = $p < 0,05$ – $0,0001$ respectively; ns = non-significant; n = number of samples; different number of samples reflect fall-out of animals during the course of the experiment. Samples at the last time-point were taken directly from the heart. SE was induced by $0.4 \mu\text{g} / 0.4 \mu\text{l}$ of kainic acid injected into the medial part of the right hippocampus (see Methods). SE was defined as continuous motor seizures for at least 10 min within 3 h after kainate injection. The mortality rate in this cohort of animals was 16% through-out the whole duration of the experiment.

4. Discussion

4.1. Col-VI modulates short-term synaptic plasticity

ECM in the brain is composed of molecules synthesized and secreted by neurons and glial cells in a cell-type-specific and activity-dependent manner (Barros et al., 2011; Dityatev and Fellin, 2008). In the mature brain, ECM undergoes a slow turnover and supports multiple physiological processes while restraining structural plasticity (Dityatev and Fellin, 2008); reviewed in (McRae and Porter, 2012). In addition, brain ECM proteins can be engaged in different aspects of epileptogenesis, such as dysregulation of excitatory and inhibitory neurotransmission, sprouting of mossy fibers, granule cell dispersion and/or gliosis, neurotrophin-mediated modulatory effects and synaptic plasticity at inhibitory neurons (Dityatev and Fellin, 2008; McRae et al., 2012; Pitkanen and Lukasiuk, 2009; Wong, 2012); reviewed in (Kim et al., 2016).

Here we demonstrate that exposure of hippocampal slices to exogenously applied Col-VI enhances PPF at Schaffer collateral-CA1 cell excitatory synapses. It has been proposed that various ECM molecules can modulate different forms of synaptic plasticity through their receptors, such as integrins, mostly by regulating NMDA and/or AMPA receptors (Dityatev and Fellin, 2008; Staubli et al., 1990). Interestingly, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are Col-VI receptors (Pfaff et al., 1993). $\alpha 2\beta 1$ integrins co-localize with epidermal growth factor receptor (EGFR) (Yu et al., 2000) and the activation of EGFR has been reported to up-regulate the NMDA receptors (NMDAR), and thereby increase.

NMDAR-mediated currents, which enhances LTP in the hippocampus (Tang et al., 2015). Co-localization of EGFR with $\alpha 2\beta 1$ integrins is also known to regulate the activity of the downstream effectors such as ERK, AKT, JNK and RhoGTPases. More details on the downstream pathways for integrins can be found in (Legate et al., 2009). Collagen VI also binds to NG2 proteoglycans, an integral membrane chondroitin sulfate proteoglycan (CSP; Stallcup et al., 1990). The CSPs are known to modulate AMPA receptor stability at the synapse (Frischknecht et al., 2009). The removal of CSPs has been reported to degrade perineuronal networks, resulting in increased excitability of parvalbumin expressing inhibitory interneurons innervating the perisomatic region of principal neurons in postnatal hippocampus (Celio et al., 1998; Dityatev et al., 2007; Yamaguchi, 2000). Taken together, all these previous studies support the idea that ECM molecules, including Col-VI, can regulate synaptic transmission predominantly by postsynaptic mechanisms affecting either AMPA/kainate or NMDA receptors, or in case of Col-VI, inhibitory interneurons.

Since PPF is inversely related to the transmitter release probability from the presynaptic terminals (Dobrunz and Stevens, 1997), our results showing increased PPF after Col-VI incubation would suggest reduced release probability at Schaffer collateral - CA1 cell synapses. Such effect would result in overall decreased glutamate release, and thereby lower

excitability of hippocampal network. This conclusion based on gain-of-function approach is also supported by the loss-of-function experiments in Col-VI $-/-$ mice demonstrating opposite effect, i.e. reversal of the PPF into PPD in Schaffer collateral-CA1 cell synapses. These new findings of Col-VI effect on potentially also on *presynaptic site* to regulate synaptic plasticity warrants more detailed investigation of its mechanisms of action. Above-mentioned integrins have also been reported to form clusters through interaction with ECM proteins and regulate intracellular Ca^{2+} levels, protein kinase signaling, and reorganize cytoskeletal filaments (Chan et al., 2006; Pfaff et al., 1993; Wu and Reddy, 2012). If such alterations in intracellular levels of Ca^{2+} would occur in presynaptic terminals, it might directly influence release probability of neurotransmitters, e.g. glutamate, and affect the paired-pulse ratio (Korber and Kuner, 2016; Rusakov, 2006). Future studies should investigate the correlation of changes in Col-VI levels with seizure frequency, duration, and severity to better understand Col-VI's role in epileptogenesis. However, measuring Col-VI levels in the brain is challenging due to the complexities of obtaining accurate and consistent measurements from brain tissue. Additionally, inducing seizures in Col-VI knock-out mice is ethically problematic due to their significantly reduced bone density, which raises the risk of fractures during seizures and convulsions. Future studies may explore the possibility of Col-VI overexpression to evaluate its role in seizure modulation without compromising animal welfare.

4.2. Col-VI expression is increased after status epilepticus in rat brain and in brain tissue of patients with epilepsy

If Col-VI levels can regulate excitatory synaptic transmission in the hippocampus, could this molecule affect network excitability, and thereby play a role in pathophysiological mechanisms of epilepsy? Integrins, which are proposed to be Col-VI receptors (Pfaff et al., 1993), have been implicated in the pathophysiology of several brain diseases, including epilepsy (McCarty et al., 2005; Wu and Reddy, 2012). Our results showing increased Col-VI mRNA and protein expression both at 4 weeks after SE in rats, an insult to the brain that leads to chronic epileptic state (Morimoto et al., 2004), and in human brain tissue derived from patients with TLE, would support this idea. The present study is also in line with previous findings in human temporal lobe epilepsy showing an increased expression of extravascular collagen I-IV which suggests their association with seizure occurrence (Veznedaroglu et al., 2002). If this is the case, what is the possible role of Col-VI in epileptogenesis? Our data of Col-VI effect on synaptic transmission would suggest that increased Col-VI expression could act as a protective/adaptive mechanism to counteract increased excitability in hippocampal circuits after brain insults (SE). The elevated levels of Col-VI observed in epilepsy patients may represent a compensatory mechanism in response to ongoing neuronal hyperexcitability, rather than a direct anti-seizure

effect. Therefore, it could represent a dynamic and long-lasting adaptive alterations in ECM, even extending into the chronic phase of epilepsy (as shown by increased Col-VI immunoreactivity at 1 year after kainate-induced SE, and in brain tissue of patients with epilepsy in the present study). The changes in the expression levels of other ECM proteins (e.g., CSP) have also been reported in kainate and kindling models of epilepsy, leading to a proposal that there seems to be an interaction between ECM, astrocytes and neurons, that is involved in regulation of gamma-oscillations and epileptogenesis in vivo (Dityatev and Fellin, 2008).

4.3. Blood levels of Col-VI after SE

Dynamic changes in Col-VI levels were observed in blood samples post-SE. It is likely that the source of this protein in the blood is central, originating from the brain. Col-VI is widely expressed in the CNS, particularly in the extracellular matrix of brain tissues, where it interacts with astrocytes, neurons, and endothelial cells (Wareham et al., 2024). These collagens can be released into the bloodstream, particularly following injury or disease that disrupts the blood-brain barrier. However, it is also possible that Col-VI in the blood could originate from peripheral sources. Col-VI is present in various tissues throughout the body, including muscle, skin, and blood vessels (Gregorio et al., 2018). Peripheral injury or pathological conditions affecting these tissues could contribute to the observed changes in blood Col-VI levels. Our novel findings of dynamic changes of Col-VI levels in blood samples taken longitudinally from animals after experiencing SE indicates that Col-VI can potentially be considered as an informative marker of brain insults, and even turn out to be a predictive factor of ongoing epileptogenesis. For example, a combination of initial decrease at 1–3 weeks and consequent increase at 4 weeks after brain insult could be predictive of developing epileptic condition, since almost all animals in the cohort developed epilepsy. While Col-VI shows potential as a functional biomarker of epileptogenesis, its lack of specificity due to elevations in other conditions such as fibrosis (Williams et al., 2022) and cancer (Chen et al., 2013) warrants caution and further validation in epilepsy-specific contexts. Further studies with more detailed analysis of Col-VI levels as biomarker of epileptogenesis is needed. These studies should also include ROC curve analysis (Soreide, 2009) to truly establish how valuable Col-VI levels in blood could be for predicting epileptogenesis, or facilitating diagnosis of certain acquired epilepsies.

4.4. Concluding remarks

Our data demonstrate that Col-VI alterations may affect presynaptic glutamate release and thereby regulate hippocampal excitability. This most likely happens during the late phase of epileptogenesis, as well as in the chronic epileptic state in animals and human brain tissue, at which timepoints we observed increased levels of Col-VI and its mRNA. We speculate that potential mechanism underlying this presynaptic action is the interaction between Col-VI and presynaptic CB1Rs exerting an inhibitory effect on glutamate release. This hypothesis is supported by earlier suggestion that the binding of Col-VI to CB1R may inhibit glutamate release (Lam et al., 2022). Future studies should investigate the involvement of CB1R in the antiepileptic effects of Col-VI, potentially using CB1R antagonists to validate this hypothesis.

Taken together, our study gives new insights on the role of ECM in regulation of synaptic transmission and epileptogenesis, and warrants further investigations to delineate more detailed mechanisms of the proposed link between ECM and network excitability, including pathophysiological states such as in epilepsy. This research may lead to novel strategies to aid treatment of this incurable disease.

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Tania Ramos-Moreno: Writing – original draft, Formal analysis. **Alexandra Cifra:** Investigation, Formal analysis. **Nikitidou Ledri Litsa:** Methodology, Investigation, Formal analysis, Data curation. **Esbjörn Melin:** Data curation. **Matilda Ahl:** Formal analysis. **Sören H. Christiansen:** Data curation. **Casper R. Gøtzsche:** Data curation. **Matilde Cescon:** Data curation. **Paolo Bonaldo:** Data curation. **Karen van Loo:** Methodology, Data curation. **Valeri Borger:** Methodology, Investigation, Data curation. **J. Anink Jasper:** Data curation. **Albert Becker:** Investigation, Formal analysis, Data curation. **Erwin A. van Vliet:** Investigation, Formal analysis, Data curation. **Eleonora Aronica:** Supervision. **David P. Woldbye:** Supervision, Data curation. **Merab Kokaia:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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