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Inhibiting cholesterol degradation induces neuronal sclerosis and epileptic activity in mouse hippocampus

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Abstract

Elevations in neuronal cholesterol have been associated with several degenerative diseases. An enhanced excitability and synchronous firing in surviving neurons are among the sequels of neuronal death in these diseases and also in some epileptic syndromes. Here, we attempted to increase neuronal cholesterol levels, using a short hairpin RNA (shRNA) to suppress expression of the enzyme CYP46A1. This protein hydroxylates cholesterol and so facilitates trans-membrane extrusion. A sh-RNA CYP46A1 construction coupled to an adeno-associated virus (AAV5) was injected focally and unilaterally into mouse hippocampus. It was selectively expressed first in neurons of the CA3a region. Cytoplasmic and membrane cholesterol increased, neuronal soma volume increased and then decreased before pyramidal cells died. As CA3a pyramidal cells died, inter-ictal EEG events occurred during exploration and non-REM sleep. With time, neuronal death spread to involve pyramidal cells and interneurons of the CA1 region. CA1 neuronal death was correlated with a delayed local expression of phosphorylated tau. Astrocytes were activated throughout the hippocampus and microglial activation was specific to regions of neuronal death. CA1 neuronal death was correlated with distinct aberrant EEG activity. During exploratory behaviour and rapid eye movement sleep, EEG oscillations at 7-10 Hz (theta) could accelerate to 14-21 Hz (beta) waves. They were accompanied by low amplitude, high-frequency oscillations of

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Supplementary material

Supplementary material available on the short hairpin RNA (shRNA) used to suppress expression of the enzyme, 24-hydroxy cholesterol coded by the gene CYP46A1 concerns choice of the sequence, details of the construction with an adeno-associated viral vector tagged with the green fluorescent protein and tests of the cell-specificity of vector uptake.

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peak power at ~300Hz and a range of 250-350 Hz. While episodes of EEG acceleration were not correlated with changes in exploratory behaviour, they were followed in some animals by structured seizure-like discharges. These data strengthen links between increased cholesterol, neuronal sclerosis and epileptic behavior

Keywords

Neurodegeneration; Cholesterol; Epilepsy; shRNA; CYP46A1

Introduction

Defects in lipid, and especially cholesterol, metabolism have been associated with some neuronal degeneration syndromes. Niemann-Pick disease type C results from an inherited defect in proteins involved in neuronal cholesterol homeostasis and extrusion (Sévin *et al.*, 2007; Lange *et al.*, 2000). Alzheimer disease, which affects neurons of the parahippocampal region and neocortex, has been associated with excessive levels of cholesterol (Simons *et al.*, 1998; Puglielli, Tanzi & Kovacs, 2003, Panchal *et al.*, 2010; di Paolo & Kim, 2011). Cholesterol elevation has also been linked to neurodegeneration of dopaminergic substantia nigra neurons in Parkinson's disease (Cheng *et al.*, 2011) and the loss of basal ganglion neurons in Huntington's disease (Karasinska & Hayden 2011).

Several neurodegenerative diseases are associated with an abnormal neuronal synchrony and enhanced excitability (Palop & Mucke, 2009). Niemann-Pick type C patients express seizures (Sévin *et al.*, 2007). In Parkinson's disease, the basal ganglia generate abnormal neuronal oscillations and population synchrony (Wichmann & Dostrovsky, 2011). Similarly, patients with, and animal models of, Alzheimer pathologies exhibit seizure-like activities (Busche *et al.*, 2008) associated with an enhanced neuronal excitability (Minkeviciene *et al.*, 2009; Verret *et al.*, 2012).

Epileptic syndromes classically reflect an enhanced neuronal excitability and aberrant synchrony. In some focal epilepsies, including those of the temporal lobe, a consistent pattern of neuronal death is evident. This hippocampal sclerosis (Blümcke, Thom & Wiestler, 2002) precedes the emergence of seizures (Pitkänen & Lukasiuk, 2009). However possible links between lipid dysregulation, neuronal death and the emergence of epileptic behaviours remain unclear.

Over-expression of cholesterol 24-hydroxylase, a cholesterol degradation enzyme coded by the gene CYP46A1, has been shown to reduce amyloid plaques in a mouse model of Alzheimer's disease (Hudry *et al.*, 2010). Conversely inhibiting CYP46A1 should enhance intraneuronal cholesterol and induce neuronal death. In this study we injected into the hippocampus a small hairpin (sh) viral construction to suppress cholesterol 24-hydroxylase expression. We asked whether it induced an increase of intra-neuronal cholesterol, and neuronal death and whether aberrant electrical activities were generated by the hippocampus.

Materials and methods

shRNA-AAV5 viral vector

Procedures for selection and testing of shRNA to inhibit CYP46A1 and construction of a tagged, viral vector are described in the Supplementary Data. Briefly, shRNA sequences were tested on 2 cell lines. Sequences that effectively suppressed CYP46A1 expression in culture (Supplementary Fig. 1) were cloned into an adeno-associated virus type 5 (AAV5) vector, with an eGFP reporter, controlled by the phosphoglycerate kinase promoter (pPGK). A vector with a scrambled shRNA sequence was used as control. The shRNA sequence causing the largest reduction of 24-hydroxycholesterol content in vivo was used subsequently. The construction was taken up by neurones but not by astrocytes or microglial cells (Supplementary Fig. 2). 24-hydroxycholesterol levels were not changed by the AAV5-scramble vector.

Intrahippocampal vector injection

The AAV-shRNA vector construction was injected into one hippocampus of adult, female C57BL/6 mice of age 3 months and weight 20-25 g (Janvier, Le Genest Saint Isle, France). Animals were housed in a 12hr light - dark cycle and work was done in accordance with the European Committee Council Directive (2010/63/UE), INSERM and local guidelines (approved by the Comité d'éthique, Ministère de l'Enseignement Supérieur et de la Recherche, Paris).

Mice were anesthetized by intraperitoneal injection of ketamine (80mg/kg) and xylazine (12mg/kg) and placed in a stereotaxic frame (D Kopf Instruments, Tujunga, USA) with all measures taken to minimise pain or discomfort during surgery and afterwards. The AAV5-vector (2 μ l, 1×10^9 vg) was injected at ~ 0.2 μ l/min from a 10 μ l syringe attached to a 30-gauge needle. Control injections consisted of the same volume of phosphate buffer or a scrambled RNA sequence. Injections were made unilaterally in the hippocampus at a site verified by injecting Fluororuby (Tetramethylrhodamine-dextran, 10 000 MW, Life Technologies, St. Aubin, France; Le Duigou *et al.*, 2005). The coordinates were: anterior-posterior, - 2 mm; medial-lateral, -1.4 / 1.2, dorsal-ventral -2 mm with respect to the bregma: corresponding to a region occupied by apical dendrites of CA1 pyramidal cells near the subiculum. Injecting at this site produced a reproducible vector uptake and electrophysiological responses. Anatomical studies were made on 12 animals, and EEG-video records were made once per week from 14 animals at 2-10 weeks after injection.

Histology and immunohistochemistry

Anatomical consequences of vector injection in the hippocampus were examined in tissue dissected from animals sacrificed at 2, 3, 4, 6, 8 and 12 weeks after injection (n=3 mice injected with the shRNA, 2 injected with the scrambled vector and 1 with PBS for each time point). Animals were anaesthetized and perfused intracardially with 4% paraformaldehyde in 0.12 M phosphate buffer (PB). After fixation in PB for 24 hr at 4°C, serial sections of thickness 50 μ m were cut with a vibratome (Oxford Inst, Oxford, UK). They were cryo-protected in 30% sucrose and 30% ethylene glycol in PB and kept at -20°C before staining.

Filipin, a fluorescent, sterol-binding molecule, was used to detect intracellular or membrane cholesterol (Cooper & McLaughlin, 1984). Filipin (Sigma, Lyon, France) was first dissolved in 2.5% DMSO at 25 mg/ml. Brain slices of thickness 50 μm , fixed with paraformaldehyde were washed and exposed to filipin, at 125 $\mu\text{g/ml}$ in phosphate buffer, for 2 hrs in darkness. Filipin staining was visualised on a confocal microscope (SP5 Leica Microsystems, Wetzlar, Germany) with excitation at 351 nm by a UV laser. A $\times 60$ oil-immersion, objective of NA 1.4 was used to collect light emitted between 400 and 680 nm (Marquer *et al.*, 2011).

Specific antibodies were used to detect GFP in cells expressing the AAV-vector construction and to recognise neurons and glial cells. Immunostaining was done after washing in PB, and immersion for 2hr in 0.2% milk powder to saturate non-specific signals and 0.3% Triton-X to permeabilise cell membranes. The following primary antibodies were applied for 12 hr at 4°C: anti-NeuN (monoclonal mouse; 1:200; Merck-Millipore, St Quentin, France), anti-GFP (monoclonal rabbit biotinylated; 1:200; Millipore), anti-IbA-1 (monoclonal rabbit; 1:250; Wako, Montbonnot, France), anti-GFAP (monoclonal rabbit; 1:1000; Millipore) and anti-phosphorylated tau (AT8, monoclonal mouse; 1:1000; Thermo Scientific, Villebon-sur-Yvette, France). The following secondary antibodies were applied for 4 hr at 20 °C: anti-IgG rabbit-Alexa 488 (1:200), anti-IgG rabbit-Cy3 (1:100), anti-IgG mice Alexa 555 (1:200) and anti-IgG rabbit-Cy5 (1:200). Sections were washed in PB saline and mounted with the anti-fade agent Prolong Gold (Invitrogen, Eugene, OR, USA).

Images were acquired with a QImaging Retiga EXI camera (Qimaging, Surrey, BC, Canada), mounted on an inverted Olympus IX81 microscope. An optigrid system (Thales Optem, Qioptik, Rochester, NY, USA) permitted 3D image reconstruction and analysis (Volocity, Improvision, Perkin-Elmer, Coventry, UK). Image stacks for reconstruction were acquired with a 10 $\times$ objective of NA 0.4, a 20 $\times$ objective of NA 0.9 or with a 40 $\times$ objective of NA 1.3. Numbers of NeuN immuno-stained elements in the st. pyramidale of the CA3a, CA3c and CA1b regions were compared in injected and contralateral hippocampal sections at 2-12 weeks after viral vector injection. Stacks were made from 5 successive 1 μm optical sections and 3 replicates were made at different z-values in 50 μm thick slices. NeuN positive elements were counted automatically from 200 μm long segments of st. pyramidale using features of Volocity (Perkin-Elmer, Coventry, UK) including 'intensity detection', 'exclude elements larger than' (typically 600 μm^2), 'exclude smaller than' (typically 50 μm^2) and 'separate touching objects'. Comparison with manual counts indicated differences of less than 20% (n=3). We also compared numbers of NeuN positive somata in st. radiatum and st. oriens of the CA1 region from contralateral and injected hippocampi. These presumed interneurons were counted from stacks of twenty 2 μm thick optical sections.

Video-EEG recordings

EEG and video recordings were made from animals injected with the AAV5-shCYP46A1 vector (n=7), with an AAV5-scrambled vector (n=3) or with phosphate buffer (n=4 mice). EEG electrodes were made from Nichrome wire of diameter 50-200 μm . Bipolar records were made with a twisted wire pair near the hippocampal site of vector injection. Monopolar records were made bilaterally with Nichrome wire from parieto-occipital cortex. A monopolar reference electrode was implanted in the cerebellum and a neutral electrode

above the olfactory cortex. Animals were implanted with the electrodes immediately after vector injection and were housed singly. EEG-video recordings of 3 hrs duration were made in the home cage of each animal once per week from 2 to 10 weeks after injection. During recording sessions, animals engaged in exploratory activities for the first hour or so, and then alternated between sleeping and waking behaviour.

EEG signals were amplified and filtered between 0.5 and 500 Hz with an EEG amplifier (V32 amplifier, NicoletOne, Natus, San Carlos, CA, USA). Observation of EEG traces and video records of animal behaviour let us distinguish between exploration or quiet awake behaviour and non-REM or REM sleep. Interictal events, including spikes, spike and wave complexes or polyspikes, were clearly distinct from background activity. Their frequency was measured during non-REM sleep. Ictal events were defined as stereotyped, organised EEG oscillations of frequency increasing from the theta to the gamma range and duration at least 5 sec.

Time-frequency plots of EEG signals were made with a Gabor wavelet of 3 cycles, (Mallat, 1999). Power spectral density was computed using an autoregressive model estimated by the Burg method, after spectral equalization to remove polynomial trends in the log-log Fourier transform (Mitra & Pesaran, 1999). An index of relative power corresponded to the power at each frequency divided by the total power across all frequencies (Fig. 6C, 7F).

Statistics

Results are given as mean $\pm$ sem. Statistical significance was determined with the t-test of Student.

Results

Initial uptake of the AAV-shCYP46A1 vector in the hippocampus

GFP fluorescence or immuno-staining with an antibody against GFP revealed a consistent initial uptake pattern for the AAV-shCYP46A1-GFP vector (Fig. 1A). At 2-3 weeks after injection, the vector was highly expressed in neurons at the border between the CA1 and CA3 regions of the hippocampus (n=4 animals). Pyramidal cell somata and apical dendrites were both stained. The rate of neuronal transduction, measured as the proportion of CA3a pyramidal cells immuno-positive for both NeuN and GFP, was 70-90% (counts from 3 slices in each of 4 animals). Staining was also detected in cells with somata in st. oriens and st. radiatum of the CA3a region which were presumed to be interneurons. Expression was low in principal cells of the CA3b- c regions, in CA1 and in the dentate gyrus (n=8). GFP expression patterns were identical for the sh-CYP46A1 vector and the scrambled version.

The AAV-shCYP46A1 vector increases neuronal cholesterol

We attempted to detect changes in intra-neuronal cholesterol following neuronal expression of the viral vector. Immuno-detection of GFP was combined with staining for filipin (Fig. 1B-E), a fluorescent molecule that binds to sterols (Cooper & McLaughlin, 1984). Filipin staining was never detected in GFP-positive cells of animals injected with the scrambled AAV5vector or in contralateral, non-injected hippocampus (not shown). Spots of filipin

staining were first detected in neurons of the CA3a region of injected hippocampus at three weeks after injection (Fig. 1B, E). They were detected in most GFP-expressing neurons (54 of 61, or 89%, of CA3a neurons in 4 slices from 2 animals at 3 weeks). Our observations suggested cholesterol levels increased at both cytoplasmic and membrane sites. Localisation of filipin staining within a volume of GFP staining for GFP was considered as cytoplasmic while that at the limit of GFP staining for a neuron was considered as indicating membrane cholesterol. The area of cytoplasmic spots was $4.6 \pm 4.4 \mu\text{m}^2$ (mean $\pm$ sem; $n=70$) and that of spots associated with neuronal membranes was $5.4 \pm 5.6 \mu\text{m}^2$ ($n=66$). At four weeks, filipin fluorescence was detected diffusely in the cytoplasm and at the membrane of GFP-positive neurons (Fig. 1C, E). Somatic size seemed to increase with time after filipin staining was detected (Fig. 1E, F). At six weeks after injecting the vector, intense staining was present throughout GFP-positive neurons (Fig. 1D, E), and signs of cellular damage included somatic shrinkage. The mean somatic area of neurons expressing GFP and filipin was $950 \pm 356 \mu\text{m}^2$ (mean $\pm$ sem; $n=54$) at 3 weeks, $1274 \pm 294 \mu\text{m}^2$ ($n=50$) at 4 weeks and $704 \pm 359 \mu\text{m}^2$ ($n=77$) at 6 weeks after injecting the vector (Fig. 1F).

Initial death of CA3a neurons - delayed death of CA1 neurons

Filipin staining revealed progressive changes in cholesterol levels in CA3a neurons expressing the AAV-shCYP46A1 vector. A comparison of GFP immuno-staining with NeuN staining showed these changes culminated in neuronal death (Fig. 2). Neuronal death occurred first in CA3a, the site of initial vector expression and spread with time to the CA1 region.

GFP was first detected at the border between the CA3 and CA1 regions at 2 and 4 weeks after vector injection (Fig. 2A). At 8 and 12 weeks after injection, the somata of CA1 pyramidal cells were immuno-positive for GFP with intensities similar to those detected in CA3a cells at 4 weeks. We therefore wished to measure uptake of the viral vector construction in CA1 cells at 2-3 weeks after injection when a strong GFP staining was detected in CA3a pyramidal cell somata. At 2-3 weeks, the mean intensity of GFP signals in CA1b pyramidal cell somata ($n=60$ cells, 3 sections, 3 animals) was 4-8 % of the mean intensity in CA3a cell somata ($n=60$ cells, same sections). These data suggest that GFP expression was initially weakly or not expressed in CA1 pyramidal cells.

GFP expression (Fig. 2A) was followed by a reduction in NeuN staining (Fig. 2B). At 4 weeks after vector injection, a neuronal loss was detected in the CA3a region, while at 12 weeks NeuN immuno-positivity was reduced in the st. pyramidale from CA3a to the far CA1 region (Fig. 2B). NeuN staining of dentate and hilar neurones was little changed at 12 weeks even though the vector was expressed at low levels in these regions (Fig. 2A).

We examined the time course of neuronal death by counting NeuN immuno-stained somata in the st. pyramidale of the CA3c, CA3a and CA1b regions at 2-12 weeks after vector injection (Fig. 2C). Neuronal densities were normalized to measurements from the same site in animals injected with the scramble vector. The density of CA3a pyramidal cell somata was reduced to 51 ± 26 % at 4 weeks after injection and to 12 ± 6 % of control levels at 12 weeks after vector injection. Neuronal loss from the CA1b region occurred with a delay. A reduction was first detected at 8 weeks when the density of CA1b neuronal somata was

reduced to 46 ± 14 %. At 12 weeks after viral vector injection the density was reduced to 34 ± 12 % of that in animals injected with the scramble vector. In contrast, the density of CA3c pyramidal cells at 12 weeks after injection was 104 ± 32 % of control values.

We asked whether interneuron survival was affected, by comparing the density of NeuN immuno-positive somata in the st. radiatum and st. oriens of the CA1b region of injected and contralateral hippocampus (Fig. 2D). At 8 weeks after vector injection, the density of presumed interneurons in CA1 st. radiatum was reduced to 33 ± 16 % of that in contralateral non-injected hippocampus at 8 weeks (n=4 slices). The density of NeuN immuno-positive somata in CA1 st. oriens was reduced to 16 ± 5 % of that in contralateral st. oriens at 8 weeks (n=4).

These data suggest that suppressing cholesterol 24-hydroxylase function increased cytoplasmic and membrane cholesterol in neurons before they died. CA3a pyramidal cells were lost first and CA1 pyramidal cells after a delay. CA1 interneurons with somata in st. oriens and st. radiatum were lost in parallel with CA1 pyramidal cells.

Astrocyte and microglial cell activation and changes in phosphorylated tau

Changes in the number and form of glial cells were correlated with neuronal degeneration. GFAP immuno-positivity for activated astrocytes was increased throughout the hippocampus at both 4 (Fig. 3A, E) and 8 weeks after vector injection (Fig. 3B). The processes of activated astrocytes increased in length and complexity. Injection of the scrambled version of the vector induced only a transient local activation of astrocytes around the injection site.

Activated microglia, evident as Iba1 immuno-positive elements with rhomboid-shaped cell bodies, proliferated in regions of pyramidal cell death. At 4 weeks after vector injection, they were limited to the CA2-CA3a region (Fig. 3C, F), while at 8 and 12 weeks, activated microglia were expressed most strongly in the CA1 st. pyramidale (Fig. 3D, G). Activated microglia were not detected in CA3c or the dentate gyrus, in contralateral, non-injected hippocampus or in the hippocampus of animals injected with the scrambled vector.

Several molecules, including phosphorylated tau (Clavaguera *et al.*, 2009), are associated with the propagation of death within a neuronal population. We compared phosphorylated tau staining in the CA1 region at 4 and 8 weeks after vector injection. At 8 weeks after vector injection, phosphorylated tau immuno-staining in the CA1 region of the injected hippocampus was increased with respect to levels signals detected in the non-injected hippocampus (Fig. 3 H, I; n=4 slices). The intensity of immuno-staining was not markedly different in ipsilateral and contralateral CA1 region at 4 weeks after vector injection (not shown).

Neuronal death in the CA3a region is correlated with interictal epileptic discharges

Aberrant EEG activities were detected during the progressive death of CA3 and CA1 neurons. Coincident with the loss of CA3a pyramidal cells (Fig. 2B), interictal like epileptic discharges including large, isolated EEG spikes, spike-and-wave motifs or polyspikes were detected. Fig. 4 shows differences in typical EEG traces recorded during exploration (Fig.

4A, B) and during non-REM sleep (Fig. 4C, D) at 2 and 6 weeks after vector injection. Interictal-like EEG events first occurred as isolated spikes and later recurred periodically during exploration (Fig. 4B) or in rhythmic bursts during non-REM sleep (Fig. 4D). The duration of isolated EEG spikes was 34.2 ± 13.4 ms while the duration of more complex events ranged up to 100-120 ms. Repetitive or complex interictal discharges could spread to ipsilateral and contralateral cortex during exploration and non-REM sleep (Fig. 4B, D). The frequency of interictal events during non-REM sleep increased from 1 ± 2 per min at 4 weeks after vector injection to reach a maximum of 68 ± 15 per min at 8 weeks (Fig. 4E; $n=7$ animals). No aberrant EEG activity was detected in animals injected with the scrambled vector.

CA1 neuronal death is correlated with accelerated EEG oscillations during exploration

A different form of aberrant EEG activity emerged at about 6 weeks after vector injection (Fig. 5), a delay corresponding to the loss of CA1 pyramidal cells. Until this point, exploration was associated with EEG oscillations in the theta range at 7-10 Hz. After 6 weeks, theta oscillations were sometimes interrupted by larger amplitude oscillations at alpha and beta frequencies (Fig. 5A-C). The duration of these episodes of frequency acceleration was 2-7 s and peak frequency was in the range 12-16 Hz. Active exploratory behaviour was maintained during episodes of EEG acceleration (Fig. 5D). Frequency accelerations typically spread to the cortical EEG (Fig. 5A), and also occurred during REM sleep ($n=7$ mice). They were detected from 6 to 10 weeks after vector injection (Fig. 5E) with a peak rate of 28.2 ± 9.3 episodes per hr. at 10 weeks after vector injection. Episodes of EEG acceleration during exploration were never observed in mice injected with the scrambled vector.

Low-voltage, high frequency EEG oscillations in the 250-500 Hz range have been associated with an epileptic brain (Bragin *et al.*, 1999; Foffani *et al.*, 2007; Jefferys *et al.*, 2012). Such oscillations (Fig. 6A, B) were evident during episodes of EEG acceleration during both exploration and REM sleep. The frequency of peak power was near 300 Hz and oscillation frequencies were distributed over a narrow band of 250-350 Hz. These high frequency oscillations (HFOs) were typically superimposed on the peaks of beta activity. HFOs were detected only after the emergence of accelerated oscillations during exploration and their occurrence increased with time. Fig. 6C shows the increase in power of high frequency oscillations in mean spectra from analysis of 2 hr EEG recordings of the same animal at 4, 5 and 7 weeks.

Ictal-like discharges

The presence of high frequency oscillations could indicate the emergence of an epileptic brain at about 6-8 weeks after vector injection. After this time point, which corresponds to the spread of neuronal death from CA3 into the CA1 region, we observed temporally organized, ictal-like EEG events, of duration greater than 5 sec, in 3 of 7 animals (Fig. 7). These stereotyped EEG events invariably emerged from episodes of accelerated oscillations during exploration (Fig. 7A-C) rather than quiet wake states or sleep. No ictal-like events were detected in animals injected with the scrambled vector.

During an ictal-like event, EEG oscillations increased gradually from 7-10 Hz up to gamma frequencies of 30-50 Hz (Fig. 7A-C, F). Ictal-like discharges were typically initiated in the injected hippocampus and propagated to the cortex. Animals continued to explore as EEG oscillations accelerated into the gamma range (Fig. 7D). Ictal events were recorded between 6 and 10 weeks after vector injection (in 2 or more recording sessions from 3 animals; Fig. 7E). The peak rate of occurrence of ictal-like events was 4.2 ± 0.4 per hr, at 8 weeks after vector injection.

Few differences were apparent between accelerations in the frequency of EEG activity that were or were not associated with ictal events. The mean duration of beta frequency oscillations followed by a seizure was 3.6 ± 0.2 s ($n=38$) while the duration of oscillations that were not succeeded by an ictal event was 4.4 ± 0.2 s ($n=42$, $p>0.05$). The mean amplitude of beta oscillations preceding an ictal event was 2312 ± 178 μ V while that for oscillations that were not succeeded by an ictal event was 2379 ± 179 μ V ($p>0.05$). The mean frequency of oscillations preceding ictal events was 14.3 ± 0.3 Hz ($n=38$) while it was 13.8 ± 0.2 Hz ($n=42$; $p>0.05$) for oscillations that were not followed by an ictal event. HFOs at 250-350 Hz (Fig. 6) were associated with EEG accelerations that were or were not followed by an ictal event.

One difference between episodes of EEG frequency acceleration that were or were not followed by an ictal-like event, was that beta oscillations were interrupted by larger amplitude EEG events of duration 150-300 ms (arrows in Fig. 7 A, C). These EEG motifs were detected before and during 33 of 38 ictal-like events but only occurred during 13 of 42 beta frequency oscillations that were not followed by ictal-like discharges (Fig. 7A, C). During an ictal-like event they were succeeded by a train of repetitive gamma oscillations. The mean number of similar large, slow EEG motifs occurring during an ictal-like event was 2.2 ± 0.2 and their mean duration was 286 ± 13 ms. The number of large, slow EEG events that interrupted an episode of beta oscillations not followed by an ictal-like event was 0.4 ± 0.1 ($p<0.001$) and their mean duration was 211 ± 17 ms ($p<0.05$).

Discussion

These data show that suppressing expression of the catabolic enzyme CYP46A1 increases cholesterol levels in hippocampal neurons (Fig. 1). An increase in cholesterol was succeeded by neuronal death (Figs. 1-2). CA3 pyramidal cells died first and pyramidal cells and interneurons in the CA1 region with a delay. Distinct, aberrant EEG activities were correlated with the progression of neuronal death (Figs. 4-7). The initial loss of CA3a neurons was associated with interictal-like events. As CA1 pyramidal cells and interneurons also died, the frequency of EEG oscillations associated with exploration sometimes accelerated from the theta range to higher frequencies. In some animals, seizure like events followed these accelerated oscillations during exploration, but not during non-REM sleep.

Vector expression patterns and kinetics

The sh-AAV5 vector was most strongly first expressed in pyramidal cells at the transition between the CA3 and CA1 regions of the hippocampus (Figs. 1A, 2A). This uptake pattern may be specific to the injection site, centred on afferent fibres from the entorhinal cortex

that innervate CA2 and CA3a pyramidal cells (Tamamaki & Nojyo, 1995; Chevaleyre & Siegelbaum, 2010). The vector was not detected in astrocytes or microglia of the same region (Hudry *et al.*, 2010). GFP staining showed that some neurons of the hilar and dentate regions (Fig. 2A) expressed the vector at low levels, but neurons in these regions largely survived.

There was a striking delay between the death of pyramidal cells in the CA3a and CA1 regions (Fig. 2B, C). Strong expression of the vector in the CA1 region occurred with a delay (Fig. 2A), although a low initial expression in CA1 pyramidal cells cannot be excluded. GFP intensity in CA1 pyramidal cells at 2 weeks after vector injection was less than 10% of that in CA3a cells. Alternatively, the delay might result from an orthograde, trans-synaptic transmission (Colle *et al.*, 2010) of the viral vector at Schaffer collateral connections made by CA3 cells in the CA1 region. Finally we demonstrated that an increase in phosphorylated tau levels in the CA1 region coincided with the onset of neurodegeneration (Fig. 3H, I). Phosphorylated forms of tau may be involved in the spread of neuronal death in several neuro-degenerative diseases (Clavaguera *et al.*, 2009; De Calignon *et al.*, 2012) and associated with increased neuronal excitability (Devos *et al.*, 2013; Holth *et al.*, 2013).

Elevation of internal cholesterol and neuronal death

The sterol marker, filipin, showed that neuronal cholesterol increased after expression of the CYP46A1 shRNA construction. CYP46A1 knockdown suppresses extrusion of cholesterol (Sodero *et al.*, 2012) in the short-term, although steady state levels of brain cholesterol of a CYP46A1 knockout animal were reportedly unchanged (Lund *et al.*, 2003). Neither neuronal survival nor electrical brain activity were monitored in CYP46A1^{-/-} animals. Possibly alternative routes of cholesterol extrusion are enhanced in knockout animals, but not in the neuron-specific knockdown used in this work.

Death induced by increased cholesterol has been linked to several cellular organelles. Cholesterol is concentrated in specific domains of neuronal membrane, lipid rafts, which are thought to contribute to the membrane distribution of signalling molecules. Accumulation of cholesterol in neurons may change membrane expression of receptors with excitotoxic effects (Del Toro *et al.*, 2010) or may activate toxic changes at intracellular sites. With filipin staining (Fig. 1B-F), we showed that neuronal cholesterol increased over 1-2 weeks, before neuronal death. The initial increase was detected at both membrane and cytoplasmic sites suggesting that actions at intracellular organelles could also contribute. Neuronal organelles where cholesterol might exert toxic actions include the endoplasmic reticulum (Feng *et al.*, 2003) and mitochondria (Celsi *et al.*, 2009; Fernandez *et al.*, 2009).

Neuronal death and aberrant EEG activities

We found distinct aberrant EEG signals were associated with the loss of CA3a neurons and the later loss of CA1 neurons. Spike-and-wave epileptiform events emerged as CA3a neurons died (Fig. 4). Accelerations of theta oscillations occurred during exploration and REM sleep (Fig. 5) as CA1 neurons were lost. At this point HFOs in the range 250-350 Hz were detected in EEG records (Fig. 6). Similar oscillations (Jefferys *et al.*, 2012) have been

recorded from the temporal lobe of epileptic patients (Staba *et al.*, 2004) and from epileptic animals (Bragin *et al.*, 1999; Foffani *et al.*, 2007), but are not detected in non-epileptic brains. The association of HFOs with the emergence of an epileptic brain was confirmed as EEG accelerations during exploration culminated in structured, seizure-like events in some animals (Fig. 7).

Measurements of changes in neuronal excitability and synaptic function could help understand the mechanisms underlying these phenomena. Nevertheless, the loss of inhibitory control (Fig. 2D) and effects on neuronal excitability consequent on glial activation (Fig. 3) may both favour generation of epileptiform synchrony (Miles & Wong, 1987; Cacheaux *et al.*, 2009; Vezzani *et al.*, 2011).

The accelerations in the frequency of EEG oscillations during exploration occurred transiently from a background of theta frequency activity and were typically associated with an increase in the amplitude of EEG activity. Theta rhythmicity is often suggested to originate from extra-hippocampal regions including the septum (Stewart & Fox, 1990; Manseau *et al.*, 2008). If so, our data suggest this externally generated rhythmicity can be modified for short time periods by intra-hippocampal circuits. These transient exploratory EEG accelerations had few or no behavioural correlates (Fig. 5D) and were also detected during REM sleep suggesting they were not initiated behaviourally. EEG oscillation amplitude increased after the transition from theta to beta frequencies, which tends to exclude mechanisms based on out of phase oscillatory firing of a neuronal population fractionated into subgroups (Ibarz *et al.*, 2010).

Seizure-like EEG discharges always emerged from accelerations of theta oscillations during exploration (Fig. 7). These events lasted for 5-15 sec, with frequencies of EEG oscillations as high as 40-50 Hz and a stereotyped, sequential pattern of oscillations and pauses consistent with an electrographic seizure. No behavioural correlate was evident, as for EEG accelerations during exploration, and as for some isolated hippocampal seizures in the human. Some intracranial EEG recordings suggest that changes in theta band oscillations may be associated with a pre-ictal period preceding temporal lobe seizures (Ebersole & Pacia, 1996; Le Van Quyen *et al.*, 2000) and modelling studies have explored mechanisms that might underly an abrupt transition from theta activity to seizure (Kispersky, White & Rotstein, 2010). Possibly large, slow EEG events occurring during EEG accelerations (Fig. 7C) were associated with the transition to seizure, but otherwise we detected few differences from EEG accelerations that were not succeeded by seizure-like activity. These slow events typically consisted of an interruption of EEG oscillations of duration 250-300 ms followed by multiple large amplitude oscillations at gamma band frequencies. They could correspond to a cellular or synaptic inhibition, followed by a rebound of excitability.

Epileptic synchrony after hippocampal sclerosis or cholesterol mediated neuronal death

There are differences and similarities in the emergence of epileptiform synchrony after hippocampal sclerosis and after intraneuronal cholesterol accumulation. Both are associated with neuronal death, restricted to CA3a and CA1 in this study presumably due to properties of viral uptake. Ictal events possessed similar durations, although their pattern seems different. The association with alert exploratory behaviour may be specific to this

cholesterol accumulation model. No initial convulsive episode was detected, either just after injection of the CYP46A1 shRNA vector construction, or later associated with neuronal death. Glial cells were activated: astrocytes throughout the hippocampus and microglia more focally at sites of neuronal death. We presume this glial activation induced an inflammatory reaction (Vezzani *et al.*, 2011; Crespel *et al.*, 2002). While more precise data would better prove the point, these results suggest that aberrant neuronal population activities emerged with a short delay after neuronal death. This timing differs from the delay observed between neuronal loss and aberrant synchrony in animal models of epilepsies in which an episode of status epilepticus is followed by hippocampal sclerosis (Pitkänen & Lukasiuk, 2009). Presumably a reactive synaptogenesis after neuronal death (Sutula & Dudek, 2007) is less important in the aberrant synchronies induced by cholesterol accumulation. Of these aberrant activities, we noted an interictal-like synchrony (Fig. 4) that emerged in parallel with a moderate loss of CA3a neurons, while accelerations of oscillations linked to exploration and ictal-like events were associated with the spread of neuronal death to the CA1 region (Figs. 5-7). Thus, interictal events occurred before, rather than between seizures.

In conclusion, cholesterol accumulation induces neuronal death and aberrant hippocampal synchronies. Further work might ask whether cholesterol is elevated during insults that induce epileptic syndromes (Taghibiglou *et al.*, 2009; Kim *et al.*, 2010) and whether aberrant neuronal synchronies contribute to symptoms of neurodegenerative diseases that have been linked to cholesterol accumulation (Karasinska & Hayden, 2011; Celsi *et al.*, 2009; Di Paolo & Pim, 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AAV5	adeno-associated virus type 5
ACSF	artificial cerebrospinal fluid
CA	cornu ammonis (hippocampus)
CYP46A1	cytochrome P450 family 46, subfamily A, polypeptide 1 gene
DMSO	dimethyl sulphoxide
EEG	electroencephalogram

GFAP	glial fibrillary acidic protein specific to astrocytes
(e)GFP	(enhanced) green fluorescent protein
IbA-1	ionized calcium binding adaptor protein specific to microglia
IgG	immunoglobulin G
NA	numerical aperture
NeuN	neuronal nuclear antigen specific to neurons
PB	phosphate buffer
REM	rapid eye movement (sleep)
shRNA	short hairpin RNA
st	stratum

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Fig. 1.

Regional uptake of the sh-CYP46A1 vector and effects on neuronal cholesterol. **(A)** Uptake of the AAV5-shRNA vector at 2 weeks after intra-hippocampal injection. The GFP (green) signal was most intense in regions occupied by the somata and apical dendrites of neurons at the border between the CA3 and CA1 hippocampal regions. **(B-D)** Neurons expressing the AAV5-shRNA vector (GFP, green) and related changes in cholesterol detected by filipin (red). The lower images are merged. **(B)** First filipin signals detected as small spots in CA3a neurones at 3 weeks after vector injection. **(C)** At 4 weeks, a membrane and diffuse

cytoplasmic filipin staining was detected at membrane and cytoplasmic sites of GFP positive neurons. **(D)** At 6 weeks, strong filipin staining was evident throughout GFP containing neurons. Signs of neuronal damage included a diminished somatic volume. **(E)** Mean somatic area of GFP-stained CA3a neurons at 3, 4 and 6 weeks.

Fig. 2.

Time course of changes in AAV5-shRNA vector expression and neuronal survival. **(A)** Regional distribution of GFP expression indicating the presence of the AAV5-shRNA vector at 2, 4, 8 and 12 weeks after injection. At 2 weeks the vector was expressed in a restricted zone at the border between the CA3 and CA1 regions. It spread to most of the CA1 region at 8 weeks. **(B)** NeuN immuno-staining at 2, 4, 8 and 12 weeks after injection. An absence of staining corresponds to a regions of neuronal loss. Neuronal death began in the CA3a region and spread to most of the CA1 region at 12 weeks. **(C)** Counts of neuronal density in the CA3c, CA3a and CA1 regions between 2 and 12 weeks after injection of the AAV5-shRNA vector. CA3a pyramidal cell numbers were reduced first, CA1b pyramidal cell death was delayed. No death of CA3c pyramidal cells, or dentate granule cells, was detected. **(D)** NeuN staining in the ipsilateral and contralateral CA1 region at 8 weeks after vector injections reveals not only a reduction of intensity in st. pyramidale, but also fewer stained somata (arrows) in the st. oriens (below st. pyramidale) and st. radiatum (above pyramidale).

Fig. 3.

Glial activation and propagating neuronal death. (A) GFAP immunostaining (red) reveals astrocytes distributed throughout the hippocampus, at 4 weeks after injection of the AAV5-shRNA vector. (B) A similar distribution of GFAP staining at 10 weeks after injection. (C) In contrast, Iba1 immuno-staining (red) was restricted to regions of neuronal death, in CA3a at 4 weeks. (D) Iba1 staining throughout the CA1 region at 8 weeks after vector injection. (E) Activated astrocytes stained with GFAP (red) at 4 weeks after vector injection. (F) Iba1-positive microglial cells (red) and GFP-immunopositive neurons (green) in the CA3a region

at 4 weeks. **(G)** Larger microglial cells (red) with weakly GFP positive neurons (green) in the CA1 region at 12 weeks. Phosphorylated tau staining in the CA1 region reveals an increase at 8 weeks **(H)** compared to levels at 4 weeks after vector injection **(I)**.

Fig. 4.

Interictal-like EEG activity emerges after neuronal death in the CA3 region. **(A)** EEG activity recorded during exploration from the injected hippocampus (H-ipsi) and from ipsilateral (C-Ipsi) and contralateral cortex (C-cont) at 2 weeks after vector injection. **(B)** Spikes emerged during exploration in the hippocampal and cortical EEG at 6 weeks after injection correlated with the initial death of CA3a pyramidal cells. **(C)** EEG activity recorded during non-REM sleep at 2 weeks after vector injection. **(D)** More complex sequences of interictal-like events in cortical and hippocampal EEG signals recorded during

non-REM sleep at 6 weeks after vector injection. **(E)** The frequency of simple or complex EEG spikes measured during non-REM sleep in records made at 2-10 weeks after vector injection.

Fig. 5.

Acceleration of EEG oscillations during exploration. **(A)** An acceleration from theta to beta frequency EEG oscillations during exploration. Records from the injected hippocampus and both ipsi- and contra-lateral cortex at 7 weeks after vector injection. **(B)** Time-frequency analysis of this EEG episode showing the frequency acceleration. **(C)** Hippocampal EEG during the acceleration on an expanded time scale. **(D)** Video records of mouse movements show that active exploration continues throughout this episode of EEG acceleration. Frames 1-8 correspond to time points 1-8 in A. **(E)** Frequency of episodes of beta oscillations during exploration with time after vector injection (n=7).

Fig. 6.

The emergence of high frequency, low amplitude EEG oscillations. **(A)** Low voltage, high frequency activity detected around the peak of beta oscillations in an hippocampal EEG record. Upper trace band-pass filtered at 0.5-500 Hz, lower trace at 200-500 Hz. **(B)** High frequency oscillations showed a peak frequency near 300 Hz and a rather narrow frequency distribution from 250-350 Hz. **(C)** They emerged with time after vector injection. Average power plotted against frequency for hippocampal EEG signals from the same animal recorded during sessions of duration 2 hours at 4, 5 and 7 weeks after vector injection.

Fig. 7.

Ictal-like EEG activity during exploration. **(A)** An episode of accelerated beta oscillations during exploration develops into an ictal-like event. Records from the injected hippocampus and both ipsi- and contra-lateral cortex at 7 weeks after vector injection. **(B)** A time-frequency plot of the event shows the EEG frequency increases into the gamma band after the theta to beta transition. **(C)** Hippocampal EEG towards the end of the seizure on an expanded time scale. The arrows in A and C indicate large, slow EEG motifs. **(D)** Video records of mouse movements before and during the seizure show that active exploration is not interrupted. Frames 1-8 correspond to time points 1-8 in A. **(E)** Frequency of ictal-like events plotted against time after vector injection (n=4 mice). **(F)** Differences in frequency content of EEG frequency accelerations during exploration and seizure-like events on a relative power graph.