Zinc regulates a key transcriptional pathway for epileptogenesis via metal-regulatory transcription factor 1

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Temporal lobe epilepsy (TLE) is the most common focal seizure disorder in adults. In many patients, transient brain insults, including status epilepticus (SE), are followed by a latent period of epileptogenesis, preceding the emergence of clinical seizures. In experimental animals, transcriptional upregulation of Ca\textsubscript{V}3.2 T-type Ca\textsuperscript{2+}-channels, resulting in an increased propensity for burst discharges of hippocampal neurons, is an important trigger for epileptogenesis. Here we provide evidence that the metal-regulatory transcription factor 1 (MTF1) mediates the increase of Ca\textsubscript{V}3.2 mRNA and intrinsic excitability consequent to a rise in intracellular Zn\textsuperscript{2+} that is associated with SE. Adeno-associated viral (rAAV) transfer of MTF1 into murine hippocampi leads to increased Ca\textsubscript{V}3.2 mRNA. Conversely, rAAV-mediated expression of a dominant-negative MTF1 abolishes SE-induced Ca\textsubscript{V}3.2 mRNA upregulation and attenuates epileptogenesis. Finally, data from resected human hippocampi surgically treated for pharmacoresistant TLE support the Zn\textsuperscript{2+}-MTF1-Ca\textsubscript{V}3.2 cascade, thus providing new vistas for preventing and treating TLE.
Epilepsy affects 1% of individuals of all ages, socioeconomic backgrounds and races, and is the second most common cause of mental disability, particularly among young adults, accounting for a worldwide disease burden similar to that of breast cancer in women and lung cancer in men¹⁻³. Chronic recurrent seizures often originate in the temporal lobe (temporal lobe epilepsy (TLE)) and are pharmacoresistant in approximately a third of the patients. Surgical removal of the epileptic focus, albeit highly effective, represents a therapy option for only a fraction of patients⁴. Often, TLE develops as a consequence of a brain disease or of an acute brain insult (acquired or ‘symptomatic’ TLE) via a multifaceted process referred to as epileptogenesis⁵. Intriguingly, a single episode of status epilepticus (SE) can induce the structural and functional alterations that lead to the emergence of chronic recurrent seizures. Identifying the key epileptogenic mechanisms is essential for devising new treatments to prevent or attenuate the development of TLE.

Recent experimental and clinical evidence suggests that acquired ‘transcriptional channelopathies’ play a role in epileptogenesis, as well as in the pathogenesis of other neurological disorders⁶⁻⁹. Thus, in rodents, SE induced chemically with pilocarpine (pilocarpine-SE) causes a marked increase in the propensity for intrinsic bursting in hippocampal CA1 pyramidal cells, particularly in the early phase of epileptogenesis¹⁰. Pharmacological analyses with subtype-selective blockers of voltage-dependent Ca²⁺ channels (VDCCs) disclosed the involvement of a Ni²⁺-sensitive T-type Ca³⁺ current (I_{CaT}) in this aberrant activity¹¹,¹². Congruently, I_{CaT} was threefold upregulated early after SE, whereas other Ca³⁺ currents were unchanged or even reduced¹². Furthermore, a significant upregulation of Ca₃.2 mRNA, but not that of other T-type Ca²⁺-channel α₁ mRNAs, was present in SE-experienced neurons and translated to an increase in Ca₃.2 protein level¹³. Interestingly, the emergence of epileptic seizures was strongly attenuated in Ca₃.2 knockout mice subjected to pilocarpine-SE¹³, indicating that transcriptional Ca₃.2 upregulation may play a pivotal role in epileptogenesis.

It has been shown that SE causes a rise in intracellular free Zn²⁺ concentrations ([Zn²⁺]ᵢ) in pyramidal cells¹⁴ and we have recently demonstrated that Zn²⁺, although acutely and reversibly blocking T-type channels¹⁵, induces a long-term upregulation of I_{CaT} in hippocampal pyramidal cells in vitro¹⁶. Therefore, we have aimed at delineating the signalling cascade linking the increase in [Zn²⁺]ᵢ to Ca₃.2 promoter activation. Our results in vitro indicate that this link is mediated by metal-regulatory transcription factor 1 (MTF1). Supporting data obtained from ‘viral transgenic’ mice and human TLE hippocampi suggest that MTF1 may be a target for treatment strategies aimed at impeding the development of epilepsy following acute brain insults.

Next, we analysed the mRNA expression levels of the neuronal L-type (Caᵥ1.2 and Caᵥ1.3), P/Q-type (Caᵥ2.1), N-type (Caᵥ2.2), R-type (Caᵥ2.3) and T-type (Caᵥ3.1, Caᵥ3.2 and Caᵥ3.3) VDCCs in NG108-15 cells incubated with basal, K⁺ or K⁺ + Zn²⁺ solutions. In basal condition, high expression levels were obtained for Caᵥ3.2 and to a lesser degree for Caᵥ2.2 and Caᵥ3.3 (Fig. 1d). Incubation with K⁺ + Zn²⁺ solution resulted in a significant upregulation of Caᵥ3.2 mRNA levels, but did not affect mRNA levels of other VDCCs that are sensitive to block by submolar concentrations of Ni²⁺ (Fig. 1e, Supplementary Fig. 1). Furthermore, incubating NG108-15 cells with K⁺ solutions containing other divalent cations (0.5 mM Ni²⁺ or 1 mM Cu²⁺) did not affect Caᵥ3.2 mRNA expression (Supplementary Fig. 2). Thus, a rise in [Zn²⁺]ᵢ uniquely and selectively enhances Caᵥ3.2 mRNA expression, an effect that is expected to cause an increase in Caᵥ3.2 protein level. Indeed, immunoblotting detected the Caᵥ3.2 protein under basal conditions and revealed a significant increase of Caᵥ3.2 protein levels after exposure of the cells to K⁺ + Zn²⁺ solution (Fig. 1fg).

**Increase of I_{CaT} by Zn²⁺.** We next used whole-cell patch-clamp recordings of Ca²⁺ currents in NG108-15 cells to investigate whether the Zn²⁺-induced increase in Caᵥ3.2 protein is reflected functionally in a larger I_{CaT}. NG108-15 cells were incubated in either K⁺ or K⁺ + Zn²⁺ solutions and Ca²⁺ currents were recorded. Representative examples of I_{CaT} in a control cell and in a cell exposed to K⁺ + Zn²⁺ solution are shown in Fig. 2a. We found that exposing the cells to K⁺ + Zn²⁺ solution, but not to K⁺ solution led to a significant increase in I_{CaT} (Fig. 2bc). This increase was not accompanied by a change in voltage dependence of activation or inactivation (Fig. 2d, Supplementary Table 1). Expectedly, application of 100 µM Ni²⁺, a dose potently inhibiting Ca₃.2 channels (grey traces in Fig. 2e), almost completely reduced I_{CaT} in both basal and K⁺ + Zn²⁺ conditions (Fig. 2f). Altogether, these findings are congruent with the notion that Zn²⁺-induced increase in I_{CaT} reflects an increase in functional Caᵥ3.2 channels.

**Zn²⁺-induced activation of the Caᵥ3.2 promoter.** We next sought to identify the molecular mechanisms underlying the Zn²⁺-induced upregulation of Caᵥ3.2. To that end, we made use of the previously identified Caᵥ3.2 promoter²⁰, the activity of which strongly correlates with endogenous Caᵥ3.2 mRNA levels. We found that treating the cells with K⁺ + Zn²⁺ solution, but not with K⁺ solution, resulted in a significant Caᵥ3.2 promoter activation (Fig. 3a). This effect was enhanced by raising Zn²⁺ concentration above 200 µM. We further examined whether Zn²⁺-induced activation of the Caᵥ3.2 promoter reverses on Zn²⁺ removal. To that end, we exposed cells having been incubated in a K⁺ + Zn²⁺ solution to N,N,N’,N’-tetraakis (2-pyridylmethyl)ethane-1,2-diamine (TPEN), a cell permeable Zn²⁺ chelator. Intriguingly, TPEN significantly reduced Caᵥ3.2 promoter activity. In contrast, basal activity of the Caᵥ3.2 promoter was unaffected by TPEN (Fig. 3b). These data suggest that enhanced activation of the Caᵥ3.2 promoter requires a sustained increase in [Zn²⁺]ᵢ.

We next delineated the region responsible for Zn²⁺-induced Caᵥ3.2 promoter activation by analysing Caᵥ3.2 promoter deletion luciferase reporter constructs (Caᵥ3.2-1020, -312 and -105) (ref. 20). Increases in [Zn²⁺]ᵢ, resulted in a significant Caᵥ3.2 promoter activity only for the longest deletion fragment (Fig. 3c) suggesting that the Zn²⁺-responsive regulatory element is located in the genomic between 312 and 1,020 upstream of the Caᵥ3.2 ATG. These findings indicate that Caᵥ3.2 gene regulation is under control of a Zn²⁺-responsive
transcription factor. The only currently known transcription factor fulfilling these criteria in mammals is MTF1.

MTF1 increases CaV3.2 promoter activation and I_CaT. Subsequent bioinformatic analyses revealed a surprising accumulation of potential binding sites (metal-responsive elements (MREs)) for MTF1 (Table 1). One of the bioinformatically detected MREs resided within the above identified Zn$^{2+}$-inducible minimal CaV3.2 promoter region (Fig. 4a), supporting a role for MTF1 in the Zn$^{2+}$-dependent CaV3.2 mRNA expression. To test if MTF1 indeed is able to stimulate the CaV3.2 promoter, we analysed CaV3.2 promoter activity after MTF1 overexpression in NG108-15 cells (Fig. 5b), supporting a role for MTF1 in the Zn$^{2+}$-dependent CaV3.2 promoter region (Fig. 4c). We therefore injected either rAAV-CMV-MTF1-IRES-Venus (MTF1) or rAAV-CMV-empty as control (Ctrl) into the somata of CA1 pyramidal cells, as well as in mice hippocampi, revealed binding of MTF1 to the Zn$^{2+}$-sensitive promoter region of the CaV3.2 promoter. To prove unequivocally that the Zn$^{2+}$-induced CaV3.2 promoter activation is mediated by MTF1, a dominant-negative form of MTF1 (MTF1ΔC) was co-transfected with the CaV3.2-1188 reporter plasmid. MTF1ΔC is still able to bind MREs but does not possess the ability to activate transcription, thereby blocking MREs from wild-type MTF1. Treatment with K$^{+}$ + Zn$^{2+}$ solution of cells overexpressing MTF1ΔC resulted in a complete repression of Zn$^{2+}$-induced CaV3.2 promoter activation (Fig. 5d). Therefore, MTF1 appears necessary and sufficient to mediate the stimulatory effects of Zn$^{2+}$ on the CaV3.2 promoter.

MTF1 upregulates hippocampal CaV3.2 mRNA in vivo. It was previously shown that pilocarpine-SE induces an increase in [Zn$^{2+}$]i in the somata of CA1 pyramidal cells, likely due to the release of intracellularly bound Zn$^{2+}$. We investigated in rats the time course of this increase using TFL staining of hippocampal slices resected from control and SE-experienced animals (Fig. 6a; CA1, CA3, hilus and granular layer of the dentate gyrus). The strongest increase in TFL staining was observed in the CA1 region (Fig. 6b). TFL-positive pyramidal cells first appeared at 1 day after SE, their incidence peaking 2–4 days after SE and declining thereafter (Fig. 6b,c). These data suggest an association between the SE-induced increase in [Zn$^{2+}$]i and the SE-induced increases in CaV3.2 mRNA and protein, as well as in I_CaT (ref. 13). To test whether MTF1 mediates between the rise in [Zn$^{2+}$]i and associated increases in CaV3.2 expression and function, we first transduced mice brains with MTF1. To that end, we injected either rAAV-CMV-MTF1-IRES-Venus (MTF1-
Figure 2 | Increase of the T-type current by Zn²⁺. (a) Analysis of I_{CaT} of NG108-15 cells stimulated with K⁺ + Zn²⁺ (50 mM/200 μM) for 4 h, measured 1 day after stimulation. Ca²⁺ currents were recorded in the presence of Na⁺ and K⁺ current blockers (tetraethylammonium 20 mM, 4-AP 4 mM and tetrodotoxin 1 μM). Cells were depolarized from a holding potential of −80 mV to various potentials ranging from −90 to +30 mV, yielding transient, predominantly T-type Ca²⁺ currents. Representative current families elicited with the voltage protocol (upper panel) under basal conditions (left) and following increases in [Zn²⁺]. (b) Quantification of the peak I_{CaT} elicited by a step to −10 mV under the different conditions; basal: n = 37 cells; K⁺: n = 11 cells; K⁺ + Zn²⁺: n = 22 cells; t-test: *P < 0.01). (c) Current-voltage relationship of T-type currents under basal conditions and following incubation with K⁺ + Zn²⁺ (filled circles basal, n = 3; open circles K⁺ + Zn²⁺, n = 3). (d) The voltage dependence of I_{CaT} activation and inactivation was unaltered in cells incubated with K⁺ + Zn²⁺ compared with untreated controls (filled circles basal, activation n = 3, inactivation n = 9; open circles K⁺ + Zn²⁺, activation n = 3, inactivation n = 6). (e) Ca²⁺ currents were elicited with a voltage step from −80 to −10 mV (upper part). Representative current traces show an increased amplitude in cells incubated with K⁺ + Zn²⁺ (lower trace right) compared with cells under basal conditions (lower trace left). Representative current traces showed the potent block by 100 μM Ni²⁺, indicating involvement of CaV₃.2 channels (grey trace). (f) Average of the transient Ca²⁺ current of all cells for control cells (basal, n = 37) and cells incubated with K⁺ + Zn²⁺ (n = 22) display I_{CaT} upregulation following incubation with K⁺ + Zn²⁺ (t-test: **P < 0.01). Average of the transient currents after application of 100 μM Ni²⁺ showed a large amplitude reduction in all recorded cells (−33.1 ± 3.0 pA, n = 37 versus −6.5 ± 1.7 pA, n = 9 for basal conditions and −52.2 ± 7.8 pA, n = 22 versus −10.4 ± 3.0 pA, n = 6 following incubation with K⁺ + Zn²⁺).

Figure 3 | Increases in [Zn²⁺] promote the CaV₃.2 promoter. (a) NG108-15 cells transfected with the 1,188-bp CaV₃.2 promoter–luciferase reporter construct²⁰ and stimulated with K⁺ + Zn²⁺ in the presence of increasing Zn²⁺ concentrations (0, 50, 100, 150, 200, 250 and 300 μM). The effect on the promoter activity was determined using a luciferase assay 4 h after stimulation (one-way analysis of variance (ANOVA); P < 0.001; F(7,16) = 25.64; Tukey’s multiple comparisons test, *P < 0.05, **P < 0.001; n = 3). (b) Activity of the CaV₃.2 promoter–luciferase reporter gene determined after stimulation of NG108-15 cells first with K⁺ or K⁺ + Zn²⁺ (50 mM/500 μM) solutions for 1 h and subsequently incubated in the presence or absence of TPEN (10 μM) for 1 h. Luciferase activity was measured 4 h after stimulation (one-way ANOVA; P < 0.001; F(5,10) = 24.63; Tukey’s multiple comparisons test, *P < 0.05, **P < 0.001; n ≥ 3). (c) Luciferase activity of three CaV₃.2 promoter deletion fragments²⁰ after stimulation with K⁺ + Zn²⁺ (50 mM/200 μM). Only the CaV₃.2-1020 deletion fragment showed significant activation of the CaV₃.2 promoter after stimulation with K⁺ + Zn²⁺. The short CaV₃.2-105 showed a significantly reduced activity after stimulation with K⁺ + Zn²⁺, likely due to the presence of Zn²⁺-inhibitory regions within this fragment (one-way ANOVA: P < 0.001; F(5,12) = 39.82; Tukey’s multiple comparisons test, **P < 0.01, ***P < 0.001; n = 3).
group) or rAAV-CMV-Venus particles (control group) into area CA1 of mice hippocampi. The mRNA isolated from hippocampi of both groups revealed significantly increased MTF1 mRNA expression in the former group, indicating efficient viral transduction. Correspondingly, we observed CaV3.2 mRNA expression to be significantly increased in the MTF1 group versus the control group (Fig. 6d). In addition, in vivo imaging using infrared fluorescent proteins (iRFP) under control of the CaV3.2 promoter (Fig. 6e–g) also showed the activation of the CaV3.2 promoter after transduction with MTF1 and a remarkably similar activation after intrahippocampal injection of Zn2+ (Fig. 6g–j). These data thus show that MTF1 and Zn2+ activate the CaV3.2 promoter also in vivo.

We next similarly injected mice with MTF1ΔC. We injected either rAAV-CMV-MTF1ΔC-ires-Venus (MTF1ΔC group) or rAAV-CMV-Venus particles (control group) into area CA1 of mouse hippocampi (Fig. 7a). Two weeks after injection, mice were either subjected to pilocarpine-SE or sham treated. The mice were killed 3 days thereafter, that is, at the time point of maximal CaV3.2 mRNA increase after SE13. Interestingly, overexpression of MTF1ΔC significantly reduced the SE-induced increase in CaV3.2 mRNA peak during early epileptogenesis (Fig. 7b), further indicating that MTF1 mediates between the SE-induced rise in [Zn2+], and the transcriptional CaV3.2 upregulation.

Given its key role in SE-induced CaV3.2 transcription, we also studied whether pilocarpine-SE affects MTF1 expression levels. We found that the levels of MTF1 mRNA significantly increased 6 and 12 h after SE, and returned to basal values thereafter (Fig. 7c).

Interfering with MTF1 attenuates seizure development. We have previously shown that mice lacking CaV3.2 manifested a much milder form of chronic TLE13. We therefore expected that interfering with the Zn2+–MTF1–CaV3.2 cascade would also exert an antiepileptogenic action. We tested this prediction in mice transduced with MTF1ΔC as described above. With respect to the acute SE induced by pilocarpine, we found no differences in electroencephalography (EEG) recordings between MTF1ΔC and control animals before, during and after the SEs (Fig. 8a). Likewise, the two groups of mice were similar with respect to the latencies to the first acute seizure and to the onset of SE (Fig. 8b). These results show that the two groups of mice likely experience SEs of identical intensities. Further EEG monitoring indicated that spontaneous seizures emerged in both groups of mice (representative examples of EEGs shown in Fig. 8c). However, seizure frequency was substantially lower in MTF1ΔC mice compared with control animals (Fig. 8d,e). Intriguingly, the extent of neurodegeneration found in MTF1ΔC mice was similar to that found in control animals, despite the lesser seizure frequency in the former group (Supplementary Fig. 3).

Increased MTF1 and CaV3.2 expression correlate in HS. To assess the potential role of the Zn2+–MTF1–CaV3.2 cascade in

| MREs located within the CaV3.2 promoter region. |
|-----------------------------------------------|
| Position | Orientation | Sequence |
|---------|-------------|----------|
| 1       | 994 bp      | TGGGCCCC |
| 2       | 275 bp      | TGGGCCGC |
| 3       | 152 bp      | TGGGCCGC |
| MRE-consensus sequence | 1,188 bp | TGCRCNC |

Figure 4 | MTF1 mediates the activation of CaV3.2 promoter by Zn2+ and increases functional expression of CaV3.2. (a) Schematic overview of the rat CaV3.2 promoter with the CaV3.2-1188 promoter-luciferase reporter construct25. The identified Zn2+-inducible region is indicated (black box) together with the three MREs. One MRE is located within the Zn2+-inducible region (black bar), whereas two MREs are located outside the Zn2+-inducible region (white bars). (b) Left panel: luciferase activity of the CaV3.2 promoter construct after transfection with MTF1 in NG108-15 cells. Right panel: luciferase activity of the CaV3.2 promoter in rat hippocampal neurons transduced with rAAV particles harbouring the rat CaV3.2 promoter luciferase reporter (rAAV-CaV3.2) (Ref. 39), a PRL-TK control promoter construct, an expression construct for MTFL (rAAV-CMV-MTF1-ires-Venus) or a control expression construct (rAAV-CMV-Venus) at DIV1 and measured at DIV15 (t-test: ***P≤0.001; n=3). (c) Ca2+ currents in NG108-15 cells were elicited with a voltage step from –80 to –10 mV (upper panel). Representative current traces show an increased amplitude in MTF1-transfected cells (lower trace) compared with controls (upper trace). (d) Average of the transient Ca2+ currents for control cells (n=37) and cells transfected with MTF1 (n=12) display the functional upregulation of T-type Ca2+ currents (t-test: ***P≤0.001). Average of the transient currents after application of 100 μM Ni2+ showed the large amplitude reduction in all recorded cells (n=9 for basal conditions and n=5 following Zn2+ application), indicating involvement of CaV3.2 channels.
Figure 5 | MTF1 binds the CaV3.2 promoter. (a) Luciferase activity of the CaV3.2 promoter deletion fragments after overexpression with MTF1. Note the almost similar activation pattern of the CaV3.2 promoter deletion fragments as seen after stimulation with K⁺ + Zn²⁺ (50 mM/200 μM; Fig. 3c). No activation was observed for the pGL3-basic control plasmid (one-way analysis of variance (ANOVA): P = 0.012; F(7,16) = 3.856; Tukey’s multiple comparisons test, **P≤0.05; n = 3). (b) Luciferase activity of the CaV3.2-1020 fragment and the CaV3.2-312 fragment with the mutated Zn²⁺-sensitive MRE-binding site (CaV3.2-1020-MRE-mut) after overexpression with MTF1. Mutation of the Zn²⁺-sensitive MRE-binding site resulted in a reduced CaV3.2 promoter activity (two-way ANOVA: P = 0.0002; F(3,8) = 38.9. (c) ChIP analysis of MTF1 binding to the Zn²⁺-sensitive MRE within the CaV3.2 promoter. PCR amplicons were generated of anti-MTF1 ChIP immunoprecipitates from NG108-15 cells and mouse hippocampi, using primer pairs spanning the Zn²⁺sensitive MRE and a control region in the CaV3.2 promoter lacking a MRE. A rabbit-IgG immunoprecipitate was used as negative control. (d) Luciferase activity of unstimulated and K⁺ + Zn²⁺-challenged (50 mM/200 μM) NG108-15 cells transfected with the full-length CaV3.2 promoter-luciferase reporter construct and MTF1 or MTF1ΔC (one-way ANOVA: P < 0.001; F(4,10) = 117.9; Tukey’s multiple comparisons test, **P≤0.01, ***P≤0.001; n ≥ 3).

Discussion

Here we describe a novel mechanism of neuronal plasticity, which we refer to as the Zn²⁺⁺-MTF1-CaV3.2 cascade, whereby a rise in [Zn²⁺⁺] activates MTF1, which then binds to MREs in the CaV3.2 gene promoter and increases transcription of this gene. The increase in CaV3.2 mRNA leads to enhanced expression of CaV3.2 channels and larger I_{CaT}. In CA1 pyramidal cells, the I_{CaT} increase causes regular firing cells to convert to burst firing, thereby enhancing the excitability of the hippocampal network. Our cumulative data suggest that this cascade may play a pivotal role in epileptogenesis triggered by pilocarpine-SE. First, pilocarpine-SE leads to a rise in [Zn²⁺⁺], in CA1 pyramidal cells, appearing within a day and persisting for at least 1 week. Second, exposure of cells to elevated [Zn²⁺⁺] induces transcriptional upregulation of CaV3.2 mediated by MTF1. Third, pilocarpine-SE causes a selective increase in I_{CaT} in CA1 pyramidal cells that is underlain by transcriptional upregulation of CaV3.2 (ref. 13). This increase is temporally correlated with the rise in [Zn²⁺⁺], and is reduced by transfection of these neurons with MTF1ΔC (shown to interfere with Zn²⁺⁺-induced CaV3.2 mRNA upregulation), suggesting a causal relationship between the two SE-induced effects. Finally, deletion of CaV3.2 markedly attenuates the emergence of recurrent seizures following pilocarpine-SE, and this antiepileptogenic effect is mimicked by transfecting the hippocampi with MTF1ΔC. Thus, the development of chronic TLE in the pilocarpine-SE model may be attenuated by targeting human TLE, we analysed hippocampal MTF1 and CaV3.2 expression levels in pharmacoresistant TLE patients with hippocampal sclerosis (HS) versus patients with ‘lesion-associated’ TLE (Supplementary Note 1). We found a strong positive correlation between MTF1 and CaV3.2 expression levels in both groups of patients (Fig. 9b, Supplementary Fig. 4). These data indicate that the correlation of expression between MTF1 and CaV3.2 is a rather stable phenomenon, especially when considering patients heterogeneity with respect to endophenotypes (for example, hippocampal damage, time point after seizure onset, etc.) and genetic background. Intriguingly, both MTF1 and CaV3.2 mRNA expression levels were substantially higher in TLE patients with HS compared with those with ‘lesion-associated’ TLE (Fig. 9c). This difference may reflect differences in seizure frequencies or intensities between the two groups or in other factors regulating [Zn²⁺⁺].
Figure 6 | The Zn$^{2+}$–MTF1–CaV3.2 cascade contributes to CaV3.2 upregulation. (a) TFL-Zn$^{2+}$ staining of the hippocampal region in the pilocarpine-SE model in rats under basal conditions (upper panel) and 2 days after SE (lower panel). White boxes represent the grids for differential counting in four distinct areas (CA1, CA3, hilus and the granular layer of the dentate gyrus (DG)). (b) Quantification of TFL-positive neurons in hippocampal CA1, CA3 and DG at various time points after SE (n = 3 rats per time point; 39 slices). (c) Representative examples of TFL-Zn$^{2+}$ staining in the CA1 area under basal conditions and 1, 2, 4 and 7 days after SE. Scale bar, 50 μm. (d) Quantitative RT-PCR on total hippocampi isolated from control, rAAV-Venus and rAAV-MTF1-Venus-injected mice. Expression levels were measured 14 days after injection, with synaptophysin as reference gene (one-way analysis of variance: P < 0.001; F(5,18) = 12.32; Tukey’s multiple comparisons test, *P ≤ 0.05, **P ≤ 0.01; n = 3). (e) Near-infrared in vivo imaging. Upper panel: representative example of in vivo iRFP signal of a recorded mouse with exposed skull. Middle panel: pseudo color visualization of iRFP signals. Lower panel: regions of interest (ROIs) were defined above the hippocampal region and the surface radiance was defined in arbitrary units (a.u.). The colour bar indicates the total fluorescence efficiency. (f) iRFP fluorescence of coronal brain slices isolated from a rAAV-CaV3.2-iRFP-injected animal (left side: iRFP fluorescence intensity; right side: reference images from the Allen Mouse Brain Atlas (© 2015 Allen Institute for Brain Science: http://mouse.brain-map.org), with the hippocampal region annotated with an asterisk (upper panel). Positions (in mm) are given relative to Bregma. (g) Representative example of a recorded rAAV-CaV3.2-iRFP mouse under basal conditions and 3 weeks after injection with rAAV-Syn-Venus (Venus) or rAAV-Syn-MTF1-IRES-Venus (MTF1). (h) Quantification of iRFP signals of rAAV-Syn-Venus (n = 10)- and rAAV-Syn-MTF1-IRES-Venus (n = 10)-injected animals (t-test: ***P ≤ 0.001). (i) Representative example of a recorded rAAV-CaV3.2-iRFP mouse under basal conditions and 3 days after injection with 1 μl 0.9% NaCl or 1 μl 100 μM ZnCl$_2$. (j) Quantification of iRFP signals of NaCl (n = 8)- and ZnCl$_2$ (n = 8)-injected animals (t-test: *P ≤ 0.05).

different components of the Zn$^{2+}$–MTF1–CaV3.2 cascade that lead to CaV3.2 upregulation.

We found that [Zn$^{2+}$], increases, acting via MTF1, upregulate CaV3.2 promoter activity but do not affect transcription of other T-type Ca$^{2+}$ channel subunits. This intriguing selectivity is due to the fact that only CaV3.2, but not CaV3.1 and CaV3.3, contain MREs in the 1.5-kb genomic region upstream of the start ATG. Indeed, gene promoters that harbour MREs are sparsely distributed throughout mammalian genomes$^{25}$. The only ones known to be regulated by MTF1 in a [Zn$^{2+}$]-dependent manner are the genes encoding for metallothioneins. These are small, cysteine-rich proteins with a high affinity for Zn$^{2+}$ and other heavy metals$^{26}$. By increasing the expression of metallothioneins, MTF1 acts in a negative feedback manner to facilitate removal of excess free Zn$^{2+}$ (ref. 27).

In the pilocarpine-SE model, deleting CaV3.2 not only reduced the frequency of recurrent seizures in the chronic stage but also strongly protected the hippocampus from SE-induced neurodegeneration$^{13}$. Here we show that transducing hippocampi with MTF1ΔC also reduced chronic seizures frequency, but did not
one-way ANOVA: MTF1–CaV3.2 cascade by inducing a rise in [Zn2+], thereby allowing tightly bound to metallothioneins may be released by action of nitric oxide whose production is markedly increased during SE. An alternative strategy to impede Zn2+–MTF1–CaV3.2 cascade would be to interfere with nitric oxide accumulation by application of nitric oxide synthase inhibitors or scavengers. Pharmacological targeting of CaV3.2 blockers early after pilocarpine-SE may also prove to be antiepileptogenic, and selective CaV3.2 blockers are becoming available. Alternatively, MTF1 may be targeted pharmacologically. The fact that activation and promoter binding of MTF1 require phosphorylation may enhance the

Figure 7 | The Zn2+–MTF1–CaV3.2 cascade in the pilocarpine-SE model. (a) Representative image of a rAAV-Venus- and rAAV-MTF1ΔC-Venus-injected animal. (b) mRNA expression of CaV3.2 in mice injected with rAAV-Venus (left) and rAAV-MTF1ΔC-Venus (right) 3 days after pilocarpine-induced SE (quantification based on synaptophysin; one-way analysis of variance (ANOVA): P = 0.0335; F_{(2,26)} = 3.374; Tukey’s multiple comparisons test, *P ≤ 0.05; n = 4). (c) MTF1 mRNA expression of hippocampal CA1 6, 12, 24, 36 and 72 h after pilocarpine-induced SE in mice (quantification based on synaptophysin; one-way ANOVA: P < 0.001; F_{(0.39)} = 21.71; Tukey’s multiple comparisons test, ***P ≤ 0.001; n ≥ 4).

Figure 8 | Emergence of spontaneous seizures is markedly attenuated in animals transduced with MTF1ΔC. (a) Representative EEG traces of a rAAV-Venus- and rAAV-MTF1ΔC-Venus-injected animal 5 min after pilocarpine application and 5, 20 and 40 min after SE onset. (b) No differences for the latency to first seizure (left) and latency to SE (right) in animals injected with rAAV-MTF1ΔC-Venus compared with animals injected with rAAV-Venus (n = 5). (c) Representative EEG recordings from SE-experienced rAAV-Venus- and rAAV-MTF1ΔC-Venus-injected animals. (d) Average number of seizures in the chronic epileptic stage in rAAV-Venus- (n = 5) and rAAV-MTF1ΔC-Venus-injected animals (n = 7; t-test: *P ≤ 0.01). (e) Spontaneous seizure activity after SE in rAAV-Venus versus rAAV-MTF1ΔC-Venus animals. The frequency of spontaneous seizures is significantly decreased in rAAV-MTF1ΔC-Venus (n = 5) versus rAAV-Venus animals (n = 7; t-test: *P ≤ 0.05).

prevent neurodegeneration. This discrepancy may be due to the fact that MTF1ΔC overexpression also interferes with MTF1-mediated upregulation of metallothioneins, thereby allowing [Zn2+] to increase to toxic levels. To overcome this side effect, and to unequivocally prove that the reduction of seizures after treatment with MTF1ΔC is due to a direct effect on the CaV3.2 promoter, the Zn2+-sensitive MRE in the CaV3.2 promoter could be genetically modified in vivo using the CRISPR/Cas complex. Subsequent analysis of these mice in the pilocarpine-SE model will then reveal whether they experience less seizure activity as well as a reduced neurodegeneration.

Our findings suggest that pilocarpine-SE evokes the Zn2+-MTF1–CaV3.2 cascade by inducing a rise in [Zn2+]. However, the mechanism coupling SE to [Zn2+] increase is yet unknown. During the intense neuronal activity underlying SE, labile Zn2+ is released from glutamatergic terminals and may enter postsynaptic neurons via multiple routes. Alternatively, Zn2+ tightly bound to metallothioneins may be released by action of nitric oxide, whose production is markedly increased during SE. In either case, another potential strategy to impede the Zn2+-MTF1–CaV3.2 cascade would be the early application of cell permeable Zn2+ chelators. However, given that Zn2+ is mandatory for many critical cell processes, its chelation might lead to deleterious effects. An alternative strategy to impede the Zn2+-CaV3.2 cascade would be to interfere with nitric oxide accumulation by application of nitric oxide synthase inhibitors or scavengers. Pharmacological targeting of CaV3.2 blockers early after pilocarpine-SE may also prove to be antiepileptogenic, and selective CaV3.2 blockers are becoming available. Alternatively, MTF1 may be targeted pharmacologically. The fact that activation and promoter binding of MTF1 require phosphorylation may enhance the
development of inhibitory drugs, for example, by small molecule library screening. As perspective, we suggest that pharmacological interventions targeting the Zn$^2\+\$–MTF1–CaV3.2 cascade may prove as intriguing future option for treating pharmacoresistant TLE.

**Methods**

**Bioinformatic analysis and plasmids.** MREs were identified using the software tool PoSSuMsearch with position-specific scoring matrices from the TRANSFAC database.

The mammalian expression vectors pCDNA3-HA-mMTF1 and pCDNA3-MTF1-EcoRI (dominant negative; MTF1AC) were kindly provided by Carl Seguin (Québec) and Guy J. Rosnan (Fred Hutchinson Cancer Center, Seattle).

The CaV3.2-1020-MRE-mut reporter plasmid was made by mutating the Zn$^2\+\$-sensitive MRE in the CaV3.2-1020 luciferase promoter fragment. For this, the first three nucleotides of the MRE consensus sequence (TGC; Table 1) were mutated into GAG, resulting in a destroyed MRE. Mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Waldbronn, Germany) with the following primers: 5'-CTCGCGGCGGGCCCTCGA GGCAGCGGCGG-3' and 5'-GGCCGCGGCGGGTAAAGGGCGGGCAGTG-3', and with conditions as follows: 2 min at 95°C, then 18 cycles of 50 s at 95°C, 50 s at 55°C and 10 min at 68°C.

For construction of the adenov-associated viral (AAV) plasmids, pAAV-CMV-MCS harbouring the AAV2 inverted terminal repeats (Stratagene, La Jolla, USA) was modified. For pAAV-CMV-MTF1-IREs-Venus, first an IRES-Venus sequence was cloned in the Sall and BglII sites of pAAV-CMV-MCS. The MTF1 sequence was amplified from pCDNA3-HA-mMTF1 using primers with BgII and BglII overhang and cloned in the BsuI and BglII digested pAAV-CMV-IREs-Venus vector, resulting in pAAV-CMV-MTF1-IREs-Venus. For pAAV-CMV-Venus, a sequence encoding the green fluorescent protein Venus was inserted in the HindIII and BglII sites of pAAV-CMV-MCS. The PAAV-hSyn-Venus construct was made from pAAV-CMV-Venus by exchange of the promoters via the MluI and BsuI sites.

The pAAV-hSyn-MTF1-IREs-Venus construct was made from pAAV-CMV-IREs-Venus by exchange of the CMV promoter for the hSyn promoter (MluI and EcoRI) and introducing MTF1AC (EcoRI and BglII/BamHI).

The pAAV-CaV3.2-luciferase construct was described previously. For the AAV-RL-TK control plasmid, the RL-TK cassette (Promega, Mannheim, Germany) was amplified with NotI overhang and cloned in NotI-digested pAAV-MCS. The pAAV-CaV3.2-iRFP713 was made by exchanging the luciferase from pAAV-CaV3.2-venus with iRFP713 (Addgene clone #31857) using HindIII and BglII restriction sites. All AAV-cloning procedures were performed in Sbi2l2 bacteria (Life Technologies, Germany) to minimize recombination events. Plasmid sequences were verified by sequencing analysis. Integrity of the inverted terminal repeats was confirmed by SmaI restriction analysis.

**Cell cultures.** Several types of cultured cells were used in this study. NG108-15 cells (American Type Culture Collection HB-12317) were maintained at 37°C and 5% CO$_2$ in DMEM supplemented with 10% (v/v) heat-inactivated FCS (Invitrogen) 100 units per ml penicillin/streptomycin, 2 mM glutamine and 1 × HAT (sodium hyposaxitidine, aminoantinopterin and thymidine; Invitrogen). If not stated otherwise, NG108-15 cells were seeded in 24-well plates with 60,000 cells per well. HEK293 cells stably transfected with human CaV3.2,3.2 (kindly provided by Ed Perez-Reyes, University of Virginia, Charlottesville, VA, USA) and HEK293-AAV cells (#240073, Stratagene, La Jolla, CA) were kept in high-glucose DMEM supplemented with 10% FCS (Invitrogen), 100 units per ml penicillin/streptomycin and 2 mM glutamine, and incubated at 37°C and 5% CO$_2$. Primary rat hippocampal neurons were prepared and kept in culture as described previously.

Zn$^2\+$ loading of NG108-15 cells. Twenty-four hours after seeding, NG108-15 cells were loaded for 30 min with calcine red-orange AM (2.5 μg per well; Invitrogen Molecular Probes). Next, the cells were incubated for 4h with one of the following solutions: (i) basal solution containing (in mM): NaCl, 140; KCl, 3; CaCl$_2$, 2; MgCl$_2$, 1; d-glucose, 25; HEPES/NaOH, 10 (pH 7.4); (ii) K$^+\$-solution, same as the basal solution but KCl concentration raised to 50 mM; (iii) Zn$^2\+$-solution, same as the basal solution but with added Zn$^{2+}$ (200μM); and (iv) K$^+\$-Zn$^{2+}$-solution, same as the K$^+\$ solution but containing also 200μM Zn$^{2+}\$. Fifteen minutes after returning the cells to the DMEM incubation medium they were exposed to the fluorescent Zn$^{2+}\$ indicator N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSLQ; 0.001% final, added from 0.5% weight per volume stock in dimethylsulfoxide). Ten minutes later, cells were examined and photographed under a fluorescence microscope (Axio Observer.A1, Zeiss). Photographs (×20) were taken under identical conditions. Background-corrected calcine red-orange and TSLQ fluorescence was quantified for single cells (regions of interest were set using a differential interference contrast image) using ImageJ software (NIH) and averaged for every field of view.

**mRNA isolation and real-time RT-PCR quantification.** mRNA from NG108-15 cell preparations and hippocampi was isolated using the Dynabeads mRNA Direct Micro Kit (Invitrogen) according to the manufacturer’s protocol. cDNA was

Figure 9 | MTF1 and CaV3.2 expression levels co-segregate and are increased in hippocampal tissue of patients with HS. (a) Epileptogenesis in a human individual without any previous neurological symptoms. The patient initially manifested clinically with SE (Supplementary Note 2). Coronal T2-weighted fast spin echo (A–C) and axial diffusion-weighted spin echo planar imaging (EPI) show the rapid development of a right-sided HS in the clinical course. Initially, there is hippocampal swelling (A; arrow) associated with cytotoxic oedema of the CA1 sector (D; arrow). Two weeks later, swelling and cytotoxic oedema are somewhat regredient but still present (E,F). Only 8 weeks later, cytotoxic oedema has disappeared (F) and hippocampal atrophy, that is, HS has manifested (C; arrow). MNI (Montreal Neurological Institute) coordinates as derived from the ‘standard brain template’ correspond to 30, −14 and 20. (b) Regression analyses of CaV3.2 mRNA versus MTF1 mRNA expression in patients with HS. A strong positive correlation between the two variables is present even in the heterogeneous group of human HS hippocampi. (c) Quantitative determination of MTF1 and CaV3.2 mRNA. MTF1 and CaV3.2 are significantly abundant expressed in hippocampal tissue of TLE patients with HS versus hippocampi from patients with lesion-associated TLE, that is, in which seizures are explained by lesions such as low-grade neoplasms and/or focal dysplasia in the immediate vicinity or even including the hippocampal formation (HS: n = 79; lesion associated: n = 35; t-test: ***P < 0.001, with synaptophysin as reference gene).
were quantified using the nanodrop (ThermoScientific) and 150 gelatin (Sigma) and then incubated for 1 h with antibodies directed against CaV3.2 membranes. Membranes were blocked for 1 h at room temperature in 2% fish standard voltage step protocols (Fig. 2a). The voltage-dependent activation of the voltage-dependent activation equation (1), and the general constant field currents were elicited with depolarizing voltage steps to 

\[ \Delta V = A_0 + \frac{A_1 - A_0}{1 + e^{(V_{1/2} - V)/a}} \tag{1} \]

where \( \Delta V \) denotes the Ca \(^{2+} \) current and \( g_{Ca} \) (the Ca \(^{2+} \) conductance) amplitude, respectively, at the membrane potential V as set by the command voltage V. \( g_{Ca} \) and \( g_{Ca} \) correspond to internal and external Ca \(^{2+} \) concentration, respectively. The values \( V_{1/2} \), membrane potential at half-maximal inactivation or activation, \( A_0 \) and \( A_1 \) (maximal and minimal conductance, respectively) were determined by the fitting procedure. \( F \) is Faraday’s constant, R is the gas constant and T is the temperature at which the measurements were conducted (22 °C on average). The conductance \( g_{Ca} \) for each potential was derived, normalized to \( A_0 \) and averaged for all cells of the same group. The voltage dependence of inactivation was derived by converting peak current to \( g_{Ca} \) and fitting these values with equation (1).

** Luciferase assay.** Transfection of the NG108-15 cells was carried out using lipofectamine (Invitrogen) following the manufacturer’s protocol. Functional titres (transducing units) of the fluorescent protein vectors were determined by coomassie blue staining of SDS–polyacrylamide gels. The viruses was validated by coomassie blue staining of SDS–polyacrylamide gels. Functional titres (transducing units) of the fluorescent protein vectors were determined by transduction of cultured primary neurons.

**Animal experiments.** Injection of AAV vectors. Mice and rats were housed under a 12 h light/dark cycle with food and water ad libitum. All experiments were performed in accordance with the guidelines of the European Union and the University of Bonn Medical Center Animal Care Committee. Adult male mice (~ 50 days, ~20 g) were obtained from Charles River (C57Bl/6-N) and were anesthetized with 6 mg kg \(^{-1} \) xylazine (Rompun; Bayer) plus 90–120 mg kg \(^{-1} \) ketamine, intraperitoneal (i.p.) (Ketavet; Pfizer). Intracerebral injection of viral particles in the right CA1 hippocampal region was performed stereotactically at the coordinates (in mm) –2, –2 lateral and 1.7 ventral relative to bregma. Holes the size of the injection needle were drilled into the skull, and 1 μl of viral suspension containing ~10\(^8\) transducing units was injected using a 10 μl Hamilton syringe at a rate of 100 nl min \(^{-1} \) using a microprocessor-controlled mini-pump (World Precision Instruments). After injection, the needle was left in place for 5 min before withdrawal. The needle was then slowly withdrawn and the incision closed.

**Near-infrared in vivo imaging.** MTFl overexpression. Mice were injected with rAAV-Ca\(_{3.2}\)-d-IRFP particles as described above. Two weeks after injection, mice were anesthetized, the skull was exposed and holes were drilled at the same location for subsequent rAAV-Syn-MTF1-IRES-eGFP or rAAV-Syn-IRES-eGFP injection. Just before injection, basal IRFP values were measured through the skull. Three weeks after injection of viruses harbouring MTF1- or Venus-expressing constructs, IRFP values were again determined.
Human TLE patients and mRNA expression analyses. For gene expression analyses, we used human hippocampal biopsy tissue from patients with hippocampal sclerosis (n = 79) versus patients with lesion-associated (low-grade neoplastic or dysplastic) (n = 35) chronic TLE who underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center due to pharmacoresistance. In all patients, presurgical evaluation using a combination of non-invasive and invasive procedures revealed that seizures originated in the mesial temporal lobe22. 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. Clinical characteristics per subgroup are described in Supplementary Table 3. mRNA analyses for Ca3.2 and MTF1 were carried out analogous to a procedure described elsewhere in detail20. Briefly, RNA from biopsies representing all hippocampal subfields served to generate 720 ng RNA used for hybridization on Human HT-12 v3 Expression BeadChips with Illumina Direct Hybridization Assay Kit (Illumina, San Diego, CA) according to standard procedures. We extracted data for Ca3.2 and MTF1 analysed by Illumina’s GenomeStudio Gene Expression Module and normalized using Illumina BeadStudio software suite by quantile normalization with background subtraction.

**Statistical analysis.** Statistical analyses were performed with GraphPad Prism 6.05 software (GraphPad Software). Sample size (n) per experiment was calculated using power analysis, with parameters set within the accuracy of the respective experiment’s t-tests and repeated measures analysis of variance followed by Bonferroni’s multiple comparisons or Tukey’s multiple comparisons tests were used to evaluate the statistical significance of the results. Values were considered significantly at P < 0.05. All results are plotted as mean ± s.e.m. All electro-physiological and animal experiments were conducted in a randomized and blinded fashion. All in vitro experiments were independently repeated at least two times.

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Author contributions

K.M.J.v.L., Y.Y. and A.J.B. conceived and planned the study; K.M.J.v.L. performed molecular and cellular experiments; C.S. and T.O. performed electrophysiological and molecular and cellular experiments; K.M.J.v.L., Y.Y. and A.J.B. wrote the manuscript.

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