On the Origin of Paroxysmal Depolarization Shifts: The Contribution of \( \text{Ca}_1.x \) Channels as the Common Denominator of a Polymorphous Neuronal Discharge Pattern

Christiane Meyer, Annika Kettner, Ulla Hochenegg, Lena Rubi, Karlheinz Hilber, Xaver Koenig, Stefan Boehm, Matej Hotka, and Helmut Kubista

Abstract—Since their discovery in the 1960s, the term paroxysmal depolarization shift (PDS) has been applied to a wide variety of reinforced neuronal discharge patterns. Occurrence of PDS as cellular correlates of electrographic spikes during latent phases of insult-induced rodent epilepsy models and their resemblance to giant depolarizing potentials (GDPs) nourished the idea that PDS may be involved in epileptogenesis. Both GDPs and – in analogy – PDS may lead to progressive changes of neuronal properties by generation of pulsatile intracellular \( \text{Ca}^{2+} \) elevations. Herein, a key element is the gating of L-type voltage gated \( \text{Ca}^{2+} \) channels (LTCCs, \( \text{Cav}_{1.x} \) family), which may convey \( \text{Ca}^{2+} \) signals to the nucleus. Accordingly, the present study investigates various insult-associated neuronal challenges for their propensities to trigger PDS in a LTCC-dependent manner. Our data demonstrate that diverse disturbances of neuronal function are variably suited to induce PDS-like events, and the contribution of LTCCs is essential to evoke PDS in rat hippocampal neurons that closely resemble GDPs. These PDS appear to be initiated in the dendritic sub-compartment. Their morphology critically depends on the position of recording electrodes and on their rate of occurrence. These results provide novel insight into induction mechanisms, origin, variability, and co-existence of PDS with other discharge patterns and thereby pave the way for future investigations regarding the role of PDS in epileptogenesis. © 2021 The Author(s). Published by Elsevier Ltd on behalf of IBRO.

Key words: paroxysmal depolarization shift, L-type voltage-gated calcium channels, epileptogenesis, seizure-like activity, hippocampal neurons, perforated patch-clamp.

INTRODUCTION

Paroxysmal depolarization shifts (PDS) were originally discovered in the early 1960ties in penicillin-treated foci of cat cortex as the cellular correlate of interictal electrographic spikes. The most commonly observed type of PDS (type 1) was described as a positive shift of membrane voltage “up to 30 mV or occasionally more” with “durations from 40 up to 400 milliseconds or more”, during which “spike generation would progressively decrease in amplitude until only small oscillations remained riding on top of the PDS” (Goldensohn and Purpura, 1963; Matsumoto and Ajmone Marsan, 1964). A second form of PDS (type 2) was seen less commonly and occurred preferentially during development and fading away of paroxysmal discharges. This latter type of PDS was described as showing “high levels of repetitive spikes with reduced amplitude, but seldom to the point of spike inactivation” (Matsumoto and Ajmone Marsan, 1964). Over the years, the term PDS has been used for a variety of similar electrical events, including epileptic bursts, segments of seizure-like activity and post-ictal...
discharges (Silva-Barrat et al., 2001; Sun et al., 2001; Martella et al., 2005; Dreier et al., 2012). Notably, these latter epileptiform events often lack the characteristic features of original PDS, e.g. in terms of event duration and spike amplitude decline during the depolarized plateau. However, the precise pattern of PDS may not be without relevance, given the intriguing hypothesis of an epileptogenic role of PDS (Staley et al., 2005, 2011). Notably, this hypothesis emerged not only from the observation of an early occurrence of electrographic spikes (the multi-unit representation of PDS) in animal models of acquired epilepsy—they were recorded as early as day one after an insult (e.g. chemically-provoked status epilepticus) (Hellier et al., 1999; White et al., 2010; Chauvière et al., 2012)—, but also from the close resemblance of the voltage trajectories of type 1 PDS and of giant depolarizing potentials (GDPs). The latter are known to govern self-organization of developing brain circuits (Ben-Ari et al., 1989, 2007; Staley et al., 2005). In acquired forms of epilepsy, PDS may have similar effects, albeit in the mature nervous system, and may thus be responsible for eventually pathogenic re-modelling processes.

Although much has been learned about the ionic basis of PDS (reviewed in Hotka and Kubista, 2019, and Kubista et al., 2019), little is known about the mechanisms of their genesis and of their origin. Interictal spikes were observed to occur at high frequencies immediately after epileptic seizures (Gotman, 1991). Hence it is possible that the basis for PDS discharge is laid during late stages of a provoked seizure. Alternatively, cellular processes that occur within hours after the seizure in post-status epilepticus (post-SE) models may also be responsible for the generation or maintenance of PDS. Such changes may comprise one or more of the following: (a) reduction of GABAergic neurotransmission (e.g. through a loss of interneurons) (Dinocourt et al., 2003; Drexel et al., 2011), (b) augmentation of glutamatergic neurotransmission, for example by phosphorylation of GluR1 AMPA receptors (AMPAR) with concomitant enhancement of AMPAR-mediated synaptic currents (Rakhade et al., 2012; Lopes et al., 2013) or up-regulation of NMDA receptors (Di Maio et al., 2013), (c) disruption of the intracellular Ca²⁺-homeostasis (Raza et al., 2004; Delorenzo et al., 2006), (d) formation of reactive oxygen species (Di Maio et al., 2011; Waldbaum and Patel, 2010), and (e) elevation of intracellular chloride (e.g. by NKCC1 up- and KCC2 down-regulation) (Li et al., 2008; Barmashenko et al., 2011). In addition, channelopathies of voltage-gated ion channels were also shown to be acquired in post-SE models of epilepsy (Lerche et al., 2013), some of them in an acute manner (Poolos and Johnston, 2012). Hence, this study was initiated to test for PDS formation using the following approaches. First, we elicited seizure-like activity by application of a nominally Mg²⁺-free external solution (“low-Mg²⁺ solution”) and scrutinized the discharge patterns—after an initial “tonic” phase had ended, and clonic-like afterdepolarizing potentials had appeared—for PDS-like events. Secondly, we derived experimental manoeuvres from findings obtained in post-SE animal models (mechanisms a-f listed above) to test for their propensities to induce PDS. A focus was put on the implication of L-type voltage-gated calcium channel (LTCCs) activity, as many (Walden et al., 1986; Witte et al., 1987; Bingmann et al., 1988; Straub et al., 1990; Gean and Chou, 1991; Moraidis et al., 1991; Schiller, 2002; Rubi et al., 2013; Stiglbauer et al., 2017) but not all (see for example Deisz and Prince, 1987; Kiura et al., 2003) of previous studies provided evidence that this channel family contributes crucially to PDS. Since LTCCs play a privileged role in coupling excitation to long-lasting neuronal changes (Ma et al., 2012), a contribution of this type of Ca²⁺ channel to the generation of PDS in the context of various precipitating pathomechanisms may be essential for their role in epileptogenesis.

**EXPERIMENTAL PROCEDURES**

**Primary cell culture of hippocampal neurons**

Hippocampi were dissected from neonatal Sprague-Dawley rats (pregnant animals were provided by the Department of Biomedical Research, Division for Laboratory Animal Science and Genetics, Himberg, Austria) which had been killed by decapitation in full accordance with all rules of the Austrian animal protection law (see http://www.ris.bka.gv.at/Dokumente/BgbAuth/BGBLA_2012_I_114/BGBLA_2012_I_114.pdf) and the Austrian animal experiment by-laws (see http://www.ris.bka.gv.at/Dokumente/BgbAuth/BGBLA_2012_I_522/BGBLA_2012_I_522.pdf) which implement European (DIRECTIVE 2010/63/EU; see http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF) into Austrian law (all information accessed on August 24th, 2020). The responsible animal welfare body is the “Ethics Committee of the Medical University of Vienna for Research Projects Involving Animals”. Primary co-cultures of hippocampal neurons and glial cells were prepared in the same manner as described in full detail previously (Hotka et al., 2020). In brief, cells were dissociated from dissected hippocampi after enzymatic digestion of the tissue for 20 min at 37 °C/5% CO₂ with papain (25 units/ml in L-15 Leibovitz medium supplemented with 2 mM kynurenate) by mechanical dissociation with Pasteur pipettes in culture medium. The culture medium was composed of Dulbecco’s Modified Eagle’s Medium (containing 25 mM glucose) supplemented with 10% γ-irradiated fetal bovine serum, 12.5 nM progesterone, 112.5 μM putrescine dihydrochloride, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml sodium selenite, 25,000 U/l penicillin and 25 mg/l streptomycin. From the resulting single cell suspension, 50,000 cells were seeded into microchambers (~45 mm²) created by glass rings positioned in the centre of 35 mm diameter poly-o-lysine-coated culture dishes. The glass rings were removed after a 2 h incubation allowed for attachment of the cells. After 24 h, the initial medium was exchanged for the same medium but without antibiotics. To reduce the proliferation of non-neuronal cells, 1 μM cytosine arabinoside was added at day 3–4 after the preparation. Neurons were cultured at 37 °C and 5% CO₂ for at least 12 days (and up to 18 days)
before experiments were performed. During that time, 200 µl autoclaved and sterile filtered purified water was added once a week to compensate for evaporation from the medium. In the course of this study, approximately 60 neonatal rat pups from 20 female rats were used for the preparation of the primary cultures. All experiments were performed ex vivo.

**Electrophysiology**

Recordings of membrane voltage were performed using a Multiclamp 700B amplifier (Axon Instruments) in the current clamp mode and Clampex 10.5 software, which is part of the pCLAMP 10 electrophysiology data acquisition and analysis software package (Molecular Devices, Sunnyvale, CA, USA). Signals were low-pass filtered at 10 kHz and digitized with a Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA, USA) at a sampling rate of 20 kHz. Patch pipettes were made of borosilicate capillaries (GB150-8P, Science Products, Hofheim, Germany) with a Sutter P97 horizontal puller (Sutter Instrument Company, Novato, CA, USA). Tip resistances lay between 3.5 and 5 MΩ. Standard pipette solutions (“7 mM chloride”) contained (in mM) 120 potassium gluconate, 1.5 sodium gluconate, 3.5 NaCl, 1.5 CaCl₂, 0.25 MgCl₂, 10 HEPES, 10 glucose and 5 EGTA. pH was adjusted to 7.3 by KOH. All recordings were made in perforated patch mode using 500 µg/ml amphotericin B (which forms chloride-permeable pores), which was added to the pipette solution just before seal formation. Experiments were started only after the series resistance had dropped to the lowest achievable level (e.g. to between 20 and 30 MΩ), which usually required ≥15 minutes. To assure that only viable cells were used, the following inclusion criteria had to be met: a membrane voltage of at least −50 mV and the capability of generating overshooting action potentials, which was always tested prior to the recordings. Experiments were performed at room temperature, and cells were superfused continuously using a DAD-12 drug application system (Adams & List, Westbury, NY, USA) with a micromanifold that held 12 channels converging into a 100 µm diameter quartz outlet (ALA Scientific Instruments, NY, USA). The tip of the outlet was positioned in close proximity (about 250 µm) to the patch-clamped cell. Standard external solution contained (in mM) 140 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 20 glucose (pH was adjusted to 7.4 by NaOH). No MgCl₂ was added to the “low-Mg²⁺” solution (i.e. nominally Mg²⁺-free), which was otherwise identical to the standard external solution. LTCC activity was modulated by application of the dihydropyridines isradipine (“isra”, LTCC antagonist) and Bay K8644 (“BayK”, LTCC agonist), both at 3 µM (dissolved from a 1 mM stock solution in dimethyl sulfoxide, DMSO). Control solution thus also contained 0.3% DMSO. In the investigation of PDS, the trigger agents (10 µM bicuculline, 1 mM caffeine, 3 µM cyclothiazide, 30 µM 4-aminopyridine) were applied first together with DMSO for 5 minutes, then together with BayK for 5 minutes and finally together with isradipine for another 5 minutes. In the comparison of internal solutions of different chloride concentrations, the DMSO application was extended to 10 minutes to allow for additional equilibration time between the pipette solution and the cytosol.

**Drugs**

4-Aminopyridine, amphotericin B (from Streptomyces sp.), BayK, bicuculline methiodide, caffeine, cyclothiazide, DMSO, isradipine and bulk chemicals were purchased from Sigma-Aldrich (Vienna, Austria).

**Data analysis**

For identification of spontaneously occurring suprathreshold excitatory events, a threshold search operation ("event detection") of Clampfit 10.5 (pCLAMP 10 electrophysiology data acquisition and analysis software package, Molecular Devices, Sunnyvale, CA, USA) was applied during one minute (analysis of seizure-like activity, see Fig. 1) or two minutes (PDS analysis) of each of the above mentioned drug application sequences (i.e. the second-last and last minute of DMSO application, after at least one minute of BayK application, and the fourth and fifth minute of isradipine application, respectively). To detect and analyse the events for area-below-the-trajectory, subsequently termed "event areas" (given in mV.ms), and for duration, the threshold was set to 35 mV and the re-arm to 5 mV (PDS analysis) or 1 mV (analysis of seizure-like activity) above baseline. Prior to event detection, traces were corrected for event-of-interest-independent changes of the membrane voltage by Clampfit 10.5 manual baseline adjustment. In the experiments with caffeine, neurons that responded with an induction or aggravation of pronounced bursting discharge activity had to be excluded from further investigation.

**Statistics**

Data were sampled in the manner indicated in the preceding section on Data analysis. The event areas from the neurons investigated (n numbers are given in the results section) were pooled for each of the three conditions of LTCC modulation (DMSO, BayK, isra). GraphPad Prism version 9.0.2 was used for preparation of graphs, and for curve fitting and for statistical testing. Statistical testing was performed using the nonparametric Kruskal–Wallis H test with Dunn’s multiple comparisons or in a few cases (as indicated) using Mann-Whitney U test. Results of the statistical analysis are reported in the respective figure captions. The results of post-hoc multiple comparisons tests are indicated in the figures using asterisks for data sets linked by horizontal brackets as follows: ***p ≤ 0.001;
**p ≤ 0.01; *p ≤ 0.05; n.s. ("not significant") indicates a p value > 0.05.

Data in Fig. 9 were fitted using non-linear least squares minimization with a two-exponential function of the form: $Y(t) = A * \exp(-t/\tau1) - (1 - A) * \exp(-t/\tau2) + 1$ with values constrained to $Y(0) = 0$. Note that $Y$ values at $t \to \infty$ are implicitly constrained to 1, i.e. $Y(t \to \infty) = 1$. $Y$, $t$, $\tau1$, $\tau2$, are the normalized event area, the interevent time, and the two time constants, respectively. $A$ is the relative amplitude of the first exponential component, while the amplitude of the second exponential component is calculated as 1-$A$.

**RESULTS**

PDS-like events associated with late stages of a seizure

Clonic-like afterdepolarizing potentials (CLADPs) are a common feature of late stages of seizure-like discharge activity (SLA) (Kovács et al., 2005). Some authors designated such events as PDS (see for example Delorenzo et al., 2007). To explore similarities between CLADPs and original PDS, SLA was evoked in hippocampal neurons by application of a nominally Mg$^{2+}$-free external solution ("low-Mg$^{2+}$" solution, see Fig. 1). Typically, SLA induced in this manner consists of a tonic-like period which is followed − within less than one minute − by CLADPs. Neurons continue to generate CLADPs as long as Mg$^{2+}$ is kept low, for example for ~20 min as in the recording depicted in Fig. 1A. CLADPs (inserts c and d in Fig. 1B) disappear and discharge activity returns to quasi normal patterns rapidly after re-addition of millimolar Mg$^{2+}$ (insert e in Fig. 1B). To investigate the contribution of LTCC-mediated Ca$^{2+}$-influx to CLADPs, we induced SLA by 3 minutes long application of low-Mg$^{2+}$ solution in the presence of either DMSO (control), 3 μM BayK or 3 μM isradipine (note that the role of LTCCs in the initial seizure stage was investigated in an earlier study by Rubi et al., 2013). Between low-Mg$^{2+}$ applications, neurons were allowed to recover for five minutes. In each experiment, two control applications of low-Mg$^{2+}$ solution plus DMSO were made, and only SLA evoked by the second application was used for analysis. This was made to ensure that only SLAs occurring after the same time of recovery were compared. CLADPs were evaluated within the third minute of low-Mg$^{2+}$ application. Statistical analysis of 15 experiments revealed an apparent ~16% decrease of the median area of events recorded when LTCC activity was potentiated (BayK), but this change was not statistically significant (Kruskal Wallis H test with Dunn’s multiple comparisons test, $p = 0.2468$). In the presence of isradipine, median event area was not statistically different from that observed in the presence of BayK ($p = 0.3000$) and remained by 16% smaller than the median area of events under control conditions (DMSO). The latter difference was statistically significant (Kruskal Wallis H test with Dunn’s multiple comparisons test, $p = 0.0023$). Hence, the statistically non-significant reduction of event areas by BayK was not reversed after LTCC blockade with isradipine (see Table 1, “all neurons”) (Kruskal Wallis H test: $H_2 = 11.30$, $p = 0.0035$). However, closer examination revealed that up- (with BayK) and down-regulation (with isradipine) of LTCCs had diverging effects on the area of CLADPs. In 9 out of 15 neurons, CLADP areas were reduced in the presence of BayK and restored in the presence of isradipine, whereas in the remaining 6 neurons CLADP areas remained largely unaffected by BayK but were decreased by isradipine. The scatter plots in Fig. 2A, C demonstrate these effects by a comparison between event areas determined under control conditions (only the solvent DMSO was added) and in the presence of BayK or isradipine. Data from 9 neurons, in which BayK caused a decrease of CLADP area (group I in Table 1), showed a significant statistical difference between solvent
and BayK treatment as well as between BayK and isradipine treatment, but not between solvent treatment and treatment with isradipine (Fig. 2A). In contrast, data from 6 neurons (group II in Table 1), in which isradipine caused a decrease of CLADP area, showed a significant statistical difference between BayK and isradipine treatment as well as between solvent and isradipine treatment, but not between solvent treatment and treatment with BayK (Fig. 2C). As can be seen in the exemplary traces depicted in Fig. 2B, D, E, there are pronounced

Table 1. Descriptive statistics of the area-below-the-curve of events during seizure-like activity (SLA). The values in italics represent mV.ms; * change refers to the factor of increase or of reduction of the median relative to the respective control value (DMSO). BayK, 3 μM Bay K8644; DMSO, 0.3% dimethyl sulfoxide; isra, 3 μM isradipine; lowMg, nominally Mg2+-free external solution.

| SLA event areas | all neurons | group I | group II |
|-----------------|-------------|---------|----------|
| Number of values | 394 (n = 15 neurons) | 412 (n = 9 neurons) | 409 (n = 6 neurons) |
| Minimum | 305.9 | 323.1 | 167.6 |
| 25% Percentile | 6629 | 5114 | 3910 |
| Median | 12,838 | 10,720 | 10,782 |
| 75% Percentile | 27,379 | 27,612 | 23,107 |
| Maximum | 230,523 | 271,699 | 230,523 |
| Range | 230,217 | 271,531 | 230,058 |
| 5% Percentile | 1240 | 1101 | 269.4 |
| 95% Percentile | 108,758 | 83,980 | 110,822 |
| change* | 0.84 | 0.84 | 0.52 |

Fig. 2. LTCC-mediated Ca2+-influx shapes clonic-like afterdischarges. (A) Scatter plot summarizing the analysis of event areas occurring in region i (see Fig. 1), with endogenous LTCC activity (DMSO control) and when LTCC activity was potentiated (BayK) or inhibited (isradipine, isra), for nine neurons in which BayK exerted an inhibitory effect. The median is indicated by grey horizontal lines. Statistical analysis was performed using Kruskal Wallis H test ($H_2 = 26.04, p < 0.0001$). The asterisks indicate the results of pairwise comparisons which were performed post-hoc using Dunn’s multiple comparison’s test (see Experimental Procedures). (B) Exemplary traces illustrating the inhibitory effect of LTCC-mediated Ca2+-influx on long-lasting CLADPs. (C) Scatter plot summarizing the analysis of event areas occurring in region i (see Fig. 1), with endogenous LTCC activity (DMSO control) and when LTCC activity was potentiated (BayK) or inhibited (isradipine), for 6 neurons in which isradipine exerted an inhibitory effect. The median is indicated by grey horizontal lines. Statistical analysis was performed using Kruskal Wallis H test ($H_2 = 38.88, p < 0.0001$). The asterisks indicate the results of pairwise comparisons which were performed post-hoc using Dunn’s multiple comparison’s test. (D, E) Exemplary traces illustrating the facilitative effect of LTCC-mediated Ca2+-influx on brief CLADPs. Inserts in E depict the events on a magnified time scale. Scale bars in the inserts represent 20 mV and 200 ms.
differences in the size of CLADPs between neurons. The reduction of CLADP area by BayK was typical for neurons with CLADP duration of several seconds - on average the duration in 9 neurons amounted to 2.85 s [1.26–4.40] (median [interquartile range]) (an example is shown in Fig. 2B), whereas reduction of CLADP area by isradipine was typical for neurons with events of about 1 s under control conditions (DMSO, on average the duration in 6 neurons amounted to 1.03 s [0.78–1.95] (examples are shown in Fig. 2D, E). Hence, LTCC-mediated Ca^{2+}-influx can exert varying effects on clonic-like afterdischarges. The inserts in Fig. 2E, which depict CLADPs on an expanded time scale, illustrate a close resemblance of brief CLADPs and PDS (type 1), as long as LTCC activity was available (DMSO and BayK traces), but not in the presence of isradipine.

**Induction of PDS-like events by mechanisms potentially arising from a preceding seizure**

Next, we selected a series of treatments to address the mechanisms (a)–(f) listed in the introduction for their propensities to induce PDS (see Fig. S1 in the supplementary material): (i) Patch pipettes filled with 25 mM instead of standard 7 mM chloride (Fig. S1A) to mimic elevation of intracellular chloride (Fig. S1B). (ii) Application of bicuculline to mimic reduction of GABAergic neurotransmission (Fig S1C). (iii) Exposure to caffeine to mimic dysregulation of the intracellular Ca^{2+}-homeostasis (Fig. S1D). (iv) Administration of cyclothiazide to mimic augmentation of glutamatergic neurotransmission (Fig. S1E). (v) Use of 4-aminoypyridine to mimic reduction of A-type potassium currents (Fig. S1F). Supplementary Fig. S1 shows exemplary traces of membrane voltage of hippocampal neurons in the absence and presence of these agents. With standard (7 mM) and high (25 mM) Cl\(^{-}\) concentrations in the recording pipettes, traces were recorded immediately and 10 min after membrane rupture to allow for Cl\(^{-}\) equilibration of the cytosol. Typical changes in discharge patterns are described in the respective sections below.

**High intracellular chloride**

Intracellular chloride has been shown to increase during seizure activity, and this change may contribute to seizure duration (Ellender et al., 2014; Raimondo et al., 2015 and Fig. S2B). Long-term increments in intracellular chloride concentration after status epilepticus (e.g. in animal models of acquired epilepsy) are brought about by persistent changes in the expression of cation chloride cotransporters, and depolarizing actions of GABA were suggested to play a role in early epileptogenesis (Li et al., 2008; Barmashenko et al., 2011).

Recordings made with electrodes containing 25 mM chloride often showed compound action potentials (Fig. S1B and Fig. 3B), which were not seen when standard internal solution was used (Fig. S1A and Fig. 3A). Potentiation of LTCCs by BayK augmented these events, and isradipine exerted an inhibitory effect (Fig. 3B, Fig. S3B and Table S1 in the supplementary material, \(n = 13\)). When electrodes filled with 7 mM chloride were used instead, BayK also led to an enhancement of suprathreshold events, but the increase caused by the LTCC agonist was less pronounced (Fig. 3A, Fig. S3A and Table S1, \(n = 11\)). In comparison to control (DMSO), BayK led to a 1.82-fold change of median event area with 25 mM Cl\(^{-}\), but to an only 1.04-fold change of median event area with 7 mM Cl\(^{-}\). In both cases, isradipine caused a return of event areas towards (25 mM chloride) or even below (7 mM chloride) control values. For a better idea on what these numbers mean with respect to the morphologies of these events, note that the areas of the exemplary traces for BayK-treated neurons in Fig. 3 amount to 3050 mV.ms (25 mM chloride) and 1170 mV.ms (7 mM chloride), respectively. Direct statistical comparison of BayK-induced events confirmed that with elevated internal chloride significantly larger events were evoked than with standard internal chloride (Mann-Whitney U test: \(U_{673, 899} = 258852\), \(p < 0.0001\), with \(n_1 = 25\) mM chloride and \(n_2 = 7\) mM chloride). Hence, raised LTCC activity caused a significant potentiation of depolarizing events only when neurons had experienced a rise in intracellular chloride.

In perforated patch recordings with electrodes containing 25 mM Cl\(^{-}\), inhibitory postsynaptic potentials (IPSPs) were absent at resting potentials, but were evident when neurons were depolarized beyond −40 mV by applying positive holding currents (see Fig. S4). In contrast, in recordings with standard intracellular Cl\(^{-}\) (7 mM), IPSPs did occur at resting potentials (see Fig. S2A). This confirms the positive shift in the Cl\(^{-}\) equilibrium potential with electrodes containing 25 mM Cl\(^{-}\).

**Bicuculline**

To mimic a reduction in GABAergic neurotransmission, 10 \(\mu\)M bicuculline (BIC) was added. PDS-like events were readily evoked after application of this GABA\(_A\) receptor antagonist under control conditions (DMSO) (Fig. S1C and Fig. 3C). Co-administration of BayK enhanced these events, whereas co-administration of isradipine led to a reduction (Fig. 3C, Fig. S3C and Table S1, \(n = 10\)). Compared to control, BayK enhanced median event areas 1.14-fold, and isradipine reduced them 0.79-fold.

**Caffeine**

Disruption of the intracellular Ca\(^{2+}\)-homeostasis by 1 mM caffeine (Verkhratsky et al., 1994) did not lead to obvious changes of the neuronal discharge pattern (Fig. S1D). In particular, PDS-like events were not evoked in the presence of solvent (DMSO), but appeared upon co-administration of BayK (Fig. 4A, Fig. S3D and Table S1, \(n = 7\)). In 5 out of 7 neurons, the event areas remained irreversibly elevated upon exchange of BayK with isradipine. An example of such partial reversibility is shown in Fig. 4A. Compared to control, BayK enhanced the median event areas 3.15-fold. Isradipine caused a reduction of event areas, but the median remained 1.80-fold larger.
than under control conditions (Table S1). Although on average PDS-like events in the presence of caffeine were smaller than those observed in bicuculline, some caffeine-induced PDS were at least as pronounced as those formed during exposure to bicuculline (illustrated in Fig. S5; note full reversibility of the BayK-induced PDS area augmentation by isradipine in this particular neuron).

Cyclothiazide

Augmentation of glutamatergic neurotransmission by 3 μM cyclothiazide (Ctz) evoked depolarizing waves that lasted on average 1.7 s (range 1.4 to 2.2 s) with large excitatory postsynaptic potentials (EPSPs) riding on top (Fig. S1E). These events displayed a tendency to be increased by BayK and were decreased in the presence of isradipine (Fig. 4B, Fig. S3E and Table S1, n = 6). Compared to control, BayK caused a 1.10-fold (although not statistically significant; p = 0.1068) increase of median event area, and isradipine led to a reduction to 0.89 of control.

4-Aminopyridine

Chemically-provoked status epilepticus induces an acute down-regulation of A-type potassium channels, e.g. K_V4.2 channels, which are known to regulate dendritic excitability and post-synaptic signal integration (Francis et al., 1997; Lugo et al., 2008). We used a low concentration of the potassium channel blocker 4-aminopyridine (4-AP, 30 μM) to mimic this situation (Fig. S1F). 4-AP evoked PDS-like events, which were augmented by co-application of BayK. When BayK was replaced by isradipine, 4-AP-induced events resembled those in the absence of LTCC modulators (Fig. 4C, Fig. S3F and Table S1, n = 6). Compared to control, BayK enhanced the median event area 1.24-fold, and in isradipine this value was 1.09-fold of control.

To summarize and to highlight the PDS induction propensities of
the aforementioned approaches, we provide a side-by-side comparison of all the data falling into the 75 to 99.5% percentile of each data set (~top 25% in event area) in Fig. 5. Full descriptive statistics of this analysis are given in Table 2.

PDS originate in the dendritic tree and co-exist with somatically triggered action potentials

It should be noted that all of the events described above occurred spontaneously without any direct stimulation, such as current injection via the recording electrode. Hence, we were interested whether PDS occurrence could be promoted by electrical depolarization. When neurons were depolarized beyond the firing threshold (e.g. –45 mV) by brief current injections applied immediately after a spontaneous PDS, action potentials were elicited, but not additional PDS (an example is illustrated in Fig. 6A). During long lasting current injections, recordings typically showed intermingled PDS and action potentials (Fig. 6B). Electrical depolarization did not promote the formation of PDS, but rather increased the likelihood of co-occurrence of action potentials (this effect was not further quantified in this study). Co-occurrence of normal (action potentials) and abnormal (PDS) discharges was also observed in the absence of current injection. An example is illustrated in Fig. 6C.

The side-by-side appearance of action potentials and PDS leads to the question of underlying mechanisms. PDS were suggested to arise in the dendritic compartment (Schiller, 2002), whereas evoked action potentials are thought to be initiated at the axon hillock. Hence, the co-occurrence of action potentials and PDS may be related to their diverging places of origin. To address this possibility, we varied the positions of recording electrodes and applied current injections to neurons exposed to BIC + BayK, but only during PDS free periods (if a PDS or synaptic activity occurred during the 2 Hz stimulation and distorted the evoked responses, this particular stimulus train was not used for further evaluation). Positions of recording electrodes were (i) the centre of a soma, (ii) a dendritic root or (iii) a proximal dendrite (approximately 50 µm away from the dendritic root, see Fig. S7E). As depicted in an exemplary recording in Fig. 7A, current injections at the

---

**Fig. 5.** Analysis of event areas of PDS induced by various epileptogenic stimuli. (A–F) Scatter plots of data obtained under the conditions indicated on top. Only data points falling into the 75–99.5% percentile of each data set are depicted. The median is indicated by grey horizontal lines. Statistical analysis was performed using Kruskal Wallis $H$ test which yielded the following omnibus test results: (A) $H_2 = 35.0, p < 0.0001$, (B) $H_2 = 260.0, p < 0.0001$, (C) $H_2 = 311.4, p < 0.0001$, (D) $H_2 = 530.3, p < 0.0001$, (E) $H_2 = 160.1, p < 0.0001$, (F) $H_2 = 132.2, p < 0.0001$. The asterisks indicate the results of pairwise comparisons which were performed post-hoc using Dunn’s multiple comparison’s test.
soma elicited one to three action potential(s) per depolarizing pulse, but no PDS-like plateau potentials, despite the presence of BIC + BayK (which did evoke PDS in the same neurons, as monitored between current injections, see below). In contrast, current injections at proximal dendrites led to responses that were characterized by single spikes followed by depolarized plateaus that sometimes outlasted current injections (Fig. 7 B). With electrodes positioned at dendritic roots (Fig. 7 C), obviously, the most pronounced depolarization shifts occurred at proximal dendrites, with one spike only and PDS areas being significantly larger than those at dendritic roots or at somata (Fig. 8 D). Frequency dependent PDS variability

Experiments employing various putative epileptogenic mechanisms revealed considerable differences in their propensities to induce PDS. Bicuculline and caffeine evoked the most pronounced effects, with potentiated LTCC activity as an important precipitating factor. But even with these compounds there was considerable variability in the PDS evoked, as indicated above for caffeine (Fig. 5B). Furthermore, differences in PDS morphology appeared to depend crucially on the intervals between the events. This was most obvious in the experiments with bicuculline, which typically evoked groups of PDS that were separated by silent periods of several seconds. Within these groups, PDS occurred in fast succession, and this was accompanied by a loss of the distinct PDS pattern, notably the disappearance of the plateau phase, which resulted in a marked decline of event areas. This is illustrated in Fig. 9A (see the

### Table 2

| Event Area | DMSO/7 Cl⁻ | BayK/7 Cl⁻ | isra/7 Cl⁻ | DMSO/25 Cl⁻ | BayK/25 Cl⁻ | isra/25 Cl⁻ | DMSO/BIC | BayK/BIC | isra/BIC |
|------------|------------|------------|------------|------------|------------|------------|----------|----------|----------|
| Minimum    | 1074       | 1094       | 901.8      | 628.7      | 1373       | 791.1      | 2566     | 3227     | 2058     |
| 25% Percentile | 1169       | 1203       | 1025      | 731.4      | 1605       | 892.3      | 2816     | 3630     | 2200     |
| Median     | 1322       | 1342       | 1159      | 890.9      | 1889       | 1037       | 3306     | 4268     | 2480     |
| 75% Percentile | 1500       | 1552       | 1498      | 1114      | 2389       | 1396       | 4001     | 7076     | 2901     |
| Maximum    | 2306       | 3080       | 2842      | 1929      | 5234       | 1947       | 7985     | 9738     | 4924     |
| Range      | 1232       | 1986       | 1940      | 1300      | 3881       | 1156       | 5419     | 6511     | 2866     |
| 5% Percentile | 1092       | 1108       | 922       | 639.5      | 1404       | 814.2      | 2617     | 3281     | 2081     |
| 95% Percentile | 1963       | 2322       | 2250      | 1495      | 4454       | 1722       | 5659     | 9271     | 4502     |

Values shown for the three electrode positions in Fig. 5C together with the statistical analysis of the elicited spikes (panels D and E). At the soma, PDS showed a series of spikes of decreasing amplitudes, which rode on a depolarizing wave (Fig. 8 A). In contrast, PDS recorded from proximal dendrites had one initial spike that was followed by a depolarized plateau (Fig. 8 B). Recordings from dendritic roots delivered PDS that resembled those of either proximal dendrites or somata. A dendritic-like PDS is depicted in Fig. 5C, but sample traces documenting the heterogeneity of PDS at dendritic roots are shown in Fig. S7 C. Obviously, the most pronounced depolarization shifts occurred at proximal dendrites, with one spike only and PDS areas being significantly larger than those at dendritic roots or at somata (Fig. 8 D).

### Full descriptive statistics of the event areas of the 75–99.5% percentile

| Event Area | DMSO/caff | BayK/caff | isra/caff | DMSO/ctz | BayK/ctz | isra/ctz | DMSO/4-AP | BayK/4-AP | isra/4-AP |
|------------|-----------|-----------|-----------|----------|----------|----------|-----------|-----------|-----------|
| Minimum    | 794.5     | 2425      | 1562      | 1907     | 2081     | 1555     | 1377      | 1800      | 1373      |
| 25% Percentile | 910.3     | 2937      | 1784      | 2070     | 2206     | 1684     | 1522      | 2035      | 1494      |
| Median     | 1063      | 3748      | 2257      | 2230     | 2357     | 1867     | 1772      | 2315      | 1768      |
| 75% Percentile | 1201      | 4739      | 3552      | 2451     | 2623     | 2101     | 2141      | 2756      | 2005      |
| Maximum    | 1679      | 9884      | 7021      | 3263     | 3825     | 3069     | 3068      | 4249      | 3030      |
| Range      | 894.6     | 7458      | 5459      | 1357     | 1744     | 1514     | 1691      | 2449      | 1657      |
| 5% Percentile | 812.2     | 2494      | 1599      | 1929     | 2104     | 1573     | 1389      | 1853      | 1386      |
| 95% Percentile | 1505      | 8099      | 6235      | 3077     | 3244     | 2641     | 2725      | 3632      | 2825      |

Values in italics represent mV.ms; * change refers to the factor of increase or of reduction of the median relative to the respective control value (DMSO). 4-AP, 30 μM 4-amaminopyridine; BayK, 3 μM Bay K8644; BIC, 10 μM bicuculline; caff, 1 mM caffeine; ctz, 3 μM cyclothiazide; DMSO, 0.3% dimethyl sulfoxide; isra, 3 μM isradipine.
The first PDS after a silent period showed the typical PDS trajectory again. From traces as the one shown in Fig. 9A, we determined the dependence of the event area on the distance from the preceding event. The relation between event area and event interval (given as inter-event time) is illustrated in Fig. 9C. Normalized event area in the presence of BayK was best described by two positive exponential components, with time constants $s_1 = 0.33$ s, and $s_2 = 4.56$ s, and amplitudes of 0.65 and 0.35 for the fast and slow component, respectively. A similar relation was identified for events recorded in the presence of isradipine (Fig. 9B and 9C). In the presence of isradipine a larger positive exponential component of 1.63 in amplitude and with a time constant of 0.59 s was followed by a negative exponential component with an amplitude of $-0.63$ and a time constant of 2.23 s. Overall, recovery of event areas was faster when LTCC activity was inhibited. Hence, the reduction in event area has an LTCC-dependent as well as an LTCC-independent component.

**DISCUSSION**

To date, the causes of PDS formation remain unclear, although experimental induction methods used by various research groups provide some insight into underlying mechanisms. Here, we compared different epileptogenesis-relevant experimental approaches regarding their propensities to initiate PDS.

Interictal spikes, the multi-unit correlate of PDS (Uva et al., 2015; Kubista et al., 2019), occur at high frequencies immediately after an epileptic seizure in humans (Gotman, 1991). In animal models of acquired epilepsy, spikes were also found to appear as early as day one after the precipitating insult (e.g. chemically-provoked status epilepticus) (Hellier et al., 1999; White et al., 2010; Chauvrière et al., 2012; Puttachary et al., 2016). For example, spike frequency amounted to about 0.5 Hz in the first 14 h after an evoked seizure in a rat kainate model and declined to about 0.05 Hz within the next couple of days (Puttachary et al., 2016). It has been hypothesized that the tonic phase of an epileptic seizure results from a melting of PDS into long lasting depolarizations. The clonic phase of an epileptic seizure was suggested to coincide with recurrent PDS that are separated by intervening repolarizations (Dreier et al., 2012; Holmes and Ben-Ari, 2001). According to this view, interictal spikes probably represent electrophysiological remnants of the preceding ictal discharge activity.

Our results confirm the presence of PDS at later stages of experimentally induced seizure-like activity. These post seizure PDS events share fundamental characteristics, including the key role of LTCC-mediated Ca$^{2+}$ influx, with those PDS events elicited by GABA$\_A$ receptor inhibition in the first experimental description thereof (Matsumoto and Ajmone Marsan, 1964; Walden et al., 1986; Witte et al., 1987; Bingmann et al., 1988; Straub et al., 1990). Thus, a restriction of GABA$\_A$ receptor-mediated inhibitory drive by intracellular Cl$^-$ accumulation may represent one mechanism leading to seizure-associated PDS.

**Comparison of PDS induction by various experimental approaches**

In line with this idea, PDS were elicited in neurons in which intracellular chloride was elevated by equilibration with perforated-patch recording pipettes containing 25 mM Cl$^-$. In addition to a rise in intracellular chloride, disruption of the intracellular Ca$^{2+}$ homeostasis by caffeine, a pharmacological gain-of-function of AMPA receptors, and inhibition of 4-aminopyridine-sensitive

---

**Fig. 6.** Co-existence of normal action potentials and PDS. (A) Recording of voltage responses of a neuron exposed to 10 $\mu$M bicuculline (BIC) plus 3 $\mu$M BayK to 2 Hz current injections, which was started immediately after a spontaneously occurring PDS. Note that the current injections generate only a single, fast decaying action potential. (B) Recording from another BIC + BayK-treated neuron before (upper trace) and during continuous depolarizing current injection (lower trace). Action potentials are marked with arrows. (C) Recording from a BIC + BayK-treated neuron, which illustrates discharges of concurrent action potentials (a, c) and PDS (b, d). The events marked by letters (a–d) are depicted on an elongated time scale in the bottom traces (Ca–Cd). Arrows indicate single action potentials.
potassium channels also evoked PDS-like events. The GABA<sub>A</sub> receptor antagonist bicuculline was the most reliable trigger of PDS. Nevertheless, induction of distinct PDS appeared to require additional concomitant factors, in particular augmented LTCC activity. The density of LTCCs in hippocampal neurons is highly variable. Voltage-gated Ca<sup>2+</sup> currents were carried by less than 10% and up to 60% by LTCCs in different neurons (Rubi et al., 2013). Hence, distinct PDS may form preferentially in neurons with high LTCC density or upon pathological up-regulation (see below). However, earlier work on primary hippocampal neurons showed that potentiation of LTCCs by BayK alone does not suffice to elicit PDS. Subthreshold spontaneous EPSPs were not at all affected by application of BayK, and suprathreshold EPSPs were augmented, but not to potentials or waveform characteristic of PDS (Rubi et al., 2013). Hence, PDS formation appears to depend on changes in LTCC activity in combination with other precipitating factors.

Caffeine treatment was exceptional in PDS generation in several aspects. First, when BayK was added, PDS emerged out of an otherwise unaltered neuronal discharge pattern. This is certainly one reason for its pronounced effect on the top 25% percentile of the event areas (3.5-fold increase, as opposed to a ~2-fold increase with 25 mM chloride, ~1.3-fold increase with bicuculline and 4-aminopyridine and hardly any increase with cyclothiazide; see Table 2). In contrast, high intracellular chloride, bicuculline, cyclothiazide and 4-aminopyridine changed the discharge pattern even prior to LTCC upregulation. Secondly, the BayK-induced increase in event area that accompanied the appearance of caffeine-induced PDS was not consistently antagonized upon inhibition of LTCCs by isradipine. Nevertheless, PDS could be inhibited significantly by isradipine in the other induction assays, e.g. in the bicuculline assay and in the 4-aminopyridine assay. One explanation for this discrepancy may be that LTCC-mediated Ca<sup>2+</sup>-influx, on the background of caffeine effects, rapidly leads to persistent functional changes, which may involve LTCC-independent enhancement of suprathreshold postsynaptic potentials. Long-term effects of PDS may depend on the presence of additional cellular alterations, e.g. interference with the close interplay between cAMP and Ca<sup>2+</sup> signaling in guiding neuronal responses (Nicol et al., 2011), and may thus be promoted by caffeine-induced inhibition of phosphodiesterases. Indeed, a recent study provided evidence for an important role of Ca<sup>2+</sup>÷/cAMP-signaling in chemoconvulsant-induced epileptogenesis (Chen et al., 2016). Although these possibilities were not addressed any further in this study, the observation of irreversible PDS effects on discharge patterns appears in line with neuropathogenic effects. However, the observed changes are much more rapid than known time courses of epileptogenesis (Dudek and Staley, 2011). It should be noted that for investigational purposes rather strong stimuli have to be applied in PDS induction studies (for example a larger than 50% inhibition of GABA<sub>A</sub> receptors with 10 μM bicuculline; Baumann et al., 2003). We propose that a combination of subtle changes in neuronal signalling (such as a rise in intracellular chloride, reduction in GABAergic inhibition; enhancement of glutamatergic excitation, altered Ca<sup>2+</sup> homeostasis, and down-regulation of dendritic K<sup>+</sup> channels) together with an augmentation of LTCC activity may lead to the appearance of PDS and potentially epileptogenic effects on the long run.

A dendritic origin of PDS

Schiller (2002) concluded that PDS have a dendritic origin. In line with this view, we recorded the most pro-

---

**Fig. 7.** Location dependent occurrence of voltage responses to current injections. (A–C) The depicted traces illustrate voltage responses to 2 Hz electrical stimulations (as indicated in grey below the traces) recorded with electrodes positioned either at the cell soma (A), a proximal dendrite (B) or a dendritic root (C) in BIC + BayK-treated neurons. (D) Scatter plot summarizing the analysis of voltage responses as in the recordings illustrated in (A–C). Data points denote the areas below the voltage trajectories and a virtual baseline set to −45 mV (dotted horizontal line) (data are from 7 to 10 different neurons). The median is indicated by grey horizontal lines. Statistical analysis was performed using Kruskal Wallis H test (H<sub>2</sub> = 191.4, p < 0.0001). The asterisks indicate the results of pairwise comparisons which were performed post-hoc using Dunn’s multiple comparison’s test.
nounced PDS when electrodes were positioned at a proximal dendrite rather than at the somatic region of the hippocampal neurons (Fig. 8 and Fig. S7). In contrast, epileptic bursts may arise predominantly from a perisomatic region where they can be readily elicited by somatic injection of depolarizing currents (reviewed in Kubista et al., 2019). In an early study of PDS in epileptic foci of anesthetized cats, Prince (1968) noted that while PDS arose spontaneously, they could not be evoked by electrical stimulation of the neuron. Here, this was confirmed in hippocampal neurons in cell culture. Experimental evidence suggests that PDS are initiated synaptically by AMPA receptor-mediated excitatory postsynaptic potentials and that they are carried mainly by ion fluxes via NMDA receptors and LTCCs (reviewed in Kubista et al., 2019).

Cav1.2 and Cav1.3 are known to have a somatodendritic localization. The first immunocytochemical studies on the neuronal distribution of LTCCs showed, besides an intense signal from the soma, dense labelling at the base of major dendrites (Westenbroek et al., 1990; Hell et al., 1993). This earlier work reported on a clustered localization of Cav1.2 and a more generalized distribution of Cav1.3 (Hell et al., 1993). However, a more recent study provided evidence that Cav1.3 may also be localized in clusters on both, dendritic shafts and spines (Stanika et al., 2016).

Hence, the dendritic origin of PDS can be explained by local AMPA receptor-mediated depolarization that enables NMDA receptor recruitment and activation of LTCCs at clusters of these ion channels. The initiation of PDS may be facilitated in the dendritic subcompartment. However, it has not been addressed so far if and how electrical compartmentalization - defined by dendritic geometry, passive membrane properties, the distribution, densities and kinetics of voltage-gated channels (Roth and Häusser, 2005) - may lead to preferential activation of PDS at the dendritic tree rather than at the soma, where LTCCs are also abundantly distributed.

The role of LTCCs in bona fide PDS

Over the last decades, many – more or less non-physiological – electrical discharge patterns have all been denominated as PDS, irrespective of their actual resemblance to the original descriptions of these abnormal discharges (i.e. type 1 and type 2 PDS, Matsumoto and Ajmone Marsan, 1964). Similar depictions of PDS have been reported around the same time by other authors (see for example Goldensohn and Purpura, 1963; Prince, 1968). Importantly, the resemblance to GDPs applies only for originally identified (e.g. type 1) PDS but arguably less for certain other discharge patterns tentatively termed PDS (see for example Silva-Barrat et al., 2001; Martella et al., 2005).

A main finding of this study was that LTCC activity is essential in the formation of type 1, GDP-like PDS. Experiments using the LTCC blocker isradipine demonstrated that without a contribution of LTCCs, suprathreshold events remained distinct from this type of PDS, irrespective of the induction method used. This indicates that LTCC-mediated Ca\(^{2+}\)-influx represents a general feature of type 1 PDS (which we consider as “ bona fide” PDS), for which an epileptogenic role has been proposed (Staley et al., 2005).

Probable causes of LTCC upregulation

PDS were readily observed upon pharmacological potentiation of LTCC activity with BayK, provided that synaptic neurotransmission or neuronal excitability was altered by application of bicuculline, caffeine, cyclothiazide, 4-aminopyridine or augmentation of intracellular chloride. Notably, several epileptogenic factors are known to positively modulate LTCCs. Hemorrhagic stroke is a common cause of acquired

Fig. 8. Location-dependent appearance of PDS. (A–C) The depicted traces illustrate PDS induced by co-administration of bicuculline (10 µM) and BayK (3 µM) and recorded with electrodes positioned either at the cell soma (A), a proximal dendrite (B) or a dendritic root (C). (D) Scatter plot depicting the areas of PDS above a virtual baseline set to −45 mV (dotted horizontal line) recorded at the three different electrode positions (data are from 9 to 10 different neurons). The median is indicated by grey horizontal lines. dR, dendritic root; pD, proximal dendrite. Statistical analysis was performed using Kruskal Wallis H test (H\(_{2}\) = 75.68, p < 0.0001). The asterisks indicate the results of pairwise comparisons which were performed post-hoc using Dunn’s multiple comparison’s test.
forms of epilepsies (Bladin et al., 2000; Benbir et al., 2006) and thrombin and thrombin-induced cytokines, such as TNF-α and IL-1β, have been found to enhance LTCC activity (Furukawa and Mattson, 1998; Yang et al., 2005). Additional epileptogenic effects may arise from the products of heme degradation, for example ferrous iron. Indeed, pronounced elevations of iron levels were found to persist for several months after the initial hemorrhagic insult (Babu et al., 2012), and LTCCs have been identified as an entry pathway for Fe²⁺ (Lockman et al., 2012). Iron is known to provoke oxidative stress, e.g. via the Fenton reaction (Pelizzoni et al., 2011). This may provide a positive feedback loop, because there is ample evidence that LTCC activity is sensitive to oxygen radicals (Ueda et al., 1997; Thomas et al., 1998; Akaishi et al., 2004; Hudasek et al., 2004). In line with this possibility, one of our previous studies (Rubl et al., 2013) showed that hydrogen peroxide can induce PDS, and that this effect requires LTCCs. Moreover, upregulation of LTCCs has been observed to occur in some brain areas as a consequence of aging, for example in the hippocampus (Veng et al., 2003; Núñez-Santana et al., 2014). Extrapolating such findings to humans, age-dependent up-regulation of LTCCs together with vascular dysfunction (note that chronic hypoxia and ischemic hypoxia followed by reperfusion also cause generation of reactive oxygen species, see Christophe and Nicolas, 2006; Fearon et al., 2006) or inflammation (TNF-α and IL-1β) may initiate epileptogenesis via promotion of LTCC-dependent PDS and consequently contribute to the enhanced incidence of epilepsies in elderly people (Hauser et al., 1993).

LTCC activity in epileptogenic effects of PDS

Oscillations of intracellular free Ca²⁺ concentrations are known to be essential for activity-dependent gene regulation (Barbado et al., 2009). In a similar manner, Ca²⁺-rises that accompany PDS may lead to stable changes of neuronal properties. Indeed, LTCCs – rather than other voltage-gated Ca²⁺ channels – were suggested to have a preferential ability to convey Ca²⁺ signals to the nucleus (Matamales, 2012). Ca²⁺ signals have been reported to exert their effects via amplitude- as well as frequency-dependent modulation (Berridge, 1997) and certain patterns of Ca²⁺ spike activity lead to specific outcomes (Rosenberg and Spitzer, 2011). Considering that long-term effects of PDS – in analogy to GDPs – may be initiated by LTCC-mediated Ca²⁺ signals, the frequency dependence of LTCC-mediated Ca²⁺-influx in PDS can be envisaged to have profound

Fig. 9. Correlation of event areas and of time intervals between single PDS. (A) Recording of PDS induced by co-administration of bicuculline (10 μM) and BayK (3 μM). Letters a–d label groups of PDS that are separated by silent periods of several seconds. The first event (or first and second event in d) of each group is depicted at a higher time resolution below (scale bars represent 20 mV and 200 ms). The insert labelled with a’ on the right depicts the group a of events at higher time resolution (scale bars represent 20 mV and 500 ms). (B) Same as in (A) but for events recorded in the presence of bicuculline (10 μM) and isradipine (3 μM). (C) Plot of normalized event areas (y-axes) versus interevent intervals (= “interevent time”, x-axes) for events recorded in the presence of bicuculline and BayK (black symbols and fitted line) and bicuculline and isradipine (grey symbols and fitted line), respectively. The area of the largest event in each recording was set to 1. Data were collected from five different neurons, and were binned into interevent intervals of 40 ms (as for 0–400 ms intervals), of 100 ms (400–1000 ms), 500 ms (1000–1500 ms) and of 1000 ms (2000–10000 ms), yielding 7–138 values per bin. Data points (depicted as the mean together with the standard error of the mean) were fitted with a two-exponential function (see Experimental Procedures).
implications on PDS associated pathogenesis. In support of this notion, LTCC-dependent PDS were found to get lost when discharges occurred in rapid succession and their recovery was considerably slower than that of the LTCC-independent events (see Fig. 9). This phenomenon can be explained by the inactivation properties of LTCCs, which have Ca$$^{2+}$$- and voltage-dependent components (Hofmann et al., 2014). Hence, it appears reasonable to assume that neuromodulatory (e.g. epileptogenic) sequelae of PDS arise in a frequency- and/or amplitude-dependent manner.

LTCCs as a therapeutic target to counteract epileptiform activity

BayK acted as an enhancer of PDS in all our assays and never led to a decline. In contrast, there was a bimodal effect of BayK on CLADPs, demonstrating antipodal roles of LTCCs in seizure associated PDS-like events. The most probable explanation for this observation is a stimulus-dependent coupling of LTCCs to de- or hyperpolarizing conductances that emanate from the depolarizing drive of Ca$$^{2+}$$-influx in primary hippocampal neurons, as described previously (Geier et al., 2011). Accordingly, blockade of LTCCs by appropriate antagonists may reduce seizure-independent (pre-seizure, interictal) PDS, but enhance seizure-associated PDS-like events. Despite these opposing actions of LTCC blockers on different types of PDS, these channels can be viewed as targets for drugs that interfere with epileptogenesis. Since seizure-independent PDS may subserve GDP-like remodeling as basis for epileptogenesis, their inhibition by dihydropyridines (such as isradipine and nimodipine) can be expected to lead to anti-epileptogenic effects. In line with this idea, acute inhibition of LTCCs with nimodipine during application of the chemoconvulsant kainate did not significantly alter acute seizures in a post-SE model. Nevertheless, this LTCC blockage sufficed to significantly reduce subsequent spontaneously recurring seizures, although the LTCC blocker was no longer present (Mikati et al., 2004).

With respect to epileptogenesis, it appears essential to reconsider carefully the exact nature of PDS under investigation. PDS as originally described in hippocampal explants from newborn rats. Exp Brain Res 72:439–442.https://doi.org/10.1007/BF00250266.

AUTHOR CONTRIBUTIONS

C.M., A. K., U. H. and L. R. performed experiments and analyzed the data. K.H., S.B. and M.H. aided in interpreting the results and revised the manuscript. H. K. performed data analysis and revised the manuscript. H. K. conceived the original idea, analyzed data, drafted and finalized the manuscript. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGEMENTS

We would like to acknowledge support of this study by grants to H.K. of the Austrian Science fund (FWF) [P-28179] and the Herzfeldersche Familienstiftung, a grant to M.H. of the Austrian Science fund (FWF) [P-33797] and support to S.B. from the interuniversity cluster project "Novel scaffolds for improved antiepileptic drugs" financed by the University of Vienna and the Medical University of Vienna. Furthermore, we thank Gabriele Gaumann for excellent technical assistance.

REFERENCES

Akashi T, Nakazawa K, Sato K, Saito H, Ohno Y, Ito Y (2004) Hydrogen peroxide modulates whole cell Ca$$^{2+}$$ currents through L-type channels in cultured rat dentate granule cells. Neurosci Lett 356:25–28. https://doi.org/10.1016/j.neulet.2003.11.012.

Babu R, Bagley JH, Di C, Friedman AH, Adamsson C (2012) Thrombin and hemin as central factors in the mechanisms of intracerebral hemorrhage-induced secondary brain injury and as potential targets for intervention. Neurosurg Focus 32:E8. https://doi.org/10.3171/2012.1.FOCUS11766.

Barbado M, Fablet K, Ronjat M, De Waard M (2009) Gene regulation by voltage-dependent calcium channels. Biochim Biophys Acta 1793:1096–1104. https://doi.org/10.1016/j.bbamcr.2009.02.004.

Barmashenko G, Hefft S, Aertsen A, Köhling R (2011) Positive shifts of the GABA$$\alpha$$ receptor reversal potential due to altered chloride homeostasis is widespread after status epilepticus. Epilepsia 52:1570–1578. https://doi.org/10.1111/j.1528-1167.2011.03247.x.

Baumann SW, Baur R, Sigel E (2003) Individual properties of the two functional agonist sites in GABA$$\alpha$$ receptors. J Neurosci 23:1158–1166. https://doi.org/10.1523/JNEUROSCI.23-35-2003.2003.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303–325. https://doi.org/10.1113/jphysiol.1989.sp017762.

Ben-Ari Y, Gaiarsa J-L, Tyzzo R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215–1284. https://doi.org/10.1152/physrev.00017.2006.

Benbir G, Ince B, Bozluolcay M (2006) The epidemiology of post-stroke epilepsy according to stroke subtypes. Acta Neurol Scand 114:8–12. https://doi.org/10.1111/j.1600-0404.2006.00642.x.

Bennett MJ (1997) The AM and PM of calcium signalling. Nature 386:759–760. https://doi.org/10.1038/386759a0.

Bingmann D, Speckmann E-J, Baker RE, Ruiter J, de Jong BM (1988) Differential antiepileptic effects of the organic calcium antagonists verapamil and flunarizine in neurons of organotypic neocortical explants from newborn rats. Exp Brain Res 72:439–442. https://doi.org/10.1007/BF00250266.

Bladin CF, Alexandrov AV, Bellavance A, Bornstein N, Chambers B, Coté R, Lebrun L, Pirisi A, et al. (2000) Seizures after stroke: a prospective multicenter study. Arch Neurol 57:1617–1622. https://doi.org/10.1001/archneur.57.11.1617.

DECLARATIONS OF INTEREST

None.
Chauvière L, Douillet T, Ghestem A, SlyouCEF SS, Wendling F, Huys R, Jirsa V, Bartolomei F, Bernard C (2012) Changes in interictal spike features precede the onset of temporal lobe epilepsy. Ann Neurol 71:805–814. https://doi.org/10.1002/ana.23549.

Chen X, Dong G, Zheng C, Wang H, Yun W, Zhou X (2016) A reduced susceptibility to chemooconvulsant stimulation in adenyl cyclase 8 knockout mice. Epilepsy Res 119:24–29. https://doi.org/10.1016/j.epilepsyres.2015.11.007.

Christophe M, Nicolas S (2006) Mitochondria: a target for neuroprotective interventions in cerebral ischemia-reperfusion. Curr Pharm Des 12:739–757. https://doi.org/10.2174/138945006777547422.

Cooper G, Kang S, Perez-Rosello T, Guzman JN, Galtieri D, Xie Z, Kondapalli J, Mordell J, Silverman RB, Surmeier DJ (2020) A single amino acid determines the selectivity and efficacy of selective negative allosteric modulators of Ca2+,1.3 L-type calcium channels. ACS Chem Biol 15:2539–2550. https://doi.org/10.1021/acschembio.0c00577.

Deiz RA, Prince DA (1987) Effect of D890 on membrane properties of neocortical neurons. Brain Res 422:83–73. https://doi.org/10.1016/0006-8993(87)90540-3.

DeLorenzo RJ, Sun DA, Blair RE, Sombati S (2007) An in vitro model for excitatory and inhibitory coupling modes of neuronal L-type calcium channels. Am J Physiol Cell Physiol 300:C307–C349. https://doi.org/10.1152/ajpcell.00219.2010.

DeLorenzo RJ, Sun DA, Deshpande LS (2006) Erratum to “Cellular mechanisms underlying acquired epilepsy: the calcium hypothesis of the induction and maintenance of epilepsy.”. Pharmacol Ther 111:288–325. https://doi.org/10.1016/j.pharmthera.2004.10.015.

DeLorenzo RJ, Sun DA, Blair RE, Sombati S (2007) An in vitro model of stroke-induced epilepsy: elucidation of the roles of glutamate and calcium in the induction and maintenance of stroke-induced epileptogenesis. Int Rev Neurobiol 81:59–84. https://doi.org/10.1016/S0074-7742(06)10180-5.

Di Maio R, Mastroberardino PG, Hu X, Montero L, Greenamyre JT (2011) Pilocarpine-induced NMDA receptor expression and function in hippocampal neurons: NADPH oxidase and ERK1/2 mechanisms. Neurobiol Dis 42:482–495. https://doi.org/10.1016/j.nbd.2011.02.012.

Di Maio R, Mastroberardino PG, Hu X, Montero LM, Greenamyre JT (2013) Thiol oxidation and altered NR2B/NMDA receptor functions in in vitro and in vivo pilocarpine models: implications for epileptogenesis. Neurobiol Dis 49:87–98. https://doi.org/10.1016/j.nbd.2012.07.013.

Dinocourt C, Petanjek Z, Freund TF, Ben-Ary Y, Esclapez M (2003) Loss of interneurons innervating pyramidal cell dendrites and axon initial segments in the CA1 region of the hippocampus following pilocarpine-induced seizures. J Comp Neurol 459:407–425. https://doi.org/10.1002/cne.10622.

Dreier JP, Major S, Pannek H-W, Woltzki J, Scheel M, Wiesental D, Martinus P, Winkler MKL, Hartings JA, Fabijancius E-J, Gorji A (2012) Spreading convulsions, spreading depolarization and epileptogenesis in human cerebral cortex. Brain 135:259–275. https://doi.org/10.1093/brain/awr303.

Drexel M, Preidt AP, Kirchmair E, Sperk G (2011) Parvalbumin-expressing interneurons and calretinin fibers arising from the thalamic subiculum during chronic hypoxia. Adv Exp Med Biol 580:197–201. https://doi.org/10.1007/978-3-787-13117-7_30.

Francis J, Jugloff DGM, Mingo NS, Wallace MC, Jones OT, McIntyre Burnham W, Eubanks JH (1997) Kainic acid-induced generalized seizures alter the regional hippocampal expression of the rat Kv4.2 potassium channel gene. Neurosci Lett 232:91–94. https://doi.org/10.1016/S0304-390X(97)00593-4.

Furukawa K, Mattson MP (1998) The transcription factor NF-kappaB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-alpha in hippocampal neurons. J Neurochem 70:1876–1886. https://doi.org/10.1046/j.1471-4169.1998.70051876.x.

Gean P-W, Chou S-M (1991) Suppression of 4-aminopyridine-induced paroxysmal depolarizing shift in rat amygdaloid neurons by diltiazem. Brain Res 560:306–310. https://doi.org/10.1016/0006-8993(91)91248-V.

Geier P, Lagler M, Boehm S, Kubista H (2011) Dynamic interplay of excitatory and inhibitory coupling modes of neuronal L-type calcium channels. Am J Physiol Cell Physiol 300:C307–C349. https://doi.org/10.1152/ajpcell.00219.2010.

Goldensohn ES, Purpura DP (1963) Intracellular potentials of cortical neurons during focal epileptogenic discharges. Science 139:840–842. https://doi.org/10.1126/science.139.3557.840.

Gotman J (1991) Relationships between interictal spiking and seizures: human and experimental evidence. Can J Neurol Sci 18:573–576. https://doi.org/10.1017/S031716710002273X.

Haeuser WA, Angenieurs JF, Kurtland LT (1993) Incidence of epilepsy and unprovoked seizures in Rochester, Minnesota: 1935–1984. Epilepsia 34:453–458. https://doi.org/10.1111/j.1528-1157.1993.tb02586.x.

Hellier JL, Patrylo PR, Dou P, Nett M, Rose GM, Dudek FE (1999) Assessment of inhibition and epileptiform activity in the septal dentate gyrus of freely behaving rats during the first week after kainate treatment. J Neurosci 19:10053–10064. https://doi.org/10.1523/JNEUROSCI.19-22-10053.1999.

Hell JW, Westenbroek RE, Warner C, Ahlijanian MK, Prystay W, Gilbert MM, Snutch TP, Catterall WA (1993) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J Cell Biol 123:949–962. https://doi.org/10.1083/jcb.123.4.949.

Hofmann F, Flockerzi V, Kahl S, Wegener JW (2014) L-type Ca(v)1.2 calcium channels: from in vitro findings to in vivo function. Physiol Rev 94:303–326. https://doi.org/10.1152/physrev.00016.2013.

Holmes GL, Ben-Anir Y (2001) The neurobiology and consequences of epilepsy in the developing brain. Pediatr Res 49:320–325. https://doi.org/10.1203/00006450-200103000-00004.

Hotka M, Kubista H (2019) The paroxysmal depolarization shift in epilepsy research. Int J Biochem Cell Biol 107:77–81. https://doi.org/10.1016/j.biocel.2018.12.006.

Hotka M, Cagaliniec M, Hilber K, Hool L, Boehm S, Kubista H (2020) L-type Ca2+ channel-mediated Ca2+ influx adjusts neuronal mitochondrial function to physiological and pathophysiological conditions. Sci Signaling 13:eaaa6923. https://doi.org/10.1126/sci signals.aeeaw6923.

Hudasek K, Brown ST, Fearon IM (2004) H2O2 regulates recombinant Ca2+ channel alpha1C subunits but does not mediate their sensitivity to acute hypoxia. Biochem Biophys Res Commun 318:135–141. https://doi.org/10.1016/j. bbr.2004.04.011.

Kovács R, Kardos J, Heinemann U, Kann O (2005) Mitochondrial calcium ion and membrane potential transients follow the pattern of epileptiform discharges in hippocampal slice cultures. J Neurosci 25:4260–4269. https://doi.org/10.1523/ JNEUROSCI.0400-05.2005.

Kubista H, Boehm S, Hotka M (2019) The paroxysmal depolarization shift: reconsidering its role in epilepsy, epileptogenesis and beyond. Int J Mol Sci 20:5077. https://doi.org/10.3390/ ijms20030577.
acute models of focal limbic seizures. J Neurosci 35:3048–3055. https://doi.org/10.1523/JNEUROSCI.3692-14.2015.

Veng LM, Mesches MH, Browning MD (2003) Age-related working memory impairment is correlated with increases in the L-type calcium channel protein alpha1D (Ca_{1.3}) in area CA1 of the hippocampus and both are ameliorated by chronic nimodipine treatment. Brain Res Mol Brain Res 110:193–202. https://doi.org/10.1016/s0169-328x(02)00643-5.

Verkhratsky A, Shmigol A, Kirschuk S, Pronchuk N, Kostyuk P (1994) Age-dependent changes in calcium currents and calcium homeostasis in mammalian neurons. Ann N Y Acad Sci 747:365–381. https://doi.org/10.1111/j.1749-6632.1994.tb44423.x.

Waldbaum S, Patel M (2010) Mitochondrial dysfunction and oxidative stress: a contributing link to acquired epilepsy?. J Bioenerg Biomembr 42:449–455. https://doi.org/10.1007/s10863-010-9320-9.

Walden J, Pockberger H, Speckmann E-J, Petsche H (1986) Paroxysmal neuronal depolarizations in the rat motor cortex in vivo: intracellular injection of the calcium agonist BAY K 8644. Exp Brain Res 64:607–609. https://doi.org/10.1007/BF00340500.

Westenbroek RE, Ahlijanian MK, Catterall WA (1990) Clustering of L-type Ca^{2+} channels at the base of major dendrites in hippocampal pyramidal neurons. Nature 347:281–284. https://doi.org/10.1038/347281a0.

White A, Williams PA, Hellier JL, Clark S, Dudek FE, Staley KJ (2010) EEG spike activity precedes epilepsy after kainate-induced status epilepticus. Epilepsia 51:371–383. https://doi.org/10.1111/j.1528-1167.2009.02339.x.

Witte OW, Speckmann E-J, Walden J (1987) Motor cortical epileptic foci in vivo: actions of a calcium channel blocker on paroxysmal neuronal depolarizations. Electroencephalogr Clin Neurophysiol 66:43–55. https://doi.org/10.1016/0013-4694(87)90137-4.

Yang S, Liu ZW, Wen L, Qiao HF, Zhou WX, Zhang YX (2005) Interleukin-1beta enhances NMDA receptor-mediated current but inhibits excitatory synaptic transmission. Brain Res 1034:172–179. https://doi.org/10.1016/j.brainres.2004.11.016.

APPENDIX A. SUPPLEMENTARY DATA
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2021.05.011.