Published in final edited form as:

Epilepsia. 2015 February ; 56(2): 297–309. doi:10.1111/epi.12883.

Muscarinic excitation of parvalbumin-positive interneurons contributes to the severity of pilocarpine-induced seizures

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SUMMARY

Objective—A common rodent model in epilepsy research employs the muscarinic acetylcholine receptor (mAChR) agonist pilocarpine, yet the mechanisms underlying the induction of pilocarpine-induced seizures (PISs) remain unclear. Global M₁ mAChR (M₁R) knockout mice are resistant to PISs, implying that M₁R activation disrupts excitation/inhibition balance. Parvalbumin-positive (PV) inhibitory neurons express M₁ mAChRs, participate in cholinergically-induced oscillations, and can enter a state of depolarization block (DB) during epileptiform activity. Here, we test the hypothesis that pilocarpine activation of M₁Rs expressed on PV cells contributes to PISs.

Methods—CA1 PV cells in PV-CRE mice were visualized with a floxed YFP or hM3Dq-mCherry adeno-associated virus, or by crossing PV-CRE mice with the RosaYFP reporter line. To eliminate M₁Rs from PV cells, we generated PV-M₁KO mice by crossing PV-CRE and floxed M₁ mice. Action potential (AP) frequency was monitored during application of pilocarpine (200 μM). In behavioral experiments, locomotion and seizure symptoms were recorded in WT or PV-M₁KO mice during PISs.

Results—Pilocarpine significantly increased AP frequency in CA1 PV cells into the gamma range. In the continued presence of pilocarpine, a subset (5/7) of PV cells progressed to DB, which was mimicked by hM3Dq activation of Gq-receptor signaling. Pilocarpine-induced depolarization, AP firing at gamma frequency, and progression to DB were prevented in CA1 PV cells of PV-

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DISCLOSURE

The authors have no conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

M₁KO mice. Finally, compared to WT mice, PV-M₁KO mice were associated with reduced severity of PISs.

Significance—Pilocarpine can directly depolarize PV+ cells via M₁R activation but a subset of these cells progress to DB. Our electrophysiological and behavioral results suggest that this mechanism is active during PISs, contributing to a collapse of PV-mediated GABAergic inhibition, dysregulation of excitation/inhibition balance, and increased susceptibility to PISs.

Keywords

pilocarpine; muscarinic; acetylcholine; hippocampus; seizures

INTRODUCTION

Systemic administration of cholinomimetic drugs has long been known to induce acute seizures.¹ Upon systemic administration of pilocarpine, the hippocampus is one of the earliest structures to exhibit electrographic seizure activity.² Seizures are also induced upon direct injection of cholinergic agonists^{3,4} or acetylcholinesterase inhibitors⁵ into the hippocampus.

A widely accepted mechanism in the generation of cholinergically-induced seizures is the pathological activation of muscarinic acetylcholine receptors (mAChRs).² Cholinergically-induced seizures can lead to long term pathological consequences, such as rewiring of hippocampal circuitry, cell death, and increased susceptibility to epileptogenesis.⁶

M₁ mAChRs (M₁Rs), which are expressed at a high density in limbic and cortical structures,⁷ are critically involved in the generation of PISs.^{8,9} Although pharmacological studies have clearly shown that seizures are generated by the recruitment of glutamatergic and GABAergic networks,³ it is still not clear how activation of M₁Rs leads to the disruption of excitation/inhibition balance. M₁Rs are expressed on both excitatory and inhibitory cell populations,^{10–12} suggesting that the mechanisms could involve both increased excitation and reduced inhibition.

One clue to the cell types activated by M₁Rs is that the muscarinic agonist carbachol can elicit hippocampal gamma oscillations (γ), which are eliminated in global M₁R knockout (M₁KO) mice.¹³ Direct M₁Rs agonists also induce γ .¹⁴ γ are thought to involve the participation of fast-spiking parvalbumin-containing (PV) interneurons.^{15,16} γ often precede epileptic discharges in vivo¹⁷ and have been suggested to indicate cholinergic overstimulation at seizure onset.¹⁸ Interestingly, recent observations indicate that interneurons are susceptible to entering a pathological state of depolarization block (DB) during ictal activity in hippocampus^{19,20} and cortex,²¹ suggesting that the overactivation of PV networks precipitate a reduction in GABA release and can contribute to seizure induction.

We have previously shown that hippocampal CA1 PV cells are more strongly depolarized by M₁R activation than other hippocampal or cortical interneuron subtypes.^{11,12} However, it is not clear whether pilocarpine can excite PV cells and whether pathological activation of

mAChRs on PV cells contributes to the induction of PISs *in vivo*. In this study, we found that bath application of pilocarpine directly excites PV cells in acute hippocampal slices. Moreover, the activation of G_q-mediated signaling in PV cells was capable of inducing a transient induction of gamma frequency firing that was followed by a progressive reduction in AP frequency and amplitude, in which PV appeared to be in a sustained state of DB. The selective elimination of M₁Rs from PV+ cells abolished pilocarpine-induced excitation of PV cells, prevented mAChR-induced entry of PV cells into DB, and reduced the severity of PISs. Therefore, we conclude that direct activation of M₁Rs on PV cells is a mechanism that contributes to the generation of PISs.

METHODS

Generation of PV-ROSA and PV-M₁KO transgenic mice

All procedures were performed in accordance with the University of Montana Institutional Animal Care and Use Committee (Animal Use Protocols 030-10 and 035-13). PV-CRE^{+/-}/Rosa26EYFP^{+/-} (PV-Rosa) mice that expressed YFP in PV cells (PV-YFP) were generated by crossing PV-CRE^{+/-} (stock #008069; Jackson Labs, Bar Harbor, ME) mice with a Rosa26EYFP^{+/-} reporter line (stock #007920; Jackson Labs).¹¹ PV-CRE mice containing floxed M₁^{-/-} (WT) or floxed M₁^{+/-22} (PV-M₁KO) genotypes were generated as previously described.¹¹ Both male and female mice were used for this study.

Stereotaxic injection of adeno-associated viruses (AAVs) into CA1 hippocampus

Floxed YFP (AAV2-EF1a-DIO-EYFP)²³ or floxed hM3Dq-mCherry^{24,25} (AAV8-hSyn-DIO-hM3Dq-mCherry) AAVs (~10E⁻¹² vc/ml) were obtained from the UNC Vector Core (Chapel Hill, NC). AAVs were injected into the ventral CA1 (anteroposterior: 2.8 mm, mediolateral: 3.3 mm, dorsoventral: 2.3 mm) of adult PV-CRE or PV-M₁KO mice (>8 weeks of age); PV-YFP or hM3Dq-mCherry-positive (PV-mCherry) cells were visualized in acute hippocampal slices from PV-M₁KO or PV-CRE mice, respectively, at least 4 weeks after surgery.¹¹

Hippocampal slice preparation

Mice were deeply anesthetized with 4% isoflurane and then rapidly decapitated. The brain was removed and placed in an ice-cold sucrose-based artificial cerebrospinal fluid (ACSF) containing (mM): 80 NaCl, 2.5 KCl, 24 NaHCO₃, 0.5 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 25 glucose, 75 sucrose, 1 ascorbic acid, 3 sodium pyruvate, saturated with 95% O₂-5% CO₂, pH 7.4. After minimizing the z-vibration of the mounted blade with the Vibrocheck device,²⁶ transverse hippocampal slices (300 μm) were obtained using a Vibratome 1200S (Leica Microsystems, Bannockburn, IL). Slices were stored in warm (36 °C) oxygenated ACSF for >30 minutes prior to use.

Electrophysiology

Hippocampal slices from PV-Rosa mice (1–3 months old) or AAV-injected mice (>2 months old, see above) were transferred to submerged recording chamber filled with extracellular solution (EC; in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄ and 20 glucose, saturated with 95% O₂-5% CO₂, pH 7.4. PV-YFP or PV-

mCherry cells were visualized using IR-Dodt contrast on an upright Zeiss microscope (AxioExaminer D1, Carl Zeiss Microscopy, Thornwood, NY) equipped with an AxioCam camera running Axiovision 4.8. Cells were visualized on a Patch Pro 2000 (Scientifica, East Sussex, UK) with 505/590 nm LEDs (LED4A4/DC 4100; Thorlabs, Newton, NJ) or an Infrapatch (Luigs and Neumann, Ratingen, Germany) with Collibri LED illumination system (505/590 nm, Carl Zeiss Microscopy). Pipettes (2–4 M Ω impedance) were generated with a PC-10 vertical puller (Narishige, East meadow, NY). For loose patch recording, suction was applied to EC-filled pipettes until a stable access resistance (R_a) of 10–200 M Ω was achieved.²⁷ Cell-attached seals were >1 G Ω prior to whole cell recording. Initial access resistances (R_a) that were higher than 20 M Ω were discarded. To allow R_a to stabilize, cells were monitored at the resting membrane potential (V_m) during an initial 2 minutes baseline period. Intracellular solution (IC) was composed of (in mM): 110 potassium gluconate, 40 KCl, 10 HEPES, 0.1 EGTA, 4 MgATP, 0.3 Na₂GTP, 10 phosphocreatine and biocytin 0.2%, pH 7.2, osmolarity 295–305 mOsm. Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices, Union City, CA), filtered at 4 KHz, and digitized at 20 KHz (Digidata 1440A, Molecular Devices). During recordings, solutions were heated to 34–35 °C with HPT-2 (Scientifica) or SH-27B/TC-324B (Warner, Hamden, CT) inline solution heaters. A total of 11 PV-Rosa (24 PV cells), 2 PV-CRE (6 PV cells) and 2 PV-M₁KO mice (6 PV cells) were used for recordings.

Chemical reagents

DL-APV was obtained from R&D Systems (Minneapolis, MN). Clozapine N-oxide (CNO) was obtained from Enzo Life Sciences (Pittsburgh, PA). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Data analysis

Electrophysiological data was acquired and analyzed using Axograph X (Axograph Scientific, Sydney, Australia). Loose patch and whole cell APs were detected with the Event Detection Plug-In program in Axograph X using the AP derivative (100–500pA/ms or 30mV/ms). During hM3Dq-mCherry activation, the level of depolarization was calculated based on a linear regression between the first derivative of the AP and V_m .¹¹ Intrinsic membrane properties were measured as described previously.¹¹

Anatomical identification

Biocytin (0.2%) was introduced into cells during whole-cell recordings. After recording, slices were fixed overnight at 4°C in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA; cat# 15714-S, Electron Microscopy Sciences, Hatfield, PA). Slices were then transferred to PBS and stored for up to 2 weeks at 4°C. After permeabilization with 0.3% Triton X-100 (cat# BP151-500; Fisher Scientific, Pittsburgh, PA) in PBS for 2 h at room temperature, slices were incubated in PBS containing Alexa 633-conjugated streptavidin (final concentration 1 μ g/ml; cat #S-21375; Invitrogen, Grand Island, NY) overnight at 16°C. Slices were cryopreserved in PBS containing 30% sucrose, and then resectioned at 100–150 μ m thickness with a sliding freezing microtome (HM430, Thermo Scientific, Waltham, MA). After staining with Neurotrace 435/455 (1:100 in PBS; cat #N21479, Invitrogen) and mounting on Colorfrost Plus slides (cat #99-910-11, Fisher

Scientific) using Vectashield HardSet Mounting Medium (NC9029228, Fisher Scientific), sections were imaged with a Fluoview FV-1000 confocal imaging system (Olympus, Center Valley, PA) with a 25× objective (XLPL25XWMP, Olympus, Tokyo, Japan).

Pilocarpine induced seizures

C57BL6 (3–4 months old, n=10), WT PV-M₁KO (2–3 months old, n=10), and homozygous PV-M₁KO (2–3 months old, n=10) mice were used in this study. Where possible, age-matched sibling littermates were used between groups. In the total population, males were heavier than females ($p=6.4E-7$, $n=46$), but the gender ratio was similar within each group, resulting in no difference in weight across groups ($p>0.05$). Mice were injected with atropine (1 mg/kg i.p. dissolved in 0.9% saline; Sigma-Aldrich, cat# A0132) to reduce effects of peripheral mAChR activation²⁸ 30 minutes prior to pilocarpine (155–200 mg/kg i.p., dissolved in 0.9% saline; Sigma-Aldrich, cat# P6503) injection. Immediately after pilocarpine injection, mice underwent open field maze (OFM) testing for 45 minutes and were then euthanized (CO₂ exposure followed by cervical dislocation). Overhead and side videos were later observed for seizure severity analysis. Seizure severity was evaluated using an adapted Racine scale: (1) stop and stare; (2) head bobbing, tail twitch, single body jerk; (3) multiple body jerks; (4) forearm clonus, wet dog shakes; (5) clonic seizures; (6) tonic-clonic seizures. The experimenter was blind to genotype and drug (saline or pilocarpine) during testing; Racine scores were further validated through blinded review of videos.

Statistical analysis

Prism software (v.5/6) was used for statistical analysis. Parametric tests were used when data passed the Shapiro-Wilk normality test; otherwise, nonparametric tests were used. For paired comparisons, a two-tailed student's t-test and Wilcoxon signed rank test were used. For unpaired tests, a student's unpaired t-test or Mann-Whitney U test was used. Multiple t-test, one-way ANOVA (with Dunn's multiple comparison's test) and two-way ANOVA was used for behavioral data. All data are displayed as mean ± SEM.

RESULTS

Pilocarpine directly excites hippocampal CA1 PV interneurons

To examine the effect of pilocarpine on the AP frequency of PV cells, we visualized PV cells under YFP fluorescence (Fig. 1A) and Dodt-IR contrast (Fig. 1B) in the CA1 pyramidal cell layer in acute hippocampal slices from PV-Rosa mice. In a representative loose patch recording from a PV-YFP cell, spontaneous firing was observed in normal ACSF (Fig. 1C), which increased in AP frequency after bath application of 200 μM pilocarpine (Fig. 1D). Spontaneous firing (1.9 ± 1.1 Hz) was observed in all (6/6) PV cells (Fig. 1C,G, blue). Two minutes after pilocarpine application, AP frequency significantly increased to 15.4 ± 3.7 Hz ($p=0.031$, $n=6$, $W_5=21$; Wilcoxon matched-pairs signed rank test, Fig. 1D,G, red). To examine whether pilocarpine increased PV cell firing frequency through direct or network-induced mechanisms, we blocked ionotropic glutamatergic transmission with AMPA and NMDA receptor antagonists DNQX (25 μM) and APV (50 μM) respectively, and GABAergic transmission with the GABA_A receptor antagonist gabazine (5

μM). In synaptic blockers (Fig. 1E,H), baseline AP frequency (3.2 ± 1.9 Hz, $n=7$) was similar to ACSF conditions ($p=0.81$, $U_{11}=19$; Mann-Whitney U test). Upon bath application of pilocarpine (Fig. 1F,H), AP frequency significantly increased within 2 minutes (from 3.2 ± 1.9 Hz to 12.3 ± 4.5 Hz, $p=0.016$, $W_6=28.0$, $n=7$; Wilcoxon matched-pairs signed rank test; Fig. 1H). Therefore, pilocarpine directly excites PV cells through a mechanism that is independent of glutamatergic synaptic excitation.

Sustained activation of G_q -mediated signaling in CA1 PV interneurons induces gamma frequency firing that progresses to a reduction in AP firing frequency and amplitude

Pilocarpine is an agonist at G_q -coupled mAChRs. We previously showed that the selective activation of the Designer Receptor Exclusively Activated by Designer Drugs (DREADD) hM3Dq-mCherry^{24,25} in hippocampal PV neurons enhanced the frequency and amplitude of inhibitory postsynaptic currents (IPSCs) in CA1 pyramidal cells.¹¹ In hippocampal PV cells maintained in current clamp at -60 mV, activation of hM3Dq modulated PV cell excitability similarly to activation of M_1 mAChRs with the full agonist muscarine. This modulation included an increase in AP frequency, conversion of the post-spike afterhyperpolarization to an afterdepolarization, and an increase holding current at -60 mV.^{11,12}

Here, we examined the direct effect of G_q -mediated signaling on V_m by expressing hM3Dq-mCherry, an evolved G_q -coupled receptor, in CA1 PV cells (Fig. 2A,B) and monitoring spontaneous firing in loose patch mode. In a representative cell (Fig. 2C,F), application of the hM3Dq agonist clozapine-N-oxide (CNO; 500 nM) increased AP firing within 1 min. The AP firing peaked at gamma frequency (Fig. 2C,G), but reduced firing frequency thereafter (Fig. 2C,H). Concomitantly, AP amplitude was progressively reduced in the presence of CNO (Fig. 2E, H). Because gamma firing induction was variable (54.0 ± 13.2 s), we aligned loose patch recordings to the onset of gamma firing. As a population, hM3Dq activation initially increased AP firing frequency into the gamma range (from 13.1 ± 5.4 to 45.4 ± 6.0 Hz, $p=0.031$, $W_5=21.0$, $n=6$; Wilcoxon matched-pairs signed rank test); however, this increase in AP frequency was followed by a progressive reduction in AP firing (from 45.4 ± 6.0 to 6.6 ± 4.7 Hz, $p=0.031$, $W_5=-21.0$, $n=6$; Wilcoxon matched-pairs signed rank test; Fig. 2I). Of 3 PV cells that fired spontaneously in control conditions, two PV cells exhibited a lower AP frequency than in control conditions after CNO-induced gamma firing (compare Fig. 2H to Fig. 2F). Concomitantly, the relative AP amplitude was progressively reduced (from 1.0 ± 0.08 to 0.31 ± 0.10 , $p=0.031$, $W_5=-21.0$, $n=6$; Wilcoxon matched-pairs signed rank test). Because we had previously examined the relationship between AP amplitude and V_m in PV cells,¹¹ we calculated that a 69% reduction in AP amplitude corresponds to a large predicted depolarization (18.1 ± 2.6 mV, Fig. 2J).

Pilocarpine induces DB in a subset of CA1 PV cells

To further investigate the relationship between pilocarpine-induced increases in AP firing and V_m , we performed whole cell recordings from CA1 PV cells in PV-Rosa mice (Fig. 3). Consistent with a previous study,¹¹ PV cells exhibited fast spiking phenotype (Table 1). In a representative PV cell recording (Fig. 3A–C), spontaneous firing was observed in control conditions (Fig. 3D). Pilocarpine administration depolarized the PV cells and increased AP frequency both early (Fig. 3E) and later (Fig. 3F) in the recording (Fig. 3G). However, in

another PV cell (Fig. 3H) pilocarpine increased AP firing initially (Fig. 3I) but progressed, within 3 minutes, to a state in which the PV cell was depolarized but did not fire APs (DB; Fig. 3J). Similar to that observed in loose patch mode (Fig. 1E,F), we found that bath application of pilocarpine depolarized PV cells (from -55.0 ± 1.5 mV to -49.8 ± 1.4 mV, $p=0.0003$, $t_{10}=5.5$, $n=11$; paired t test; Fig. 3L) and increased the average AP frequency within the first 1–2 min (from 0.04 ± 0.04 Hz to 6.9 ± 3.1 Hz, $p=0.0078$, $W_{10}=36$, $n=11$; Wilcoxon matched-pairs signed rank test, Fig. 3M). In 2/7 PV cells that exhibited APs during the first 1–2 min, AP firing was sustained or increased later in the recording (at 3–4 min; Fig. 3D–F, M, open triangles). However, in 5/7 PV cells, AP frequency was reduced later in the recording (Fig. 3H–K, M, open squares). Four cells were depolarized by pilocarpine but did not fire during the measured window (Fig. 3M, open circles). In 82% (9/11) of recorded cells in PV-*rosa* mice, pilocarpine increased AP frequency into gamma range (onset at 53.4 ± 7.9 s). In summary, pilocarpine-induced depolarization persisted in most PV cells, a substantial subset of PV cells progressed to DB, a state associated with a reduction or cessation in AP firing.

M₁Rs expressed on CA1 PV cells mediate pilocarpine-induced depolarization, induction of gamma firing, and mAChR-induced progression to DB

To determine the specific role of M₁Rs in pilocarpine excitation of PV cells, we utilized the PV-M₁KO mouse that eliminated M₁Rs specifically in PV cells.¹¹ In a representative PV-YFP cell (Fig. 4A,B), spontaneous firing was observed under baseline conditions (Fig. 4C). Pilocarpine had no appreciable effect on the level of depolarization or AP firing frequency both early (Fig. 4D) and later (Fig. 4E) in the recording (Fig. 4G), with no obvious change in AP amplitude. Baseline AP frequency in PV cells from PV-M₁KO mice ($n=6$) and PV-*Rosa* mice ($n=11$) were similar ($p=0.12$, $U_{15}=19.5$, Mann-Whitney test). In PV-M₁KO mice, V_m (-51.7 ± 2.2 mV to -49.4 ± 1.4 mV, $p=0.31$, $W_5=11$, $n=6$; Wilcoxon matched-pairs signed rank test; Fig. 4G) and AP frequency (from 2.1 ± 1.2 Hz to 3.7 ± 1.9 Hz, $p=0.25$, $W_5=8$, $n=6$; Wilcoxon matched-pairs signed rank test; Fig. 4H) were not significantly altered by pilocarpine. Notably, in PV-M₁KO mice, 2 of 6 cells that did not fire spontaneously also did not depolarize appreciably (Fig. 4H, open circles). Finally, pilocarpine induced AP frequency in the gamma range in only 1 of 6 recorded cells in PV-M₁KO mice (Fig. 4H). These observations suggest that the elimination of M₁Rs from PV cells blocks both pilocarpine-induced firing at gamma frequency and prevents entry of PV cells into DB.

The severity of PISs is reduced in PV-M₁KO mice

Previous studies have shown that global M₁ knockout mice are highly resistant to PISs.^{8,9} Moreover, administration of the selective M₁ muscarinic antagonist VU0255035 has been shown to reduce the severity of PISs.²⁹ To test the hypothesis that the elimination of M₁Rs expressed in PV cells plays a role in PISs, we acutely administered pilocarpine to WT and PV-M₁KO mice while tracking position and rating seizure severity in an OFM (Fig. 5). A previous study demonstrated that deletion of M₁Rs from PV cells does not interfere with normal locomotion.¹¹

We first examined the effect of saline (Fig. 5A) or pilocarpine (Fig. 5B) on locomotion (Fig. 5E–H) and seizure development (Fig. 5I) in C57BL6 mice. In C57BL6 mice injected with

saline (Fig. 5A), mice freely explored all areas of the OFM (Fig. 5E–H). Over the course of 45 minutes, saline-injected mice ($n=5$) exhibited a progressive reduction in line crossings ($p<0.05$ for 25–45 min compared to the first 5 min; Fig. 5E, black) and speed ($p<0.05$ for 35–45 min compared to the first 5 min; Fig. 5F, black), and an increase in the time immobile ($p<0.05$ for 35–45 min compared to the first 5 min; Fig. 5G, black). No significant change was observed in rearing behavior over 45 min (Fig. 5H, black; $p>0.05$). Within the first 5 minutes after the administration of pilocarpine ($n=5$), line crossings (211.6 ± 5.2 vs. 48.8 ± 10.4 ; $p=6.4E-7$; multiple t-test for saline vs. pilocarpine at 0–5 min bin; Fig. 5B, E, red) and speed (0.058 ± 0.004 vs. 0.012 ± 0.003 m/s; $p=2.8E-5$; multiple t-test for saline vs. pilocarpine at 0–5 min bin; Fig. 5B, F, red) were dramatically reduced, indicating a severe impairment of locomotor behavior. Pilocarpine administration also increased time immobile (15.8 ± 3.0 vs. 214.3 ± 15.5 ; $p=1.5E-6$; Fig. 5B, G, red) and reduced rearing behavior (22.4 ± 2.6 vs. 3.8 ± 1.3 ; $p=0.00019$; Fig. 5B, G, red) at the 0–5 minute time point (multiple t-test for saline vs. pilocarpine). All measures of locomotion fell to a minimal level 10 min after injection of pilocarpine, which were significantly different than in saline-injected mice ($p<0.05$; multiple t-tests; Fig. 5E–H). Importantly, robust increases in seizure behavior were observed in all mice injected with pilocarpine, as measured on a modified Racine scale (see Methods), progressing to status epilepticus in 5 of 5 mice (Fig. 5I, red). While no seizure symptoms were detected with vehicle alone (Fig. 5I, black), pilocarpine induced a detectable increase in seizure behavior at all time points ($p<0.01$, multiple t-test; Fig. 5I).

We then performed a similar experiment in WT (Fig. 5C) and homozygous PV-M₁KO (Fig. 5D) mice. Measures of locomotion during pilocarpine administration were not significantly different between WT and PV-M₁KO mice ($p>0.05$; two-way ANOVA; Fig. 5E–H). However, PV-M₁KO mice (3.9 ± 0.5 , $n=10$; Fig. 5J, green) exhibited reduced seizure severity in the 30–45 minute time window ($p=0.0024$, Mann-Whitney test) compared to WT controls (5.5 ± 0.2 , $n=10$; Fig. 5J, blue). Maximum seizure severity was also lower in PV-M₁KO mice (4.6 ± 0.5 , $n=10$) than in WT controls (5.9 ± 0.1 , $n=10$) over the entire 45 minutes ($p=0.026$, Mann-Whitney test). Specifically, the percentage of mice that exhibiting tonic-clonic seizures (Racine score of 6) was significantly different in WT (90%, 9/10) than in PV-M₁KO (40%, 4/10) mice ($p=0.029$, one sample t-test, Fig. 5K). In conclusion, consistent with electrophysiological measures showing that elimination of M₁Rs protects PV cells from entering a state of mAChR-induced gamma firing and DB, we find that PV-M₁KO mice exhibit reduced severity to PISs.

DISCUSSION

The precise balance of inhibitory and excitatory transmission in the mammalian hippocampus is maintained, in part, by the participation of PV cells in network dynamics that provide both feedforward and feedback inhibition to glutamatergic principal cells.^{15,16} Additionally, one observation in pilocarpine models of epilepsy is that γ , which are thought to be generated by PV cells,¹⁵ precede seizure onset.¹⁷ Therefore, it is possible that a mAChR-induced collapse of PV-mediated inhibition contributes to cholinergically-induced seizures.

In the present study, we gained three key insights into the underlying mechanisms of PISs. First, G_q -coupled receptor signaling, either through pilocarpine activation of M_1 mAChRs, or through CNO activation of hM3Dq-mCherry, directly depolarizes PV cells, which is capable of inducing firing of PV cells at gamma frequency. Second, sustained G_q -coupled receptor signaling in PV cells can induce DB, whereby PV cells remain in a depolarized state but firing is impaired or eliminated altogether. Finally, PV- M_1 KO mice exhibit reduced seizure severity, as would be expected if DB occurred *in vivo* during PISs.

Pilocarpine excitation of PV cells is dependent upon M_1 Rs

In this study, we found that pilocarpine administration increased AP frequency in hippocampal CA1 PV cells even when excitatory and inhibitory transmission were blocked. Consistent with previous studies using muscarine,^{11,12} this result demonstrates that pilocarpine acts to enhance PV cell excitability through the direct activation of mAChRs. Interestingly, we found that a substantial subset of PV cells were initially excited by pilocarpine but progressed to DB. Two other studies have shown that PV cells are particularly vulnerable to DB during epileptic conditions in both *in vitro* animal models^{20,21} and in humans with generalized tonic-clonic seizures.³⁰ The cholinergically-induced DB phenotype observed here was most often seen as bursts of APs at gamma frequency followed by a momentary or complete cessation of firing (Fig. 3). Using the selective expression of hM3Dq-mCherry in PV cells (Fig. 2) and the use of PV- M_1 KO mice (Fig. 4), we confirmed that the underlying mechanism of DB involves the activation of G_q -mediated signaling pathways. The effectors of G_q -signaling in PV cells are still poorly understood, but most likely involve inhibition of $Kv7.2/Kv7.3$ channel mediated current (I_M), inhibition of calcium-activated potassium channel-mediated current (I_{AHP}), and an increase in calcium-dependent non-specific cationic current (I_{CAT})^{31,32} possibly mediated by TRPC6³³ or TRPC7³⁴ channels. All of these effects would act to persistently depolarize PV cells and weaken the ability of PV cells to repolarize. Since I_M can contribute to I_{AHP} in some cell types,^{35,36} further work is needed to distinguish components of the I_{AHP} that are inhibited by M_1 Rs. Finally, we noted that a small subset of PV cells were excited by pilocarpine in PV- M_1 KO mice (Fig. 4H). Although we have demonstrated that PV BCs lack M_3 mAChRs,¹² it is possible that PV bistratified cells, which are also strongly excited by mAChR activation,¹¹ express both M_1 and M_3 mAChRs.

A subset of PV cells did not enter DB. We determined whether there was any defining physiological characteristic (i.e. input resistance, resting V_m , basal firing rates, AP half-width, AHP depth) that could distinguish DB from non-DB cells. Although we did not find a significant difference in these parameters ($p > 0.05$, Mann-Whitney test), we noted that in 3 cells that showed input resistance higher than $120\text{ M}\Omega$, all of them entered DB, while that in 8 cells showed input resistance lower than $120\text{ M}\Omega$, only 2 cells entered DB. Because PV bistratified cells tend to have a higher input resistance than PV basket cells,¹¹ we suspect that they are more susceptible to cholinergically-induced DB. We look forward to testing this hypothesis in a future study.

Muscarinic excitation of PV cells during PISs

Previous studies have shown that M₁ mAChRs are important for the generation of γ *in vitro*.^{13,14} M₁Rs are the major postsynaptic mAChR subclass expressed on PV cells.^{11,37} These findings led us to investigate their role in pilocarpine-induced PV cell excitation. Pilocarpine neither depolarized nor increased AP firing in PV-M₁KO mice (Fig. 4). Moreover, DB was not observed in PV cells from PV-M₁KO mice. Thus, elimination of the M₁Rs conferred protection of PV cells from becoming overexcited by pilocarpine. Our behavioral results showing that PV-M₁KO mice are associated with reduced seizure severity compared to WT controls (Fig. 5) are consistent with this mechanism. Therefore, we propose that pilocarpine overexcitation of PV cells is a major mechanism that leads to an impairment of inhibitory transmission, causing excitation/inhibition imbalance, and precipitating seizure induction. While the generation of γ itself may contribute to the loss of excitation/inhibition balance through short-term depression (STD) and the depletion of vesicular GABA³⁸ ictogenesis seems to be better timed to the induction of the DB state than gamma firing itself in fast-spiking PV cells.²¹ It is not clear whether STD of PV-mediated IPSCs alone can disrupt excitation/inhibition balance.³⁹ Interestingly, presynaptic M₂ mAChRs, which may also be stimulated during PISs, induce greater presynaptic inhibition at inhibitory than excitatory hippocampal synapses.⁴⁰ There is no obvious seizure phenotype in global M₂ knockout mice,⁹ but it is possible that elimination of both M₁ and M₂ mAChRs from PV cells may confer higher protection from PISs.

Although our studies clearly reveal that pilocarpine excitation of PV interneurons contribute to the severity of PISs, PV-M₁KO mice are not completely protected from PISs. Therefore, other cellular and synaptic mechanisms must play a role in PISs. More complete protection from PISs is observed in global M₁KO mice^{8,9} than in PV-M₁KO mice (Fig. 5), implying that there are non-PV circuit elements activated by M₁Rs that confer further vulnerability to PISs. First, activation of M₁Rs in non-PV interneurons, such as CCK interneurons¹² and O-LM cells³¹ may contribute to the loss of inhibition during PISs. Second, given that M₁Rs are expressed on principal cells,¹⁰ pilocarpine is likely to increase the cellular excitability of glutamatergic neurons through G_q-mediated activation of TRPC7 channels,³⁴ which could indirectly exacerbate depolarization of PV cells through the activation of NMDA receptors,³ kainate receptors,^{4,41} and G_q-coupled metabotropic glutamate receptors.^{42,43} However, by comparing the extent of mAChR-induced depolarization in the presence and absence of the glutamate receptor antagonists DNQX and APV, we find that direct activation of M₁Rs either by muscarine¹¹ or by pilocarpine (Fig. 1) to be a primary mechanism by which mAChRs control the cellular excitability of PV cells. Finally, given that pilocarpine is a fairly nonspecific agonist at all mAChRs,⁴⁴ we cannot exclude the possibility that the activation of other mAChR subtypes could play supporting roles in promoting or sustaining PISs. Thus, while our study shows that M₁Rs on PV cells play a role in PISs, the maintenance of PISs likely involves the temporal coordination of many different circuit elements.

Could cholinergically-induced impairment of PV cells be a mechanism that could contribute to epileptogenesis?—The long-term upregulation of M₁Rs⁴⁵ and recruitment of M₁R-mediated cholinergic potentials⁴⁶ after experimental seizures raises the

possibility that circuit alterations could abnormally activate M₁Rs on PV cells, leading to enhanced susceptibility to seizures in the epileptic hippocampus. Interestingly, a recent study in a tetanus toxin model of temporal lobe epilepsy found that seizure induction is most often observed during exploratory wake and REM sleep,⁴⁷ conditions that are associated with cholinergic activation⁴⁸ and elevated hippocampal acetylcholine levels.⁴⁹ These observations are consistent with a contribution of overexcitation of M₁R on PV cells prior to seizure onset, followed by a reduction of firing frequency during ictal activity.¹⁷ Optogenetic stimulation of PV networks can reduce the emergence of behavioral seizures,⁵⁰ suggesting that PV cells can be recruited to restore excitation/inhibition balance. However, based on our results, the fraction of PV cells available to participate in network activity will likely depend on the extent of G_q receptor activation in PV cells.

Implications for organophosphate-induced seizures

Cholinergic dysfunction is thought to be a factor in diseases such as organophosphate exposure induced neuronal death, where excessive cholinergic activation of central cholinergic receptors causes seizures.^{51,52} In this study, we have gained valuable insights into the mechanisms of pilocarpine-induced modulation of PV cell excitability. PV cells are also excited by bath application of muscarine through M₁R activation.^{11,12} Compared to pilocarpine, which is a partial agonist at M₁Rs, ACh is a full agonist. Therefore, prolonged acetylcholinesterase inhibition induced by centrally acting nerve agents would be expected to induce a more severe DB in PV cells than pilocarpine itself. In addition, a pathological rise in ACh would also be expected to inhibit GABA release from PV terminals via the activation of M₂ mAChRs,^{53,54} which could further impair PV circuit function during organophosphate-induced seizures.⁵⁵ Therefore, anticonvulsive mechanisms that prevent both cholinergic overexcitation and presynaptic inhibition of PV cells could be useful therapeutic avenues to explore in the future.

ACKNOWLEDGEMENTS

We thank Drs. Susumu Tonegawa and David Gerber (MIT) for floxed M₁ mice, Ed Calloway (Salk Institute) and Silvia Arber (University of Basel, Switzerland) for PV-CRE mice, Dr. Bryan Roth (University of North Carolina-Chapel Hill) for hM3Dq-mCherry AAV, Miriam Rose Baker for preliminary data on PIS experiments, Alison Hixon for help with genotyping, and Drs. Laura Ewell, Debbie Smith, and Neil Nathanson for critically reviewing the manuscript. Funding was from NIH R01 NS069689 (JLL), NCRR P20RR015583 (JLL), the Epilepsy Foundation (JLL), a Davidson Honors College Undergraduate Research Award (ED), NSF EPSCOR (KES), the University of Montana Small Grants Program (JLL and KES), and the Montana State University Mountains and Minds Research Experiences for Undergraduates Program (NSF REU 1156855; EM). ED was a Center for Structural and Functional Neuroscience Summer Undergraduate Research Fellow. NCRR P20RR015583, P20RR017670, and P20GM10356 grants supported core imaging and behavioral facilities.

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Figure 1. Hippocampal CA1 PV interneurons are directly excited by pilocarpine

(A) Live YFP (505 nm) fluorescence and (B) live IR Dodt contrast images of a PV-Rosa cell in hippocampal CA1. Representative loose patch recording obtained under (C) ACSF and (D) bath application of 200 μM pilocarpine. E and F were similar experimental design but were conducted in the continuous presence of synaptic blockers (ionotropic glutamate receptor antagonists 50 μM APV, 20 μM DNQX, and 5 μM gabazine). Cumulative probability distributions of instantaneous AP firing frequencies (2 Hz bin width) of population data showing a significant difference between (blue) control (red) and pilocarpine in (G) ACSF ($p < 0.05$ at 2–22 Hz, $n = 5$) and (H) synaptic blockers ($p < 0.05$ at 0–18 Hz, $n = 9$). The inset in G represents the average AP frequency changes in a 1 min window (blue) before and (red) 2–3 min after pilocarpine administration ($p = 0.031$, $n = 5$). In the inset in H, a similar analysis was performed in synaptic blockers ($p = 0.017$, $n = 9$).

Figure 2. Sustained activation of Gq-mediated signaling in CA1 PV interneurons induces gamma frequency firing that progresses to a reduction in AP firing frequency and amplitude

(A) Live mCherry (590 nm) fluorescence and (B) live IR Dodt contrast images of a PV cell expressing hM3Dq-mCherry in the CA1 pyramidal layer. (C) Voltage response during CNO application (red bar) while monitoring the same cell as in A–B in loose patch mode. (D) Instantaneous (grey) and 6-s binned average (blue) firing frequency during CNO application, aligned to the onset of APs in C. The blue arrow denotes onset of gamma firing (threshold at 6-s average of 20 Hz). (E) Individual (grey) and average 6-s (blue) binned AP amplitudes, aligned to the onset of APs in C. (F–H) Expanded 2-s regions (F) before, (G) during firing at gamma firing, or (H) after gamma firing in corresponding regions in C. Individual overlaid APs (grey) and the average (blue) in each 2 s region are shown to the right, demonstrating a progressive reduction in AP amplitude. (I) Population data (averaged in 6 s bins) showing the time course of firing frequency in CNO, aligned to the onset of gamma frequency firing (blue arrow at time 0). There was a significant increase ($p=0.031$) followed by a decrease ($p=0.031$) in AP frequency (Wilcoxon matched pairs signed rank test). (J) Relative AP amplitudes and the corresponding predicted depolarization (averaged in 6 s bins) in CNO, normalized to the first detected AP amplitude in control or CNO

conditions. There was a significant decrease ($p=0.031$) in AP amplitude (Wilcoxon matched pairs signed rank test), corresponding to a large depolarization¹¹.

Figure 3. Pilocarpine induces DB in a subset of CA1 PV cells

(A) Live YFP (505 nm) fluorescence and (B) live IR Diodt contrast images of a PV-Rosa cell in hippocampal CA1. (C) Biocytin labeling (red) of the same cell as in A–B, counterstained with Neurotrace 435/455 (blue). Representative traces (2 sec long) in baseline (D, t1: -1–0 min before pilocarpine), early phase (E, t2: 1–2 min after pilocarpine) and late phase (F, t3: 3–4 min after pilocarpine). (G) Cumulative probability plots of interspike interval (ISI) for t1–t3. (H–K) Representative traces (2 sec long) for a different cell in t1–t3 conditions. Population data (n=11) for (L) V_m and (M) AP frequency in t1–t3 conditions. DB (open squares), non-DB (open triangles), and non-firing cells (open circles) are indicated.

Figure 4. M_1 R_s expressed on CA1 PV cells mediate pilocarpine-induced depolarization, induction of gamma firing, and mAChR-induced progression to DB

(A) Live YFP (505 nm) fluorescence of a YFP-expressing PV cell in a PV- M_1 KO mouse. Inset, live Dodt contrast image. (B) Biocytin labeling (red) of the same cell as in A, counterstained with (blue) Neurotrace 435/455. Stratum oriens (SO), stratum pyramidale (SP), and stratum radiatum (SR) are indicated. Note that the axon arborizes in SP and on the SP/SO border, identifying this cell as a PV basket or axo-axonic cell. Representative traces (2 sec long) in baseline (C, t1: -1-0 min before pilocarpine), early phase (D, t2: 1-2 min after pilocarpine) and late phase (E, t3: 3-4 min after pilocarpine). (F) Cumulative probability plot of ISI for t1-t3. Population data (n=6) of (G) V_m and (H) AP frequency in t1-t3 conditions. Cells that fired in pilocarpine were labeled as open triangles; cells that did not fire were labeled as open circles.

Figure 5. The severity of PISs is reduced in PV-M₁KO mice

Track plots of locomotion in the OFM at 0–5 min and 40–45 min after administration of (A) saline in a C57BL/6 mouse, (B) pilocarpine in a C57BL/6 mouse, (C) pilocarpine in a WT PV-M₁KO mouse, or (D) pilocarpine in a homozygous PV-M₁KO mouse. Note that pilocarpine induces a severe impairment in locomotion. (E–H) Population data for saline-treated C57BL/6 (black, n=5), pilocarpine-treated C57BL/6 (red, n=5), WT PV-M₁KO (blue, n=10), and homozygous PV-M₁KO (green, n=10) mice showing time course of (E) line crossings, (F) speed, (G) time immobile, and (H) rearings (binned in 5 min increments). The

saline-treated C57BL6 group was significantly different than other groups at every time point ($p < 0.05$; two-way ANOVA, post-hoc Sidak's multiple comparison test). **(I)** Time course of seizure severity, as rated on a Racine scale (see Methods), after injection of saline (black, $n=5$) or pilocarpine (red, $n=5$) in C57BL6 mice. **(J)** Time course of seizure severity after pilocarpine injection in WT ($n=10$) and homozygous ($n=10$) PV-M₁KO mice. **(K)** Ranked histogram for maximum seizure severity showing that homozygous PV-M₁KO mice exhibited less seizure severity (4.6 ± 0.5) compared to WT PV-M₁KO (5.9 ± 0.1) ($p=0.029$, one sample t test).

Table 1

Intrinsic membrane properties of hippocampal PV cells.

Property	PV-ROSA DB (n=5)	PV-ROSA non-DB (n=6)	Total PV-ROSA (n=11)	PV-M ₁ KO (n=6)
R _{in} (MΩ)	122.5 ± 27.1	81.5 ± 11.6	100.1 ± 14.6	167.1 ± 23.6
AP half-width (ms)	360.0 ± 39.3	352.5 ± 41.1	355.9 ± 27.3	449.7 ± 54.3
AP height (mV)	49.4 ± 6.8	47.5 ± 4.1	48.4 ± 3.6	62.6 ± 2.1
C _m (pF)	99.6 ± 8.4	123.8 ± 23.3	114.1 ± 14.4	94.4 ± 12.4
τ (ms)	13.0 ± 2.7	9.6 ± 2.3	11.0 ± 1.7	15.5 ± 2.1
AHP (mV)	-4.2 ± 2.1	-2.9 ± 0.4	-3.5 ± 0.9	-3.1 ± 0.8
I _{hold} (pA)	-66.4 ± 27.5	-50.0 ± 38.4	-57.4 ± 23.3	-74.5 ± 37.7
AP Frequency at 700 pA (Hz)	182.2 ± 35.5	225.0 ± 42.6	199.3 ± 26.7	94 ± 8.4

Abbreviations: R_{in}, input resistance; AP, action potential; C_m, capacitance; τ, membrane time constant; AHP, afterhyperpolarization; I_{hold}, bias current introduced to maintain the cell at -60mV.