Gq-Coupled Muscarinic Receptor Enhancement of KCNQ2/3 Channels and Activation of TRPC Channels in Multimodal Control of Excitability in Dentate Gyrus Granule Cells

Chase M. Carver and Mark S. Shapiro

Department of Cellular and Integrative Physiology, University of Texas Health San Antonio, Texas 78229

KCNQ (Kv7, “M-type”) K^+ channels and TRPC (transient receptor potential, “canonical”) cation channels are coupled to neuronal discharge properties and are regulated via $G_{q/11}$ -protein-mediated signals. Stimulation of $G_{q/11}$ -coupled receptors both consumes phosphatidylinositol 4,5-bisphosphate (PIP_2) via phospholipase $C\beta$ hydrolysis and stimulates PIP_2 synthesis via rises in Ca^{2+}_i and other signals. Using brain-slice electrophysiology and Ca^{2+} imaging from male and female mice, we characterized threshold K^+ currents in dentate gyrus granule cells (DGGCs) and CA1 pyramidal cells, the effects of $G_{q/11}$ -coupled muscarinic M_1 acetylcholine (M_1R) stimulation on M current and on neuronal discharge properties, and elucidated the intracellular signaling mechanisms involved. We observed disparate signaling cascades between DGGCs and CA1 neurons. DGGCs displayed M_1R enhancement of M-current, rather than suppression, due to stimulation of PIP_2 synthesis, which was paralleled by increased PIP_2 -gated G-protein coupled inwardly rectifying K^+ currents as well. Deficiency of KCNQ2-containing M-channels ablated the M_1R -induced enhancement of M-current in DGGCs. Simultaneously, M_1R stimulation in DGGCs induced robust increases in $[Ca^{2+}]_i$, mostly due to TRPC currents, consistent with, and contributing to, neuronal depolarization and hyperexcitability. CA1 neurons did not display such multimodal signaling, but rather M current was suppressed by M_1R stimulation in these cells, similar to the previously described actions of M_1R stimulation on M-current in peripheral ganglia that mostly involves PIP_2 depletion. Therefore, these results point to a pleiotropic network of cholinergic signals that direct cell-type-specific, precise control of hippocampal function with strong implications for hyperexcitability and epilepsy.

Key words: electrophysiology; hippocampus; hyperexcitability; muscarinic receptors; potassium channel; signal transduction

Significance Statement

At the neuronal membrane, protein signaling cascades consisting of ion channels and metabotropic receptors govern the electrical properties and neurotransmission of neuronal networks. Muscarinic acetylcholine receptors are G-protein-coupled metabotropic receptors that control the excitability of neurons through regulating ion channels, intracellular Ca^{2+} signals, and other second-messenger cascades. We have illuminated previously unknown actions of muscarinic stimulation on the excitability of hippocampal principal neurons that include M channels, TRPC (transient receptor potential, “canonical”) cation channels, and powerful regulation of lipid metabolism. Our results show that these signaling pathways, and mechanisms of excitability, are starkly distinct between peripheral ganglia and brain, and even between different principal neurons in the hippocampus.

Introduction

Voltage-gated “M-type” (KCNQ, Kv7) K^+ channels are ubiquitously expressed in the nervous system, where they regulate neuronal resting membrane potential, spike-frequency adaptation

(SFA), bursting, and hyperexcitatory states (Cooper and Jan, 2003; Brown and Passmore, 2009; Gamper and Shapiro, 2015; Greene and Hoshi, 2017). M-channel function is modulated by metabotropic G-protein-coupled receptors, providing a powerful mechanism by which neuronal networks alter signaling functions. M-current (I_M) was named due to its depression by stimulation of $G_{q/11}$ -coupled muscarinic acetylcholine receptors (mAChRs) in sympathetic ganglia (Brown and Adams, 1980;

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Correspondence should be addressed to Mark S. Shapiro at shapiro@uthscsa.edu.

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Constanti and Brown, 1981), where the principal mechanism of action is hydrolysis and depletion of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C β (PLC) (Zhang et al., 2003; Suh and Hille, 2007). Stimulation of other G_{q/11}-coupled receptors also depresses I_M, but involves second messengers such as Ca²⁺/calmodulin without depletion of PIP₂ (Gamber et al., 2003; Bal et al., 2010; Kosenko and Hoshi, 2013), as well as Ca²⁺-driven stimulation of PIP₂ synthesis, a pathway long well described in nonexcitable cells (Tuosto et al., 2015). Similar pleiotropic signaling has been shown in heart, governing multimodal regulation of the G-protein-activated inward-rectifier K⁺ (GIRK-type, Kir3) channels, which are under tight control by PIP₂ in cardiac cells and neurons (Logothetis et al., 2015).

Regulation of excitability and circuit behavior has been thoroughly studied in the hippocampus due to its fundamental roles in learning, memory, and cognition (Nicoll, 2017) and abnormally in epilepsy (Poolos and Johnston, 2012; Takano and Coulter, 2012; Dengler et al., 2017). The dentate gyrus (DG) filters external afferent inputs into the hippocampal circuit as a “gatekeeper” of excitability, which is crucial to the processing of physiological (Coulter and Carlson, 2007; Madroñal et al., 2016) and pathophysiological network functions (Krook-Magnuson et al., 2015). Because DG granule cells (DGGCs) are typically quiescent at rest, robust excitatory signaling at these neurons dramatically increases the activity of the hippocampus (Lübke et al., 1998; Kobayashi and Buckmaster, 2003; Hester and Danzer, 2013; Scharfman and Myers, 2016). I_M activity strongly modulates DG excitability, inherent in afterhyperpolarization and spike-frequency adaptation (SFA) (Peters et al., 2005; Singh et al., 2008; Tzingounis and Nicoll, 2008; Petrovic et al., 2012; Soh et al., 2014). The hippocampus is strongly innervated by cholinergic inputs from the medial septum/diagonal band of the basal forebrain (Nyakas et al., 1987; Sotter et al., 2003) and M₁Rs are abundant in neurons of the hippocampus (Levey et al., 1995; Volpicelli and Levey, 2004). The cholinergic/M-channel axis represents a significant mechanism of control of DGGC excitability (Martiniello et al., 2015). Moreover, long-term plasticity and ion channel dysregulation during mAChR stimulation may have consequences on the inhibitory function of I_M in DG and hippocampus. Significant increase to transcription of KCNQ2/3 mRNA in the hippocampus after chemoconvulsant-induced seizures suggests long-term linkage of temporal-lobe states of excitability with M-channel expression and regulation (Zhang and Shapiro, 2012). Loss-of-function mutations in M channels underlie inherited epilepsy disorders (Singh et al., 1998; Biervert et al., 1998) and KCNQ2 or KCNQ3 channel suppression in mice generates epileptic phenotypes (Peters et al., 2005; Singh et al., 2008).

In this study, we sought to elucidate the actions of mAChR stimulation on I_M and delineate the intracellular signaling mechanisms underlying effects on threshold K⁺ currents in the hippocampus. Using brain-slice electrophysiology and Ca²⁺ imaging, we unexpectedly found highly disparate mechanisms regulating excitability between DGGC and CA1 pyramidal neurons. Astonishingly, I_M was strongly enhanced by stimulation of G_{q/11}-coupled mAChRs in DGGCs, as was I_{GIRK} in parallel. Paradoxically, stimulation of mAChRs also increased excitability. We delineate the mechanisms of such phenomena as being via Ca²⁺-mediated stimulation of PIP₂ synthesis upon muscarinic stimulation coupled with simultaneous activation of TRPC (transient receptor potential, “canonical”) cation channels by PLC. Whereas the net effect of muscarinic stimulation in DGGCs is markedly increased excitability, this is due to synergy between G_{q/11}-driven signals involving two opposing ion channels, Ca²⁺

arises from multiple sources and regulation of lipid metabolism. These novel findings reveal intricate but pleiotropic signaling systems in the hippocampus and perhaps elsewhere in the brain, in which input/output coupling of neuron circuitry is crucial to cerebral function and to halting adverse spread of hyperexcitability that, among other events, promotes seizures.

Materials and Methods

Animals. Wild-type (WT) adult male C57BL/6 mice (RRID:IMSR_JAX:000664) between 2 and 4 months of age were used for the electrophysiology experiments in this study. Muscarinic receptor 1 knock-out (M1 KO) mice were also used (Hamilton et al., 1997) and were kindly provided initially by Jurgen Wess (National Institutes of Health). Muscarinic DREADD (Designer Receptors Exclusively Activated by Designer Drug) mice were generated by crossing hMDq-mCitrine (Tg(CAG-CHRM3*-mCitrine)1Ute/J, Alexander et al., 2009; The Jackson Laboratory, RRID:IMSR_JAX:026220) mice with Cre-POMC mice (Tg(Pomc1-cre)16Lowl/J; The Jackson Laboratory, RRID:IMSR_JAX:010714) for DGGC-specific expression (McHugh et al., 2007). KCNQ2 flox/flox mice were acquired as a kind gift from Anastasios Tzingounis (University of Connecticut). All mice were housed in an environmentally controlled animal facility with a 12 h light/dark cycle and had access to food and water *ad libitum*. Animals were cared for in compliance with the guidelines in the National Institutes of Health's *Guide for Care and Use of Laboratory Animals*. All animal procedures were performed in a protocol approved by the Institutional Animal Care and Use Committee of UT Health SA.

Hippocampal slice preparation. Transverse slices (300 μ m) of hippocampus were cut with a vibratome (Thermo Scientific, Microm HM650V) from mice using standard techniques with minor modifications, as reported previously (Carver et al., 2014). Mice were anesthetized with isoflurane and brains excised and placed in artificial CSF (ACSF) at 3.5°C composed of the following (in mM): 126 NaCl, 3 KCl, 0.5 CaCl₂, 5 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 15 glucose, and 0.3 kynurenic acid, with pH adjusted to 7.35–7.40, with 95% O₂–5% CO₂, 305–315 mOsm/kg. Hippocampal slices were maintained in oxygenated ACSF at 30°C for 60 min and experiments were performed at 25°C.

Patch-clamp electrophysiology. Patch pipettes were pulled from borosilicate glass capillaries (KG-33) using a Flaming/Brown micropipette puller P97 (Sutter Instruments) and had resistances between 5 and 7 M Ω . The internal pipette solution consisted of the following (in mM): 110 KCl, 2 EGTA, 2 MgCl₂, 10 HEPES, 4 Na₂-ATP, 2 Na₂-GTP, 10 Tris, phosphocreatine, and 10 tetrapotassium pyrophosphate adjusted to 7.3 pH using KOH at 280 mOsm. In some recordings, a 10 μ M concentration of the cell-impermeant Ca²⁺ indicator calcium green-1 hexapotassium salt (Invitrogen) was added to the internal pipette solution to obtain simultaneous imaging/electrophysiology experiments. Electrophysiological recordings in the slice were performed in the whole-cell patch-clamp configuration. Neurons were visually identified with a Nikon FN-1 microscope equipped with a 40 $\times$ water-immersion objective and infrared differential interference contrast optics and camera. Contrast dye loaded into the pipette was used to routinely confirm intact dendrites and axons of the neurons. Neurons without visible axons or with shortly-cut axons or were not examined. Current and voltage recordings were acquired using a HEKA EPC-9 amplifier and Pulse software (HEKA/Instrutech). Membrane capacitance, series resistance, and input resistance were monitored by applying 5 mV depolarizing voltage steps. Drugs were delivered to the bath chamber using a multichannel perfusion system. All solutions were continuously bubbled with 95% O₂/5% CO₂.

Voltage-clamp protocol. For voltage-clamp recordings, DGGCs were held at –75 mV and CA1 pyramidal cells were held at –65 mV. Whole-cell current signals were digitized at 5–10 kHz and filtered at 2 kHz. The bath recording solution consisted of the following (in mM): 124 NaCl, 3 KCl, 1.5 MgCl₂, 2.4 CaCl₂, 1.25 Na₂H₂PO₄, 26 NaHCO₃, and 15 glucose, adjusted to 7.4 pH and 295–305 mOsm. When adding tetraethylammonium (TEA)-chloride to the external bath solution, it replaced an equivalent amount of NaCl to maintain proper osmolarity. The Na⁺ channel blocker tetrodotoxin (TTX, 1.0 μ M) was added to the bath during

voltage-clamp recordings. M currents were studied in hippocampal neurons with pipette access resistances that averaged $\sim 16\text{ M}\Omega$ under whole-cell mode. Mature granule cells selected for analysis exhibited input resistances of $350\text{--}450\text{ M}\Omega$. For M-current recordings, the potential was held at -25 mV , followed by 600 ms hyperpolarizing steps decrementing from -30 to -80 mV . M-current amplitude was measured at -55 mV (the maximum amplitude of the deactivating current) as the $\sim 100\text{ ms}$ relaxation of the deactivating current sensitive to the M-channel specific blocker XE-991 ($20\text{ }\mu\text{M}$) (Zaczek et al., 1998). E-4031, which binds to the intracellular domain of ERG channels, was routinely supplied both in the internal pipette solution and bath solution ($10\text{ }\mu\text{M}$) to quantify the contribution of ERG channels to the deactivating currents (Cui and Strowbridge, 2018), so E-4031 block could only be quantified as population averages between control and E-4031-perfused neuron groups.

Due to the typical nonspherical shape of neurons in brain slices, series resistance compensation, such as that used in experiments of dissociated neurons is of limited use. The product of the series resistance ($10\text{--}20\text{ M}\Omega$) multiplied by the prepulse current clamped at -20 mV ($200\text{--}300\text{ pA}$) yields an estimate of the whole-cell voltage error, which we estimate to average $2\text{--}6\text{ mV}$. For pipette and bath solutions for which the major cations are K^+ and Na^+ , respectively, the junction potential error (which was not corrected) is typically between -2 and -4 mV (Bernheim et al., 1991). Therefore, the true voltage at which the deactivating relaxation was quantified was estimated to be between -54 mV and -60 mV for each cell, very near the voltage classically used to quantify M current in peripheral neurons (Beech et al., 1991). We found that using careful vibratome technique, but similar pipette solutions used previously (Gamper et al., 2003), except with the inclusion of phosphocreatine, allowed stable whole-cell recordings of I_M in cells with series resistances $<20\text{ M}\Omega$ over a sufficient time period to allow examination of brain slice-perfused drug actions (Kim et al., 2016a,b). Either the general muscarinic receptor agonist oxotremorine methiodide (N,N,N -Trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butan-1-ammonium iodide, oxo-M) or 77-LH-28-1 (1-[3-(4-butylpiperidin-1-yl)propyl]-3,4-dihydroquinolin-2-one oxalate) were used as agonists of endogenous M_1 Rs. M-current sweeps were acquired every 10 s and currents quantified 5 min after the start of bath-application of ACSF to the slice. In control experiments, we estimate the time required to perfuse the slice from initiation of bath-application to be $\sim 1\text{ min}$.

Current-clamp protocol. To derive active discharge properties of neurons, holding current was injected to maintain a membrane potential of -75 mV and square waves of current added in $+20\text{ mV}$ steps for 500 ms with 10 s between each step up to a maximum of 300 pA . The individual properties of action potential waveforms were analyzed using AxoGraph and in-house software. Action potentials were detected with a derivative threshold of 20 mV/ms . Ten to 90% peak rise times were calculated and action potential width measured at 10% of the peak. After hyperpolarization (AHP) amplitudes were measured as the maximum negative trough following an action potential minus the threshold of action potential initiation.

Ca^{2+} imaging. Concurrent with patch-clamp electrophysiology, neurons in slices were also imaged (Nikon FN-1, $40\times$ water-immersion objective) via the fluorescent Ca^{2+} -reporter dye calcium green-1. As a hexapotassium salt, the dye ($10\text{ }\mu\text{M}$) was added to the intracellular patch pipette solution and neurons were patched under whole-cell clamp, as described above. Cells were loaded for 10 min before image acquisition. Fluorescence intensity was obtained using a SOLA Light Engine illumination source (Lumencor) with an output of 3.5 W through a 3-mm -diameter liquid light guide through a Nikon ET-GFP filter. Nikon Elements software was used for image acquisition through a QIClick CCD camera (QImaging). Images were acquired with an exposure time of 200 ms every 2 min using a single-channel, 12-bit rate and no binning. The shutter was closed between each acquisition. For each cell, the corrected total cell fluorescence was measured as the integrated density intensity $-(\text{cell area} \times \text{mean background emission})$. Ca^{2+} signals were measured as the change in fluorescence relative to the baseline fluorescence ($\Delta F/F_0$). Averages of four images were thresholded at 10% , masked using a highly averaged image of the cell, and encoded with a pseudocolor

look-up table. Group cell fluorescence data were averaged in comparison with normalized intensity at each time point.

Behavioral seizure studies. The muscarinic agonist pilocarpine was used to induce seizures *in vivo*. Two-month-old mice were injected with scopolamine methylsulfate (1 mg/kg ; MP Biomedicals) to block peripheral mAChRs 30 min before administration of pilocarpine hydrochloride (280 mg/kg ; Sigma-Aldrich). Drugs were delivered via intraperitoneal injection in 0.9% sterile saline at a total volume equivalent to 1% of the mouse body weight. Control mice were given the same dose of scopolamine followed by vehicle saline 30 min later. Mice were observed for 2 h and all seizure activity was visualized and scored in severity according to the modified Racine scale for mice (Racine, 1972). Within 15 min , mice exhibited continuous tremor activity and tail rigidity. Peak seizure activity was observed 60 min after pilocarpine delivery with occasional forelimb clonus.

Immunohistochemistry. KCNQ2 and KCNQ3 channels were visualized via immunohistochemistry of brain slices of $40\text{--}100\text{ }\mu\text{m}$ thickness, cut with a vibratome, and fixed with methanol for 10 min . Although we and others have found that such slices fixed with paraformaldehyde are not reliable for KCNQ channel detection, so unfixed brain slices were used in the past (Cooper et al., 2001; Pan et al., 2006; Klinger et al., 2011), our control experiments and others (Battfeld et al., 2014) indicate that slices fixed in methanol allow reliable immunodetection of KCNQ2 and KCNQ3 proteins. Free-floating slices were blocked with 8% donkey serum and 0.2% Triton-X in PBS for 1 h . Rabbit α -KCNQ2 antibody (RRID:AB_2314688) and guinea pig α -KCNQ3 antibody (RRID:AB_2314689) were kindly supplied by Ed Cooper (Baylor College of Medicine). Experiments probing with antibodies rabbit α -muscarinic receptor type 1 (Millipore, RRID:AB_91713), goat α -PROX1 (R&D Systems, RRID:AB_2170716), and mouse α -HA(12CA5) (Roche Diagnostics, RRID:AB_514505) were also conducted. Slices were incubated in a $1:1000$ primary antibody dilution for 12 h at room temperature. After $3\times$ PBS washes for 5 min , donkey α -rabbit rhodamine red and donkey α -guinea pig FITC secondary IgG affinis purified antibodies (Jackson ImmunoResearch) were applied $1:250$ for 2 h at room temperature. Sections were mounted with Vectashield reagent (Vector Laboratories) and imaged using a Nikon Eclipse FN1 swept-field confocal upright microscope. Images were obtained at a fixed exposure of 500 ms and a gain of 270 in a 10 MHz , 14-bit readout mode using an Andor iXon3 DU-897 EMCCD camera. FITC and TRITC excitation were performed at 80% laser power using 488 and 561 nm laser lines (MIC 400B; Agilent Technologies). KCNQ channel expression is represented as the mean $\pm$ SEM of the ratio of the emissions from 488 and 561 nm excitation.

Drugs. Chemicals used in electrophysiology experiments were from Sigma-Aldrich unless otherwise specified. XE-991 and xestospongin C were from Abcam. The M_1 R-specific allosteric agonist 77-LH-28-1 was from Aobious. Kynurenic acid, E-4031, ICA-069673, ZD7288, ML204, and M084 were from Tocris Bioscience. Retigabine dihydrochloride was from AdooQ Bioscience.

Experimental design and statistical analysis. Group data are expressed as the mean $\pm$ SEM. Each electrophysiology experimental group was acquired from cells of multiple mice. Statistical comparisons of parametric measures, including electrophysiology data, were performed using an independent two-tailed Student's *t* test, followed by Tukey's HSD test *post hoc*. In all statistical tests, the criterion for statistical significance was $p < 0.05$ unless otherwise specified.

Results

M channels and ERG channels have differential fractional contributions to the threshold K^+ current of DGCCs and CA1 pyramidal neurons

Hippocampal neurons express multiple classes of slowly deactivating, voltage-gated K^+ channels including M channels (KCNQ2/3) and ERG channels (KCNH2, $\text{K}_v11.1$) that activate at potentials near threshold and contribute to regulation of neuronal discharge properties (Saganich et al., 2001; Hu et al., 2002; Jan and Jan, 2012; Mateos-Aparicio et al., 2014; Kim et al., 2016a,b). To determine the contribution of I_M in hippocampal principal

Figure 1. Contribution of I_M and I_{ERG} to the threshold K^+ conductances of DGGCs and CA1 pyramidal cells under brain-slice voltage-clamp conditions. **A**, Hyperpolarizing 10 mV steps of currents from a DGGC under whole-cell voltage-clamp conditions using the protocol shown in the inset. **B**, DGGC voltage-clamp recordings of deactivating currents from -25 mV to -55 mV, with only ACSF bath-application over the indicated time. Scale bars, 100 pA, 0.4 s. **C**, Summarized time courses of current amplitude from the neuron recorded in **B**, with sweeps taken every 30 s. XE-991 ($20 \mu\text{M}$) significantly reduced the deactivating current. **D**, Representative expanded current traces at -55 mV, of full current traces shown in the inset, from a DGGC bathed in control ACSF, or when XE991 ($20 \mu\text{M}$) was added to the bath. Inset scale bars: 50 pA, 500 ms. **E**, Bars summarize amplitudes of the deactivating current relaxation at -55 mV in control solution (ACSF) or when XE-991 ($20 \mu\text{M}$) or E-4031 ($10 \mu\text{M}$) was added to the bath. $n = 20$ –22 cells. $*p < 0.05$ vs ACSF. **F**, Averaged current relaxations as in **D** in control ACSF or in the presence of XE-991 ($20 \mu\text{M}$) or E4031 ($10 \mu\text{M}$). The ordinates are normalized current (left) or cumulative probability of decay (right). The averaged currents in the presence of either blocker were fit by a single exponential relation. **G**, Expanded views of representative deactivating currents from the step from -25 mV to -55 mV step from a CA1 pyramidal cell. The inset shows the full current traces. Inset scale bar: 200 pA, 0.2 s. **H**, Bars summarize the amplitudes of the deactivation currents from CA1 neurons in control (ACSF) or in the presence of XE-991 ($20 \mu\text{M}$) or E-4031 ($10 \mu\text{M}$); $n = 16$ cells. $*p < 0.05$ versus ACSF. **I**, Averaged deactivation currents from CA1 neurons as in **F**. The total current was well fit by a double exponential for which I_M (XE-991-sensitive) contributed to the τ_1 and I_{ERG} channels (E-4031-sensitive) contributed to the τ_2 . **J**, Summary of τ_1 deactivation current kinetics between DGGCs and CA1 neurons from **F** and **I**. **K**, Summary of XE-991 sensitive I_M current between DGGCs and CA1 as in **E** and **H**.

neurons, we examined the deactivating current relaxation at a range of voltages -85 mV to -35 mV (see Materials and Methods) from a holding potential of -25 mV, at which voltages most other K^+ conductances fully inactivate (Fig. 1A). The deactivating current amplitudes and kinetics were examined under whole-cell voltage-clamp electrophysiology in brain slices. In ACSF

recording conditions, I_M amplitude persisted stably for >10 min and negligible run-down of I_M was observed (Fig. 1B,C), suggesting an abundant and stable available pool of PIP_2 or other necessary cytoplasmic molecules. The addition of lipid-kinase substrates such as myo-inositol, which could compromise our experiments, was not required. Likewise, inclusion of Na^+ or

thovanadate (0.1 mM) or pyrophosphate (10 mM) in the pipette solution to inhibit PIP₂ phosphatase activity (Huang et al., 1998) did not significantly alter tonic deactivating current amplitudes nor affect their stability (control: 33.4 ± 1.9 pA, with VPP: 38.3 ± 5.2 pA; $n = 12$ –20 cells per group, $t_{(30)} = 1.05$, $p = 0.3$).

In WT DGGCs, the fraction of current blocked by a saturating concentration of 20 μ M XE-991 (here referred to as I_M) had a current density of 3.60 ± 0.3 pA/pF (Fig. 1D–F). Deactivating currents were well fit by a single exponential with a time constant $\tau = 98 \pm 3$ ms ($n = 15$ cells), which closely corresponds to the kinetics found previously in sympathetic neurons and in hippocampus (Beech et al., 1991; Gamper et al., 2003; Lawrence et al., 2006a). The total current density was 4.42 ± 0.32 pA/pF and the residual current density was significantly reduced to 0.90 ± 0.08 pA/pF after addition of XE-991, indicating that $78.3 \pm 1.8\%$ of the total deactivating current was due to I_M ($t_{(54)} = 10.6$, $p < 0.0001$), with the rest displaying much slower kinetics (Fig. 1E, F). This residual current was also well fit by a single exponential, with a time constant >5 -fold slower (0.6 ± 0.08 s). The pharmacological data are summarized in Figure 1E, which also give an indication of the variation in block of the relaxation current after XE-991 in these experiments. In patch-clamp experiments on dissociated peripheral ganglia neurons or on tissue-culture cells heterologously expressing KCNQ2–4 channels, complete block of current is routinely achieved by XE-991 at 10 μ M (Shapiro et al., 2000; Zhang et al., 2011); however, the multicell diffusion barrier inherent in brain-slice electrophysiology makes such assumptions difficult. As in previous slice work, to determine fractional I_M (Battfeld et al., 2014; Kim et al., 2016a,b; Niday et al., 2017), we can estimate the fraction to be very large in DGGCs. Therefore, we define the total XE-991-sensitive current as the fractional current of KCNQ2/3 channels contributing to I_M (Kim et al., 2016a,b). Although the residual current displayed slower deactivation kinetics that might presume involvement of ERG channels (Wang et al., 1998; Gustina and Trudeau, 2009; Larsen, 2010), addition of the ERG channel blocker E-4031 (10 μ M) to the bath and internal pipette solutions did not significantly alter that current amplitude ($t_{(28)} = 0.6$, $p = 0.55$) nor the deactivation currents ($t_{(28)} = 0.98$, $p = 0.65$, $n = 15$ cells) in DGGCs (Fig. 1E, F), perhaps suggesting another member of the EAG channel family that is insensitive to E-4031, such as Elk2 (KCNH3/K_v12.2) (Saganich et al., 2001; Gessner and Heinemann, 2003).

In CA1 pyramidal neurons, I_M plays a variety of roles in determining active and passive discharge properties, including intrinsic excitability, SFA, bursting behavior, synaptic integration, and resonance (Yue and Yaari, 2004, 2006; Gu et al., 2005; Hu et al., 2007; Tzingounis and Nicoll, 2008; Leão et al., 2009; Fidzinski et al., 2015). As a result, M-channels in CA1 neurons contribute heavily to regulation of neurotransmitter release and synaptic plasticity (Vervaeke et al., 2006; Petrovic et al., 2012) and as a brake to hyperexcitability (Otto et al., 2002). EAG family (KCNH) channels are also expressed in CA1 neurons (Saganich et al., 2001), which are likely responsible for part of the threshold K⁺ current (Cui and Strowbridge, 2018). We performed parallel experiments in CA1 pyramidal neurons in the testing of threshold K⁺ currents under whole-cell voltage-clamp conditions (Fig. 1G, H). The amplitude of the total deactivating current in CA1 was much larger than in DGGCs. In addition, the fraction due to I_M was less, consistent with previous reports of multiple types of threshold K⁺ currents present in CA1 neurons (Saganich et al., 2001; Shah et al., 2008; Klinger et al., 2011; Fano et al., 2012; Zhou et al., 2013). The total deactivating current density was

14.5 ± 1.2 pA/pF and current ascribed to I_M by XE-991 block (20 μ M) was 9.5 ± 1.1 pA/pF ($t_{(30)} = 3.07$, $p = 0.0045$; Fig. 1H). This corresponds to I_M accounting for $64.0 \pm 3.3\%$ of the total deactivating current amplitude, which averaged 51 ± 7 pA. In a separate population of cells, inclusion of E-4031 (10 μ M) in the bath and pipette solutions reduced the average deactivating current amplitude to 7.9 ± 0.4 pA/pF ($t_{(20)} = 3.29$, $p = 0.0036$; Fig. 1H). Deactivating current kinetics in CA1 neurons were best fit with a biexponential function, with values of 95 ± 7 ms and 1.25 ± 0.25 s for τ_1 and τ_2 , respectively (Fig. 1I). The τ_1 exponent was eliminated after application of XE-991, but as for DGGCs, a relaxation with much slower kinetics remained. However, in CA1 neurons, E-4031 effectively eliminated the slow τ_2 component of the relaxation, whereas the fast τ_1 component remained unaltered (Fig. 1I). These results reinforce earlier studies suggesting the partial contribution of the threshold K⁺ current in CA1 pyramidal neurons to be from ERG channels. The faster τ_1 component of the current relaxation in CA1 neurons was indistinguishable from the single-exponent τ determined for the relaxation current in DGGCs (Fig. 1J). We conclude that approximately two-thirds of the deactivating current in CA1 pyramidal neurons is due to I_M and the remainder mostly due to I_{ERG} ; therefore, both conductances in these neurons contribute to the threshold K⁺ current (Fig. 1K).

G_{q/11}-coupled muscarinic receptor stimulation enhances I_M in DGGCs, but suppresses I_M in CA1 pyramidal neurons

Through comparative examination of DGGCs and CA1 pyramidal neurons, the nature of G_{q/11}-coupled mAChR action on the threshold K⁺ channels was examined. We focused on the modulation of I_M , and on the signal transduction mechanisms involved. As above, neurons were studied under whole-cell brain-slice voltage-clamp conditions. From the robust literature documenting M₁R-mediated depression of I_M in multiple types of peripheral neurons and the known increase in neuronal excitability in hippocampus and other brain regions resulting from mAChR stimulation (Bymaster et al., 2003; Shen et al., 2005; Sheffler et al., 2009; Chiang et al., 2010), we expected parallel results from DGGCs and CA1 neurons. However, this was not the case.

In DGGCs, bath-application of the nonselective mAChR agonist oxotremorine methiodide (oxo-M) enhanced I_M , rather than suppressing it, in a concentration-dependent manner (Fig. 2A–C). Maximal enhancement of I_M current density reached 2.43 ± 0.25 -fold at 10 μ M oxo-M (3.27 ± 0.39 vs 7.15 ± 0.70 pA/pF; $t_{(40)} = 4.84$, $p < 0.0001$, $n = 21$ cells). Due to the unanticipated and astonishing nature of these results, we also tested the effects of alternative muscarinic agonists. The muscarinic agonist pilocarpine (10 μ M) also increased I_M in granule cells by 2.45 ± 0.13 -fold (3.8 ± 0.6 pA/pF vs 9.3 ± 1.8 pA/pF; $t_{(6)} = 2.90$, $p = 0.03$, $n = 4$ cells). Although unlikely, it is conceivable that such a result could be due to stimulation of G_{i/o}-coupled M₂/M₄ mAChRs via some unknown pathway. Therefore, we also tested the M₁R-specific allosteric agonist, 77-LH-28-1 (Langmead et al., 2008; Thomas et al., 2008). Bath-application of 77-LH-28-1 over a range of concentrations produced a similar increase of I_M in DGGCs, up to 2.41 ± 0.21 -fold at 10 μ M (Fig. 2D, E). We performed similar experiments using M₁R germline knock-out (M₁KO) mice (Hamilton et al., 1997) and DGGCs with M₁R deletion did not exhibit a significant change in I_M amplitude by bath-application of 77-LH-28-1 (ACSF: 3.2 ± 0.6 pA/pF; 77-LH-28-1: 3.3 ± 0.6 pA/pF, $t_{(8)} = 0.12$, $p = 0.91$, $n = 5$ cells). Given previous findings of muscarinic suppression of I_M in rat DG

Figure 2. Stimulation of $G_{q/11}$ -coupled muscarinic receptors enhances I_M in DGGCs but suppresses I_M in CA1 pyramidal cells. **A**, Superimposed traces of currents in control (ACSF) or in the presence of oxo-M ($10 \mu\text{M}$) from a DGGC under whole-cell clamp. The deactivating relaxations are shown expanded in the inset. **B**, Bars summarize the deactivating current densities from DGGCs in control or in the presence of oxo-M ($10 \mu\text{M}$) or XE-991 ($20 \mu\text{M}$). **C**, Plotted is the enhancement of I_M amplitude as a function of oxo-M or 77-LH-28-1 concentrations. The data were fit by a Hill equation with parameters for oxo-M (EC_{50} : $0.92 \pm 0.36 \mu\text{M}$; $\text{coff} = 0.9 \pm 0.2$) or 77-LH-28-1 (EC_{50} : $1.18 \pm 0.38 \mu\text{M}$; $n = 0.9 \pm 0.2$). **D**, Superimposed traces of deactivating currents in control or in the presence of 77-LH-28-1 ($10 \mu\text{M}$) or XE-991 ($20 \mu\text{M}$). The deactivating relaxations are shown expanded in the inset. **E**, Bars summarize the data for 77-LH-28-1 as in **D**. **F**, Bars summarize effect on I_M by bath-application of 77-LH-28-1 ($3 \mu\text{M}$) in the presence of the PLC-blockers edelfosine or U73122. **G**, Bars summarize the effect of inclusion of Ca^{2+} channel blockers on I_M in DGGC before and after muscarinic stimulation with 77-LH-28-1 ($3 \mu\text{M}$). The bath solution included a mixture of nifedipine ($10 \mu\text{M}$), agatoxin ($0.5 \mu\text{M}$), and conotoxin GVIA ($1 \mu\text{M}$; $n = 8$ cells) or verapamil ($20 \mu\text{M}$; $n = 6$ cells). In the presence of the mixture or verapamil, 77-LH-28-1 induced 1.55 ± 0.05 -fold or 1.83 ± 0.10 -fold increases to I_M , respectively. **H**, Summary of the effect of bath-application of oxo-M ($10 \mu\text{M}$) on suppression of I_M identified as the faster deactivating component of the current relaxation in CA1 pyramidal neurons. On the right are superimposed current traces in the presence of oxo-M or E-4031. * $p < 0.05$; ** $p < 0.001$.

(Martinello et al., 2015), we also performed similar electrophysiology experiments in adult rat DGGCs. Contrary to previous findings, M_1 R stimulation with 77-LH-28-1 ($3 \mu\text{M}$) resulted in robust enhancement of I_M (1.80 ± 0.28 fold, $n = 5$ cells) in the rat slice preparation, similar to what we observed in the mouse (1.93 ± 0.09 fold, as in Fig. 2). Together, these data confirm that the observed enhancement of I_M in DGGCs by mAChR stimulation is indeed driven by $G_{q/11}$ -coupled M_1 Rs.

Given such novel and provocative results, we verified that enhancement of I_M in DGGCs involved activation of PLC, as is usually the case with $G_{q/11}$ actions. Preincubation of slices with the broad-spectrum PLC inhibitor, U73122, blocked any effect on I_M of muscarinic stimulation by 77-LH-28-1 ($10 \mu\text{M}$) and did

not significantly alter I_M amplitudes ($t_{(8)} = 0.21$, $p = 0.83$, $n = 5$ cells; Fig. 2F). However, because previous studies have demonstrated that U73122 induces a negative shift in the voltage dependence of activation of M-channels (Horowitz et al., 2005), we also tested the effects of edelfosine ($10 \mu\text{M}$), a more specific inhibitor of PLC with no agonist activity or observed nonspecific effects on M-channel gating (Horowitz et al., 2005). Similar to U73122, incubation of slices with edelfosine completely abolished M_1 R stimulation-induced enhancement of I_M ($t_{(8)} = 0.94$, $p = 0.38$, $n = 5$ cells; Fig. 2F). Furthermore, previous work has shown that sustained Ca^{2+} influx by voltage-gated Ca^{2+} channels can facilitate I_M by stimulating PKA activity in hippocampal neurons (Wu et al., 2008). To test whether the prepulse depolarization of

DGGCs used in our voltage protocol provoked effects of Ca^{2+} currents through voltage-dependent Ca^{2+} channels on M_1R -mediated enhancement of M current, we tested the effects of voltage-gated Ca^{2+} channel blockers during M_1R stimulation (Fig. 2G). We bath-applied a mixture of nifedipine (10 μM), ω -agatoxin IVA (0.5 μM), and ω -conotoxin GVIA (1 μM) to block L-type, P/Q-type, and N-type channels, respectively. There were no significant differences in the XE-991-sensitive I_M between ACSF (2.9 ± 0.3 pA/pF) and in the presence of the Ca^{2+} channel-blocker mixture (3.0 ± 0.2 pA/pF, $t_{(14)} = 0.278$, $p = 0.78$). However, in the presence of the Ca^{2+} channel blockers, there was significant potentiation of I_M by 77-LH-28-1 (4.2 ± 0.3 pA/pF, $t_{(14)} = 3.32$, $p = 0.004$, $n = 8$ cells), resulting in a 1.55 ± 0.05 -fold increase in the current. We also tested verapamil (20 μM), which has high affinity for L-type and T-type channels at depolarized potentials (Bergson et al., 2011). Again, there were no significant differences of I_M comparing between control (2.9 ± 0.2 pA/pF) and ACSF + verapamil (3.1 ± 0.4 pA/pF; $t_{(10)} = 0.45$, $p = 0.66$), but 77-LH-28-1 application still significantly increased I_M in the presence of verapamil (5.1 ± 0.2 pA/pF; $t_{(10)} = 4.47$, $p = 0.0012$, $n = 6$ cells), resulting in a 1.8 ± 0.1 -fold increase in I_M . Therefore, voltage-gated Ca^{2+} channel blockade does not significantly alter the facilitation of I_M by stimulation of M_1Rs .

To determine whether the resulting potentiation that we observed was unique to DGGCs or is generalized throughout the hippocampus, we then investigated the effects of stimulation of M_1Rs on I_M in CA1 pyramidal neurons. In CA1, previous evidence has shown I_M to be suppressed in pyramidal neurons by muscarinic stimulation (Madison et al., 1987; Fiszman et al., 1991; Rouse et al., 2000). In parallel experiments to those performed on DGGCs, we assayed the effect of M_1R stimulation of CA1 neurons under whole-cell brain-slice voltage-clamp conditions (Fig. 2H). Bath-application of oxo-M (10 μM) suppressed the deactivating current amplitude by $79.1 \pm 2.8\%$ ($n = 10$ cells), as defined by the faster deactivating τ_1 of ~ 100 ms, similar to previous reports of I_M suppression in the slice (Rouse et al., 2000). In the presence of muscarinic agonists to CA1 neurons, there was a persistent > 1 s deactivating current, which was eliminated in the presence of 10 μM E-4031. Similarly, bath-application of 77-LH-28-1 to CA1 induced a suppression of I_M (oxo-M: $78.3 \pm 2.4\%$; 77-LH-28-1: $81.6 \pm 2.3\%$, $n = 5$ –7 cells per group), confirming the involvement of M_1Rs . XE-991 blocked the total deactivating current in CA1 neurons by 9.45 ± 1.01 pA/pF, leaving a residual current of 5.07 ± 0.55 pA/pF, whereas oxo-M application resulted in a 10.34 ± 0.89 pA/pF suppression of the total deactivating current, leaving a residual current of 2.91 ± 0.42 pA/pF. Therefore, stimulation of M_1Rs in CA1 pyramidal neurons profoundly suppresses I_M similar to the effect in peripheral ganglia. Therefore, the functional actions of $\text{G}_{q/11}$ -coupled M_1Rs on M-channels are starkly divergent between DGGCs and CA1 pyramidal neurons.

PIP₂ synthesis is strongly enhanced by stimulation of $\text{G}_{q/11}$ -coupled muscarinic receptors in DGGCs

Because stimulation of M_1Rs in DGGCs resulted in enhancement, rather than suppression of I_M , we hypothesized an increase in PIP_2 abundance to be responsible for the increase in the current far above its tonic level. In a variety of tissues, stimulation of $\text{G}_{q/11}$ -coupled receptors stimulates PIP_2 synthesis concurrently with PLC hydrolysis, driven by rises in $[\text{Ca}^{2+}]_i$ (Lassing and Lindberg, 1990; Racaud-Sultan et al., 1993; Loew, 2007). Both physiological data in cerebellar neurons and biophysical model-

ing (Finch and Augustine, 1998; Takechi et al., 1998; Brown et al., 2008) indicate that stimulation of $\text{G}_{q/11}$ -coupled receptors must increase PIP_2 abundance in dendrite spine membrane by 2.5-fold to account for accumulation of local intracellular IP_3 . In sympathetic neurons, subcellular microdomain organization precludes M_1R stimulation from inducing IP_3 -mediated $[\text{Ca}^{2+}]_i$ rises, allowing substantial PIP_2 depletion, whereas bradykinin B_2R stimulation, for example, provokes sufficient rises in $[\text{Ca}^{2+}]_i$ and stimulation of PIP_2 synthesis to compensate for any decrease in PIP_2 abundance by PLC hydrolysis (Falkenburger et al., 2010). Whereas it is estimated that the abundance of PI in the plasma membrane of mammalian cells is $\sim 100,000$ molecules/ μm^2 , the tonic abundance of PIP_2 is ~ 1000 – 5000 molecules/ μm^2 (Xu and Loew, 2003; Xu et al., 2003; Suh et al., 2004; Winks et al., 2005; Hilgemann, 2007). At this tonic abundance of PIP_2 , a large body of work indicates M channels composed of KCNQ2/3 heteromers to be well under full saturation by PIP_2 interactions (Suh et al., 2004; Hernandez et al., 2009; Falkenburger et al., 2010), consistent with a maximal open probability P_o of KCNQ2/3 heteromers of ~ 0.3 , as experimentally measured at the single-channel level (Selyanko et al., 2001; Li et al., 2004). Indeed, this must be the case to make possible regulation of membrane proteins by PIP_2 because, otherwise, physiological changes in either PIP_2 abundance or PIP_2 affinity would have little effect (Hernandez et al., 2008a,b).

To test the hypothesis that stimulation of PIP_2 synthesis occurs subsequent to M_1R stimulation to DGGCs, we probed GIRK channels that have been demonstrated to require PIP_2 interactions for gate opening (Huang et al., 1998; Glaaser and Slesinger, 2017) and are highly expressed in hippocampus (Karschin et al., 1996; Ponce et al., 1996). Previous work in CA1 pyramidal cells demonstrated that M_1R stimulation reduces GIRK current, presumably via depletion of PIP_2 (Sohn et al., 2007), but to our knowledge, such actions on GIRK channels in DG have not been investigated (Lüscher et al., 1997; Lüscher and Slesinger, 2010). Therefore, using receptor-induced, endogenous GIRK current amplitudes as a PIP_2 “biosensor,” an increase of PIP_2 subsequent to M_1R stimulation should also increase GIRK currents in DGGCs. We measured GIRK currents elicited by stimulation of GABA_B receptors (Lüscher et al., 1997) using baclofen (100 μM) and GIRK amplitudes compared between control and 10 min after bath-application of muscarinic agonist to the slice. These experiments were performed in high $[\text{K}^+]_o$ (20 mM) for GIRK currents to be inward at a holding potential of -75 mV and of sufficient amplitude to reliably measure (Slesinger et al., 1997). We found that DGGCs from slices perfused with oxo-M (10 μM) displayed significantly greater GABA_B -activated GIRK currents (oxo-M + baclofen, $\Delta 110.0 \pm 12.5$ pA) compared with control ACSF condition (baclofen alone, $\Delta 31.0 \pm 6.6$ pA, $t_{(10)} = 5.59$, $p = 0.002$, $n = 6$ cells; Fig. 3A,B). Likewise, specific stimulation of M_1Rs with 77-LH-28-1 enhanced the amplitude of baclofen-elicited GIRK currents (77-LH-28-1 + baclofen, $\Delta 123.1 \pm 12.9$ pA; baclofen alone ($\Delta 19.3 \pm 2.8$ pA, $t_{(4)} = 7.86$, $p = 0.01$). Furthermore, incubation of slices with edelfosine ablated the changes to GIRK amplitudes normally stimulated by M_1Rs ($t_{(8)} = 0.94$, $p = 0.373$; Fig. 3B). Therefore, I_M and GIRK currents respond in parallel fashion to $\text{G}_{q/11}$ -coupled mAChR stimulation, which is wholly consistent with increase in PIP_2 as the underlying modulatory mechanism.

PIP_2 is synthesized by sequential phosphorylation of phosphatidylinositol (PI) and PI 4-phosphate (PIP) by PI-4 kinase and phosphatidylinositol-4-phosphate-5 kinase (PIP5K), respectively (Di Paolo et al., 2004; Balla et al., 2008; Volpicelli-Daley et

Figure 3. Stimulation of PIP₂ synthesis after mAChR stimulation underlies the enhancement of I_M in DGGCs. **A**, Gap-free current recordings before and after stimulation of GABA_B receptors with baclofen (100 μ M) to evoke inward currents from GIRK channels with 20 mM [K⁺] in the bath. **B**, Bars summarize the baclofen-evoked current at the holding potential of -75 mV before (control, white bars) and after bath-application of oxo-M or 77-LH-28-1 (10 μ M; gray bars) or in the presence of edelfosine (10 μ M; striped bar). * $p < 0.05$ versus control; # $p < 0.05$ versus + mAChR agonist; $n = 5$ –8 cells per group. **C**, Superimposed are deactivating I_M currents from DGGC in the presence of the PIP5K inhibitor UNC3230 (3 μ M) over a range of 0–4 min after bath-application of 77-LH-28-1 (3 μ M). **D**, Bars summarize the XE-991-sensitive M-current density in the presence of UNC3230 before and after bath-application of 77-LH-28-1. * $p < 0.05$; $n = 7$ cells.

al., 2010). Furthermore, tonic PIP₂ abundance is maintained by the homeostatic activity of lipid kinases and phosphatases, reflecting basal turnover (Falkenburger et al., 2010). We assayed the effect of M₁R stimulation on I_M in DGGCs using UNC 3230, a small-molecule inhibitor of PI(4)P 5-K1C/PI4 K2C kinases (Wright et al., 2014; Fig. 3C,D). Dialysis of cells with UNC 3230 (3 μ M, pipette solution) resulted in significantly reduced basal I_M amplitudes (2.39 ± 0.46 pA/pF, $n = 7$ cells, $t_{(33)} = 3.06$, $p = 0.008$). In addition, stimulation of M₁Rs by 77-LH-28-1 (3 μ M) now resulted in significant suppression of I_M by $61.8 \pm 5.3\%$ (0.99 ± 0.11 pA/pF, $t_{(12)} = 2.96$, $p = 0.012$, $n = 7$ cells). Therefore, upon inhibition of PIP5-K1C/PI-4K2C, M₁R stimulation suppressed I_M in DGGCs, which our evidence shows to occur by depletion of PIP₂, due to the impairing of provoked PIP₂ synthesis.

KCNQ2-deficient DGGCs exhibit altered gating properties and hyperexcitability

Given that most M channels in the nervous system are heteromers of KCNQ2/3 of varying stoichiometry (Gamper and Shapiro, 2015), we exploited the divergent sensitivity of KCNQ2 and KCNQ3 homomers and KCNQ2/3 heteromers to block by TEA (Shapiro et al., 2000; Hadley et al., 2000) to estimate the contribution of KCNQ2 and/or KCNQ3 to I_M in DGGCs. We found the IC₅₀ for block of I_M by TEA to be 6.5 ± 0.4 mM (Fig. 4A) in control DGGCs. At 30 mM TEA, there was $84.0 \pm 3.0\%$ reduction in the fast component of M current and 100 mM TEA was sufficient to completely abolish the current with a mean $98.8 \pm 0.7\%$ reduction (Fig. 4A). This is consistent with I_M in DGGCs being

almost wholly due to KCNQ2/3 heteromers. Although KCNQ5-containing channels have been described in CA1 (Tzingounis et al., 2010), they display a much lower sensitivity to TEA block than do KCNQ2/3 heteromers (Schroeder et al., 2000) and are therefore not likely to significantly contribute to I_M in DGGCs. Our TEA data are consistent with the previous observation that KCNQ5 expression in the DG is very low (Tzingounis et al., 2010). To further confirm that the current in DGGCs that we ascribe to I_M is indeed composed of KCNQ2 and KCNQ3 subunits, we investigated DGGC-selective deletion of KCNQ2 by crossing KCNQ2^{lox/lox} mice with Cre-POMC mice (McHugh et al., 2007). Analysis of KCNQ2^{lox/lox}/Cre-POMC⁺ mice (KCNQ2-deficient) and their control KCNQ2^{lox/lox}/Cre-POMC[−] (non-Cre, KCNQ2-control) littermates revealed Cre-recombination to be not 100% efficient in all animals, resulting in a strong knock-down, but not “knock-out,” of KCNQ2 subunits as detected by immunoblot measurement of protein ($t_{(8)} = 4.31$, $p = 0.003$) and immunohistochemistry (Fig. 4B,C). We used the TEA dose–response relation to estimate the degree of KCNQ2 knock-down in these neurons. The sensitivity of I_M to TEA current block was significantly reduced compared with non-Cre control DGGCs (Fig. 4A). In KCNQ2-deficient DGGCs, the best estimate of TEA block was IC₅₀ of 82 ± 11 mM TEA, significantly right-shifted in potency compared with KCNQ2-control DGGCs ($t_{(13)} = 6.39$, $p < 0.0001$, $n = 8$ cells). Using the previously described binomial distribution model for TEA block of KCNQ2- and KCNQ3-containing tetramers (Shapiro et al., 2000), we determined I_M in KCNQ2-control mice to be composed of channels within 1% of a 1:1 ratio of KCNQ2 and

Figure 4. KCNQ2-containing M-channels are required for the mAChR action on I_M and excitability in DGGCs. **A**, TEA sensitivity of deactivating currents from DGGCs of KCNQ2-control (squares) or KCNQ2-deficient (circles) DGGCs. The data were fit by a Hill equation with the coefficient unconstrained; with values for KCNQ2-normal neurons of IC_{50} : 6.5 ± 0.4 mM; coefficient: 1.1 ± 0.1 and for KCNQ2-deficient neurons of IC_{50} : 82 ± 11 mM; coefficient: 1.0 ± 0.3 , $n = 7$ –8 cells per group. **B**, Summarized semiquantified values of DG-localized, KCNQ2 protein immunoblots from Cre-POMC⁺/KCNQ2^{flx/flx} (KCNQ2-control) or Cre-POMC⁺/KCNQ2^{flx/flx} (KCNQ2-deficient) mice normalized to GAPDH ($n = 3$ mice per group). **C**, Confocal 3D renderings of KCNQ2 immunostaining of fixed DG slices from KCNQ2-control and KCNQ2-deficient mice. Right, Line-scan plots of KCNQ2-labeled fluorescence intensity from either KCNQ2-control (Cre-) or KCNQ2-deficient (Cre+) DG granule layers. **D**, Bars summarize I_M density (pA/pF) in KCNQ2-control and KCNQ2-deficient mice. $*p < 0.05$; $n = 13$ –15 cells per group. **E**, Bars summarize the deactivating current density of KCNQ2-deficient DGGCs in control or bath-application of 77-LH-28-1 (3μ M) or XE-991 (20μ M); $n = 11$ cells. $*p < 0.05$ versus ACSF condition. **F**, Summarized deactivating current amplitudes of DGGCs from KCNQ2-control (closed squares) and KCNQ2-deficient (open circles) mice in control, in the presence of 77-LH-28-1 (3μ M), or TEA over the range from 0.3 – 10 mM. $*p < 0.05$ versus KCNQ2-deficient. **G**, Bars summarize the potentiation of I_M by the M-channel opener retigabine (10μ M) in DGGCs from KCNQ2-control or KCNQ2-deficient mice. $*p < 0.05$ versus KCNQ2-control. **H**, Representative current-clamp recordings of evoked action potentials in DGGCs from KCNQ2-control and KCNQ2-deficient mice. **I**, Summarized action-potential spike frequencies as a function of injected current. $*p < 0.05$; $n = 8$ –14 cells per group. **J**, Values of summarized initial spike intervals (ISIs) and final spike intervals (FSIs) during evoked action potentials by current injection of 100 or 200 pA from KCNQ2-control and KCNQ2-deficient DGGCs. $*p < 0.05$ versus KCNQ2-control.

KCNQ3 subunits, whereas I_M from KCNQ2-deficient DGGCs was estimated to be due to channels containing a 0.15:0.85 ratio of KCNQ2 to KCNQ3 subunits, which means that there will be many KCNQ3 homomers, and many heteromers containing only one KCNQ2 subunit, but few others compositions. In DGGCs from KCNQ2-deficient mice, I_M current density at -55 mV was significantly reduced, to 2.2 ± 0.2 pA/pF ($n = 13$), compared with 3.6 ± 0.3 pA/pF DGGCs from non-Cre littermates ($t_{(28)} = 3.88$, $p = 0.0006$, $n = 15$ cells; Fig. 4D). We were unable to detect any significant differences in the rate constants of the current relaxations between KCNQ2-normal and KCNQ2-deficient neurons, consistent with KCNQ2 and KCNQ3 displaying similar deactivation current kinetics (Gamper et al., 2003). Given that macroscopic currents from recombinant KCNQ3 homomers is ~ 6 -fold less than that of KCNQ2/3 heteromers (Li et al., 2005; Zaika et al., 2008), we were surprised by the modest nature of the reduction in I_M current density in KCNQ2-deficient DGGCs. Nonetheless, the data presented below are wholly consistent with the degree of knock-down suggested by the TEA dose-response, immunostaining, and immunoblot results. We suspect that the KCNQ3-containing channels produce much larger currents when endogenously expressed in brain than when heterologously expressed; however, there is little evidence that KCNQ3 channels traffic as homomers in neurons (Chung et al., 2006a,b). Previous work in cultured superior cervical ganglion neurons found that removal of KCNQ2 leads to increase in KCNQ3 mRNA (Robbins et al., 2013). The mechanisms of compensatory expression upon KCNQ2 deletion may be distinct between peripheral ganglia and brain and even further down to cell-type differences in transcriptional regulation within a given brain region. We observed a modest increase in the protein levels of KCNQ3 subunits in KCNQ2-deficient mice, relative to KCNQ2-normal DGGCs ($t_{(8)} = 2.32$, $p = 0.048$; Fig. 4B) and axonal KCNQ3 expression remained robust in KCNQ2-deficient mice (Fig. 4C).

Muscarinic enhancement of I_M is ablated in KCNQ2-deficient DGGCs

If stimulation of M_1 Rs in DGGCs enhances I_M due to increased PIP_2 abundance, then another diagnostic test of this hypothesis would be to determine whether KCNQ2 deficiency limits the M_1 R-induced enhancement of I_M . The apparent affinity of PIP_2 for KCNQ2 homomers is nearly 80-fold lower than for KCNQ3 homomers, with KCNQ2/3 heteromers intermediate, and the open probabilities at saturating voltages are 0.14 for KCNQ2 homomers, ~ 0.3 for KCNQ2/3 heteromers and >0.9 for KCNQ3 homomers at normal basal PIP_2 intracellular concentrations (Li et al., 2004, 2005; Telezhkin et al., 2012). Therefore, an increase in tonic PIP_2 abundance can significantly increase macroscopic KCNQ2 currents and could increase KCNQ2/3 currents, but has little effect on currents from KCNQ3 channels, the latter being already fully saturated with PIP_2 (Li et al., 2004). Therefore, we compared I_M of DGGCs from KCNQ2-deficient mice with KCNQ2-normal littermate controls. Whereas stimulation of M_1 Rs in non-Cre controls with 77-LH-28-1 ($3 \mu M$) resulted in a 1.9 ± 0.1 -fold increase in I_M density (3.27 ± 0.28 pA/pF, $n = 12$), stimulation of M_1 Rs in DGGCs from KCNQ2-deficient mice did not significantly affect I_M density (before stimulation: 2.21 ± 0.21 pA/pF vs after stimulation: 2.13 ± 0.29 pA/pF; $t_{(22)} = 0.22$, $p = 0.82$; Fig. 4E). However, I_M from KCNQ2-deficient DGGCs was strongly blocked by XE-991 ($20 \mu M$; Fig. 4E). To confirm the strong knock-down of KCNQ2 in these experiments and to determine whether the mAChR-mediated enhanced I_M in control neurons is from KCNQ2/3 heteromers, we examined block by

TEA for both groups of neurons in the presence of 77-LH-28-1 (Fig. 4F). For the neurons with unaltered expression of KCNQ2 subunits, the TEA IC_{50} blockade of the enhanced current occurred at 3 mM and above, indicating that the bulk of current was from KCNQ2/3 heteromers. However, for the KCNQ2-deficient DGGCs, in which I_M was not affected by mAChR stimulation, the current was highly insensitive to TEA, as expected and representative of KCNQ3 homomers.

Retigabine (RTG) is the prototypic anticonvulsant compound that targets M-channels (Rundfeldt and Netzer, 2000). Based on the above findings based on increases in tonic PIP_2 , we wished to compare the action of RTG on I_M between DGGCs of KCNQ2-control mice with those from KCNQ2-deficient mice. RTG is known to enhance M-channel opening in a bimodal fashion: by shifting the voltage dependence of activation to more negative potentials and by increasing channel open probability at all voltages (Tatulian et al., 2001; Tatulian and Brown, 2003). Whereas RTG ($10 \mu M$) enhanced I_M in KCNQ-control DGGCs, it did not significantly alter I_M amplitudes in KCNQ2-deficient neurons (Fig. 4G). These results are consistent with those of Tatulian et al. (2001), who showed that RTG does not increase the fractional voltage dependence of KCNQ3 channels at -25 mV, the voltage tested here, but does shift the voltage dependence of KCNQ2 channels, which is therefore consistent with the different open probabilities of KCNQ2, KCNQ3, and KCNQ2/3 channels (Selyanko et al., 2001; Li et al., 2004).

Only a 25% reduction in I_M in brain is sufficient for the manifestation of epileptic seizures (Schroeder et al., 1998). Therefore, we investigated the effects of KCNQ2 deficiency on DGGC discharge properties. Under whole-cell current-clamp conditions, KCNQ2-deficient granule cells displayed greater excitability properties, including increased action potential frequency, reduced SFA, and increased interspike intervals (Fig. 4H–J). In addition, the rheobase for action potential firing was of significantly smaller amplitude for KCNQ2-deficient DGGCs (KCNQ2-normal: 73.5 ± 3.0 pA; KCNQ2-deficient: 33.4 ± 4.6 pA; $t_{(20)} = 7.62$, $p < 0.0001$). All of these altered properties are consistent with reduced I_M amplitudes, underscoring the sensitivity of DGGCs to even modest changes in I_M . They also predict that M-channel openers such as RTG are likely to be less efficacious in the clinical setting to treat hyperexcitability syndromes resulting in decreased expression of KCNQ2 gene products such as reported in animal pain models (Mucha et al., 2010), but more efficacious for disorders such as idiopathic epilepsy, in which KCNQ2 expression in brain may be selectively upregulated (Zhang and Shapiro, 2012).

Effects of mAChR stimulation on I_M of DGGCs do not correlate with the alteration in neuronal excitability

DGGCs exhibit a very low basal firing rate (Jung and McNaughton, 1993). Furthermore, mAChR stimulation increases DGGC firing and mossy fiber neurotransmission to CA3 and CA1 (Vogt and Regehr, 2001). Our data here show an increase in I_M upon M_1 R stimulation, which would ordinarily be expected to decrease excitability, presenting us with an apparent conundrum. To parse this question, we first assayed the changes in active and passive discharge properties of DGGCs in response to M_1 R stimulation in the slice (Fig. 5). Bath-application of oxo-M ($10 \mu M$) shifted the action potential threshold of DGGCs from WT mice by -5.6 ± 1.0 mV (ACSF: -46.3 ± 0.8 mV, oxo-M: -51.3 ± 1.2 mV; $t_{(16)} = 3.47$, $p = 0.004$, $n = 9$ cells), whereas in DGGCs from M_1 R KO mice, the mean shift of -0.9 ± 0.5 mV was not significantly different (ACSF: -47.2 ± 1.7 mV, oxo-M: -48.1 ± 1.8

Figure 5. Hyperexcitability of DGGCs induced by stimulation of M₁ AChRs. **A, B**, Representative current-clamp recordings of evoked action potentials in DGGCs from WT (**A**) or M₁R KO (**B**) mice in response to mAChR stimulation by oxo-M (10 μ M). **C, D**, Plots summarize the change in action potential frequency induced by application of oxo-M (10 μ M) or XE-991 (20 μ M) in DGGCs from WT (**C**; $n = 10$ cells) or M₁R KO (**D**; $n = 7$ cells). Scale bars, 100 ms, 50 mV. Action potential properties derived from current-clamp experiments are listed in Figure 5-1, available at <https://doi.org/10.1523/JNEUROSCI.1781-18.2018.f5-1>. **E**, Representative action potential waveforms before (black) and after mAChR stimulation with oxo-M (blue). **F**, Summarized membrane potential values (voltage floor) over ranges of current injection (0–280 pA) from neurons before (ACSF) or after stimulation of mAChRs with oxo-M (10 μ M). $n = 7$ cells. * $p < 0.05$ versus ACSF.

mV; $t_{(12)} = 0.36$, $p = 0.72$, $n = 7$ cells). During whole-cell current-clamp recordings with zero injected current (i.e., at resting membrane potential, V_m), DGGCs did not spontaneously fire. However, bath-application of oxo-M (10 μ M) depolarized

V_m significantly and increased spontaneous action potential firing. Input/output relationships of DGGCs were compared between control conditions, during M₁R stimulation, or when I_M was blocked. Action potential firing rate was quantified as a func-

tion of injected current over a range from 0 to 250 pA. During control conditions, neurons exhibited pronounced SFA, thought to be heavily dependent on I_M . Stimulation of M_1 Rs with oxo-M (10 μ M) strongly increased the action potential frequency at all values of injected current and abolished SFA to the point at which by 140 pA, the firing rate could not increase further due to “depolarization block” (accumulated inactivation of Na^+ channels; Fig. 5A, C). Interestingly, under block of I_M with XE-991 (20 μ M), the rate of firing significantly increased and SFA was reduced, but both effects were less compared with muscarinic agonist, suggesting that the latter involves other factors in addition to control of I_M .

Parallel experiments were performed on slices from M_1 KO mice. Compared with WT, basal excitability of neurons was reduced, and SFA was more pronounced, possibly incident of a low level of tonic activity of M_1 Rs in slices from WT mice or to greater expression of M channels in M_1 KO mice. As expected, DGGCs from M_1 KO mice did not display changes to neuronal firing as a result of bath-application of oxo-M (Fig. 5B, D). However, block of I_M with XE-991 (20 μ M) strongly increased action potential frequency and significantly decreased SFA (Fig. 5C). In slices from WT mice, action potential width was significantly increased upon stimulation of neurons by oxo-M (ACSF: 4.01 ± 0.17 ms; oxo-M: 4.67 ± 0.27 ms, $t_{(12)} = 2.07$, $p = 0.03$). The AHP displayed a significantly slower rate of decay (12.9 ± 1.4 vs 18.8 ± 0.4 ms, $t_{(12)} = 4.05$, $p = 0.001$, $n = 7$ cells), resulting in a significantly larger area of the AHP (defined as $mV \cdot ms$; 173 ± 23 vs 270 ± 12 ; fold change: 1.79 ± 0.23 , $n = 7$ cells, $t_{(12)} = 3.74$, $p = 0.002$; Fig. 5E and Fig. 5-1, available at <https://doi.org/10.1523/JNEUROSCI.1781-18.2018.f5-1>). A complementary parameter is the mean potential of the cell during the time of current injection, called the “voltage floor.” In these experiments, a tonic level of current was first injected to maintain the cell at -75 mV to isolate voltage changes due to action potential firing. Because the voltages involved are in the pivotal range for action potential initiation, these magnitudes in ΔV are highly significant for affecting excitability. At nearly all values of injected current, stimulation of M_1 Rs caused a significant depolarization of the mean potential, ranging from 4 to 10 mV (Fig. 5F). As a measure of the instantaneous SFA, the initial interspike interval was significantly increased after bath-application of oxo-M at the lowest current that elicited two action potentials (Fig. 5-1, available at <https://doi.org/10.1523/JNEUROSCI.1781-18.2018.f5-1>), meaning that muscarinic stimulation induced hyperexcitability of DGGCs.

Selective stimulation of M_1 Rs with 77-LH-28-1 (0.3 or 3 μ M) resulted in similar increase to the excitability of DGGCs in slices from WT mice (Fig. 6A). Action-potential threshold was significantly reduced at 3 μ M 77-LH-28-1 (-44.7 ± 0.9 mV vs -51.9 ± 1.7 mV; $t_{(10)} = 3.74$, $p = 0.0038$, $n = 6$ cells). As a complementary experiment to those above in voltage clamp that suggested M_1 R-induced stimulation of PIP_2 synthesis, we performed current-clamp recordings in the presence of UNC 3230 (3 μ M) to determine whether block of PIP_2 synthesis would affect the intrinsic firing properties of DGGCs accordingly. In the presence of the PI(4)P-5K inhibitor, DGGCs demonstrated greater action potential frequency (Fig. 6B) and the AHP amplitude was significantly reduced after M_1 R stimulation compared with action potentials in control conditions ($t_{(24)} = 9.57$, $p < 0.0001$; Fig. 6C). Therefore, the current-clamp and voltage-clamp recordings under block of PI(4)P-5 kinases are consistent with a reduction of I_M . Blockade of nicotinic acetylcholine receptors with the antagonist hexamethonium (50 μ M; Papke et al., 2010) did not significantly affect DGGC excitability (Fig. 6D).

Exogenous G_q -coupled muscarinic DREADD receptors enhance I_M in DGGCs

As another way to isolate the G_q -coupled muscarinic receptor signaling input onto DGGCs, we used DREADDs, which allow tissue- and cell-specific interrogation of signal transduction when driven behind the appropriate promoter for expression of Cre-recombinase (Roth, 2016). We inserted exogenous G_q -coupled muscarinic hM3Dq receptors (Alexander et al., 2009) in the brains of mice to determine whether selective stimulation with its cognate synthetic agonist, clozapine-*N*-oxide (CNO), would affect I_M similarly to stimulation of endogenous M_1 Rs in DGGCs. To produce germline mice expressing hM3Dq receptors exclusive to DGGCs, we crossed floxed hM3Dq-HA mice with mice expressing Cre driven by the POMC promoter (Alexander et al., 2009; Zhu et al., 2016; Fig. 7A). Specific expression was confirmed via visualization of the mCitrine fluorescent tag of the DREADDs in brain slices during electrophysiology recordings (Fig. 7B). Upon bath-application of CNO to DREADD-expressing DGGCs (Cre⁺/DREADD^{+/−}), I_M amplitude was maximally increased 2.51 ± 0.26 -fold by 1 μ M CNO, similar to the effect observed upon stimulation of endogenous M_1 Rs (Fig. 7D). As a negative control, we recorded from DGGCs of DREADD transgenic mice without Cre-recombinase (Cre[−]/DREADD^{+/−}) that did not express hM3Dq receptors. CNO had no significant effect on I_M in DGGCs from Cre[−]/DREADD^{+/−} mice; however, oxo-M application still significantly enhanced I_M amplitude ($t_{(12)} = 2.50$, $p = 0.028$, $n = 7$ cells; Fig. 7D). Therefore, stimulation of exogenous G_q -coupled mAChRs in DGGCs enhances I_M similarly to native M_1 Rs. Furthermore, in DGGCs from Cre⁺/DREADD^{+/−} mice, bath-application of CNO (0.1 or 0.3 μ M) hyperpolarized the action potential threshold (Fig. 7C) and significantly increased the action potential frequency (Fig. 7E). Together, these data indicate that stimulation of G_q -coupled mAChRs robustly increases DGGC excitability and that there must be other downstream effectors of PLC activation that contribute to this effect, in seeming counteraction to the potentiation of I_M .

M-channel openers enable I_M to counteract the M_1 R-induced hyperexcitability of DGGCs

Stimulation of mAChRs in GABAergic neurons in the *stratum oriens* of CA1, which spontaneously fire rapid action potentials, are quickly silenced via drug-induced augmentation of I_M (Lawrence et al., 2006a,b,c). We investigated whether first enhancing I_M activity with M-channel openers before muscarinic stimulation would counteract the excitability while still enabling greater I_M augmentation in DGGCs (Fig. 8). To accomplish this, we acquired current-clamp recordings of evoked action potentials in the presence of RTG (10 μ M) or the KCNQ2/3-selective compound, ICA-069673 (10 μ M). Following this, we bath-applied 77-LH-28-1 (3 μ M) for 10 min and then again recorded evoked action potentials. The presence of RTG or ICA-069673 maintained low spiking frequency of DGGCs after mAChR stimulation compared with the robust hyperexcitability observed during 77-LH-28-1 without preapplication of M-channel openers (Fig. 8A). Whereas control stimulation of M_1 Rs with 77-LH-28-1 significantly reduced the rheobase of current required for evoking action potential firing ($t_{(8)} = 5.71$, $p = 0.0005$), in the presence of either RTG or ICA-069673, the rheobase did not significantly change after application of 77-LH-28-1 (RTG: $t_{(10)} = 2.11$, $p = 0.06$; ICA-069673: $t_{(10)} = 0.69$, $p = 0.50$; Fig. 8B). Therefore, preapplication of M-channel openers before mAChR stimulation

Figure 6. Selective stimulation of endogenous M_1 muscarinic receptors induces DGGC hyperexcitability, mediated by stimulation of PIP_2 synthesis. **A**, Summarized action potential frequencies before (ACSF, filled circles) and after bath-application of 77-LH-28-1 at 0.3 μM (open squares) or 3.0 μM (filled triangles; $n = 6–8$ cells per group, $*p < 0.05$ versus ACSF). **B**, Representative current-clamp recordings of action potentials in the presence of UNC3230 (3 μM) before (control) and after bath-application of 77-LH-28-1 (3 μM). **C**, Bars summarize the AHP amplitudes before and after 77-LH-28-1 application in the presence of UNC3230. Blockade of PIP_2 synthesis during mAChR stimulation resulted in hyperexcitability of DGGCs, consistent with M-current suppression. $*p < 0.05$. **D**, Summarized action potential frequencies plots before (ACSF, filled squares) and after bath-application of hexamethonium chloride (50 μM , open circles; $n = 4$ cells).

enables I_M to inhibit and fully oppose the hyperexcitability produced by mAChRs.

Stimulation of $G_{q/11}$ -coupled M_1 Rs of DGGCs induces increases in $[\text{Ca}^{2+}]_i$, mostly via TRPC channels, the activity of which mediates increased excitability

Our data in DGGCs thus far indicate that M_1 R stimulation enhances I_M amplitude, yet robustly increases active and passive excitability, conferring higher rates of action potential firing. We hypothesized the involvement of another excitatory ion channel that would be activated downstream of $G_{q/11}$ activation and PLC. We investigated whether stimulation of M_1 Rs could provoke rises in $[\text{Ca}^{2+}]_i$ because such rises have been shown to be the determining factor in whether $G_{q/11}$ -coupled receptor stimulation depletes PIP_2 in sympathetic neurons (Hernandez et al., 2008a,b). Indeed, cholinergic stimulation has been shown to increase $[\text{Ca}^{2+}]_i$ in the soma and mossy fiber axons of DGGCs (Itou et al., 2011). To investigate $[\text{Ca}^{2+}]_i$ dynamics of DGGCs in slices, we performed Ca^{2+} imaging in whole-cell mode with the cell-

impermeant fluorescent reporter calcium-green 1 in the internal pipette solution (Fig. 9A). In response to M_1 R stimulation by 77-LH-28-1 (3 or 10 μM), peak fluorescence increased 2.91 ± 0.41 -fold or 4.27 ± 0.95 -fold, respectively (Fig. 9B). The Ca^{2+} signal began to increase within 1 min after muscarinic agonist application to the slice, concurrent with the time at which granule cell action potential firing frequency increased, as well as enhancement of I_M amplitude. $[\text{Ca}^{2+}]_i$ reached a maximum, as detected by dye fluorescent emission, 10 min after application of muscarinic agonist. Whereas calibration of the intensity dye is not feasible (Zhou and Neher, 1993), we estimate, based on our previous work, that the maximum $[\text{Ca}^{2+}]_i$ rise to be well above 1 μM . When slices were preincubated with edelfosine (10 μM), 77-LH-28-1 had no significant effect on $[\text{Ca}^{2+}]_i$ (maximum dye emission = 1.15 ± 0.08 -fold over that before agonist; Fig. 9A,B). An important question is whether these $[\text{Ca}^{2+}]_i$ rises evoked by muscarinic stimulation require the release of Ca^{2+} from intracellular ER stores. To address this, we repeated Ca^{2+} imaging experiments using the IP_3 receptor inhibitor xestospongine C (10 μM)

Figure 7. Stimulation of exogenous G_q -coupled muscarinic DREADD receptors enhances I_M in DGGCs. **A**, Confocal images of hippocampal slices immunostained for HA-tagged hM3Dq receptors (green) and counterstained with PROX1 (red), a cellular marker of DGGCs, from a Cre POMC⁺ mouse. The hM3Dq-HA tagged fluorescence was present in the molecular (dendrites) and granule (soma) layers of the DG, but not the hilus (mossy fiber axons). CrePOMC⁺ hM3Dq mice displayed exclusive expression of DREADD receptors to the somatodendritic compartment of DGGCs. The CA1 and CA3 regions did not demonstrate hM3Dq expression. Inset, Confocal micrograph from a CrePOMC[−] hippocampus negative control demonstrating no hM3Dq receptor expression. **B**, Fluorescent images show either CrePOMC⁺ hM3Dq-mCitrine fluorescence (top) or CrePOMC[−] (bottom) granule layer live slices used for electrophysiology recordings obtained using a 488 nm emission filter. **C**, Summarized current-clamp action potential thresholds of hM3Dq-expressing DGGCs before (ACSF) and after bath-application of CNO (0.1 or 0.3 μ M; $n = 7$ cells, $*p < 0.05$). **D**, Bars summarize the enhancement of I_M by bath-application of CNO to DGGCs with transgenic insertion of hM3Dq DREADDs (Cre⁺ DREADD^{+/−}, gray solid bars) specific to DG or in control littermate DGGCs not expressing hM3Dq-DREADDs from mice that lack the Cre gene (Cre[−] DREADD^{+/−}, striped bars). The dashed line represents mean I_M amplitude for ACSF (control) in Cre⁺ DREADD^{+/−} mice. **E**, Summarized current-clamp action potential spike frequencies from hM3Dq-expressing DGGCs ($n = 7$ cells).

to block IP₃ receptor-mediated release of Ca²⁺ (Gafni et al., 1997). We found that despite blockade of IP₃ receptors, mAChR stimulation still provoked substantial rises in [Ca²⁺]_i (Fig. 9A, B) and increase to DGGC hyperexcitability was still observed upon mAChR stimulation, as indicated by increased action potential firing frequency (Fig. 9C).

What excitatory ion channel could be activated downstream of mAChR-induced PLC activity, act as a source of sustained rise in [Ca²⁺]_i, and induce marked increases in excitability of DGGCs? TRPC cation channels are activated by PLC-mediated signals (Strübing et al., 2001), permeant to Ca²⁺ influx, and are known to be highly expressed in DG and hippocampus, consisting of TRPC1/4/5 heteromers (Chung et al., 2006a,b; Ramsey et al., 2006; Fowler et al., 2007; Wu et al., 2010; He et al., 2012; Bröker-Lai et al., 2017). These facts suggest a putative link for TRPC conductances to serve as a potent mechanism that could modulate neurotransmitters and excitability in brain via metabotropic receptors, similar to the regulatory control of M channels. Interestingly, TRPC channel opening is also facilitated by depletion of intracellular Ca²⁺ stores (Boulay et al., 1999; see Discussion). Therefore, we hypothesized TRPC channel contribution to

the mAChR-induced hyperexcitability of DGGCs. Indeed, in the presence of saturating concentrations of TRPC4/5 blockers (M084 and ML204, 10 μ M; Miller et al., 2011; Zhu et al., 2015), increases in [Ca²⁺]_i by muscarinic stimulation were mostly but not completely blunted, again, as detected by calcium-green 1 emission (maximum 1.61 $\pm$ 0.21-fold; Fig. 9A, B). To further investigate whether activation of TRPC channels is the mechanistic basis for the muscarinic increase in DGGC excitability, we performed parallel current-clamp recordings as before, comparing the muscarinic response in the presence or absence of the TRPC4/5 blocker M084 (10 μ M). In this series of experiments, M₁R stimulation with 77-LH-28-1 (3 μ M) again significantly increased the action potential firing rate (Fig. 9D). However, under conditions of TRPC4/5 channel blockade, bath-application of M₁R agonist did not increase the firing rate of action potentials (Fig. 9D and Fig. 9-1, available at <https://doi.org/10.1523/JNEUROSCI.1781-18.2018.f9-1>), nor was there a significant shift in the action potential threshold (M084 only: -45.4 ± 1.3 mV, M084 + 77-LH-28-1: -46.1 ± 1.5 mV, $t_{(14)} = 0.35$, $p = 0.73$, $n = 8$ cells) or the average voltage floor across the range of voltages tested (Fig. 9E). However, blockade of TRPC channels did not

Figure 8. Pretreatment with M-channel openers prevents mAChR-induced hyperexcitability in DGGCs. **A**, Preapplication of either RTG or ICA-069673 ablated increases to DGGC spike frequency due to muscarinic stimulation by 3 μ M 77-LH-28-1 in the slice. **B**, Summarized current amplitudes required to evoke a single action potential (rheobase) in current-clamp recordings of DGGCs. Control condition indicates rheobase in the presence of either ACSF or M-channel opener application before muscarinic stimulation (white bars) compared with 10 min after application of 77-LH-28-1 (striped bars). * $p < 0.05$ versus control; $n = 7$ –10 cells per group.

prevent the enhancement of I_M amplitude by M_1R stimulation. With TRPC4/5 channels blocked, M_1R stimulation (3 μ M 77-LH-28-1) resulted in a 1.83 ± 0.14 -fold enhancement of I_M amplitude ($n = 6$ cells), which was similar to the enhancement observed in the absence of TRPC4/5 channel blockers (1.92 ± 0.10 -fold). Analysis of AP properties during current injection with TRPC4/5 channels blocked revealed increases in the decay time constant of the AHPs induced by M_1R stimulation (14.8 ± 0.6 vs 17.1 ± 0.8 ms, $t_{(10)} = 2.30$, $p = 0.044$, $n = 6$ cells) and in the mean area of the AHPs (197 ± 11 vs 265 ± 22 ms $\cdot$ mV, $t_{(10)} = 2.76$, $p = 0.02$), again, consistent with M_1R -mediated potentiation of I_M (Fig. 9-1, available at <https://doi.org/10.1523/JNEUROSCI.1781-18.2018>). However, with TRPC4/5 channels blocked, stimulation of M_1R s had no significant effect on either parameter at each value of injected current, consistent with no change to excitability under those conditions (Fig. 9E). The mechanistic reasons for this and their implications are elaborated on in the Discussion. We wondered whether there were microdomain-localized Ca^{2+} signals mediated by IP_3 Rs driving stimulation of PIP_2 synthesis, as has been proposed in sympathetic ganglia (Delmas and Brown, 2002; Winks et al., 2005; Zaika et al., 2007). However, inclusion of xestospongine C (10 μ M) in the pipette, as before, retained M_1R -mediated enhancement of I_M (1.67 ± 0.14 -fold, $n = 8$ cells).

Our findings are consistent with our hypothesis of TRPC4/5 (likely coupled with TRPC1) activation downstream of activation of PLC and PIP_2 hydrolysis, mediating at least most of the increased excitability of DGGCs upon muscarinic stimulation that has been observed by us and by others. We note here that such stimulatory input must be quite strong because it opposes and clearly overwhelms the decrease in excitability that should otherwise occur upon enhancement of I_M . Feedback rises in $[Ca^{2+}]_i$ corroborate the likelihood of this scenario (Ordaz et al., 2005). However, the contribution of PLC-produced diacylglycerol (DAG) in particular remains unclear given previous evidence of inactivation of TRPC4/5-containing channels by DAG (Venkatachalam et al., 2003). With PIP_2 synthesis increased by several-

fold upon muscarinic stimulation, production of DAG and its density in the membrane should correspondingly increase as well. The TRPC4/5-activated augmentation of the excitatory drive of DGGCs would thus be the result of the combinatorial actions of M_1R -mediated stimulation of PIP_2 synthesis, amplified PLC-mediated PIP_2 hydrolysis, release of $[Ca^{2+}]_i$, and subsequent feedforward amplification of TRPC channels (Fowler et al., 2007; see Discussion).

The hyperexcitability that we ascribe to activation of TRPC4/5 channels by M_1R stimulation in DGGCs led us to wonder whether TRPC4/5 channels could be key regulators of the spread of epileptogenic seizures. Prior evidence suggests the involvement of TRPC family channels in the control of excitability in other distinct neurons in the hippocampus (Strübing et al., 2001; Michel et al., 2005; Tai et al., 2011; Bröcker-Lai et al., 2017). Moreover, TRPC4/5 blockade has been demonstrated to suppress depression-like and anxiety behaviors of mice *in vivo* (Yang et al., 2015), similar to profiles of several classes of anticonvulsant drugs with mechanisms of action that target neuronal inhibition. Our Ca^{2+} -imaging experiments showed that TRPC4/5 blockade ablates most of the M_1R -induced rise in $[Ca^{2+}]_i$, suggesting that global TRPC4/5 blockade in the brain might preclude seizures mediated by undue Ca^{2+} influx. To test this hypothesis *in vivo*, we investigated TRPC4/5 channel block in the prevention of chemoconvulsant-induced seizures in mice using the well established pilocarpine seizure model (Fig. 9F). This model has the advantage of widespread muscarinic receptor stimulation as the trigger for spreading hyperexcitability. After administration of scopolamine (1 mg/kg), control mice were pretreated with saline vehicle and then were administered pilocarpine (280 mg/kg) 30 min later. All control animals developed strong progression of seizures that resulted in status epilepticus (mean maximum Racine seizure stage: 4.8 ± 0.2). However, littermates that were treated with the TRPC4/5 antagonist M084 (10 mg/kg) 30 min before the same dose of pilocarpine demonstrated significantly reduced seizure behavior (mean maximum Racine seizure stage: 1.1 ± 0.5) and status epilepticus was not observed in any animal

Figure 9. Muscarinic stimulation of DGGCs induces intracellular rises in $[Ca^{2+}]$ that is mostly due to TRPC4/5 channels, which underlie M_1 R-induced hyperexcitability of DGGCs. **A**, Calcium green-1 fluorescence before and after bath-application of 77-LH-28-1 (3 μ M) alone or in the presence of edelfosine (10 μ M), the TRPC channel blockers M084 and ML204 (10 μ M), or with the IP_3 R blocker, xestospongine-C (10 μ M), in the internal pipette solution. Images depict 16-color pseudocolor heat maps of fluorescence intensity. **B**, Summarized changes in fluorescence intensity over 0–12 min after perfusion of slices with 77-LH-28-1. $n = 5$ –7 cells per group. $*p < 0.05$ versus 77-LH-28-1 + edelfosine; $\dagger p < 0.05$ versus 77-LH-28-1 alone. **C**, Summarized evoked action potential frequencies before (filled squares) and after application of 77-LH-28-1 (open circles) in the presence of xestospongine-C. **D**, Summarized evoked action potential frequencies during TRPC blockade with M084 and ML204 before (filled circles) and after bath-application of 77-LH-28-1 (open triangles) $*p < 0.05$ versus TRPC block, control. **E**, Plotted are summarized membrane potential values (voltage floor) in the presence of the TRPC4/5 channel antagonist M084 (10 μ M). There were no significant differences in any values between those obtained in control ACSF and during application of 77-LH-28-1 (3 μ M). $*p < 0.05$; $n = 8$ cells. Action potential properties derived from current-clamp recordings in presence of TRPC block are listed in Figure 9-1, available at <https://doi.org/10.1523/JNEUROSCI.1781-18.2018.f9-1>. **F**, *In vivo* pretreatment with TRPC channel antagonists confers protection from pilocarpine-induced chemoconvulsant seizures. Plots summarize the Racine seizure stage progression of adult mice that were treated with vehicle or M084 (10 mg/kg) to block TRPC4/5 channels 30 min before challenge with pilocarpine (280 mg/kg). $*p < 0.05$ versus vehicle control; $n = 8$ mice per group.

($n = 8$ mice per group; Fig. 9F). Because TRPC4/5 blockade suppressed chemoconvulsant seizures throughout the brain in this assay, these results are suggestive of a widespread role of TRPC channels in epileptogenesis (Zheng, 2017). Therefore, TRPC channel inhibitors may represent a mode of anticonvulsant therapeutic control of seizures and retardation of the development of epilepsy disease from a variety of causes.

Discussion

The modulation of M-type K^+ channels by $G_{q/11}$ -coupled mAChRs is a key regulatory mechanism of excitability, discharge properties, and circuit function of the hippocampus (Gamper and Shapiro, 2015). However, neither the precise contribution of I_M to the threshold K^+ current nor the effects of $G_{q/11}$ -coupled mAChR stimulation on I_M has been comparatively described among hippocampal principal neurons. Our whole-cell recordings afforded excellent voltage control of the soma and AIS of the neurons and we were able to take advantage of the signature voltage dependence, pharmacology, and kinetics of M channels to isolate I_M for analysis. Moreover, neuron-specific knock-down of KCNQ2 via transgenic mice allowed us to offer conclusive evidence for the identity of the K^+ channels under investigation.

We focused on DGGCs and CA1 pyramidal neurons to probe mAChR-related neuromodulation of the hippocampus. Although stimulation of M_1 Rs in DGGCs increased excitability in accordance with previous reports (Tzingounis and Nicoll, 2008; Mateos-Aparicio et al., 2014; Soh et al., 2014; Martinello et al., 2015), the effect on I_M was a robust potentiation of conductance, rather than its depression, suggesting mAChR-mediated stimulation of PIP_2 synthesis as the most likely mechanism. To support this hypothesis, we investigated the well known sensitivity of GIRK channels to PIP_2 abundance (Huang et al., 1998; Hilgemann et al., 2007) and, indeed, I_{GIRK} was increased in a closely parallel manner as I_M in the same cells. We did not observe a transient decrease in I_M , such as due to Ca^{2+} /calmodulin action, before the stimulation of PIP_2 synthesis that we ascribe to I_M augmentation. In CA1 pyramidal cells, Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels was shown to potentiate I_M (Wu et al., 2008). However, we demonstrate in DGGCs that the enhancement of I_M occurs independently of VGCC currents (Fig. 2G). Loew and colleagues previously showed that, in the case of bradykinin-evoked action on I_M in neuroblastoma cells, stimulation of PIP_2 synthesis occurs (Xu and Loew, 2003; Xu et al., 2003) and I_M increases (Xu and Loew, 2003) before suppression of current occurs. Recent evidence suggests that the plasma membrane pools of PI(4)P and PIP_2 may be independently regulated by PLC-dependent, G-protein-coupled receptor stimulation and that the replenishment of PIP_2 does not underlie receptor-stimulated depletion of PI(4)P from the plasma membrane (de Rubio et al., 2018). In lymphocytes, in which PLC-directed stimulation of PIP_2 synthesis has long been known to occur (Lassing and Lindberg, 1990; Racaud-Sultan et al., 1993), the increase of PIP_2 abundance is due to a positive feedback amplification loop of hydrolysis followed by PI(4)P-5K-dependent synthesis (Xu et al., 2017). We posit that the accumulation of PIP_2 we observed in DGGCs may proceed via a similar mechanism, given that PI(4)P-5K inhibition eliminated the PLC-driven enhancement of I_M (Fig. 3).

Given that an enhancement in I_M should lead to a decrease in excitability, the fact that we observed a muscarinic-induced increase in excitability led us to investigate whether an excitatory current might be concomitantly activated downstream of M_1 R stimulation and we hypothesized significant contribution of

TRPC channels as the underlying target. In stark contrast, stimulation of M_1 Rs in CA1 pyramidal neurons depressed I_M similar to the effect in peripheral ganglia, suggesting the absence of concomitant stimulation of PIP_2 synthesis and consequential PIP_2 depletion in those neurons (Fig. 2H). Therefore, the $G_{q/11}$ -coupled mAChR, PLC-dependent gating of M channels, which is present in both types of hippocampal neurons, are modulated in dramatically distinct ways. Our results emphasize the stark contrast in muscarinic signaling between DG and CA1 that have strong influences on neuronal discharge properties and hippocampal function.

M-channel modulation has been most prevalently studied in sympathetic neurons. There, receptor-specific stimulation of PIP_2 synthesis has been shown to depend on a parallel receptor-specific rise in $[Ca^{2+}]_i$ from IP_3 -gated stores (Gamper et al., 2004; Winks et al., 2005; Zaika et al., 2011). Ca^{2+} imaging of DGGCs in brain slices revealed a strong increase in $[Ca^{2+}]_i$ that persisted during maintained M_1 R agonist exposure (Fig. 9) even after the agonist was washed off. What is the source of the relevant Ca^{2+} signal? In most excitable cells, this is a complex issue because cytoplasmic rises in $[Ca^{2+}]$ can be due to opening of IP_3 Rs or ryanodine receptors and influx of Ca^{2+} through several different types of Ca^{2+} -permeable channels, including TRP-family types (Ramsey et al., 2006; Prakriya and Lewis, 2015). Moreover, these Ca^{2+} sources are typically not fully independent because Ca^{2+} ions originating from one source have been shown to influence the opening of the others. Ca^{2+} -induced Ca^{2+} release is closely involved with ryanodine receptors, which are critical to memory formation in hippocampus (Baker et al., 2013). Here, we found stimulation of M_1 Rs in DGGCs to induce a prolonged rise in $[Ca^{2+}]_i$ that was only slightly reduced by blockade of IP_3 Rs and strongly attenuated by blockade of TRPC channels (Fig. 7B). In sympathetic and sensory neurons, receptor-induced stimulation of PIP_2 synthesis is entirely dependent upon IP_3 Rs, as are the profound $[Ca^{2+}]_i$ rises in spines of cerebellar Purkinje neurons by stimulation of mGluRs (Finch and Augustine, 1998; Takechi et al., 1998; Loew, 2007). We found that, for DGGCs, the greater fraction of the M_1 R-mediated $[Ca^{2+}]_i$ rises are due to TRPC channels, with a much smaller fraction due to IP_3 Rs, yet blockade of neither affected potentiation of I_M . Because the rate-limiting step in PIP_2 synthesis is PI 4-kinase activity and stimulation of PI 4-kinase is closely associated with intracellular Ca^{2+} signals (Balla et al., 2008), we are reluctant to exclude Ca^{2+} signaling from this process in DGGCs. In addition, activation of protein kinase C recruited by A-kinase anchoring protein 79/150 accessory to M-channels (Hoshi et al., 2003, 2005; Zhang et al., 2011; Kosenko et al., 2012) was not tested in the neurons. From the data in this study, we demonstrate that stimulation of PIP_2 synthesis must be involved, because blockade of PI(4)P 5-kinases converted enhancement of I_M to its suppression (Figs. 3, 6).

We found that TRPC4/5 channel blockade abolished the increase in neuronal excitability by muscarinic agonists, yet excitability did not decrease with TRPC channels blocked and I_M enhanced. However, some thought explains this. Due to the relatively high input resistance of DGGCs, their resting potential is normally quite negative to the threshold for activation of M channels and their basal rate of firing is low. Additionally, the activation kinetics of M channels are an order of magnitude slower than the time scale of an action potential (Gamper and Shapiro, 2015). Therefore, enhancement of I_M by stimulation of PIP_2 synthesis cannot alter excitability until excitatory stimulation becomes strong enough to foster rapid firing and greater membrane depolarization during more rapid action potential firing, resulting in

greater opening of M-channels, promoting two opposing drives on excitability. A decrease in excitability is favored, based on the laws of Goldman–Hodgkin–Katz; that is, a greater K^+ conductance opposing depolarization away from E_K . However, the strong correlation between I_M amplitudes and the AHP area evident in our data indicate that the contribution of M channels to AHPs in DGGCs is particularly substantial. Therefore, sustained firing of action potentials in DGGCs produces a strong depolarization of the average potential (voltage floor; Fig. 5) facilitated by the high impedance of DGGCs, inducing partial depolarization block via Na^+ channel inactivation. The M channel-mediated augmentation in AHP area thus promotes greater excitability from relief of accumulated inactivation of Na^+ channels. This paradoxical effect is reminiscent of the results of Vervaeke et al. (2006), studying the relationship of I_M and excitability in CA1 pyramidal neurons, in which they mimicked a depolarized voltage floor produced by sustained action potentials with modestly elevated $[K^+]_o$ and found relief of depolarization block by greater I_M activation to increase excitability. Our analysis shows that, with both M-channels and TRPC4/5 channels activated, the resulting voltage floor is significantly depolarized (Fig. 5F) with overall greater excitability as the result. However, with TRPC4/5 channels blocked, there was no net change in the voltage floor (Fig. 9E) even though I_M is still enhanced. Furthermore, when M-channel openers were applied to negatively shift the voltage dependence of KCNQ2-containing channels, I_M enhancement substantially counteracted the M_1R -induced cascade of excitability in DGGCs (Fig. 8). Therefore, the two opposing actions of I_M on excitability, which clearly depend on the degree of excitation of the neurons, are likely to closely balance themselves out in DGGCs.

A previous study (Martinello et al., 2015) examined cholinergic/muscarinic actions of DGGCs both from stimulation of cholinergic fibers in the slice and from stimulation of mAChRs by exogenous agonists, the latter of which we do here. Their study and ours both demonstrate a pronounced increase in excitability of DGGCs subsequent to stimulation of $G_{q/11}$ -coupled M_1R s. We also find a strong increase in excitability if M channels are totally blocked by XE-991, yielding concordant conclusions as to the critical role of I_M in DGGC firing. However, our results strikingly diverge in that we demonstrate convincing evidence that alteration in I_M from M_1R stimulation depends on PIP_2 abundance and, moreover, we find that I_M is enhanced by M_1R agonists as opposed to M channels being inhibited. We replicated our findings in both mouse and rat DGGCs, observing equal enhancement of I_M in both at room temperature. We also performed all of our voltage-clamp experiments under whole-cell mode with much more favorable access resistance for the required biophysical analysis of the K^+ currents (Kim et al., 2016a,b). Martinello and colleagues found T-type Ca^{2+} channels to be involved in mAChR modulation of DGGCs, but we did not observe any effect of T-type channels on the enhancement of I_M (Fig. 2G).

Our data point to TRPC4/5 channel activation as the source of the observed persistent rises in $[Ca^{2+}]_i$ upon stimulation of mAChRs rather than any type of VGCCs. The evidence includes that somatic Ca^{2+} influx occurred upon M_1R stimulation during voltage clamp of DGGCs at -75 mV; however, we realize that, under those conditions, we are unable to effectively voltage clamp the distal axon as well. The bulk of the $[Ca^{2+}]_i$ rise that we observed was ablated by blocking TRPC4/5 channels. TRPC channels are regulated by the very downstream products of PLC activation subsequent to $G_{q/11}$ -mAChR stimulation and are therefore a plausible underlying source of both the pronounced

increase in neuronal excitability and most of the increase in $[Ca^{2+}]_i$. It is important to note that the time course of changes in I_M did not include a transient suppression of I_M before its enhancement (Fig. 2), suggesting either a lack of close proximity of M_1R s and M channels, as opposed to their intimate association in sympathetic ganglia (Zhang et al., 2016), or an abundant pool of PIP_2 in DGGCs that is insulated from any initial PLC-induced PIP_2 hydrolysis. We have yet to do advanced microscopy and microdomain analysis of the subcellular localization of these signaling molecules required to answer such questions.

The exquisite, multimodal neuromodulation by M_1R s in DGGCs that we report here highlights the pivotal role of the DG as a filter for the hippocampus in controlling excitability. An equally interesting line of investigation would be to determine whether pathophysiological cholinergic challenges to the DG are sufficient to promote epileptogenic propagation of seizures throughout the brain (Sloviter et al., 2012), also mediated by regulation of TRPC channel activation. Finally, these regulatory cascades may be instrumental to disorders of hyperexcitability or hypoexcitability, such as epilepsy or cognitive dysfunction.

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