

The mechanisms shaping CA2 pyramidal neurons action potential bursting induced by muscarinic acetylcholine receptor activation

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Running Title: High cholinergic tone alters CA2 pyramidal neuron activity.

Key Points:

This is the first examination of the effect of muscarinic receptor activation on hippocampal CA2 pyramidal neuron activity.

Carbachol application causes CA2 pyramidal neurons recorded in acute hippocampal slices to depolarize and fire bursts of action potentials.

Synaptic transmission shapes the timing of bursting dynamics in area CA2 and is also attenuated by activation of M1 and M3 muscarinic receptors.

T-type calcium channels, D-type potassium channels and SK channels all contribute to regulating the action potential bursts of CA2 pyramidal neurons.

eTOC Summary

Robert et al. reveal that muscarinic receptor activation causes hippocampal CA2 pyramidal neurons to depolarize and fire bursts of action potentials. The properties of these bursts are regulated by synaptic inputs, as well as T-type calcium channels, D-type potassium channels, and SK channels.

Abstract:

Recent studies have revealed that hippocampal area CA2 plays an important role in hippocampal network function. Disruption of this region has been implicated in neuropsychiatric disorders. It is well-appreciated that cholinergic input to the hippocampus plays an important role in learning and memory. While the effect of elevated cholinergic tone has been well studied in areas CA1 and CA3, it remains unclear how changes in cholinergic tone impact synaptic transmission and the intrinsic properties of neurons in area CA2. In this study we applied the cholinergic agonist carbachol and performed on-cell, whole-cell and extracellular recordings in area CA2. We observed that under conditions of high cholinergic tone, CA2 pyramidal neurons depolarized and rhythmically fired bursts of action potentials. This depolarization depended on the activation of M1 and M3 cholinergic receptors. Furthermore, we examined how the intrinsic properties and action-potential firing were altered in CA2 pyramidal neurons treated with 10 μ M carbachol. While this intrinsic burst firing persisted in the absence of synaptic transmission, bursts were shaped by synaptic inputs in the intact network. We found that both excitatory and inhibitory synaptic transmission were reduced upon carbachol treatment. Finally, we examined the contribution of different channels to the cholinergic-induced changes in neuronal properties. We found that a conductance from Kv7 channels partially contributed to carbachol-induced changes in resting membrane potential and membrane resistance. We also found that D-type potassium currents contributed to controlling several properties of the bursts, including firing rate and burst kinetics. Furthermore, we determined that T-type calcium channels, and small conductance calcium-activated potassium channels play a role in regulating bursting activity.

Introduction

While hippocampal area CA2 was first described by Lorente de No over 70 years ago (No, 1934), relatively little is known about the cellular properties of the neurons in this region. Recent studies using *in vivo* recording methods have revealed that neurons in area CA2 are playing potentially important roles in hippocampal network activity including initiation of sharp wave ripples (Oliva *et al.*, 2016) and spatial encoding during immobility (Kay *et al.*, 2016). Lesion studies have found that synaptic transmission from area CA2 is required for social recognition memory (Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014). Furthermore, there is mounting evidence that this area undergoes detrimental changes during schizophrenia (Benes *et al.*, 1998; Piskorowski *et al.*, 2016), Alzheimer's disease (Ransmayr *et al.*, 1989), Parkinson's disease (Liu *et al.*, 2019) and other neuropsychiatric and neurodegenerative diseases (Chevalyere and Piskorowski, 2016).

In order to understand the cellular mechanisms underlying these phenomena, the physiological properties of CA2 pyramidal neurons require further examination. There is mounting evidence indicating that these neurons have numerous physiological properties that are markedly different from neighboring CA1 and CA3. The calcium buffering and G-protein signaling cascades in these neurons have been shown to actively prevent the expression of stereotypical post-synaptic NMDA-mediated long-term potentiation (Simons *et al.*, 2009; Vellano *et al.*, 2011; Zhao *et al.*, 2007). Additionally, CA2 pyramidal neurons have been shown to have a very different composition of ion channels and dendritic excitability than neighboring regions (Palacio *et al.*, 2017; Srinivas *et al.*, 2017; Sun *et al.*, 2014; Dudek *et al.*, 2016). CA2 pyramidal neurons have also been shown to be modulated in the hippocampus by several neuromodulators including oxytocin (Tirko *et al.*, 2018) and vasopressin (Pagani *et al.*, 2014).

The septo-hippocampal cholinergic projection plays a critical role in hippocampal memory formation. Acetylcholine released from septal fibers acts as both a neurotransmitter as well as a modulator of cellular excitability and synaptic transmission in the hippocampus, thus having wide-ranging effects on hippocampal oscillatory dynamics and synaptic plasticity. In this study, we examine how intrinsic excitability and synaptic transmission are modulated in CA2 pyramidal neurons under conditions of increased muscarinic tone. We found that under conditions that approximately correspond to awake, exploration *in vivo* (McIntyre *et al.*, 2003; Yamamura *et al.*, 1974), CA2 pyramidal neurons depolarize and rhythmically fire bursts of action potentials. Activation of M1 and M3 muscarinic receptors is required for the depolarization. We found that excitatory and inhibitory synaptic inputs contributed to the shape

and timing of these action potential bursts. We observed that burst firing persisted when all synaptic transmission was blocked, indicating that activation of muscarinic acetylcholine receptors altered currents that underlie this phenomenon. To investigate this further, we identified the contribution of K_v7 potassium channels, T-type calcium channels, D-type potassium channels and SK channels to the depolarization and bursting activity of CA2 PNs.

Material & Methods:

All procedures involving animals were performed in accordance with institutional regulations.

Slice preparation: Transverse hippocampal slices were prepared from 6-9-week-old SWISS mice. Animals were anaesthetized with ketamine (100 mg/kg), xylazine (7 mg/kg) and isoflurane, and perfused transcardially with a N-methyl-D-glucamin-based (NMDG) cutting solution containing the following (in mM): NMDG 93, KCl 2.5, NaH_2PO_4 1.25, NaHCO_3 30, HEPES 20, glucose 25, thiourea 2, Na-ascorbate 5, Na-Pyruvate 3, CaCl_2 0.5, MgCl_2 10. Brains were then rapidly removed, hippocampi were dissected and placed upright into an agar mold and cut into 400 μm thick transverse slices (Leica VT1200S) in the same cutting solution at 4 °C. Slices were transferred to an immersed-type chamber and maintained in artificial cerebral spinal fluid (ACSF) containing the following (in mM) : NaCl 125, KCl 2.5, NaH_2PO_4 1.25, NaHCO_3 26, glucose 10, Na-pyruvate 2, CaCl_2 2, MgCl_2 1. Slices were incubated at 32 °C for approximately 20 minutes then maintained at room temperature for at least 45 minutes. Prior to recording, slices were transferred to a recording chamber allowing dual-sided perfusion with ACSF at 5 mL/min at 30 °C.

Electrophysiological recordings: All on-cell and whole-cell recordings of CA2 pyramidal neurons were performed “blind.” Area CA2 has extremely dense extracellular matrix, making visually guided recordings very challenging. A recording pipette with positive pressure was inserted deeply into the pyramidal cell layer parallel to stratum so that the tip of the recording pipette was even with the end of the mossy fibers. The pipette was then stepped deeper into the slice while a voltage step was delivered through the pipette. The current was monitored and used to determine when the pipette was close to a neuron, when positive pressure was released, and a giga-ohm seal formed. Cell-attached recordings of CA2 PNs were obtained with ACSF in the recording pipette that had resistances between 2-5 $\text{M}\Omega$. Whole-cell recordings were performed with potassium- or cesium-based intracellular solutions containing the following (in mM): K- or Cs-methyl sulfonate 135, KCl 5, EGTA-KOH 0.1, HEPES 10, NaCl 2, MgATP 5,

Na₂GTP 0.4, Na₂-phosphocreatine 10 and biocytin (4 mg/mL). Series resistance was < 20 M Ω and was monitored throughout the recordings. Data was discarded if the series resistance changed more than 10% during the experiment. For synaptic response measurements in voltage clamp mode, no compensation was applied. In current clamp mode, the bridge balance was measured every 20 seconds and compensated with internal circuitry and monitored throughout experiments as well as before and after gap-free recordings. The liquid junction potential was not corrected for. Data were obtained using a Multiclamp 700B amplifier and digitized using a Digidata 1550 ADDA board. Data were sampled at 10 kHz. pClamp10 software was used for data acquisition.

On-cell and whole-cell current clamp recordings of spontaneous activity were acquired in gap-free mode in sweeps of 10 minutes. For measurements of intrinsic properties in current clamp mode, direct current (DC) current was injected and monitored as necessary to maintain an initial V_M of -70 mV. Action potential firing properties were measured with a series of 1-second-long depolarizing current steps.

For voltage-clamp experiments examining evoked and spontaneous synaptic transmission, cesium internal solution was used. Stimulating pipettes filled with ACSF were placed in *stratum radiatum* (SR) of CA1 to antidromically excite CA3-CA2 synapses and in *stratum lacunosum moleculare* (SLM) to stimulate distal dendritic inputs in area CA2. Synaptic currents were evoked with a constant voltage stimulating unit (Digitimer Ltd.) set at 0.1 msec at a voltage range of 5 to 30 V. For minimal stimulation experiments, synaptic responses were evoked by a patch pipette filled with ACSF placed at the same depth as the recording pipette in close proximity of the apical dendrite of the CA2 pyramidal neuron. The stimulus strength (2-10V, 0.1 ms) was adjusted to elicit small amplitude EPSCs (<20 pA) with >10% failures. Extracellular field potentials were recorded with patch pipettes filled with 1M NaCl placed in CA2 SR and SLM for detecting fEPSPs, or in the CA2 pyramidal layer for monitoring spontaneous activity. Pharmacological agents were added to the ACSF at the following concentrations (in μ M): 10 CCh to activate cholinergic receptors, 10 NBQX and 50 D-APV to block AMPA, NMDA and KA receptors, 1 SR95531 and 2 CGP55845A to block GABA_A and GABA_B receptors, 10 XE-991 to block the M current, 2 TTA-A2 to block T-type voltage-gated calcium channels, 100 4-AP to block voltage-activated potassium currents, 0.1 α -Dendrotoxin (α -DTX) to block D-type potassium currents, 0.1 apamin to block SK channels, 10 pirenzepine to block M1 muscarinic receptors, 10 AF-DX 116 to block M2 muscarinic receptors, 1 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) to block M3 muscarinic receptors,

10 mecamylamine to block nAChRs and 0.1 TTX to block voltage-activated sodium channels. Voltage clamp recordings of miniature excitatory post-synaptic currents (mEPSC) were performed with 100 nM TTX and 4 mM CaCl₂ in ACSF.

Immunocytochemistry and cell identification: Post-hoc confirmation of all cells was performed by reconstruction of neuronal morphology. Following overnight incubation in 4% paraformaldehyde in phosphate buffered saline (PBS), slices were permeabilized with 0.2 % triton in PBS and blocked for 48 hours with 3% goat serum in PBS with 0.2 % triton. Alexa-546-conjugated streptavidin (life technologies, dilution 1:300) incubation was carried out in block solution for 4 hours at room temperature. Slices were mounted in prolong diamond mounting medium, resulting in a partial clarification of tissue following incubation at room temperature for 24 hrs. Images were collected with a Zeiss 710 laser-scanning confocal microscope.

Data analysis: Electrophysiological recordings were analyzed using custom-written macros with IgorPro (Wavemetrics) and Axograph software. Action potentials (APs) were detected automatically by threshold crossing with Igor TaroTools followed by visual inspection to define AP bursts. In all whole-cell recordings, bursts were defined by clear depolarization and repolarization of the V_m flanking AP firing. For analysis of cell-attached data, bursts were defined based on the shortest bursts with lowest inter-burst interval seen in whole-cell recordings. This was defined as groups of at least 2 APs occurring less than 1 second apart from one another and separated from other groups of APs by at least 1 second. While this definition is broad, we used it to avoid constraining the analysis of cell-attached data. Kinetics of membrane potential variations were measured by linear fits of the V_m trace as it changed during the initial depolarization following CCh application, and from inter-burst-V_m plus 5 mV to burst-V_m minus 5 mV. R_M measurements were calculated from linear fits of the voltage responses to current step injections. Action potential firing properties were measured on a positive current step 1.5 times over rheobase causing similar V_m depolarization for each cell and condition, typically 200 pA over rheobase. The threshold was measured at the first AP at rheobase. The AP full-width at half-maximal amplitude (AP width) and after-hyperpolarizing potential (AHP) amplitude were measured for the first AP at threshold. The latency of the first AP and number of APs was measured with increasing current steps over rheobase. The instantaneous firing frequency and amplitude of the first 6 APs were measured at current injection steps 1.5 times over rheobase. The amplitude of the after depolarization (ADP) was measured 125 ms after step termination of a current 1.5 times over rheobase. Sag potentials

were measured with a protocol injecting current as necessary to maintain a V_M of -70 mV with a 1-second-long negative current step resulting in a membrane hyperpolarization of -100 mV. The sag was calculated as the difference between the peak and steady-state voltage during the 1 second hyperpolarization.

Statistics: Results are reported \pm SEM. Statistical significance of linear variables was assessed using paired or unpaired Student's T test, one sample T test, Mann-Whitney U test, Wilcoxon signed-rank test, Kruskal-Wallis test, one-way ANOVA, repeated-measures ANOVA, or Friedman ANOVA where appropriate. Normality was tested with the Jarque-Bera test and homoscedasticity was tested using Barlett's test to choose between parametric and non-parametric statistical analysis.

RESULTS

Carbachol induces depolarization and action potential bursting in CA2 PNs.

Modulation of hippocampal neuronal activity by cholinergic agonists has been extensively described for pyramidal neurons in areas CA3 and CA1. However, little is known about how activity in area CA2 is affected by increased muscarinic tone. In order to answer this question, we performed on-cell electrophysiological recordings in CA2 pyramidal neurons in acute hippocampal slices from adult mice and monitored how the activity of the cells changed upon the application of 10 μ M Carbamoylcholine chloride, or carbachol (CCh). This compound is the carbamate analog of acetylcholine, allowing it to resist hydrolysis by acetylcholinesterase. This compound is a nonselective agonist for muscarinic acetylcholine receptors and a weaker nonselective agonist for nicotinic acetylcholine receptors in the brain. We found that before CCh application, only one out of 15 cells spontaneously fired action potentials. Following application of 10 μ M CCh, 8 of 15 cells displayed regularly repeating bursts of action potentials (Figure 1A1-A2). Within each burst, the cells fired 14.6 ± 2.2 action potentials at a frequency of 14.3 ± 4.2 Hz. Each burst lasted 6.4 ± 2.1 seconds with inter-burst interval of 90 ± 38 seconds.

The spontaneous rhythmic firing of bursts of action potentials that we observe in area CA2 are similar to previous reports of CA3 PNs following application of CCh (MacVicar and Tse, 1989; Williams and Kauer, 1997; Cobb et al., 1999; Cobb and Davies, 2005). We postulated that high muscarinic tone may be causing a change in the resting membrane potential in CA2 PNs, as has previously been reported in area CA3 (Dodd and Kelly, 1981; Cobb et al., 1999; Cobb and Davies, 2005) and in CA2 pyramidal cells in slice cultures (Fischer, 2003). In

order to answer this question, we performed whole-cell current clamp recordings before and after bath-application of 10 μ M CCh. Upon CCh application, we observed a depolarization of CA2 PN membrane potential (V_M) from rest at -75 ± 1.1 mV to -65 ± 1.0 mV (Figure 1B1-B3; $n = 29$; paired-T test, $p < 0.001$). CCh application caused V_M depolarization at a rate of 6.0 ± 0.7 mV/min ($n = 29$) before reaching a steady-state level. This depolarization was followed by rhythmic bursts of action potential firing in 25 out of 29 cells with properties similar to what we observed in cell-attached recordings. We restricted the analysis of our whole-cell data to 15 minutes following CCh application because the on-cell data from bursting cells showed that cells would burst during this time window. Furthermore, we wanted to avoid variation of cellular properties due to dialysis of the cytoplasm from whole-cell recording conditions. The fraction of cells bursting recorded in cell-attached ($n = 8 / 15$) and whole-cell ($n = 25 / 29$) configurations were not statistically different (Chi-square test, $p = 0.127$). Bursting occurred 10.3 ± 1.9 minutes ($n = 8$) after CCh application in cell-attached mode and after 6.4 ± 0.7 minutes ($n = 25$) in whole-cell recordings (Figure 1C). Bursts occurred with an inter-burst interval of 100 ± 14.7 s (Figure 1D) and lasted 3.8 ± 0.5 seconds (Figure 1E). During these bursts, CA2 PNs fired 22 ± 2.5 action potentials paced at 12.6 ± 1.9 Hz (Figure 1F-G). Importantly, none of the measurements reported above differed between cell-attached and whole-cell conditions, thus validating our approach. Following each burst of AP-firing, the V_M returned to an inter-burst potential that was significantly more depolarized than before CCh application of -62.3 ± 0.8 mV (paired-T test, $p < 0.001$).

We further studied the CCh-induced initial depolarization seen in whole-cell recordings prior to bursting. 10 μ M CCh depolarized CA2 PN V_M within minutes of application (Figure 2A, $n = 25$ cells that subsequently fire bursts of APs; Figure 2B, $n = 29$ cells recorded in 10 μ M CCh). This depolarization of the membrane potential following application of 10 μ M CCh is likely acting via mAChRs, G-protein coupled metabotropic receptors that are expressed in the soma, dendrites and axonal terminals of hippocampal pyramidal neurons (Levey et al., 1995a; Pitler and Alger, 1990). We found that prior application of 10 μ M pirenzepine, which will preferentially act on M1 receptors with partial antagonism of M3 receptors, CCh application resulted in a minor membrane depolarization of 1.6 ± 0.7 mV that was significantly lower than the 10.0 ± 0.9 mV depolarization observed in CCh alone (Figure 2B). Further, application of 10 μ M AF-DX 116, an M2 receptor antagonist, had no significant effect on the CCh-induced depolarization (Figure 2B; 7.7 ± 1.1 mV). Application of 1 μ M 4-DAMP, which should primarily act on M3 mAChRs but also a fraction M1 mAChRs, also effectively prevented CCh-induced depolarization, resulting in a small depolarization (Figure 2B; 2.1 ± 0.5 mV).

Consequently, CCh-induced AP bursting of CA2 PNs was blocked by pirenzepine and 4-DAMP but not by AF-DX 116 (Figure 2C). These results were consistent with the $G_{q/11}$ coupled action of M1 and M3 receptors, which is known to inhibit potassium permeability, leading to membrane depolarization (Brown and Adams, 1980).

Next, we explored the dose-dependent effects of CCh on CA2 PNs and performed experiments with CCh concentrations ranging from 0.5 μ M to 20 μ M (Figure 2D1-D3). These experiments showed that, although application of 10 μ M CCh robustly depolarized CA2 PNs, CCh concentrations as little as 0.5 μ M significantly depolarized CA2 PNs (Figure 2E-F). However, action potential bursts were observed consistently with 10 μ M and higher CCh concentration (Figure 2G). While burst firing was observed with lower concentrations, these occurred only in a fraction of experiments. Measurements of acetylcholine that have been performed in the hippocampus *in vivo* indicate that the concentration of acetylcholine during awake exploration is in the range of 0.5 – 1 μ M (10 - 20 pmol acetylcholine / 20 μ L CSF) (McIntyre *et al.*, 2003). Because CCh affinity for mAChRs is 10-fold lower than that of acetylcholine (Yamamura *et al.*, 1974), and because 10 μ M CCh falls within this physiological range and consistently provokes bursts of AP firing, we used this concentration for the rest of the study.

To better understand how CA2 pyramidal neuron intrinsic properties and action potential firing are altered by muscarinic tone, we performed whole-cell current clamp experiments. Current step injections before and after 10 μ M CCh application revealed that many aspects of CA2 PNs intrinsic properties and action potential firing behavior were modified by CCh (Figure 3A1-A4). For these experiments we took care to inject DC current as necessary in order to maintain an initial V_M of -70 mV. First, we found that CCh application significantly increased CA2 PNs membrane resistance, R_M , from 42 ± 2.8 MOhm to 90 ± 8.0 MOhm (Figure 3B). We examined several aspects of action potential (AP) firing. We observed an increased AP threshold, from -47.0 ± 0.9 mV to -44.0 ± 0.8 mV (Figure 3C). Furthermore, we saw a decreased AP width, from 1.13 ± 0.04 ms to 1.02 ± 0.04 ms (Figure 3D), and an increased after hyperpolarization (AHP) from 6.2 ± 0.6 mV to 8.3 ± 0.4 mV (Figure 3E). In addition, the latency of first AP firing during a step was increased following CCh application from 83 ± 11 ms to 156 ± 15 ms. The latency was compared at a current injection step 200 pA over rheobase (Figure 3F). At the same current injection, the number of APs per step was also increased from 5.0 ± 0.5 to 11.9 ± 1.1 (Figure 3G). This increase is consistent with a decrease in AP duration and increase in AHP. CCh application also resulted in an increased instantaneous AP frequency, which was measured from the first and second AP, from 15.9 ± 1.8 Hz to 19.6

± 2.1 Hz (Figure 3H). With this, we noticed that CCh prevented adaptation of AP firing frequency with increasing current injection steps. We measured a decreased AP amplitude, from 77.5 ± 1.4 mV to 71.1 ± 2.4 mV following CCh application (Figure 3I). Following the termination of current steps eliciting AP firing, we observed an increased after-depolarization (ADP) of 7.0 ± 0.9 mV in CCh compared to 1.6 ± 0.3 mV in ACSF (Figure 3J-K). Finally, we observed that the sag potential measured following a hyperpolarizing step to -100 mV in CA2 PNs was increased by CCh application from 1.6 ± 0.1 mV to 4.5 ± 0.5 mV (Figure 3L-M). These results suggest that several conductances are modulated by CCh in CA2 PNs.

CA2 PN action potential bursts are shaped by synaptic inputs.

Muscarinic AChR activation has been shown to alter both synaptic transmission as well as ionic conductances that regulate intrinsic excitability in pyramidal neurons and interneurons (Cobb and Davies, 2005; McQuiston, 2014). In area CA3, many of the properties of rhythmic burst-firing have been shown to be shaped by changes in the local inhibitory and recurrent excitatory network (Williams and Kauer, 1997; McMahan et al., 1998). For area CA2, the effect of CCh on both the local network activity and intrinsic properties have never been examined. In order to better understand the CCh-induced spontaneous activity in area CA2, we set out to evaluate the contribution of synaptic input to several properties of spontaneous AP bursting (Figure 4A). First, we assessed the contribution of excitatory transmission in CA2 PN bursting by blocking fast glutamatergic transmission with $10 \mu\text{M}$ NBQX and $50 \mu\text{M}$ APV. We saw that under these conditions, CA2 PNs continued to burst (Figure 4A2). We observed a significantly shorter inter-burst interval (Figure 4B; Table 1) and postulate that this is due to the significantly more depolarized membrane potential between bursts (Figure 4G; Table 1). Furthermore, with the block of excitatory transmission, we observed a significant decrease in the number of action potentials per burst (Figure 4D; Table 1), indicating that synaptic input from CA2 and CA3 pyramidal neurons play a significant role in the spontaneous activity. A possible consequence of blocking excitatory transmission is that less sustained AP firing during bursts would cause a decrease in activation of hyperpolarizing conductances, potentially leading to reduced repolarization of CA2 PN V_M following bursts.

Area CA2 has been shown to have a very high density of interneurons with unique morphologies and axonal projection patterns (Mercer *et al.*, 2007; 2012; Botcher *et al.*, 2014). In order to assess the contribution of the local inhibitory network to CCh-induced CA2 PN spontaneous bursting, we blocked all GABA_A and GABA_B receptors by applying $1 \mu\text{M}$

SR95531 and $2\ \mu\text{M}$ CGP55845A. We observed several notable changes that occurred following CCh application under these conditions. The burst duration was significantly shorter (Figure 4C; Table 1) resulting in a trend for less AP fired per burst (Figure 4D; Table 1) although the firing rate during a burst was significantly higher (Figure 4E; Table 1) than in control conditions with GABAergic transmission intact. Furthermore, the rate of depolarization preceding a burst, burst rise rate, was significantly faster (Figure 4H; Table 1) and the rate at which the membrane potential hyperpolarized following a burst, the burst decay rate, tended to be faster although the difference was not significant (Figure 4I; Table 1). Our interpretation of these results is that local inhibition is acting to pace action potential firing during bursts in CA2 PNs in the presence of CCh. Furthermore, the local field potential (LFP) recorded nearby in the pyramidal layer of area CA2 bore signatures of epileptiform-like events (Figure 4A3). These events consisted of brief ($0.8 \pm 0.3\ \text{s}$, $n = 5$) bursts of 3.3 ± 1.3 synaptic potentials with superimposed population-spike-like signals. We detected these events in 5 out of 6 CA2 PNs recorded in the presence of GABA blockers. In these PNs, epileptiform-like discharges (EDs) accompanied $70 \pm 8\ \%$ of the AP bursts, which they tended to precede by $32 \pm 12\ \text{ms}$. Our interpretation of this activity is that with inhibitory transmission blocked, PNs in the network undergo bursting that is not regulated by feed-forward and feed-back inhibition. Hyper-synchrony likely emerges in the network leading to very short and sudden bursts of action potentials instead of paced activity.

When we blocked all excitatory and inhibitory synaptic transmission (Figure 4A4-I), we saw that following CCh application, the spontaneous rhythmic bursts of CA2 PNs were very similar to what we observed with NBQX and APV with only the inter-burst- V_M , inter-burst intervals and number of APs per burst different from control conditions (Figure 4B, D and G; Table 1). Thus, while the CA2 PN bursting activity is shaped by both excitatory and inhibitory synaptic input, the intrinsic conductance of these neurons permits them to undergo rhythmic bursts of action-potential firing in the presence of $10\ \mu\text{M}$ CCh.

CCh affects transmission onto CA2 PNs

High cholinergic tone has been shown to suppress synaptic transmission in the hippocampus in a laminar-selective way (Hasselmo and Schnell, 1994). These observations form a basis for computational models describing cholinergic modulation of hippocampal networks during learning. As this has never been explored in area CA2 and because we found that synaptic transmission shapes burst firing, we decided to examine how excitatory and

inhibitory transmission is altered in this area by recording synaptic currents before and after application of 10 μ M CCh.

Our first strategy was to use extracellular field recordings to examine evoked excitatory synaptic transmission in area CA2 at proximal inputs in *stratum radiatum* (SR) as well as distal inputs in *stratum lacunosum moleculare* (SLM) in the presence of GABA receptor blockers (Figure 5A-B). We observed that application of 10 μ M CCh reduced fEPSP amplitudes at both proximal (Figure 5A1-3) and distal (Figure 5B1-3) inputs. The reduction in fEPSP at SR was larger than that measured in SLM, and this difference was statistically significant ($p = 0.0079$, 2-way ANOVA, repeated measure). These results are very similar to what has been observed in areas CA1 (Hasselmo and Schnell, 1994) and CA3 (Kremin and Hasselmo, 2007).

We postulate that the application of 10 μ M CCh is likely acting on CA2 pyramidal neurons via activation of mAChRs. Our finding that application of the M1 and M3 receptor antagonists, pirenzepine and 4-DAMP prevent the large membrane depolarization and AP bursting supports this conclusion. However, in the hippocampus, nAChRs have been found to be located both at pre- and post-synaptic compartments and can modulate glutamatergic transmission (McGehee et al., 1995; Halff et al., 2014). In order to explore a possible contribution of nAChR participation in synaptic transmission in area CA2, we applied 10 μ M of the nAChR blocker mecamylamine and measured the resulting CCh-induced decrease in synaptic transmission at SR (Figure 5A3) and SLM (Figure 5B3) inputs. We found that blocking nAChRs had no impact on the CCh-induced reduction in synaptic transmission, indicating that these receptors are likely not contributing to the CCh-induced change in synaptic transmission as it is being measured here.

As shown in figure 2B, Application of M1/M3 mAChR antagonists prevented CCh-induced membrane depolarization. In order to determine if activation of these receptors is also a requirement for the attenuation of synaptic transmission, we performed extracellular recordings of synaptic transmission in the presence of either pirenzepine (10 μ M), AF-DX 116 (10 μ M) or 4-DAMP (1 μ M) mAChR blockers. We found that with either the M1 or M3 mAChRs blocked, CCh application had no effect on the amplitude of evoked EPSPs at synapses in both SR and SLM (Figure 5A3 and 5B3). This data indicates that M1 and M3 receptors are primarily responsible for mediating the CCh-induced decrease in fEPSP amplitude. With M2 receptors blocked with AF-DX 116, we observed that the fEPSP amplitude remaining after CCh application was similar to control in both SR and SLM.

In order to better examine the effect of CCh application on synaptic transmission independently from membrane depolarization effects, we performed whole-cell recordings of CA2 pyramidal neurons with cesium intracellular solution. We voltage clamped the cells at -70 mV and evoked excitatory post-synaptic currents (EPSCs) from CA2 PNs with stimulating electrodes in SR and SLM in the presence of GABA receptor blockers. Consistent with the field recordings, we observed a decrease in EPSC amplitude following CCh application in both SR and SLM inputs (Figure 5C-D). However, in contrast to the field recordings, this reduction of EPSC amplitude did not significantly differ between SR and SLM inputs (T test between proportion of control response in SR and SLM, $p = 0.47$). Thus, we conclude that CCh application decreases excitatory transmission in addition to depolarizing CA2 PNs. We observed an increase in the paired-pulse ratio of evoked EPSCs in SR and SLM (Figure 5C3-D3), consistent with a pre-synaptic effect of CCh on synaptic vesicle release probability.

In order to determine whether there is also a post-synaptic change in excitatory transmission, we monitored spontaneous EPSCs (sEPSCs). We found that the sEPSC frequency increased within 5 minutes following CCh application (Figure 6A-C). We hypothesize that this increase in frequency results from enhanced network activity, as connected synaptic inputs will be undergoing spontaneous bursts of action potential firing. We did not observe a change in the amplitude of individual synaptic events (Figure 6D), consistent with the hypothesis that CCh did not act post-synaptically to reduce synaptic transmission. To further confirm the presynaptic action of CCh and decouple the network activity from synaptic transmission, we performed this experiment in the presence of 100 nM TTX and 4 mM extracellular CaCl_2 , permitting us to record miniature EPSCs (mEPSCs). We did not detect a change in mEPSC frequency before and after application of CCh (Figure 6E-G). Furthermore, we observed no change in mEPSC quantal size (Figure 6H), consistent with a lack of postsynaptic effect of CCh. While these results do support the hypothesis that CCh is acting pre-synaptically to reduce EPSC amplitude, the addition of TTX prevented over 90% of spontaneous synaptic events, resulting in a very low mEPSC frequency. While this value is very similar to what has recently been reported by others for area CA2 (Modi et al., 2019), this very low event frequency may be problematic in detecting changes in release probability. Furthermore, these events are not input-specific. Thus, we used a third method to investigate a potential pre-synaptic site of action of CCh, performing minimal stimulation of SR inputs while recording CA2 PNs before and after CCh application (Figure 6I-J). We observed that the amplitude of synaptic events decreased when failures were included (Figure 6K) but were not changed when failures were excluded (Figure 6L).

Consistently, when the amplitudes of the first and second stimulation were averaged with failures included, CCh application resulted in an increase in the PPR (Figure 6M), indicating that release probability is decreased with CCh. Likewise, following CCh application, we observed an increase in failure rate (Figure 6N) as well as in the coefficient of variation (CV, Figure 6O) consistent with a decrease in release probability.

We examined inhibitory transmission in area CA2 by using the same voltage clamp approach but held the CA2 PNs at +10 mV in the absence of GABA blockers. We observed that CCh application reduced inhibitory post-synaptic current (IPSC) amplitudes evoked by both SR and SLM input stimulation (Figure 7A-C). This reduction of IPSC amplitude was largest in SR as compared to SLM (T-test between proportion of control response in SR and SLM, $p = 0.028$). Paired-pulse ratios of evoked IPSCs in SR and SLM were also increased after CCh application (Figure 7B3-C3). We then gauged how inhibitory transmission was affected by CCh by recording spontaneous inhibitory post-synaptic currents (sIPSCs). Monitoring sIPSCs by holding CA2 PNs at a potential of +10 mV showed that CCh application increased the sIPSC frequency within 5 minutes (Figure 7D-F). However, the sIPSC amplitude distribution was not changed by CCh (Figure 7G), indicating that CCh acts presynaptically to reduce inhibitory transmission by decreasing the probability of GABA release. Altogether, these results show a drastic influence of CCh over synaptic transmission in area CA2.

Contribution of M-current to CCh-induced depolarization.

Because CA2 PNs can fire bursts of action potentials in the absence of synaptic transmission, we examined which ionic conductance underlies the depolarization and spontaneous AP bursting in the presence of CCh. Kv7 channels, which underlie the M-current, have been shown to be modulated by acetylcholine and play a central role in controlling resting V_M , repetitive and burst firing in CA3 and CA1 PNs (Cobb *et al.*, 1999; Hönigsperger *et al.*, 2015). We hypothesize that this current may be playing a similar role in CA2 PNs. To test this, we looked at the effect of blocking the M-current with 10 μ M XE-991 on CA2 PN intrinsic properties (Figure 8A). For these experiments, care was taken to inject DC current as needed in order to keep the initial membrane potential at -70 mV. Data was acquired after 20 minutes of bath application of XE-991. We confirmed that the drug was active by performing whole-cell recordings in area CA1 and reproducing previously published changes in EPSP summation and intrinsic properties (Hönigsperger *et al.*, 2015). First, we examined the effects of XE-991 on CA2 PN intrinsic excitability. Consistent with previous observations that the M-current is open

at rest in CA2 PNs (Tirko *et al.*, 2018), we observed an increase of membrane resistance in CA2 PNs following XE-991 application, with subsequent application of CCh causing further R_M increase (Figure 8B). Application of X-991 did not change AP threshold (Figure 8C) but did reduce AP width (Figure 8D) and increased AHP (Figure 8E). These effects were unchanged or attenuated by further application of CCh (Figure 8C-E). Application of XE-991 increased the latency of AP onset (Figure 8F) as well as the number of APs per step, albeit only at current injections well-above threshold (Figure 8G) and occluded the effects of CCh application of these measurements. The milder effect of XE-991 alone compared to XE-991 plus CCh on AP number at low current injections indicates that CCh increases CA2 PN excitability by acting on additional conductances beside the M-current. Although the instantaneous firing frequency tended to be higher and to increase with AP number in XE-991 with CCh, no significant effects were seen in this data set (Figure 8H). XE-991 application alone significantly increased AP amplitude and addition of CCh reversed that effect, suggesting an M-current-independent effect of CCh in reducing spike amplitude (Figure 8I). The ADP following current step termination was reduced in XE-991 compared to ACSF, and subsequent application of CCh led to a further increase (Figure 8J-K). Application of XE-991 led to an increase in sag potential (Figure 8L). Our explanation of this finding is as follows: block of the M-current results in a depolarization of the membrane, strongly supporting the conclusion that a fraction of these channels is open at resting V_M . In normal ACSF, the sag current is a result of two processes: the opening of HCN channels, resulting in I_h , and the closing of the Kv7 channels, resulting in a reduction of M-current. In our protocol we are injecting DC current to keep the initial starting membrane potential at -70 mV. We then inject the necessary amount of current to bring the membrane potential to -100 mV. The reversal potential for potassium is above 100 mV, so any M-current will be expected to be depolarizing and counteract the non-selective I_h . Thus, in the presence of XE-991, the M-current open at rest is blocked, and the step to -100 mV only activates I_h , which appears larger, because it is not attenuated by the closing M-current. Presented differently, the membrane resistance will be higher with XE-991 present, allowing a greater current at -100 mV, thus making the “sag” measurement larger. Further application of CCh in the presence of XE-991 resulted in similar levels of sag potential (Figure 8M). Altogether, this data shows that the application of the Kv7 blocker XE-991 partially recapitulated and/or occluded many of the effects of CCh on CA2 PN intrinsic properties including AHP amplitude, number of APs per current step and sag current. This suggests a contribution of M-current closing to the effects of CCh on CA2 PN V_M .

During these experiments, we also investigated the consequences of M-current block by XE-991 on CA2 PN V_M by performing current-clamp recordings without injection of DC current. We found that application of XE-991 led to a significant depolarization of CA2 PNs. This depolarization was insufficient to elicit bursting, and subsequent application of CCh brought further depolarization and bursting (Figure 9A). XE-991 has been found to be a voltage-dependent blocker of Kv7.2 channels exogenously expressed in Chinese hamster ovary cells (Greene *et al.*, 2017). Thus, we performed several experiments in which DC current was injected to just below threshold to increase the probability of channel block. No difference was observed in the V_M following this manipulation. When compared with CCh-induced initial V_M depolarization of CA2 PNs, the XE-991 effect was smaller and did not bring V_M to depolarized levels sufficient to bursting (Figure 9B-D). We further analyzed experiments in which the recording conditions stayed of sufficiently high quality to allow the changes in membrane resistance before addition of XE-991, during and following the addition of CCh with XE-991 (Figure 9E). With this, we consistently observed an increase in the R_M following XE-991 application, indicating that a proportion of these channels are open at rest (Figure 8B). Therefore, this result shows that the M-current is likely open at rest in CA2 PNs and blocking it leads to a partial depolarization of 3.2 ± 0.6 mV ($n = 8$). However, addition of CCh resulted in a further increase of R_M , indicating a separate conductance contributing to membrane depolarization, and that M-current block alone is not sufficient to elicit bursts of AP firing in CA2 PNs in our experiments.

CA2 PN AP firing and CCh-induced bursting are paced by D-type potassium currents.

D-type potassium currents have been shown to regulate the AP timing and synchrony in hippocampal pyramidal neurons (Storm, 1988; Cudmore *et al.*, 2010). It may be possible that this current is also participating in CCh-induced effect of AP bursting in CA2 pyramidal cells, as CCh application induces a delay in AP firing with current step injection. Furthermore, activation of M1 and M3 muscarinic receptors can result in depletion of phosphatidylinositol 4,5-bisphosphate (PIP₂) in hippocampal pyramidal neurons (Hackelberg and Oliver, 2018). This results in decreased M-current, but also potentially an increase in Kv1.2 current (Rodríguez-Menchaca *et al.*, 2012). To examine this further, we blocked D-type potassium currents with 100 nM alpha-dendrotoxin (α -DTX), a compound that specifically blocks Kv1.1, 1.2 and 1.6 voltage-gated ion channels. We then injected current steps to measure CA2 PN AP firing

properties before and after the application of CCh to determine the contribution of this family of ion channels to mAChR-induced changes in intrinsic properties.

First, we assessed the role of D-type potassium currents on CA2 PN intrinsic properties and their contribution to CCh-induced changes by recording in whole-cell mode and injecting current steps before and after 100 nM α -DTX application alone followed by further application of 10 μ M CCh (Figure 10A), similar to figure 8. We found that α -DTX application alone did not significantly change R_M but subsequent addition of CCh led to a significant increase (Figure 10B). AP threshold, however, was decreased following application of α -DTX and even further by subsequent CCh (Figure 10C). AP width was unaffected by α -DTX alone or combined with CCh (Figure 10D). Application of α -DTX had no effect on the AHP of the initial AP, and further application of CCh led to a significant decrease (Figure 10E). As a consequence of lowered threshold, AP firing occurred earlier during current step injection in the presence of α -DTX, leading to a reduced AP latency that prevented any further effect by the subsequent addition of CCh (Figure 10F). In addition, the number of APs per step was significantly increased by α -DTX, but subsequent addition of CCh resulted in a further increase, indicating the contribution of other channels in regulating the AP firing (Figure 10G). Further evidence of this was shown by the instantaneous AP firing frequency. Block of D-type channels by α -DTX resulted in a significant increase, but further application of CCh caused an even greater increase in firing frequency (Figure 10H). AP amplitude, however, was not altered by α -DTX alone or combined with CCh (Figure 10I). These data show a role for D-type potassium currents in regulating the firing pattern of CA2 PNs when V_M reaches depolarized levels nearing threshold.

We also explored the contribution of voltage-activated potassium channels further by applying 100 μ M 4-AP. This compound at this concentration will effectively block all D-type potassium currents as well as potentially a fraction of A-type potassium currents. Hippocampal area CA2 has an unusually dense extracellular matrix, and we record very deeply within the slice to have data from healthy neurons. Both of these factors make this region very challenging for pharmacology with large peptides. Unlike α -DTX, 4-AP is a small molecule that washes into the slice very quickly, allowing us to confirm the effect of α -DTX as well as examining any potential contribution from A-type currents in CA2 PN intrinsic properties before and after CCh application. When we performed the same experiment with 100 μ M 4-AP, we observed that AP firing occurred rapidly following current injection and occurred in bursts (Figure 10J). Apart from this major difference in initial firing pattern, we found generally similar results with 4-AP as we did with α -DTX for R_M (Figure 10K), AP threshold (Figure 10L), AP width (Figure

10M) and number of APs per step (Figure 10P). Furthermore, we observed effects from 4-AP application that were likely not due to D-type potassium channel block. Strikingly, the AHP was abolished (Figure 10N) which likely led to the high-frequency AP bursting early in the current step. We also observed a very short AP latency (Figure 10O) and very high initial firing rate (Figure 10Q). Consistent with the AP threshold markedly lowered by 4-AP and 4-AP plus CCh, the amplitude of the first AP was higher in these conditions (Figure 10R). We conclude from this dataset that 100 μ M 4-AP likely acts on D-type and other potassium currents that contribute to the firing pattern of CA2 PNs.

Next, we assessed the contribution of voltage-activated potassium current to spontaneous burst firing by recording CA2 PNs in passive current-clamp mode and applying 100 nM α -DTX or 100 μ M 4-AP followed by 10 μ M CCh (Figure 11A). Consistent with D-type potassium channels being opened at only depolarized V_M levels (Storm, 1988), application of α -DTX or 4-AP did not change the resting membrane potential while subsequent application of CCh caused an initial depolarization similar to control conditions (Figure 11B1-B2). The main effect of blocking D-type potassium currents was an accelerated rise of V_M at the onset of bursts seen with both α -DTX and 4-AP (Figure 11C; Table 2). Other effects with 4-AP potentially not restricted to D-type potassium currents were a faster burst decay rate (Figure 4D; Table 2) and increased firing rate during bursts (Figure 4E; Table 2). Other burst parameters were unchanged by α -DTX or 4-AP (Table 2). Altogether, these experiments show a key role of D-type potassium currents in controlling the early phase of burst firing induced by CCh in CA2 PNs.

T-type calcium currents control length of CA2 PN AP burst duration

T-type calcium channels are activated at membrane potentials near rest and cause transient calcium influx that depolarizes neurons. These channels are involved in setting the resting V_M in cortical neurons (Hu and Bean, 2018) and can be activated by cholinergic stimulation in the dentate gyrus granule cells (Martinello *et al.*, 2015). We hypothesize that these channels may be expressed in CA2 pyramidal neurons and may play a role in regulating the rhythmic bursting activity of these cells. These channels are expressed widely in the hippocampus, including area CA2 (Tang *et al.*, 2011). In order to determine how T-type calcium channels contribute to the intrinsic properties of CA2 PNs, we performed whole-cell current clamp experiments and injected current steps before and after blocking T-type channels with 2 μ M TTA-A2 (Figure 12A). We found that TTA-A2 caused an increase in membrane resistance

(Figure 12B). Blocking these channels had no effect on AP threshold (Figure 12C) but did result in a decrease in the AP width (Figure 12D), whereas there was no change in AHP (Figure 12E). Furthermore, we observed no change in AP latency or number of APs (Figure 12 F-G). After blocking T-type channels, we did observe a small but significant change in firing frequency and an increase in AP amplitude (Figure 12H-I). From these experiments, we can conclude that T-type calcium channels are expressed by CA2 PNs and contribute to several aspects of their intrinsic AP firing properties, including the timing of AP firing when the cell becomes depolarized.

Given these observations, we hypothesize that blocking T-type calcium channels may potentially alter the properties of CCh-induced rhythmic AP bursting. In order to test this, we first performed current clamp experiments in which we held the cells at -70 mV and progressively injected depolarizing steps of current in the presence of CCh alone or with application of CCh plus 2 μ M TTA-A2 (Figure 13A). Blocking T-type channels significantly increased AP latency (Figure 13B) and effectively prevented the CCh-induced increase in AP firing (Figure 13C). Therefore, T-type calcium channels are likely involved in the modification of AP firing induced by CCh in CA2 PNs, opening with membrane depolarization and contributing to further depolarization and AP firing. Blocking these channels reveals this contribution. The decrease in the number of APs during the 1 second current injection step in the presence of CCh could potentially be due to the increased AP latency.

We next examined the effects of TTA-A2 on CCh-induced spontaneous AP bursts in CA2 PNs (Figure 13D). TTA-A2 block of T-type channels did not significantly change the resting V_M level of CA2 PNs and did not affect their initial depolarization by CCh (Figure 13E). However, we found that application of TTA-A2 prior to CCh application resulted in a significant increase in the duration of AP bursts (Figure 13F; Table 3) while all other burst properties were unchanged (Table 3). Therefore, we conclude that T-type calcium channels are involved in controlling the duration of bursts, potentially by regulating calcium influx prior to and during AP burst firing that would eventually contribute to ending the burst by activating calcium-dependent potassium channels.

Small-conductance calcium-activated K⁺ channels contribute to CA2 PN V_M repolarization between bursts.

Sustained AP firing and V_M depolarization as seen in CA2 PNs during CCh-induced AP bursting can lead to calcium influx and activation of calcium-activated potassium channels. In several types of neurons, SK channels frequently underlie a major component of the after-

hyperpolarizing current following single or bursts of APs, acting to regulate burst firing (Stocker, 2004). In CA1 hippocampal pyramidal cells, these channels have been shown to be less important than KV7 channels in regulating excitability (Chen *et al.*, 2014). However, it has been shown that transient activation of M1 mAChRs does activate store-release IP3-mediated pathway that acts via SK channels to transiently control membrane excitability (Dasari *et al.*, 2016). To examine this further, we first sought to establish the presence of SK channels in CA2 PNs and their contribution to AP firing by injecting current steps before and after application of 0.1 μ M apamin (Figure 14A). While we found no change in R_M (Figure 14B), we did measure a small but significant decrease in AP threshold (Figure 14C), as well as a decrease in AP width (Figure 14D) and an increase in AHP amplitude (Figure 14E). This change in AHP is puzzling to us, and merits further study. Blocking SK channels had fairly minor or no effect on other aspects of CA2 PN firing (Figure 14F-I).

While apamin had minimal effects in CA2 PNs, they are consistent with an increased membrane resistance that would be expected when blocking a potassium channel, thus warranting the study of SK channel contributions to the effects of CCh on CA2 PNs. In order to examine this further, we recorded pyramidal cells in area CA2 and injected depolarizing current steps in the presence of 10 μ M CCh and 10 μ M CCh plus 0.1 μ M of apamin (Figure 15A). Block of SK channels by apamine resulted in an increased AP latency in the presence of CCh (Figure 15B). Furthermore, blocking these channels appeared to prevent the CCh-induced increase in AP number with each current injection step (Figure 15C). The increased latency that we observed following CCh application when SK channels are blocked is a challenge to understand. Perhaps the block of SK allows increased influx of calcium that permits the activation of large-conductance calcium-activated potassium channels (BK channels), further hyperpolarizing the cells and delaying AP firing.

In order to determine how SK channels potentially contribute to CCh-induced spontaneous bursting of CA2 PNs, we recorded from these cells in the presence of apamin followed by CCh application (Figure 15D). Block of SK channels by apamin alone did not depolarize CA2 PNs, and subsequent application of CCh resulted in a significant depolarization (Figure 15E) that is very similar to what we observed with CCh application alone (Table 4). Nearly all properties of the AP bursts were unchanged by the presence of apamin (Table 4). However, we did observe an increase in the V_M between bursts, indicating that blocking SK channels disrupts the repolarization of the membrane (Figure 15F). Thus, our observations are very similar to reports for CA1 pyramidal neurons (Chen *et al.*, 2014), showing that SK

channels contribute in a relatively minor way to regulating spontaneous AP bursting under conditions following mACh activation.

DISCUSSION

Using patch-clamp recordings in acute hippocampal slices from adult mice, we report several changes in hippocampal CA2 PN properties following mAChR activation. In summary, application of $10\ \mu\text{M}$ CCh led to a depolarization of the membrane potential and rhythmic bursts of action potential firing. We found that the membrane depolarization would occur with $1\ \mu\text{M}$ CCh, whereas the burst firing required higher concentrations. $10\ \mu\text{M}$ CCh altered several intrinsic properties of CA2 PNs, including the R_M , AP threshold, AP width, AHP, latency, AP number AP firing frequency and sag current. While several aspects of the spontaneous AP bursting kinetics are shaped by excitatory and inhibitory synaptic transmission, CA2 PNs were found to be capable of spontaneously firing bursts of APs with all transmission blocked. Upon examination of synaptic transmission, we observed that CCh application attenuated both excitatory and inhibitory transmission. Lastly, we examined the contributions of the potassium M-current from K_v7 ion channels, T-type calcium channels, D-type potassium channels, and SK channels to the intrinsic properties and AP bursting of CA2 PNs, finding that they play roles in regulating the excitability and AP firing of these cells.

The influence of the muscarinic tone on hippocampal physiology has long been recognized and extensively studied in area CA3 and CA1. Application of cholinergic agonists or stimulation of cholinergic inputs in acute hippocampal slices has been found to result in the depolarization of resting membrane potential and an increase in membrane resistance in CA3 and CA1 PNs (Dodd et al., 1981; Cole and Nicoll, 1983; 1984). In area CA2, we found that low concentrations of CCh resulted in membrane potential depolarization. We also found that blocking the M1 and M3 muscarinic receptors effectively prevented depolarization and all spontaneous bursting activity. This is consistent with what has previously been described in CA3 pyramidal neurons (Williams and Kauer, 1997). Furthermore, these findings are consistent with immunohistological evidence that M1 and M3 receptors are expressed in area CA2 (Levey et al., 1995b). The pharmacological methods that we used in this paper supports the premise that M1/M3 mChRs are expressed in CA2 PNs, as application of $1\ \mu\text{M}$ 4-DAMP and $10\ \mu\text{M}$ Pirenzepine prevents the large depolarization of the resting V_M by CCh application and completely prevents spontaneous AP bursting. At $1\ \mu\text{M}$, a small fraction of M1 mAChRs would be expected to be blocked by 4-DAMP (Caulfield and Birdsall, 1998), and concrete discrimination between these two subtypes with our dataset cannot be done. Closer examination

of RNA levels for different muscarinic receptors subtypes available on the Allen Brain Atlas (<http://mouse.brain-map.org/gene/show/12456>) show that the relative levels of the M3 mAChR are lower in area CA2 as compared to areas CA1 and CA3. This difference is intriguing, as different effects on PN depolarization and plasticity have previously been attributed to M3 and M1 mAChRs (Pitler and Alger, 1990; Auerbach and Segal, 1996). Furthermore, our results indicate that potential differences in downstream Gq/11 downstream signalling cascades in area CA2 merit further investigation. There is already evidence from studies examining RGS14, and its role in preventing NMDA-mediated long term plasticity, that indicate intracellular signalling pathways diverge in area CA2 as compared to CA1 and CA3 (Lee et al., 2010; Evans et al., 2018).

These results demonstrate that CA2 PNs express muscarinic acetylcholine receptors that inhibit the M-current upon activation by CCh. We found that pharmacologically blocking the M-current led to a statistically significant depolarization of the membrane potential, albeit only a fraction of the depolarization observed with CCh. The extent of the depolarization that we observed following application of XE-991 is slightly lower than what had been reported in another study (3.2 ± 0.6 as compared to 4.2 ± 0.5 mV) (Tirko *et al.*, 2018). In our experiments, we used a K-methanesulfonate based solution while the Tirko *et al.* paper used a K-gluconate based intracellular solution. We hypothesize that this difference in intracellular solution may underlie the differences in cellular excitability that we observe following XE-991 application. Intracellular gluconate has been shown to inhibit several currents, including I_K , I_{AHP} , I_h and I_{Ca} currents, resulting in higher membrane resistance and altered AP firing properties (Velumian et al., 1997). Furthermore, intracellular gluconate has been shown to alter the calcium dynamics of the calyx of held, indicating that this counter ion potentially buffers intracellular calcium (Woehler et al., 2014). Consistent with this, we measured membrane resistance values in CA2 PNs appreciably lower than those reported in Tirko, *et al.* and the resting membrane potential was also more hyperpolarized. Thus, with the difference in membrane resistance and lower starting value, our blocking of the M-current with XE-991 did not sufficiently depolarize the membrane to allow spontaneous AP bursting.

Subsequent application of CCh, led to further membrane potential depolarization of approximately 6 mV, indicating the contribution of an additional current following muscarinic activation. The facts that we had a valid positive control for XE-991 application, and that several actions of CCh were occluded by XE-991 application suggest that the drug was fairly efficient at blocking Kv7. It has been reported that activation of M1 mAChRs can activate a non-selective cation conductance (Shalinsky et al., 2002; Haj-Dahmane and Andrade, 1996) that

could readily result in membrane depolarization. Furthermore, a clear link has been made between M1 mAChR activation and IP₃-mediated signalling (Dasari et al., 2016; Dasari and Gullledge, 2011). We postulate here that these mechanisms may also be at play in CA2 pyramidal neurons. One major weakness of our work in this study is prolonged application of CCh to activate all mAChRs. While this does allow for the fundamental molecular players to be identified, ACh is released in a pulsatile manner in vivo, and there is likely more complex signalling occurring at short time dynamics that merit future investigation. Differences have been reported between the phasic cholinergic signalling in areas CA1 and CA3 (Gullledge and Kawaguchi, 2007), and it would be interesting to know how CA2 responds under these conditions. From our results here, we would predict that CA2 PNs would look more like CA1 than CA3, but further investigation is needed. With the use of ACh, a second future question worth investigating is the potential role of nicotinic acetylcholine receptors in synaptic plasticity in area CA2. It has previously been shown that activation of $\alpha 7$ nAChRs leads to post-synaptic plasticity in hippocampal CA1 PNs neurons (Halff et al., 2014). As the calcium dynamics in area CA2 have been shown to prevent NMDA-mediated long term potentiation (Simons et al., 2009), determining if and how nAChR activation participates in synaptic plasticity in this area is an interesting avenue of research.

In addition to the M-current, we also explored a potential role of D-type potassium current in regulating CA2 cellular excitability and spontaneous bursting in high muscarinic tone. Activation of M1 muscarinic receptors results in depletions of PIP₂ (Hackelberg and Oliver, 2018). This PIP₂ depletion can potentially result in the modification of gating of several voltage-gated potassium currents (as reviewed by (Rodríguez-Menchaca *et al.*, 2012)). It is possible that several of the effects of CCh on the AP firing and bursting may be due to an increase in D-type current. This quickly-activating but slowly inactivating current has been shown to be very important for regulating AP timing and network synchrony in areas CA1 and CA3 (Storm, 1988; Cudmore *et al.*, 2010). Our results indicate that in area CA2, this current is indeed playing a considerable role in regulating spike precision as well as several aspects of CCh-induced AP bursting.

For CA3 and CA1 PNs, several different effects of CCh application on AP firing have been reported. These include spontaneous burst firing (Cobb *et al.*, 1999), transformation of current injection-evoked single spikes into bursts (Yue *et al.*, 2005) and induction of persistent firing after current injection-evoked depolarization (Jochems and Yoshida, 2013; Knauer et al., 2013). Our experiments in CA2 PNs demonstrate that CCh application causes CA2 PNs to fire

bursts of APs in a way that is very similar to CA3 PNs under similar conditions (Cobb *et al.*, 1999). In this study, we demonstrated the role of T-type calcium channels in controlling the duration of these AP bursts in CA2 PNs. This finding is consistent with other cells types, as calcium currents have been known to be important for CA3 PN AP bursting (MacVicar and Tse, 1989). Furthermore, T-type calcium channels are partially opened near resting V_M levels in cortical neurons (Hu and Bean, 2018) and their activation by muscarinic stimulation causes calcium entry in dentate gyrus granule cells (Martinello *et al.*, 2015). In dopaminergic midbrain neurons, intrinsic bursting is regulated by coupled activity of T-type and SK channels (Wolfart and Roeper, 2002). In CA2 PNs, we show that SK channels are involved in the repolarization of V_M following bursts. Thus, it may be possible that a similar mechanism is at play in these cells: that prolonged action potential firing during bursts allows calcium influx that activates calcium-activated potassium channels, terminating the burst. Further work is required to better understand the calcium channel composition and contribution to excitability in CA2 pyramidal cells.

We did not investigate the potential role of large-conductance calcium activated potassium channels in regulating the CCh-induced burst firing in this study. BK channels have been shown to be important for enabling high-frequency AP firing and early spike frequency adaptation in CA1 pyramidal neurons (Gu *et al.*, 2007), two properties that are nearly absent in CA2 pyramidal neurons. That does not mean, however that these channels are not contributing in a central way to the active properties of CA2 PN dendrites. In fact, given the unusual calcium buffering of CA2 PNs (Simons *et al.*, 2009; Evans *et al.*, 2018) and its implications for synaptic plasticity, the role of BK channels, and their possible interaction with SK channels in regulating AP firing in CA2 merits further investigation.

Of note, we saw that synaptic transmission played a role in shaping bursting frequency, duration and membrane potential between bursts. In our acute slice preparations, excitatory inputs to area CA2 originate from CA3 PNs, from recurrent connections from CA2 PNs and from the EC. When these inputs are blocked, CA2 PNs fire fewer APs per burst and repolarize less after bursts. Excitatory transmission is likely important to sustain AP firing during bursts which potentially contributes to allow calcium influx in CA2 PNs as well as promote subsequent repolarization of the V_M to start a new burst. Removal of inhibitory transmission transformed bursts of APs into sudden brief AP discharges in which APs fired at higher frequency and rapidly hyperpolarized. Blocking excitatory transmission alone appeared to be indistinguishable to blocking all synaptic transmission. Clearly, feed-forward and feed-back

inhibition is playing a central role in regulating overall of excitability and AP firing in area CA2.

Interestingly, AP burst firing by CA2 PNs has recently been described to be induced by oxytocin (Tirko *et al.*, 2018). These bursts of APs were mediated by PLC-mediated reduction of the M-current and activation of PKC. The OXTR-mediated AP bursts described by Tirko *et al.* lasted on the order of 2-3 seconds and had a firing frequency of around 14 Hz, similar to the CCh-induced bursts that we report here. A question for future research will be to determine the downstream intracellular signalling pathways activated by M1 and M3 muscarinic receptors in CA2 PNs and to determine how they are similar to those of other receptors. This question is very pertinent for CA2 PNs, as numerous G-protein and growth factor receptors are either enriched or exclusively expressed in this region. These include the Adenosine A1 receptor (Ochiishi *et al.*, 1999), Vasopressin 1b receptor (Young *et al.*, 2006) and the epidermal growth factor receptor (Tucker *et al.*, 1993). Thus, having a better understanding of how the activity of CA2 PNs is altered in the presence and absence of high muscarinic tone is an important first step in fully understanding how this hippocampal area is affected by these signaling molecules.

The hippocampus receives a major cholinergic input from the septum (Amaral and Kurz, 1985), therefore the hippocampal cholinergic tone is likely high when septal cholinergic neurons are active, during active exploration. It is well-accepted that area CA2 plays a critical role in social memory formation (Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014) and we predict that during social exploratory behaviors, cholinergic tone is likely to be elevated, influencing the cellular properties of CA2 PNs in a way similar to what we characterize in this paper. Major questions remain concerning how social information is encoded by the hippocampus, and the precise role of area CA2 in this process. During social exploration, CA2 PNs likely receive theta-locked inputs from the entorhinal cortex, the septum, the supramammillary nucleus, and area CA3 (reviewed in (Robert *et al.*, 2018)). Our results indicate that changes in the membrane potential resulting from M1 and M3 activation in high cholinergic tone suppress CA3 inputs more than cortical inputs, as has been observed in other CA regions (Kremin and Hasselmo, 2007; Hasselmo and Schnell, 1994). These diverse inputs would be summated and the resulting CA2 output will then impact area CA1, CA3 and extra-hippocampal structures. Our results will be a necessary tool for modeling and *in vivo* studies that consider area CA2 in the hippocampus.

Lastly, understanding the function and physiological mechanisms of CA2 PNs is important in the context of psychiatric and neurological disorders, as this region has been shown

to undergo changes in human post-mortem studies and animal model of schizophrenia (Benes *et al.*, 1998; Piskorowski *et al.*, 2016) and several other neuropsychiatric and neurodegenerative diseases (Chevaleyre and Piskorowski, 2016). Notably, the density of cholinergic input as well as acetylcholinesterase has been found to be decreased in patients with Alzheimer's disease most dramatically in area CA2 as compared to other hippocampal areas (Ransmayr *et al.*, 1989). Furthermore, it has recently been proposed that cholinergic dysfunction contributes to cognitive decline in Parkinson's disease and is associated with increased Lewy pathology within the CA2 region (Liu *et al.*, 2019).

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Figure Legends:

Figure 1. Carbachol application induces V_M depolarization and burst firing in CA2 PNs.

A. (A1) Sample trace of action potential bursts recorded in cell-attached configuration from a putative CA2 PN following application of $10 \mu\text{M}$ CCh in acute hippocampal slice. (A2) Expanded view of a single burst of action potentials. **B.** (B1) Sample trace of bursts recorded in whole-cell current-clamp configuration from a CA2 PN upon application of $10 \mu\text{M}$ CCh in acute hippocampal slice, and (B2) expanded views of the V_M initial depolarization and (B3) of a burst of action potentials. **C. – G.** Summary graphs of burst properties recorded in cell-attached (grey, $n = 8$) and whole-cell current-clamp (black, $n = 25$) configuration from CA2 PNs with application of $10 \mu\text{M}$ CCh (individual cells shown as dots, population averages shown as thick lines, error bars represent SEM). **C.** Bursting onset (cell-attached: 10.3 ± 1.9 min; whole-cell: 6.4 ± 0.7 min, T test, $p = 0.088$). **D.** Inter-burst interval (cell-attached: 90 ± 38 s; whole-cell: 100 ± 14.7 s, T test, $p = 0.80$). **E.** Burst duration (cell-attached: 6.4 ± 2.1 s; whole-cell: 3.8 ± 0.5 s Mann-Whitney U test, $p = 0.48$). **F.** Number of APs per burst (cell-attached: 14.6 ± 2.2 ; whole-cell: 22 ± 2.5 , Mann-Whitney U test, $p = 0.17$). **G.** Firing rate during burst (cell-attached: 14.3 ± 4.2 Hz; whole-cell: 12.6 ± 1.9 Hz, Mann-Whitney U test, $p = 0.71$).

Figure 2. Depolarization of CA2 pyramidal neuron resting membrane potential by CCh.

A. Time course of the initial V_M depolarization leading to bursting onset following $10 \mu\text{M}$ CCh application ($n = 25$; population average shown as black line, grey region represents SEM). **B.** Summary graph of V_M depolarization before and after application of $10 \mu\text{M}$ CCh in control conditions or with muscarinic acetylcholine receptors M1, M2 or M3 blocked by $10 \mu\text{M}$ pirenzepine, $10 \mu\text{M}$ AF-DX 116, or $1 \mu\text{M}$ 4-DAMP, respectively. The depolarization induced by CCh (black, $n = 29$) was significantly reduced by pirenzepine (red, Mann-Whitney U test, $p = < 0.001$, $n = 12$) and by 4-DAMP (blue, Mann-Whitney U test, $p < 0.001$, $n = 15$) but not by AF-DX 116 (green, Mann-Whitney U test, $p = 0.185$, $n = 12$; individual cells shown as dots, population averages shown as thick lines, error bars represent SEM). **C.** Fraction of cells that burst following application of $10 \mu\text{M}$ CCh and in the presence of pirenzepine, AF-DX 116 or 4-DAMP (Chi-squared test, $p < 0.001$). **D.** (D1) Sample trace recorded in whole-cell current-clamp configuration from a CA2 PN and expanded view (D2) showing a small V_M depolarization without bursts during application of $1 \mu\text{M}$ CCh followed by further depolarization (D3) and action potential bursts upon subsequent increased CCh concentration to $10 \mu\text{M}$ in acute hippocampal slice. **E. – G.** Summary graphs of the initial V_M depolarization and subsequent bursting as a function of increasing CCh concentrations ($0.5 \mu\text{M}$, $n = 9$; $1 \mu\text{M}$,

n = 10; 2 μ M, n = 5; 5 μ M, n = 6; 10 μ M, n = 29; 20 μ M, n = 5; individual cells shown as dots, population averages shown as thick lines, error bars represent SEM). **E.** Initial V_M depolarization induced by CCh (One sample T tests: 0.5 μ M, p = 0.0079; 1 μ M, p = 0.0022; 2 μ M, p = 0.010; 5 μ M, p = 0.0026; 10 μ M, p < 0.001; 20 μ M, p = 0.0056). **F.** Initial V_M depolarization rate during CCh application. **G.** Fraction of cells bursting during CCh application (Chi-square test, p = 0.002).

Figure 3. Modification of CA2 PNs intrinsic properties by CCh. **A.** Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from a CA2 PN in control (**A1**, grey) and 10 μ M CCh (**A2**, black), expanded view of the first action potential in each condition (**A3**) and corresponding phase plane plot (**A4**). **B – I.** Summary graphs of CA2 PNs action potential firing properties in control (grey) and 10 μ M CCh (black) (individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **B.** Membrane resistance (pre-CCh: 42 ± 2.8 MOhm; post-CCh: 90 ± 8.0 MOhm, Wilcoxon signed-rank test, p < 0.001, n = 26). **C.** Action potential threshold (pre-CCh: -47.0 ± 0.9 mV; post-CCh: -44.0 ± 0.8 mV, Wilcoxon signed-rank test, p = 0.017, n = 34). **D.** Action potential width at half-maximal amplitude (pre-CCh: 1.13 ± 0.04 ms; post-CCh: 1.02 ± 0.04 ms, Wilcoxon signed-rank test, p = 0.005, n = 34). **E.** After-hyperpolarization potential (pre-CCh: 6.2 ± 0.6 mV; post-CCh: 8.3 ± 0.4 mV, Wilcoxon signed-rank test, p = 0.002, n = 29). **F.** Latency to fire first action potential per current step as a function of current injection over rheobase (200 pA over rheobase, pre-CCh: 83 ± 11 ms; post-CCh: 156 ± 15 ms, Wilcoxon signed-rank test, p < 0.001, n = 34). **G.** Number of action potentials fired per current step as a function of current injection over rheobase (200 pA over rheobase, pre-CCh: 5.0 ± 0.5 ; post-CCh: 11.9 ± 1.1 , Wilcoxon signed-rank test, p < 0.001, n = 34). **H.** Instantaneous firing frequency of action potentials as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first couple of APs, pre-CCh: 15.9 ± 1.8 Hz, post-CCh: 19.6 ± 2.1 Hz, Wilcoxon signed-rank test, p = 0.027, n = 31). **I.** Action potential amplitude as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first AP, pre-CCh: 77.5 ± 1.4 mV; post-CCh: 71.1 ± 2.4 mV, paired-T test, p = 0.020, n = 29). **J.** Sample traces of the ADP following a depolarizing current step injection in a CA2 PN recorded in whole-cell current-clamp configuration in control (grey) and 10 μ M CCh (black). **K.** Summary graph of CA2 PNs ADP in control (grey) and 10 μ M CCh (black) (pre-CCh: 1.6 ± 0.3 mV; post-CCh: 7.0 ± 0.9 mV,

paired-T test, $p < 0.001$, $n = 34$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **L.** Sample traces of voltage sag in response to hyperpolarizing current step injections recorded in whole-cell current-clamp configuration from a CA2 PN in control (grey) and 10 μM CCh (black). **M.** Summary graph of CA2 PNs voltage sag following hyperpolarization to -100 mV in control ACSF (grey) and 10 μM CCh (black) (pre-CCh: 1.6 ± 0.1 mV; post-CCh: 4.5 ± 0.5 mV, Wilcoxon signed-rank test, $p < 0.001$, $n = 31$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM).

Figure 4. Contribution of synaptic transmission to CCh-induced action potential burst firing in CA2 PNs.

A. Sample trace of bursts recorded in whole-cell current-clamp configuration from a CA2 PN upon application of 10 μM CCh in acute hippocampal slice in control condition (**A1**, 10 μM CCh, black) or application of 10 μM NBQX and 50 μM APV (**A2**, red), 1 μM SR95531 and 2 μM CGP5584A (**A3**, green), and 10 μM NBQX, 50 μM APV, 1 μM SR95531 and 2 μM CGP5584A (**A4**, grey). Panel A3 shows an additional trace of an epileptiform-like discharge recorded in CA2 *stratum pyramidale* (LFP, lower trace). **B – I.** Summary graphs of burst characteristics with application of 10 μM CCh in control (black, $n = 25$; same CCh data shown in Figure 4B, 4C, 4D and 4E as whole-cell data in Figure 1D, 1E, 1F and 1G, respectively), 10 μM NBQX and 50 μM APV (red, $n = 7$), 1 μM SR95531 and 2 μM CGP5584A (green, $n = 6$), and 10 μM NBQX, 50 μM APV, 1 μM SR95531 and 2 μM CGP5584A (grey, $n = 6$) (individual cells shown as dots, population averages shown as thick lines, error bars represent SEM). **B.** Inter-burst interval (Kruskal-Wallis test, $p = 0.006$). **C.** Burst duration (Kruskal-Wallis test, $p = 0.037$). **D.** Number of action potentials per burst (Kruskal-Wallis test, $p = 0.008$). **E.** Action potential firing frequency during bursts (Kruskal-Wallis test, $p = 0.012$). **F.** V_M level during bursts (one-way ANOVA test, $p = 0.41$). **G.** V_M level in between bursts (one-way ANOVA test, $p < 0.001$). **H.** V_M depolarization rate during the burst rising phase (Kruskal-Wallis test, $p < 0.001$). **I.** V_M repolarization rate during the burst decaying phase (Kruskal-Wallis test, $p = 0.087$).

Figure 5. CCh attenuates excitatory synaptic transmission onto CA2 PNs.

A. Diagram illustrating extracellular recording configuration with recording and stimulation electrodes in *stratum radiatum* (SR). (**A1**) Sample traces of field responses to electrical stimulation of SR input to area CA2 before (grey) and after 10 μM CCh application (black) in

the presence of GABA receptors antagonists (1 μM SR95531 and 2 μM CGP5584A). **(A2)** Summary graph of the proportion of control response amplitude remaining in SR after CCh with increasing stimulation intensities (n = 10). **(A3)** Summary graph of the proportion of fEPSP amplitude remaining with 20V stimulation in SR after application of 10 μM CCh alone (control, grey; n = 10, $57.2 \pm 2.5 \%$, One sample T test, $p = 2.8 \cdot 10^{-7}$) or in the presence of 10 μM mecamylamine, a nicotinic acetylcholine receptor blocker (turquoise; n = 10, $54.2 \pm 3.6 \%$, One sample T test, $p = 4.8 \cdot 10^{-7}$), 10 μM pirenzepine, a M1 muscarinic receptor blocker (red; n = 7, $92.6 \pm 6.0 \%$, One sample T test, $p = 0.26$), 10 μM AF-DX 116, a M2 muscarinic receptor blocker (green; n = 6, $53.8 \pm 3.1 \%$, One sample T test, $p = 2.5 \cdot 10^{-5}$) and 10 μM 4-DAMP, an M3 muscarinic receptor blocker (blue; n = 6, $105 \pm 3.7 \%$, One sample T test, $p = 0.23$).

B. Diagram illustrating extracellular recording configuration with recording and stimulation electrodes in *stratum lacunosum moleculare* (SLM). **(B1)** Same as A1, except with SLM input stimulation. **(B2)** Summary graph of the proportion of control response amplitude remaining in SLM after CCh with increasing stimulation intensities (n = 10). **(B3)** Summary graph of the proportion of fEPSP amplitude remaining with 20V stimulation in SLM after 10 μM CCh application (control, grey; n = 10, $68.9 \pm 2.2 \%$, One sample T test, $p = 8.8 \cdot 10^{-7}$) or in the presence of 10 μM mecamylamine (turquoise; n = 10, $70.9 \pm 3.8 \%$, One sample T test, $p = 3.8 \cdot 10^{-5}$), 10 μM pirenzepine (red; n = 7, $97.7 \pm 7.5 \%$, One sample T test, $p = 0.766$), 10 μM AF-DX 116 (green; n = 6, $63.46 \pm 5.0 \%$, One sample T test, $p = 7.9 \cdot 10^{-4}$) and 10 μM 4-DAMP (blue; n = 6, $98.2 \pm 3 \%$, One sample T test, $p = 0.567$).

C. Diagram illustrating whole cell recording configuration from CA2 pyramidal neurons with SR input stimulation in acute hippocampal slices. **(C1)** Sample traces of evoked EPSC recorded in a CA2 PN held at -70 mV with electrical stimulation of SR input before (grey) and after 10 μM CCh application (black) in the presence of GABA receptors antagonists (1 μM SR95531 and 2 μM CGP5584A). **(C2)** Summary graph of the proportion of control response amplitude remaining after CCh with increasing stimulation intensities in SR (n = 6; percent remaining at 20 V stimulation = $57.3 \pm 10.2 \%$, One sample T test, $p = 0.008$). **(C3)** Paired-pulse ratio at 20 V stimulation (n = 6; pre-CCh = 1.5 ± 0.1 , post-CCh = 1.8 ± 0.1 , percent increase = $19 \pm 7 \%$, paired-T test, $p = 0.041$).

D. Same as C with SLM input stimulation. **(D1)** Same as in C1 except with SLM input stimulation. **(D2)** Summary graph of the proportion of control response amplitude remaining after CCh with increasing stimulation intensities in SLM; n = 6; percent remaining at 20V stimulation = $49.1 \pm 8.0 \%$, One sample T test, $p = 0.001$). **(D3)** Paired-pulse ratio at 20 V stimulation (n = 5; pre-CCh = 1.6 ± 0.1 , post-CCh = 1.8 ± 0.1 , percent increase = $13 \pm 3 \%$, paired-T test, $p = 0.024$).

Figure 6. CCh decreases excitatory transmission through a presynaptic mechanism.

A. Sample traces of spontaneous EPSCs (sEPSCs) recorded in whole-cell voltage-clamp configuration from a CA2 PN before (top, grey) and after (bottom, black) application of 10 μ M CCh on acute hippocampal slice. **B.** Summary graph of the time course of sEPSC frequency recorded in whole-cell voltage-clamp configuration from CA2 PNs upon 10 μ M CCh wash-in ($n = 8$; error bars represent SEM). **C.** Summary graph of the sEPSC frequency recorded in whole-cell voltage-clamp configuration from CA2 PNs before and after 10 μ M CCh application ($n = 8$; pre-CCh: 1.6 ± 0.4 Hz; post-CCh: 7.0 ± 1.1 Hz, paired-T test, $p = 0.002$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **D.** Normalized histograms of the sEPSC amplitude recorded in whole-cell voltage-clamp configuration from CA2 PNs before (grey) and after (black) 10 μ M CCh application ($n = 7$; first peaks in amplitude distributions: pre-CCh: 25.1 ± 0.6 pA; post-CCh: 23.9 ± 1.1 pA, paired-T test, $p = 0.27$, shaded areas represent SEM). **E.** Sample traces of miniature EPSCs (mEPSCs) recorded in presence of 100 nM TTX and 4 mM CaCl_2 before (top, grey) and after (bottom, black) application of 10 μ M CCh. **F.** Summary graph of the time course of mEPSC frequency recorded in voltage clamp configuration from CA2 PNs upon 10 μ M CCh application ($n = 9$, error bars represent SEM). **G.** Summary graph of the mEPSC frequency recorded before and after 10 μ M CCh application ($n = 9$; pre-CCh: 0.27 ± 0.05 Hz; post-CCh: 0.35 ± 0.05 Hz, paired-T test, $p = 0.312$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **H.** Normalized histograms of the mEPSC amplitude recorded before (grey) and after (black) 10 μ M CCh application ($n = 9$; first peaks in amplitude distributions: pre-CCh: 9.0 ± 0.6 pA, post-CCh: 8.6 ± 0.9 pA, paired-T test, $p = 0.489$; shaded areas represent SEM). **I.** Averaged sample traces of EPSCs evoked with minimal stimulation of CA3 inputs and recorded in CA2 PNs before (black trace) and after (grey trace) 10 μ M CCh application. **J.** Example data from a single experiment using minimal stimulation in SR to evoke EPSCs in CA2 PNs. Each EPSC amplitude is plotted over time before and during CCh application. **K-O.** Summary graphs of **K.** EPSC amplitude including failures ($n = 5$; paired-T test, $p = 0.023$), **L.** the amplitude excluding failures (paired-T test, $p = 0.555$), **M.** the paired pulse ratio (PPR, paired-T test, $p = 0.0241$), **N.** the failure rate (paired-T test, $p = 0.007$) and **O.** the coefficient of variation (CV, paired-T test, $p = 0.028$) measured before and after 10 μ M CCh application (individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM).

Figure 7. CCh alters inhibitory synaptic transmission onto CA2 PNs.

A. Diagram illustrating whole cell recording configuration from CA2 pyramidal neurons with SR and SLM input stimulation in acute hippocampal slices. Recordings were performed at +10 mV with intracellular cesium in the pipette. **B.** Sample traces (**B1**) of evoked IPSCs recorded in a CA2 PN with electrical stimulation of SR input before (grey) and after 10 μ M CCh application (black) and summary graphs (**B2**) of the proportion of control response amplitude remaining after CCh with increasing stimulation intensities (n = 6; percent remaining at 20 V stimulation = 31.9 ± 7.3 %, One sample T test, p < 0.001). (**B3**) Paired-pulse ratio at 20 V stimulation (n = 6; pre-CCh: 0.9 ± 0.1 ; post-CCh: 1.2 ± 0.1 , percent increase = 32 ± 5 %, paired-T test, p = 0.001). **C.** Same as **B.** with SLM input stimulation (**C1**; sample traces), (**C2**; summary graph; n = 6; percent remaining at 20V stimulation = 67.1 ± 11.2 %, One sample T test, p = 0.033). (**C3**) Paired-pulse ratio at 20 V stimulation (n = 6; pre-CCh: 0.7 ± 0.1 ; post-CCh: 1.0 ± 0.1 ; percent increase: 39 ± 19 % by CCh, paired-T test, p = 0.045). **D.** Sample traces of spontaneous IPSCs (sIPSCs) recorded in whole-cell voltage-clamp configuration from a CA2 PN before (top, grey) and after (bottom, black) application of 10 μ M CCh on acute hippocampal slices. **E.** Summary graph of the time course of sIPSC frequency recorded in whole-cell voltage-clamp configuration from CA2 PNs upon 10 μ M CCh wash-in (n = 7; error bars represent SEM). **F.** Summary graph of the sIPSC frequency recorded in whole-cell voltage-clamp configuration from CA2 PNs before and after CCh application (n = 7; pre-CCh: 15 ± 0.9 Hz, post-CCh: 19 ± 0.9 Hz, paired -T test, p = 0.026; individual cells shown as grey lines, population averages shown as thick lines, error bars represent SEM). **G.** Normalized histograms of the sIPSC amplitude recorded in whole-cell voltage-clamp configuration from CA2 PNs before (grey) and after (black) 10 μ M CCh application (n = 7; first peaks in amplitude distributions: pre-CCh: 36.0 ± 2.0 pA, post-CCh: 40.4 ± 2.6 pA, paired-T test, p = 0.29; shaded areas represent SEM).

Figure 8. Contribution of the M-current to CCh effects on CA2 PNs intrinsic properties.

A. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from a in CA2 PN in control (**A1**, grey), 10 μ M XE-991 (**A2**, purple) and 10 μ M XE-991 and 10 μ M CCh (**A3**, orange), expanded view of the first action potential in each condition (**A4**) and corresponding phase plane plot (**A5**). **B – I.** Summary graphs of CA2 PNs action potential firing properties in control (grey), 10 μ M XE-991 (purple) and 10 μ M XE-991 and 10 μ M CCh (orange) (n = 15; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **B.** Membrane resistance (pre-XE-991: 46 ± 2.6 MOhm, post-XE-991: 53 ± 2.3 MOhm, post-XE-991 and CCh

= 79 ± 4.5 MOhm, Friedman ANOVA, $p < 0.001$). **C.** Action potential threshold (pre-XE-991: -46.3 ± 0.9 mV, post-XE-991: -47.9 ± 1.4 mV, post-XE-991 and CCh: -47.7 ± 1.2 mV; Friedman ANOVA, $p = 0.37$). **D.** Action potential width at half-maximal amplitude (pre-XE-991: 1.13 ± 0.03 ms, post-XE-991: 1.06 ± 0.03 ms, post-XE-991 and CCh: 1.17 ± 0.04 ms, repeated-measures ANOVA, $p = 0.002$). **E.** After-hyperpolarization potential (pre-XE-991: 6.7 ± 0.7 mV, post-XE-991: 9.1 ± 0.4 mV, post-XE-991 and CCh: 8.0 ± 0.5 mV, repeated-measures ANOVA, $p < 0.001$). **F.** Latency to fire first action potential per current step as a function of current injection over rheobase (200 pA over rheobase: pre-XE-991: 100 ± 7.6 ms, post-XE-991: 132 ± 14.0 ms, post-XE-991 and CCh: 147 ± 19.7 ms, Friedman ANOVA, $p = 0.006$). **G.** Number of action potentials fired per current step as a function of current injection over rheobase (200 pA over rheobase, pre-XE-991: 6 ± 1 , post-XE-991: 12 ± 2 , post-XE-991 and CCh: 15 ± 3 , Friedman ANOVA, $p = 0.27$). **H.** Instantaneous firing frequency of action potentials as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first couple of APs frequency, pre-XE-991: 18 ± 1.2 Hz, post-XE-991: 18 ± 1.2 Hz, post-XE-991 and CCh: 20 ± 2.4 Hz, repeated-measures ANOVA, $p = 0.55$). **I.** Action potential amplitude as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first AP amplitude, pre-XE-991: 86 ± 1.9 mV, post-XE-991: 93 ± 1.7 mV, post-XE-991 and CCh: 87 ± 2.5 mV, repeated-measures ANOVA on first AP amplitude, $p = 0.044$). **J.** Sample traces of the ADP following a depolarizing current step injection in a CA2 PN recorded in whole-cell current-clamp configuration in control (grey), 10 μ M XE-991 (purple) and 10 μ M XE-991 and 10 μ M CCh (orange). **K.** Summary graph of CA2 PNs ADP in control (grey), 10 μ M XE-991 (purple) and 10 μ M XE-991 and 10 μ M CCh (orange) ($n = 14$, pre-XE-991: 2.1 ± 0.3 mV, post-XE-991: 0.5 ± 0.5 mV, post-XE-991 and CCh: 5.8 ± 0.7 mV, Friedman ANOVA, $p < 0.001$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **L.** Sample traces of voltage sag in response to hyperpolarizing current step injections recorded in whole-cell current-clamp configuration from a in CA2 PN in control (grey), 10 μ M XE-991 (purple) and 10 μ M XE-991 and 10 μ M CCh (orange). **M.** Summary graphs of CA2 PNs voltage sag following hyperpolarization to -100 mV in control (grey), 10 μ M XE-991 (purple) and 10 μ M XE-991 and 10 μ M CCh (orange) ($n = 12$, pre-XE991: 2.1 ± 0.2 mV, post-XE-991: 3.5 ± 0.3 mV, post-XE-991 and CCh: 4.2 ± 0.5 mV, Friedman ANOVA, $p < 0.001$; individual cells shown as grey lines, population averages shown as thick lines, error bars represent SEM).

Figure 9. Reduction of the M-current alone does not underlie the CCh-induced action potential bursting in CA2 PNs.

A. Sample trace recorded in whole-cell current-clamp configuration from a CA2 PN and expanded views showing only minimal V_M depolarization without bursts during application of 10 μM of the M-current blocker XE-991 (**A1**) followed by increased depolarization (**A2**) and bursts (**A3**) upon subsequent application of 10 μM CCh in acute hippocampal slice. **B – D.** Summary graphs of CA2 PN V_M changes with application of 10 μM CCh (black, $n = 29$; same CCh data shown in Figure 8C and 8D as 10 μM CCh in Figure 2E and 2F, respectively) or 10 μM XE-991 (purple, $n = 8$) (individual cells shown as dots, population averages shown as thick lines, error bars represent SEM). **B.** V_M levels at resting and after the initial depolarization induced by CCh or XE-991 (pre-CCh: -75.4 ± 1.1 ; post-CCh: -65.4 ± 1.0 ; pre-XE-991: -76.7 ± 2.7 mV, post-XE-991: -73.6 ± 2.8 mV, paired-T test pre-CCh and post-CCh, $p < 0.001$; paired-T test pre-XE-991 and post-XE-991, $p = 0.001$; T test pre-CCh and pre-XE-991, $p = 0.65$; T test post-CCh and post-XE-991, $p = 0.023$). **C.** V_M initial depolarization magnitude upon CCh or XE-991 application (CCh: 10.0 ± 0.9 mV, XE-991: 3.2 ± 0.6 mV, Mann-Whitney U test, $p < 0.001$). **D.** V_M initial depolarization rate upon CCh or XE-991 application (CCh: 6.0 ± 0.7 mV/min, XE-991: 0.8 ± 0.3 mV/min, Mann-Whitney U test, $p < 0.001$). **E.** V_M levels following sequential addition of XE-991 and XE-991 plus CCh (pre-XE-991: -74.0 ± 3.4 mV, post-XE-991: -70.9 ± 3.6 mV, post-XE-991 and CCh: -61.1 ± 3.2 mV, repeated-measures ANOVA, $p = 0.001$, $n = 5$).

Figure 10. Contribution of voltage-activated potassium currents to CCh-induced effects on CA2 PN intrinsic properties.

A. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from a CA2 PN in control ACSF (**A1**, grey), 100 nM α -Dendrotoxin (α -DTX, **A2**, orange) and 100 nM α -DTX and 10 μM CCh (**A3**, green), expanded view of the first action potential in each condition (**A4**) and corresponding phase plane plot (**A5**). **B – I.** Summary graphs of CA2 PNs action potential firing properties in control (grey), 100 nM α -DTX (orange) and 100 nM α -DTX and 10 μM CCh (green) ($n = 12$ for pre- and post- α -DTX, $n = 9$ for post- α -DTX and CCh; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **B.** Membrane resistance (pre- α -DTX: 70.9 ± 6.5 MOhm, post- α -DTX: 73.1 ± 6.6 MOhm, post- α -DTX and CCh: 92.2 ± 9.4 MOhm, repeated-measures ANOVA, $p = 0.0028$) **C.** Action potential threshold (pre- α -DTX: -42.9 ± 0.8 mV, post- α -DTX: -44.7 ± 1.1 mV, post- α -DTX and CCh: -48.5 ± 1.1 mV, repeated-measures ANOVA, $p < 0.001$). **D.** Action potential

width at half-maximal amplitude (pre- α -DTX: 1.36 ± 0.05 ms, post- α -DTX: 1.34 ± 0.05 ms, post- α -DTX and CCh: 1.38 ± 0.05 ms, repeated-measures ANOVA, $p = 0.71$). **E.** After-hyperpolarization potential (pre- α -DTX: 7.0 ± 0.7 mV, post- α -DTX: 6.9 ± 0.9 mV, post- α -DTX and CCh: -4.3 ± 1.1 mV, repeated-measures ANOVA, $p = 0.0021$). **F.** Latency to fire first action potential per current step as a function of current injection over rheobase (200 pA over rheobase, pre- α -DTX: 180.5 ± 22.5 ms, post- α -DTX: 138.1 ± 23.1 ms, post- α -DTX and CCh: 91.0 ± 20.3 ms, repeated-measures ANOVA, $p = 0.0016$). **G.** Number of action potentials fired per current step as a function of current injection over rheobase (200 pA over rheobase, pre- α -DTX: 5.5 ± 0.6 , post- α -DTX: 7.5 ± 1.0 , post- α -DTX and CCh = 15.1 ± 2.1 , Friedman ANOVA, $p < 0.001$). **H.** Instantaneous firing frequency of action potentials as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first couple of APs: pre- α -DTX: 12.9 ± 1.8 Hz, post- α -DTX: 18.7 ± 2.6 Hz, post- α -DTX and CCh: 25.3 ± 3.6 Hz, repeated-measures ANOVA, $p < 0.001$). **I.** Action potential amplitude as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first AP: pre- α -DTX: 70.0 ± 3.4 mV, post- α -DTX: 70.7 ± 4.1 mV, post- α -DTX and CCh: 69.3 ± 6.6 mV, repeated-measures ANOVA, $p = 0.59$).

J. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from a CA2 PN in control ACSF (**J1**, grey), 100 μ M 4-AP (**J2**, blue) and 100 μ M 4-AP and 10 μ M CCh (**J3**, pink), expanded view of the first action potential in each condition (**J4**) and corresponding phase plane plot (**J5**). **K – R.** Summary graphs of CA2 PNs action potential firing properties in control (grey), 100 μ M 4-AP (blue) and 100 μ M 4-AP and 10 μ M CCh (pink) ($n = 10$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **K.** Membrane resistance (pre-4-AP: 44.6 ± 5.7 MOhm, post-4-AP: 55.3 ± 8.0 MOhm, post-4-AP and CCh: 61.0 ± 6.3 MOhm, repeated-measures ANOVA, $p = 0.029$). **L.** Action potential threshold (pre-4-AP: -42.8 ± 1.3 mV, post-4-AP: -52.4 ± 1.4 mV, post-4-AP and CCh: -54.9 ± 1.5 mV, repeated-measures ANOVA, $p < 0.001$). **M.** Action potential width at half-maximal amplitude (pre-4-AP: 1.3 ± 0.1 ms, post-4-AP: 1.2 ± 0.04 ms, post-4-AP and CCh: 1.1 ± 0.05 ms, repeated-measures ANOVA, $p = 0.27$). **N.** After-hyperpolarization potential (pre-4-AP: 5.9 ± 1.4 mV, post-4-AP: -0.4 ± 1.4 mV, post-4-AP and CCh: -2.5 ± 1.2 mV, repeated-measures ANOVA, $p < 0.001$). **O.** Latency to fire first action potential per current step as a function of current injection over rheobase (200 pA over rheobase, pre-4-AP: 152 ± 33.9 ms, post-4-AP: 48 ± 5.1 ms, post-4-AP and CCh: 43 ± 3.5 ms, Friedman ANOVA, $p = 0.018$). **P.** Number of action potentials fired per

current step as a function of current injection over rheobase (200 pA over rheobase, pre-4-AP: 4.0 ± 0.7 , post-4-AP: 6.5 ± 0.7 , post-4-AP and CCh = 10.5 ± 1.6 , Friedman ANOVA, $p < 0.001$). **Q.** Instantaneous firing frequency of action potentials as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first couple of APs, pre-4-AP: 11.3 ± 3.1 Hz, post-4-AP: 84.9 ± 11.3 Hz, post-4-AP and CCh: 106.7 ± 10.9 Hz, Friedman ANOVA, $p < 0.001$). **R.** Action potential amplitude as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first AP, pre-4-AP: 76 ± 3.6 mV, post-4-AP: 89 ± 2.4 mV, post-4-AP and CCh: 88 ± 2.2 mV, repeated-measures ANOVA, $p < 0.001$).

Figure 11. Voltage-gated potassium channels pace AP firing during CCh-induced bursts in CA2 PNs. **A.** Sample trace of bursts recorded in whole-cell current-clamp configuration from a CA2 PN (**A1**) and expanded view of a burst (**A2**) upon application of $10 \mu\text{M}$ CCh in acute hippocampal slice with D-type potassium channels blocked by application of 100 nM α -Dendrotoxin (α -DTX). (**A3**) Sample trace of bursts and an expanded view (**A4**) recorded in the same configuration, except with the non-selective K_V channel blocker 4-AP applied at $100 \mu\text{M}$ with $10 \mu\text{M}$ CCh. **B.** (**B1**) V_M levels at rest (grey) after application of $100 \mu\text{M}$ 4-AP (blue) and after further application of $10 \mu\text{M}$ CCh (pink) (pre-4-AP: -76.7 ± 1.6 mV, post-4-AP: -75.6 ± 1.5 mV, post-4-AP and CCh: -65.7 ± 1.5 mV, $n = 10$; repeated-measures ANOVA, $p < 0.001$). (**B2**) V_M levels at rest (grey) and after application of α -DTX (orange) and further application of CCh (green). (pre- α -DTX: -72.9 ± 0.8 mV, post- α -DTX : -71.3 ± 1.1 mV, post- α -DTX and CCh: -64.7 ± 0.7 mV, $n = 10$; repeated-measures ANOVA, $p < 0.001$). **C - E.** Summary graphs of burst characteristics with application of $10 \mu\text{M}$ CCh in control (black, $n = 25$; same CCh data shown in Figure 12C-E as CCh in Figure 4H, 4I and 4E respectively), 100 nM α -DTX (green, $n = 9$) and $100 \mu\text{M}$ 4-AP (pink, $n = 8$) (individual cells shown as dots, population averages shown as thick lines, error bars represent SEM). **C.** Burst rise rate (Kruskal-Wallis test, $p < 0.001$). **D.** Burst decay rate (Kruskal-Wallis test, $p = 0.008$). **E.** Firing rate during burst (Kruskal-Wallis test, $p = 0.027$).

Figure 12. Influence of T-type calcium channels on CA2 PNs action potential firing.

A. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from a CA2 PN in control (**A1**, grey) and $2 \mu\text{M}$ TTA-A2 (**A2**, magenta), expanded view of the first action potential in each condition (**A3**) and corresponding phase plane plot (**A4**). **B - I.** Summary graphs of CA2 PNs action potential firing properties in control (grey) and $2 \mu\text{M}$ TTA-A2 (magenta) ($n = 9$; individual

cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **B.** Membrane resistance (pre-TTA-A2: 59.2 ± 8.3 MOhm, post-TTA-A2: 66.8 ± 9.2 MOhm, paired-T test, $p = 0.028$). **C.** Action potential threshold (pre-TTA-A2: -43.1 ± 2.0 mV, post-TTA-A2: -44.4 ± 2.0 mV, paired-T test, $p = 0.57$). **D.** Action potential width at half-maximal amplitude (pre-TTA-A2: 1.16 ± 0.02 ms, post-TTA-A2: 1.05 ± 0.02 ms, paired-T test, $p = 0.001$). **E.** After-hyperpolarization potential (pre-TTA-A2: 1.5 ± 1.2 mV, post-TTA-A2: 10.5 ± 1.2 mV; paired-T test, $p = 0.54$). **F.** Latency to fire first action potential per current step as a function of current injection over rheobase (200 pA over rheobase: pre-TTA-A2: 276.8 ± 57.4 ms; post-TTA-A2: 119.2 ± 33.8 ms; paired-T test, $p = 0.077$). **G.** Number of action potentials fired per current step as a function of current injection over rheobase (200 pA over rheobase, pre-TTA-A2: 4.1 ± 0.9 ; post-TTA-A2: 3.4 ± 0.4 , paired-T test, $p = 0.37$). **H.** Instantaneous firing frequency of action potentials as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first couple of APs, pre-TTA-A2: 5.6 ± 0.5 Hz, post-TTA-A2: 4.0 ± 0.9 Hz, paired-T test, $p = 0.046$). **I.** Action potential amplitude as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first AP, pre-TTA-A2: 77.0 ± 3.1 mV; post-TTA-A2: 85.9 ± 2.9 mV, Wilcoxon signed-rank test, $p = 0.012$).

Figure 13. T-type voltage-gated calcium channels control the duration of CCh-induced bursts in CA2 PNs.

A. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from CA2 PNs in $10 \mu\text{M}$ CCh (**A1**, black) or $2 \mu\text{M}$ TTA-A2 and $10 \mu\text{M}$ CCh (**A2**, blue). **B – C.** Summary graphs of CA2 PNs intrinsic properties in $10 \mu\text{M}$ CCh (CCh black, ACSF grey $n = 34$; For reference, same CCh and control data as shown in Figure 3F and 3G), and $2 \mu\text{M}$ TTA-A2 and $10 \mu\text{M}$ CCh (blue, $n = 9$) (population averages shown as thick lines, error bars represent SEM). **B.** Latency to fire first action potential per current step by CA2 PNs as a function of current injection over rheobase (200 pA over rheobase, CCh: 156.0 ± 15.0 ms; CCh and TTA-A2: 438.4 ± 77.7 ms, Mann-Whitney U test, $p < 0.001$). **C.** Number of action potentials fired per current step by CA2 PNs as a function of current injection over rheobase (200 pA over rheobase, CCh: 11.9 ± 1.1 , CCh and TTA-A2: 5.8 ± 1.2 , T test, $p = 0.001$). **D.** Sample trace of bursts recorded in whole-cell current-clamp configuration from a CA2 PN (**D1**) and expanded view of a burst (**D2**) upon application of $10 \mu\text{M}$ CCh in acute hippocampal slice with T-type voltage-gated calcium channels blocked by application of $2 \mu\text{M}$ TTA-A2. **E.** V_M levels at rest (grey), after application

of 2 μM TTA-A2 (magenta) and after further application of 10 μM CCh (blue) (pre-TTA-A2: -73.1 ± 2.9 mV; post-TTA-A2: -73.9 ± 3.0 mV; post-TTA-A2 and CCh V_m : -61.7 ± 1.8 mV, $n = 9$; repeated-measures ANOVA, $p < 0.001$). **F.** Summary graphs of CA2 PN action potential burst duration with application of 10 μM CCh in control (black, $n = 25$; same CCh data shown in Figure 14F as 10 μM CCh in Figure 1E) and 2 μM TTA-A2 (blue, $n = 9$) (Mann-Whitney U test, $p = 0.006$) (individual cells shown as dots, population averages shown as thick lines, error bars represent SEM).

Figure 14. Influence of SK channels on CA2 PNs action potential firing.

A. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from a in CA2 PN in control (**A1**, grey) and 0.1 μM apamin (**A2**, blue), expanded view of the first action potential in each condition (**A3**) and corresponding phase plane plot (**A4**). **B – I.** Summary graphs of CA2 PNs action potential firing properties in control (grey) and 0.1 μM apamin (blue) ($n = 10$; individual cells shown as thin lines, population averages shown as thick lines, error bars represent SEM). **B.** Membrane resistance (pre-apamin: 52.2 ± 5.0 MOhm; post-apamin: 55.9 ± 3.8 MOhm, paired-T test, $p = 0.053$). **C.** Action potential threshold (pre-apamin: -41.7 ± 1.6 mV; post-apamin: -44.7 ± 1.0 mV, paired-T test, $p = 0.010$). **D.** Action potential width at half-maximal amplitude (pre-apamin: 1.29 ± 0.05 ms; post-apamin: 1.16 ± 0.03 ms, Wilcoxon signed-rank test, $p = 0.006$). **E.** After-hyperpolarization potential (pre-apamin: 6.6 ± 1.1 mV; post-apamin: 8.7 ± 0.7 mV; paired-T test, $p = 0.013$). **F.** Latency to fire first action potential per current step as a function of current injection over rheobase (200 pA over rheobase, pre-apamin: 144.9 ± 18.5 ms; post-apamin: 191.3 ± 25.6 ms, paired-T test, $p = 0.087$). **G.** Number of action potentials fired per current step as a function of current injection over rheobase (200 pA over rheobase, pre-apamin: 5.1 ± 0.9 ; post-apamin: 5.4 ± 1.0 , paired-T test, $p = 0.60$). **H.** Instantaneous firing frequency of action potentials as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first couple of APs, pre-apamin: 13.2 ± 2.8 Hz; post-apamin: 14.2 ± 2.8 Hz, paired-T test, $p = 0.74$). **I.** Action potential amplitude as a function of action potential number during a current step of intensity chosen 1.5 times above rheobase (first AP, pre-apamin: 74.7 ± 3.0 mV; post-apamin: 79.0 ± 2.1 mV, Wilcoxon signed-rank test on first AP amplitude, $p = 0.13$).

Figure 15. SK channels contribute to CA2 PN V_m repolarization at the end of AP bursts.

A. Sample traces of action potential firing in response to depolarizing current step injections recorded in whole-cell current-clamp configuration from CA2 PNs in 10 μM CCh (**A1**, black) or 0.1 μM apamin and 10 μM CCh (**A2**, green). **B – C.** Summary graphs of CA2 PNs intrinsic properties in 10 μM CCh (CCh black, ACSF grey $n = 34$; For reference, same CCh and control data as shown in Figure 3F and 3G), and 0.1 μM apamin and 10 μM CCh (green, $n = 10$) (population averages shown as thick lines, error bars represent SEM). **B.** Latency to fire first action potential per current step by CA2 PNs as a function of current injection over rheobase (200 pA over rheobase, CCh: 156 ± 15.0 ms; CCh and apamin: 379 ± 64 ms, Mann-Whitney U test, $p < 0.001$). **C.** Number of action potentials fired per current step by CA2 PNs as a function of current injection over rheobase (200 pA over rheobase, CCh: 11.9 ± 1.1 , CCh and apamin: 4.0 ± 0.7 , Mann-Whitney U test, $p < 0.001$). **D.** Sample trace of bursts recorded in whole-cell current-clamp configuration from a CA2 PN (**D1**) and expanded view of a burst (**D2**) upon application of 10 μM CCh in acute hippocampal slice with SK channels blocked by application of 0.1 μM apamin. **E.** V_M levels at resting (grey), after application of 0.1 μM apamin (blue) and after further application of 10 μM CCh (green) (pre-apamin V_M : -76.8 ± 2.2 mV; post-apamin V_M : -75.1 ± 2.2 mV, post-apamin and CCh V_M : -66.0 ± 2.3 mV, $n = 7$; repeated-measures ANOVA, $p < 0.001$). **F.** Summary graph of CA2 PN V_M level in between bursts with application of 10 μM CCh in control (black, $n = 25$; same CCh data shown in Figure 14F as CCh in Figure 4G) and 0.1 μM apamin (green, $n = 10$) (T test, $p = 0.035$) (individual cells shown as dots, population averages shown as thick lines, error bars represent SEM).

Table 1. Effect of synaptic transmission on charbachol-induced CA2 PN spontaneous activity.

	Control (10 μ M CCh) (n = 25)	Excitatory transmission blocked (10 μ M CCh, 10 μ M NBQX, 50 μ M APV) (n = 7)	Inhibitory transmission blocked (10 μ M CCh, 1 μ M SR95531, 2 μ M CGP5584A) (n = 6)	Excitatory and inhibitory transmission blocked (10 μ M CCh, 10 μ M NBQX, 50 μ M APV, 1 μ M SR95531, 2 μ M CGP5584A) (n = 6)	Statistics
ACSF- V_M (mV) (before CCh application)	-75 \pm 1.2	-72 \pm 3.1	-80 \pm 1.9	-70 \pm 4.1	1-way ANOVA test, p = 0.054
CCh- V_M (mV)	-64 \pm 1.0	-62 \pm 2.1	-64 \pm 1.5	-57 \pm 2.9 p = 0.025 vs CT	1-way ANOVA test, p = 0.036 Tukey test post hoc
$\Delta_{\text{ACSF-CCh } V_M}$ (mV)	11 \pm 1.0	9.5 \pm 1.5	16 \pm 1.9	13 \pm 1.4	1-way ANOVA test, p = 0.065
depolarization rate (mV/min)	6.5 \pm 0.8	9.2 \pm 3.4	9.4 \pm 2.3	8.6 \pm 2.0	Kruskal-Wallis test, p = 0.52
burst onset (min)	6.4 \pm 0.7	4.6 \pm 1.8	4.7 \pm 1.2	3.5 \pm 0.6	1-way ANOVA test, p = 0.21
inter-burst interval (s)	100 \pm 14.7	36 \pm 8.3 p < 0.05 vs CT	113 \pm 41.9	36 \pm 5.6 p < 0.05 vs CT	Kruskal-Wallis test, p = 0.006 Dunn-Holland- Wolfe post hoc
burst- V_M (mV)	-42 \pm 1.0	-36 \pm 1.6	-42 \pm 2.2	-40 \pm 3.2	1-way ANOVA test, p = 0.41
inter-burst- V_M (mV)	-62 \pm 0.8	-56 \pm 1.4 p = 0.001 vs CT p = 0.013 vs SR	-63 \pm 1.9	-56 \pm 1.1 p = 0.006 vs CT p = 0.033 vs SR	1-way ANOVA test, p < 0.001 Tukey post hoc
$\Delta_{\text{burst-IBI } V_M}$ (mV)	21 \pm 1.2	18 \pm 2.6	22 \pm 3.7	14 \pm 2.3	1-way ANOVA test, p = 0.11
burst rise rate (mV/s)	3.5 \pm 0.5	5.9 \pm 1.1	59 \pm 26 p < 0.05 vs CT	3.6 \pm 0.6	Kruskal-Wallis test, p < 0.001 Dunn-Holland- Wolfe post hoc
burst decay rate (mV/s)	-4.6 \pm 0.8	-4.3 \pm 0.7	-13.7 \pm 4.0	-4.8 \pm 0.6	Kruskal-Wallis test, p = 0.087
burst duration (s)	3.8 \pm 0.5	2.6 \pm 0.7	1.1 \pm 0.3 p < 0.05 vs CT	2.4 \pm 0.6	Kruskal-Wallis test, p = 0.037 Dunn-Holland- Wolfe post hoc
Number of APs / burst	22 \pm 2.5	11 \pm 1.8 p < 0.05 vs CT	13 \pm 2.3	11 \pm 2.5 p < 0.05 vs CT	Kruskal-Wallis test, p = 0.008 Dunn-Holland- Wolfe post hoc
Firing frequency in burst (Hz)	13 \pm 1.9	9.5 \pm 1.4	36 \pm 7.9 p < 0.05 vs CT	9.7 \pm 4.5	Kruskal-Wallis test, p = 0.012

					Dunn-Holland- Wolfe post hoc
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Table 2. Effect of 4-AP and α -DTX on charbachol-induced CA2 PN spontaneous activity.

	Control (n = 25)	non-selective block of voltage-gated potassium channels (100 μ M 4-AP) (n = 8)	D-type potassium channels blocked (100 nM α -DTX) (n = 9)	Statistics
ACSF- V_M (mV)	-75 \pm 1.2	-76 \pm 1.5	-71 \pm 1.1	1-way ANOVA test, p = 0.152
CCh- V_M (mV)	-64 \pm 1.0	-66 \pm 1.5	-65 \pm 0.7	Kruskal-Wallis test, p = 0.613
$\Delta_{\text{ACSF-CCh } V_M}$ (mV)	11 \pm 1.0	10 \pm 1.5	7 \pm 0.9	Kruskal-Wallis test, p = 0.058
depolarization rate (mV/min)	6.5 \pm 0.8	5.4 \pm 0.8	3.4 \pm 0.6	Kruskal-Wallis test, p = 0.091
burst onset (min)	6.4 \pm 0.7	4.4 \pm 1.0	6.1 \pm 0.9	1-way ANOVA test, p = 0.078
inter-burst interval (s)	100 \pm 14.7	119 \pm 26.2	136 \pm 33.0	1-way ANOVA test, p = 0.512
burst- V_M (mV)	-42 \pm 1.0	-37 \pm 3.2	-44 \pm 0.9	Kruskal-Wallis test, p = 0.067
inter-burst- V_M (mV)	-62 \pm 0.8	-66 \pm 1.5	-64 \pm 1.2	1-way ANOVA test, p = 0.079
$\Delta_{\text{burst-IBI } V_M}$ (mV)	22 \pm 1.3	29 \pm 3.2 p = 0.046 vs CT	20 \pm 1.8	1-way ANOVA test, p = 0.034 Tukey post hoc
burst rise rate (mV/s)	3.5 \pm 0.5	97.5 \pm 38.0 p < 0.05 vs CT	65.6 \pm 33.4 p < 0.05 vs CT	Kruskal-Wallis test, p < 0.001 Dunn-Holland-Wolfe post hoc
burst decay rate (mV/s)	-4.6 \pm 0.8	-21.6 \pm 5.0 p < 0.05 vs CT	-13.3 \pm 6.5	Kruskal-Wallis test, p = 0.008 Dunn-Holland-Wolfe post hoc
burst duration (s)	3.8 \pm 0.5	1.9 \pm 0.7	6.2 \pm 1.9	Kruskal-Wallis test, p = 0.070
Number of APs / burst	22 \pm 2.5	25 \pm 7.7	54 \pm 19.9	Kruskal-Wallis test, p = 0.266
Firing frequency in burst (Hz)	13 \pm 1.9	30 \pm 7.1 p < 0.05 vs CT	16 \pm 3.9	Kruskal-Wallis test, p = 0.027 Dunn-Holland-Wolfe post hoc

Table 3. Effect of TTA-A2 on charbachol-induced CA2 PN spontaneous activity.

	Control (n = 25)	T-type voltage-gated calcium channels blocked (2 μ M TTA-A2) (n = 9)	Statistics
ACSF- V_M (mV)	-75 \pm 1.2	-72 \pm 2.3	T test p = 0.28
CCh- V_M (mV)	-64 \pm 1.0	-62 \pm 1.8	T test p = 0.22
$\Delta_{\text{ACSF-CCh}} V_M$ (mV)	11 \pm 1.0	11 \pm 1.0	Mann-Whitney U test p = 0.89
depolarization rate (mV/min)	6.5 \pm 0.8	4.6 \pm 0.9	T test p = 0.12
burst onset (min)	6.4 \pm 0.7	6.0 \pm 1.0	T test p = 0.78
inter-burst interval (s)	100 \pm 14.7	109 \pm 18.4	T test p = 0.74
burst- V_M (mV)	-42 \pm 1.0	-42 \pm 0.7	Mann-Whitney U test p = 0.74
inter-burst- V_M (mV)	-62 \pm 0.8	-60 \pm 1.2	T test p = 0.17
$\Delta_{\text{burst-IBI}} V_M$ (mV)	21 \pm 1.2	18 \pm 0.9	Mann-Whitney U test p = 0.13
burst rise rate (mV/s)	3.5 \pm 0.5	2.8 \pm 0.5	Mann-Whitney U test p = 0.69
burst decay rate (mV/s)	-4.6 \pm 0.8	-5.1 \pm 1.1	Mann-Whitney U test p = 0.83
burst duration (s)	3.8 \pm 0.5	8.9 \pm 1.9	Mann-Whitney U test p = 0.006
Number of APs / burst	22 \pm 2.5	31 \pm 6.4	Mann-Whitney U test p = 0.072
Firing frequency in burst (Hz)	13 \pm 1.9	10 \pm 1.9	Mann-Whitney U test p = 0.66

Table 4. Effect of apamin on charbachol-induced CA2 PN spontaneous activity.

	Control (n = 25)	SK channels blocked (0.1 μ M apamin) (n = 10)	Statistics
ACSF- V_M (mV)	-75 \pm 1.2	-75 \pm 2.2	T test p = 0.95
CCh- V_M (mV)	-64 \pm 1.0	-66 \pm 2.3	T test p = 0.49
$\Delta_{\text{ACSF-CCh}} V_M$ (mV)	11 \pm 1.0	9.1 \pm 1.3	Mann-Whitney U test p = 0.55
depolarization rate (mV/min)	6.5 \pm 0.8	5.3 \pm 1.6	T test p = 0.50
burst onset (min)	6.4 \pm 0.7	6.5 \pm 1.3	T test p = 0.93
inter-burst interval (s)	100 \pm 14.7	120 \pm 31.2	T test p = 0.58
burst- V_M (mV)	-42 \pm 1.0	-42 \pm 1.2	T test p = 0.41
inter-burst- V_M (mV)	-62 \pm 0.8	-59 \pm 1.3	T test p = 0.035
$\Delta_{\text{burst-IBI}} V_M$ (mV)	21 \pm 1.2	17 \pm 1.9	T test p = 0.045
burst rise rate (mV/s)	3.5 \pm 0.5	5.6 \pm 1.2	Mann-Whitney U test p = 0.06
burst decay rate (mV/s)	-4.6 \pm 0.8	-4.7 \pm 1.0	Mann-Whitney U test p = 0.91
burst duration (s)	3.8 \pm 0.5	5.4 \pm 1.9	Mann-Whitney U test p = 0.97
Number of APs / burst	22 \pm 2.5	16 \pm 2.0	Mann-Whitney U test p = 0.20
Firing frequency in burst (Hz)	13 \pm 1.9	12 \pm 3.0	Mann-Whitney U test p = 0.94

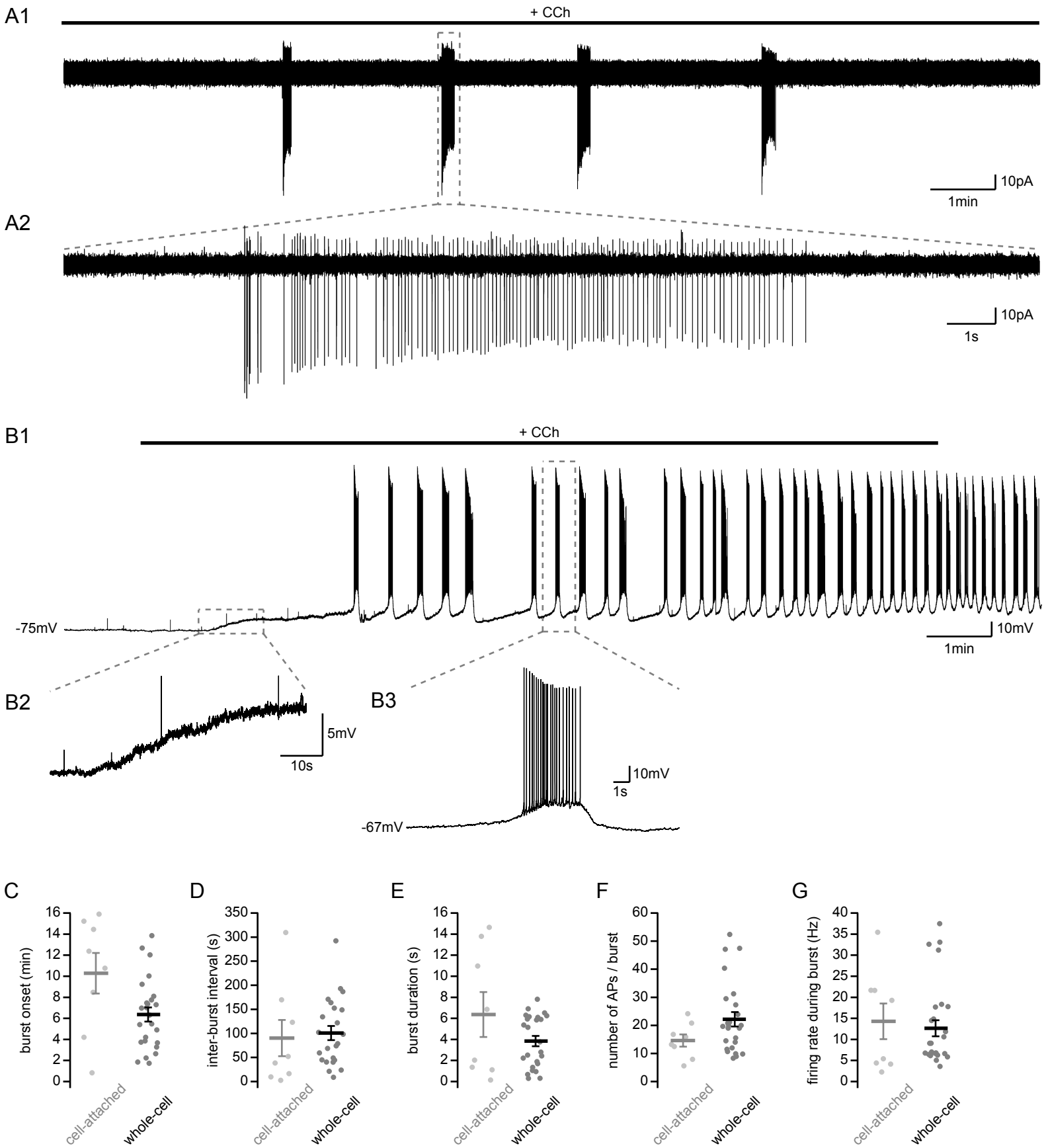


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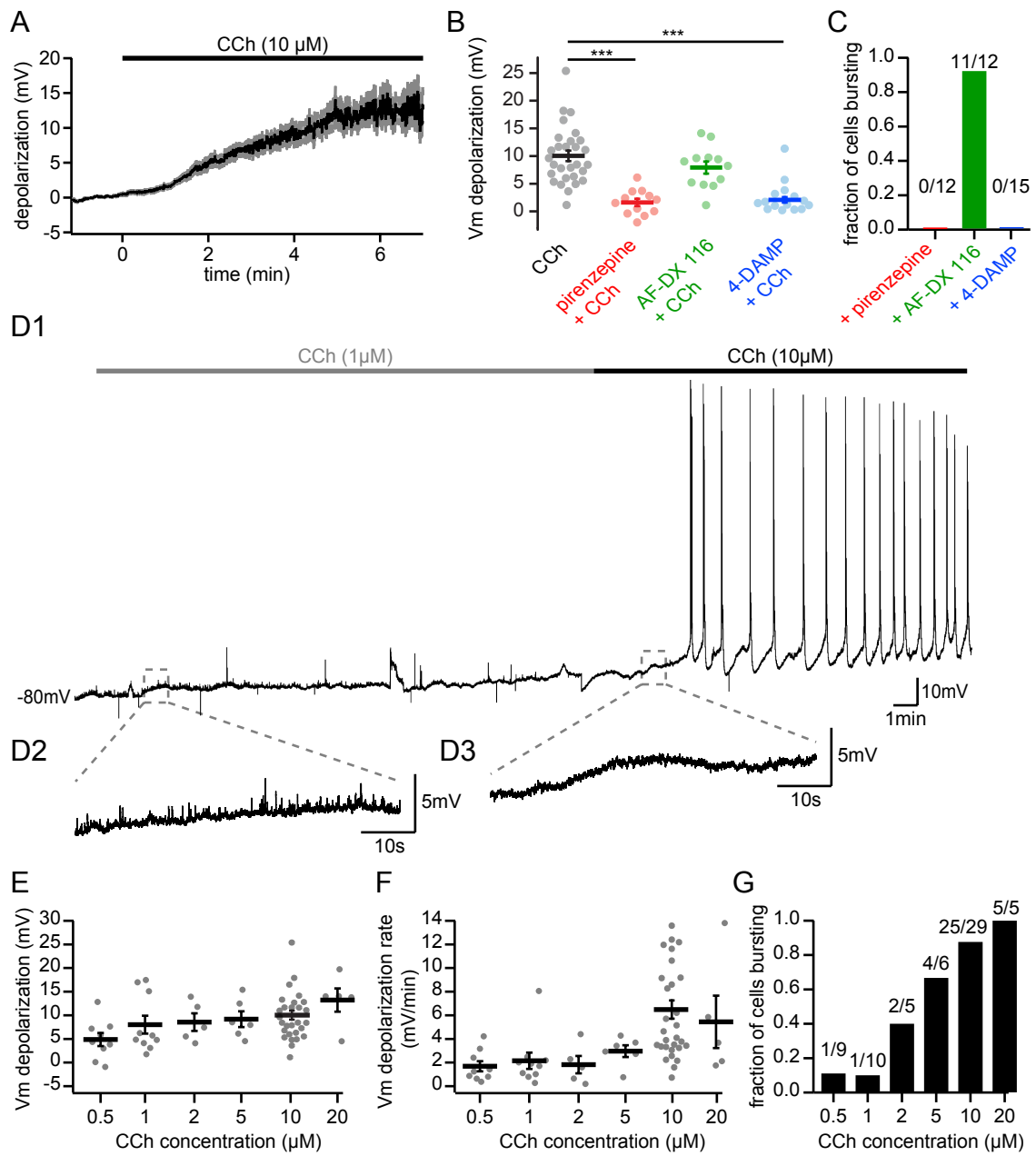


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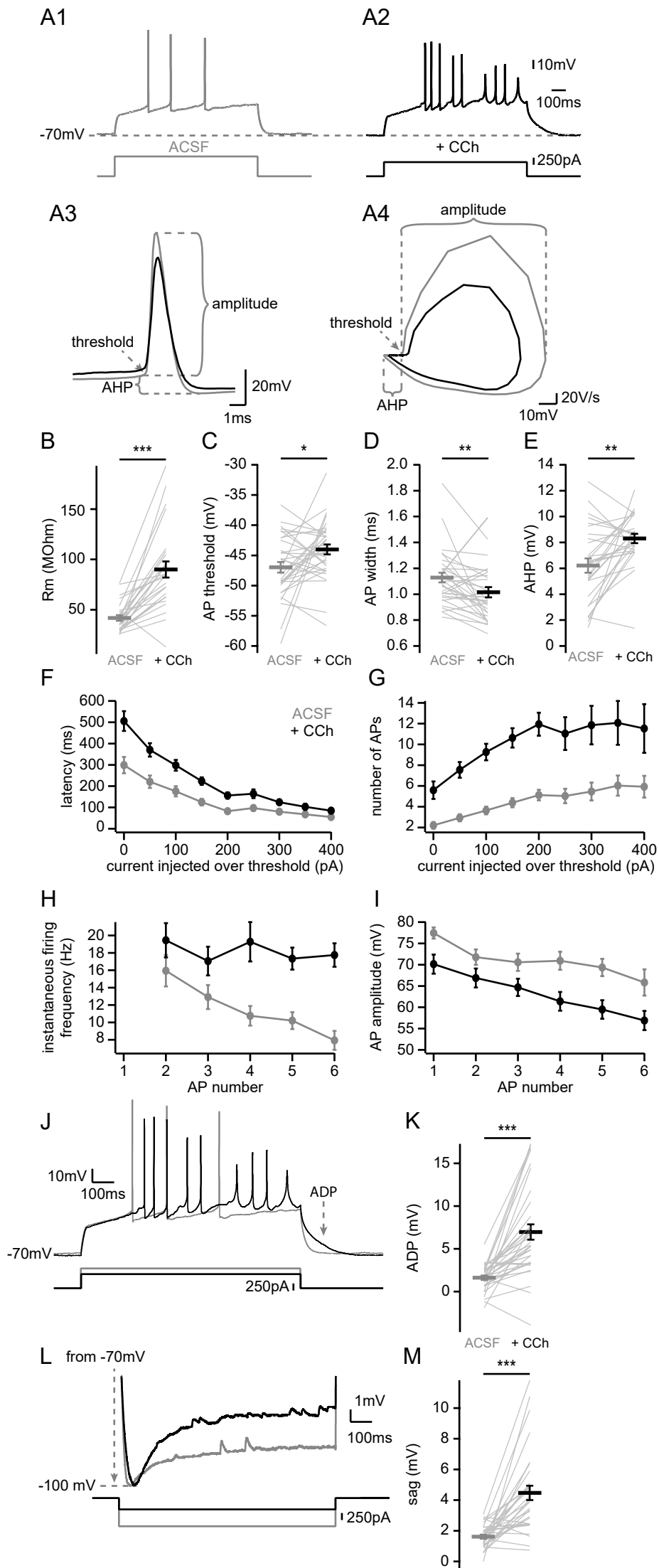


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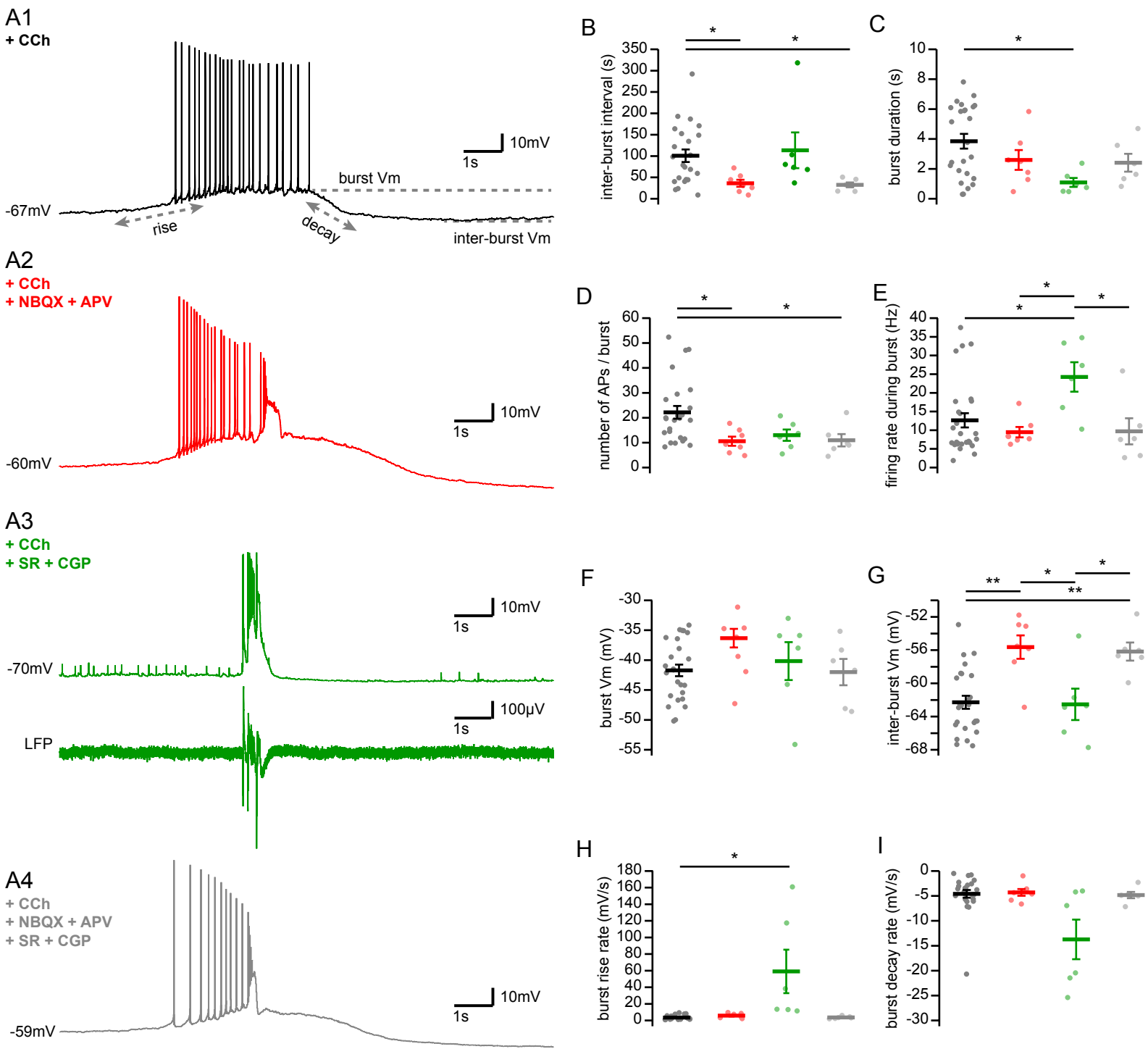


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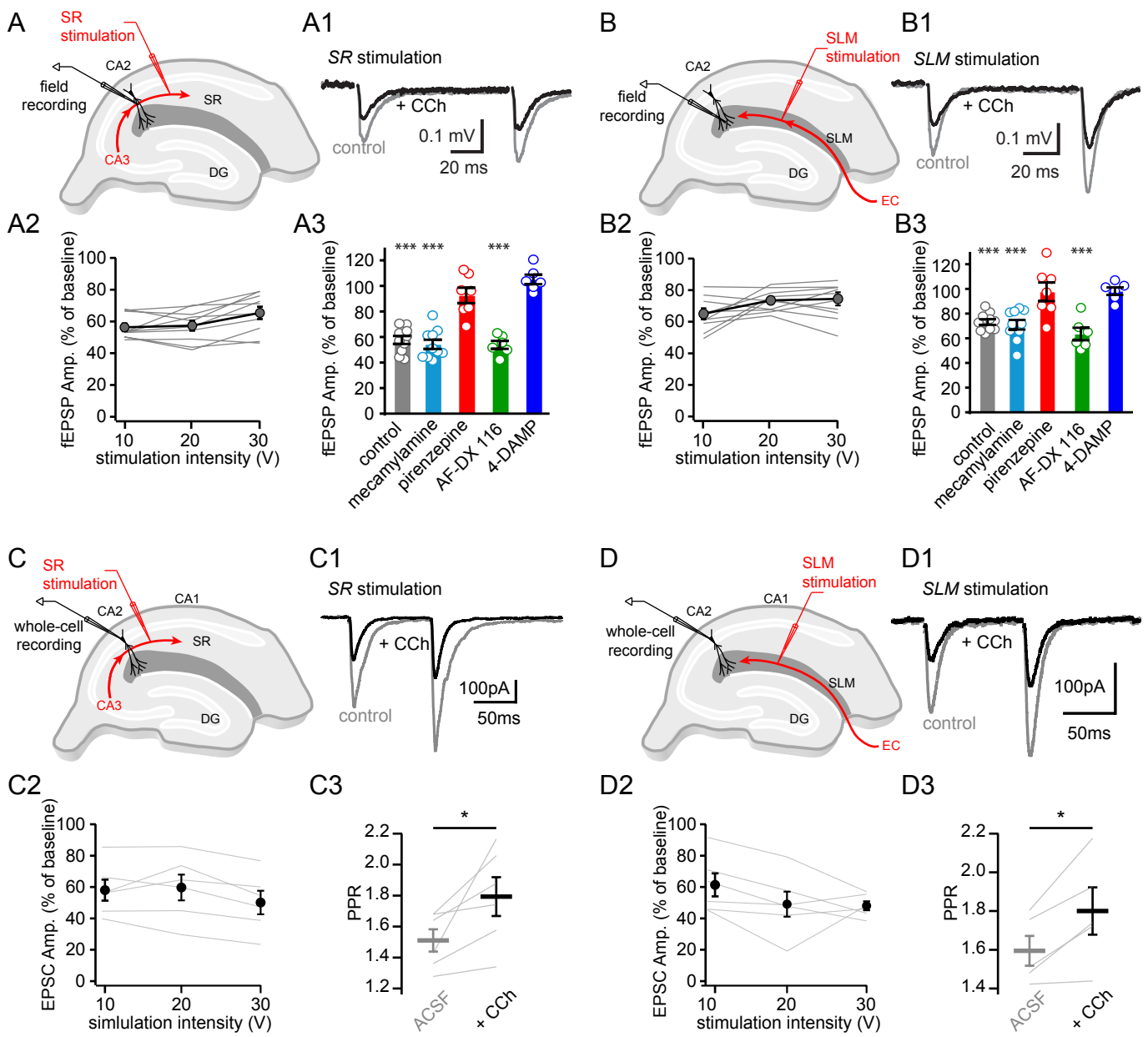


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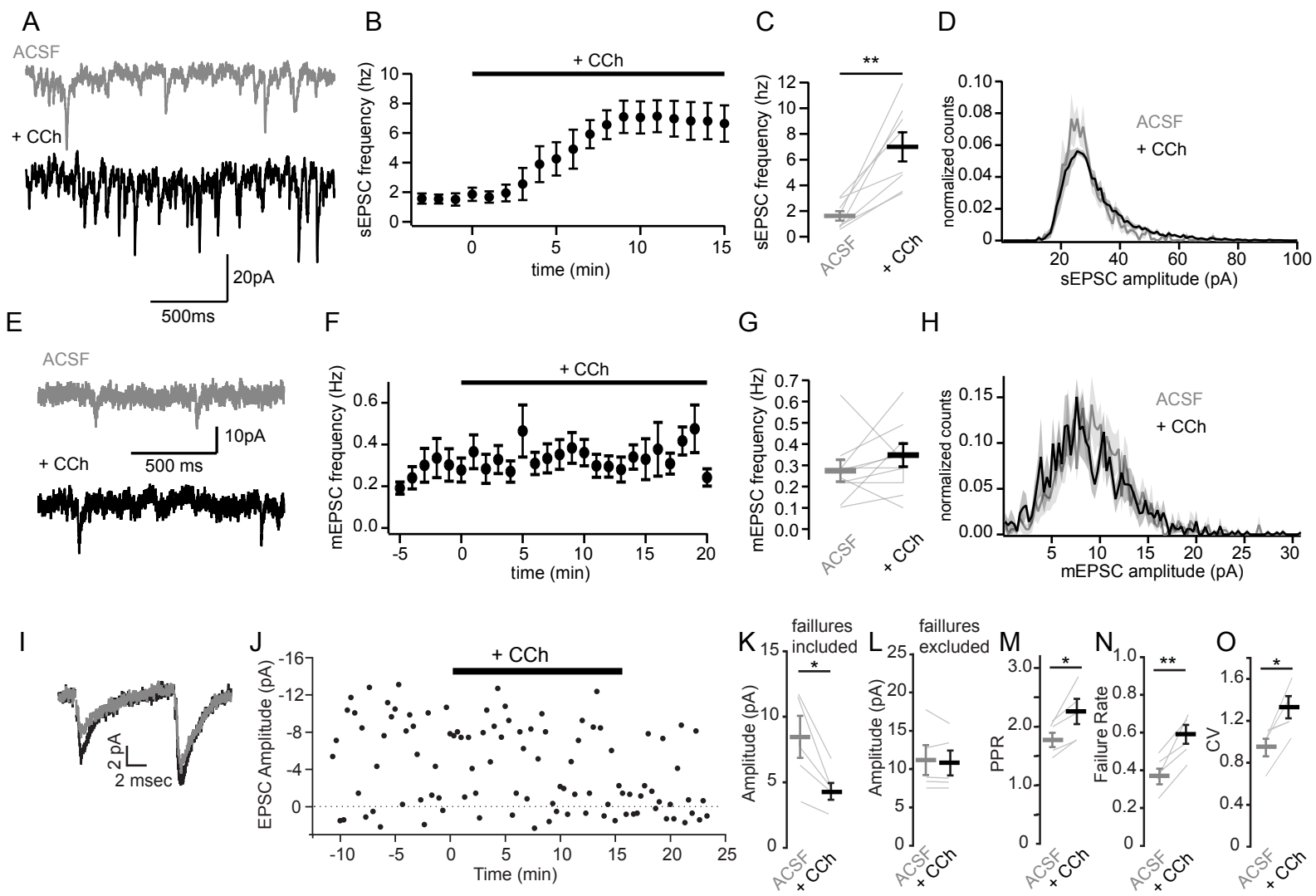


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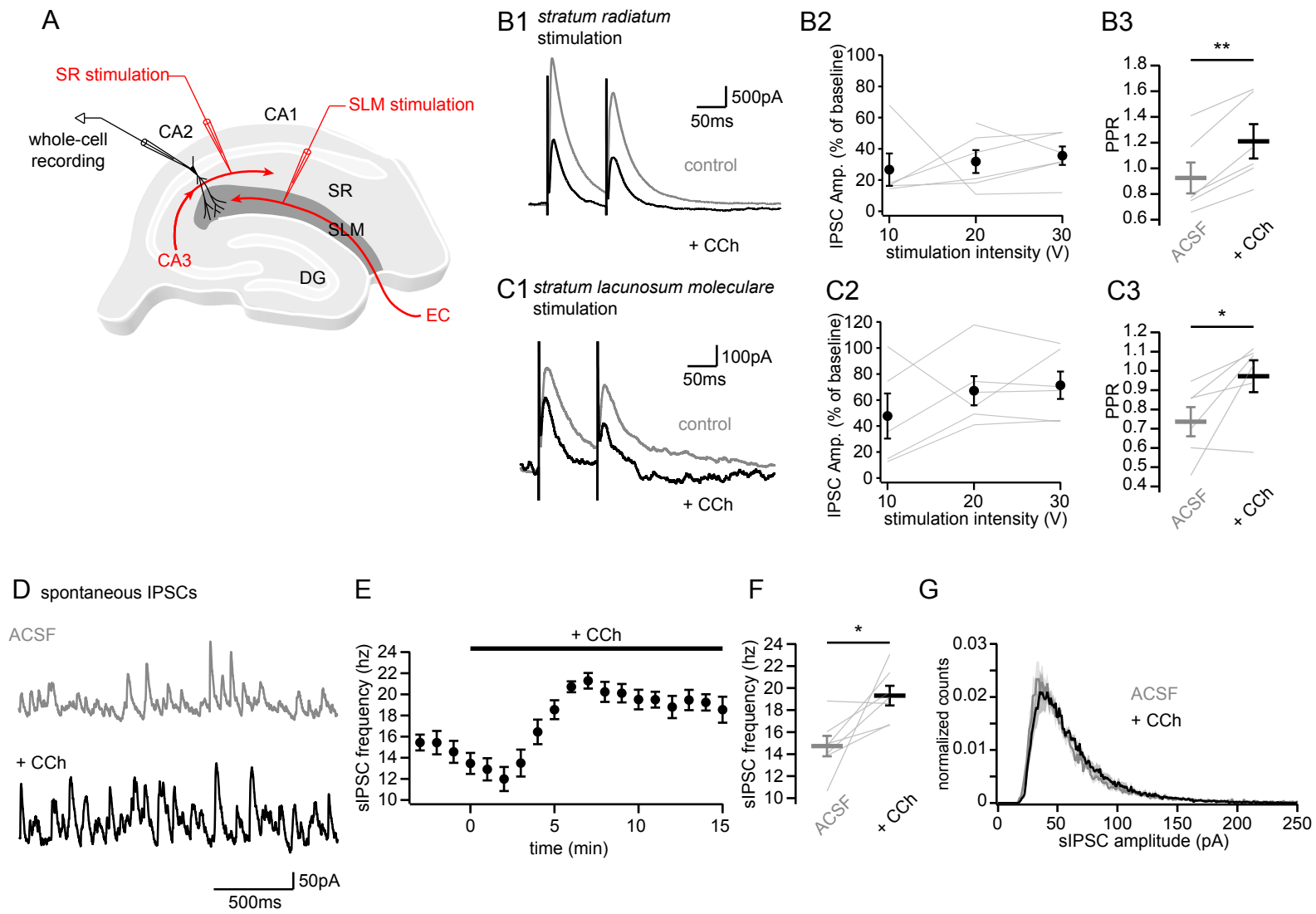


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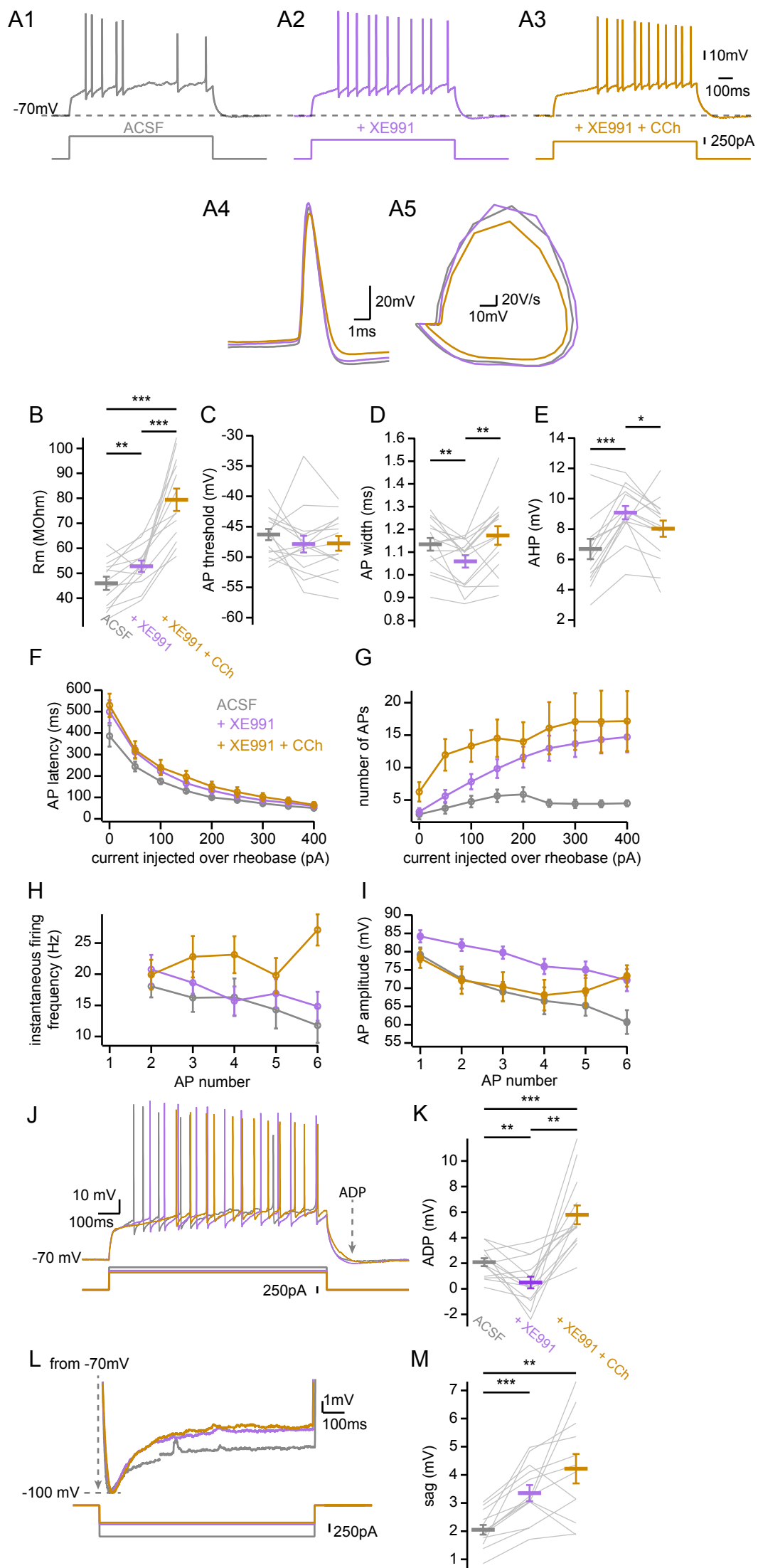


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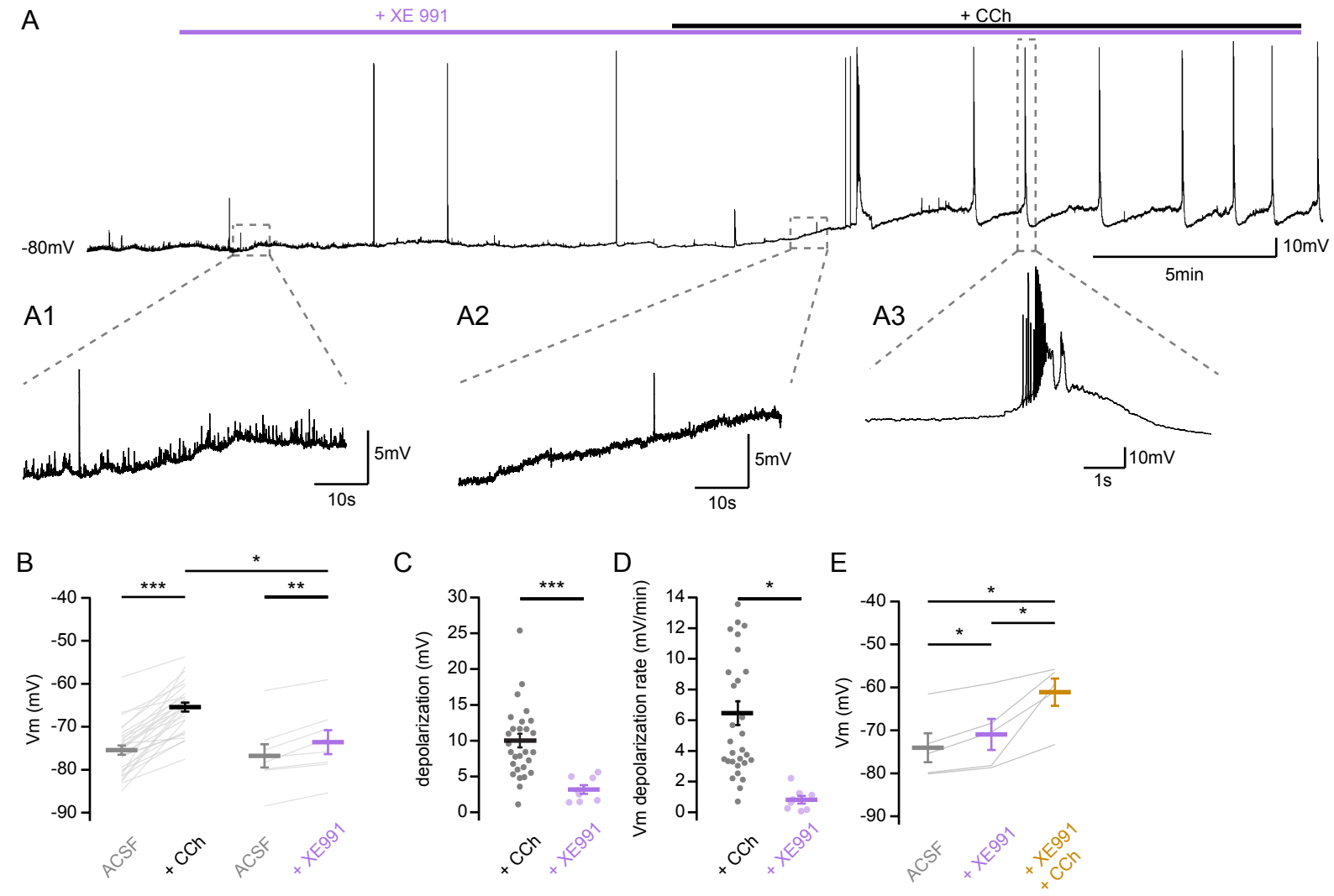


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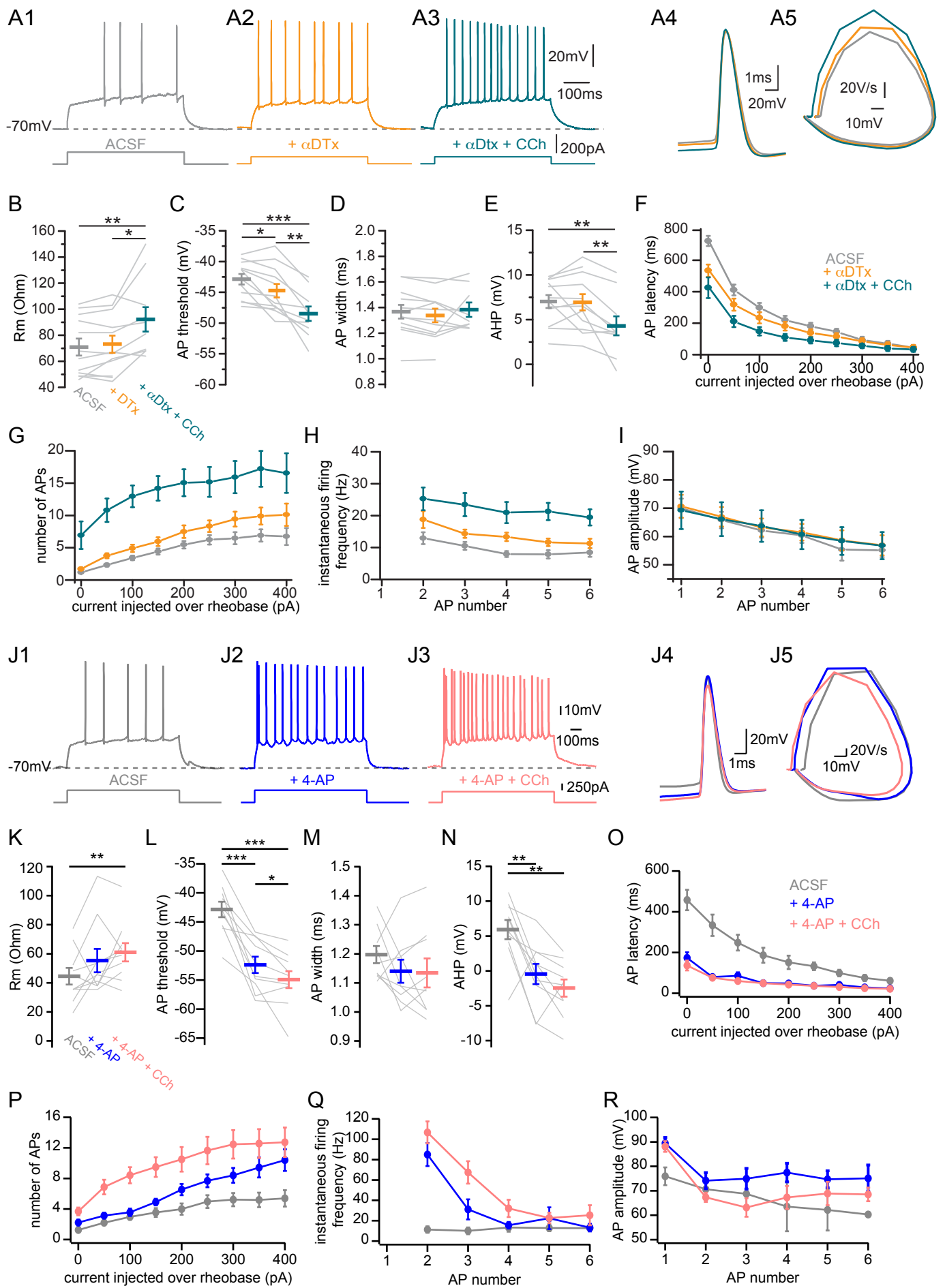


Figure 10.

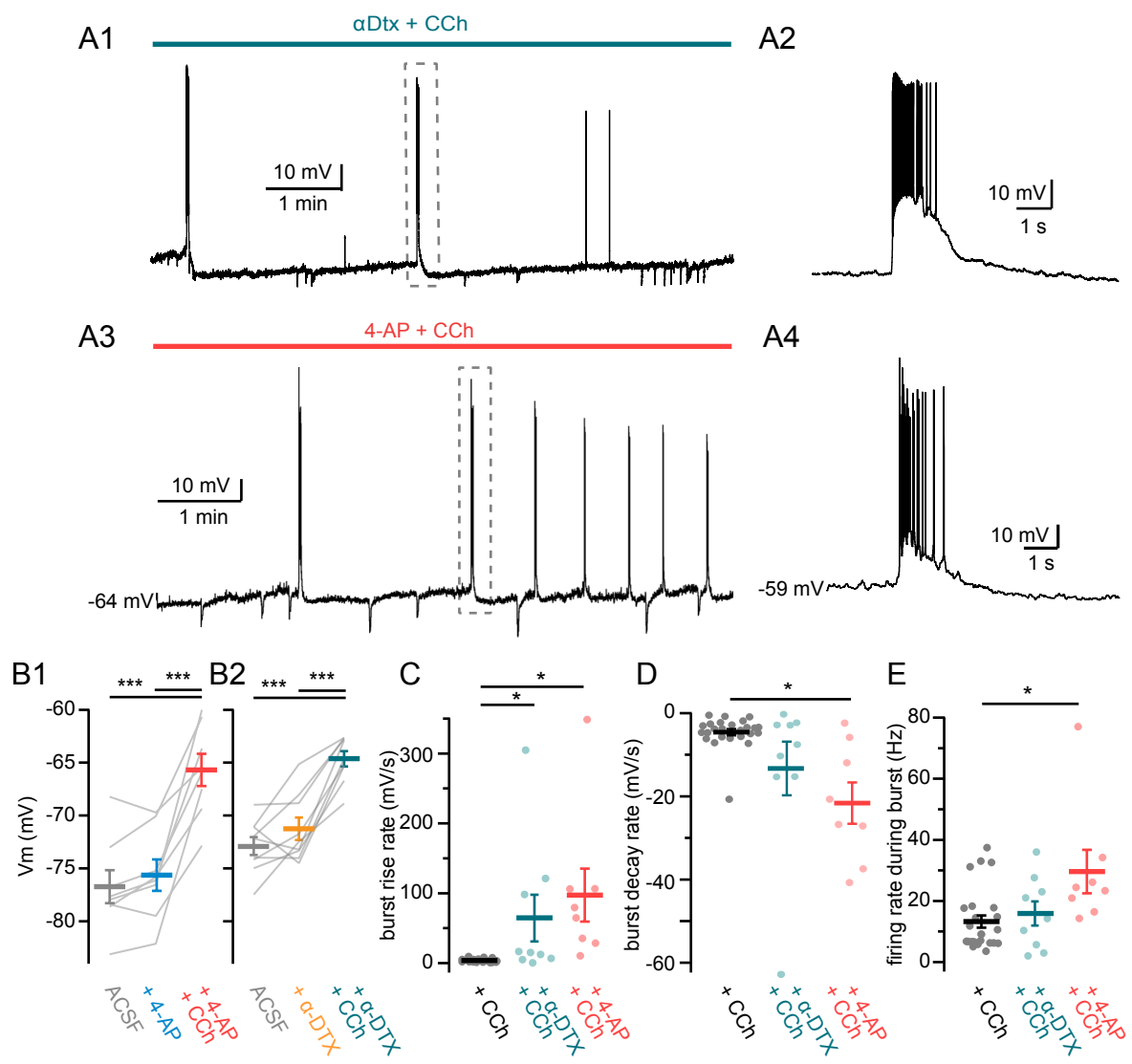


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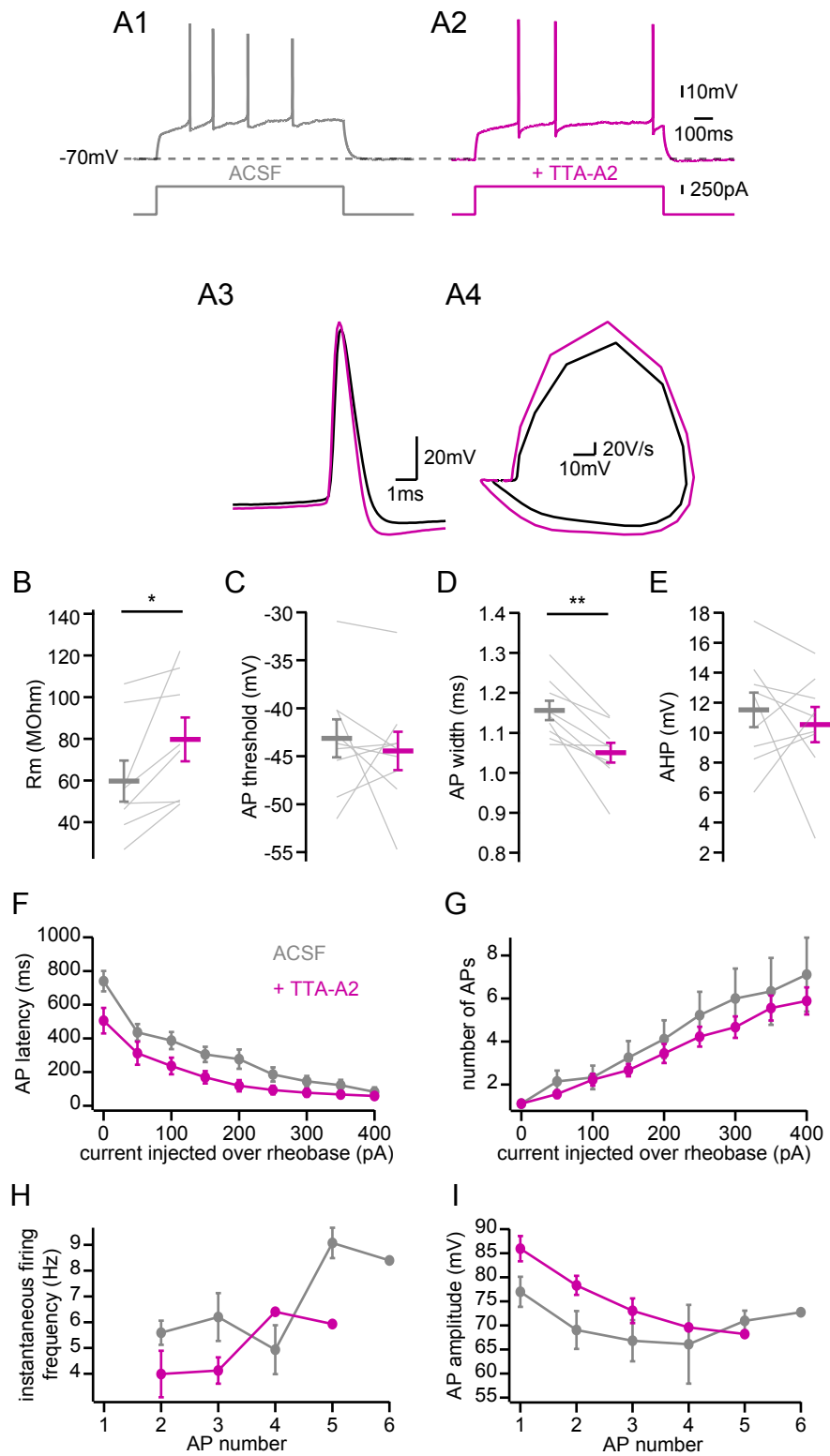


Figure 12.

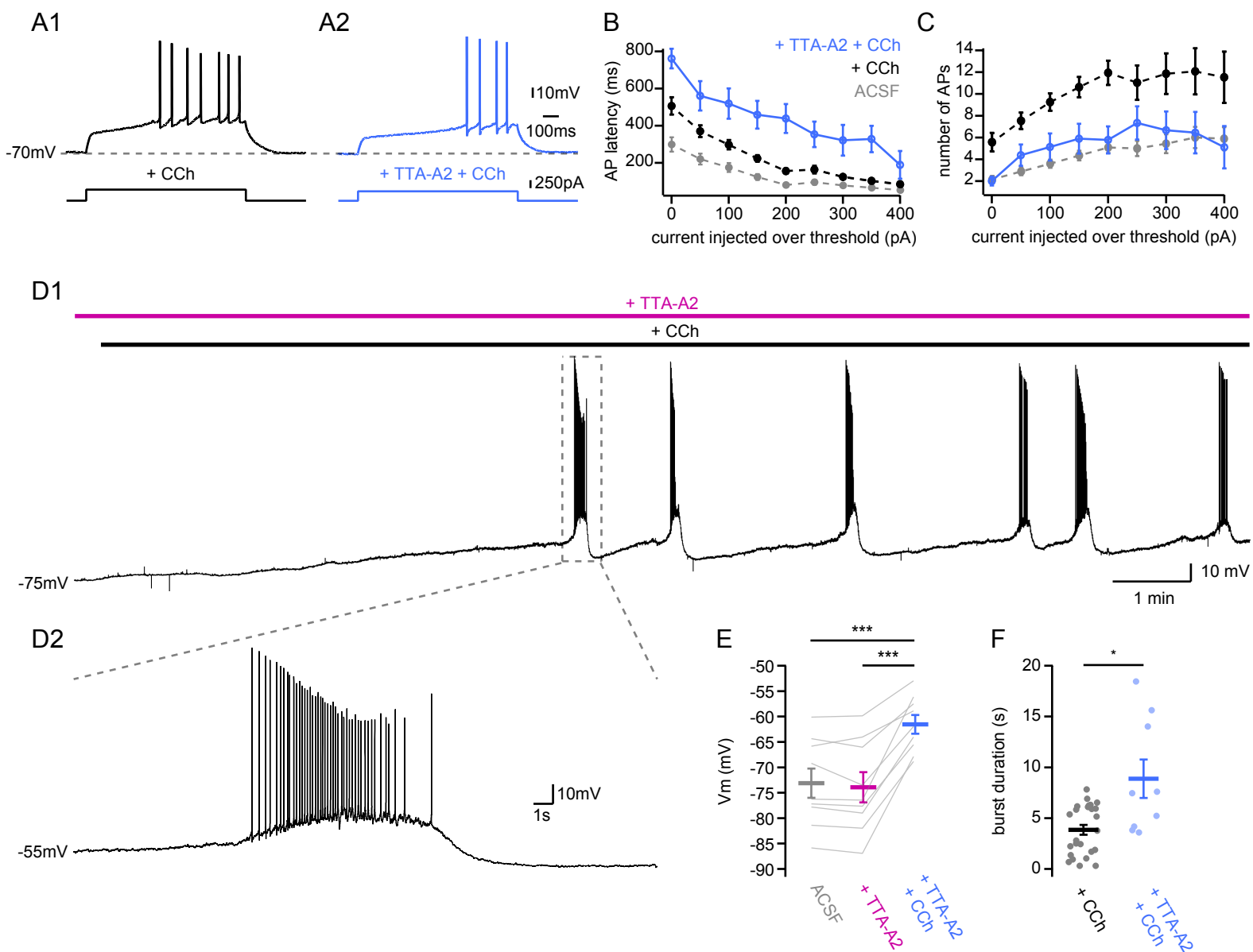


Figure 13.

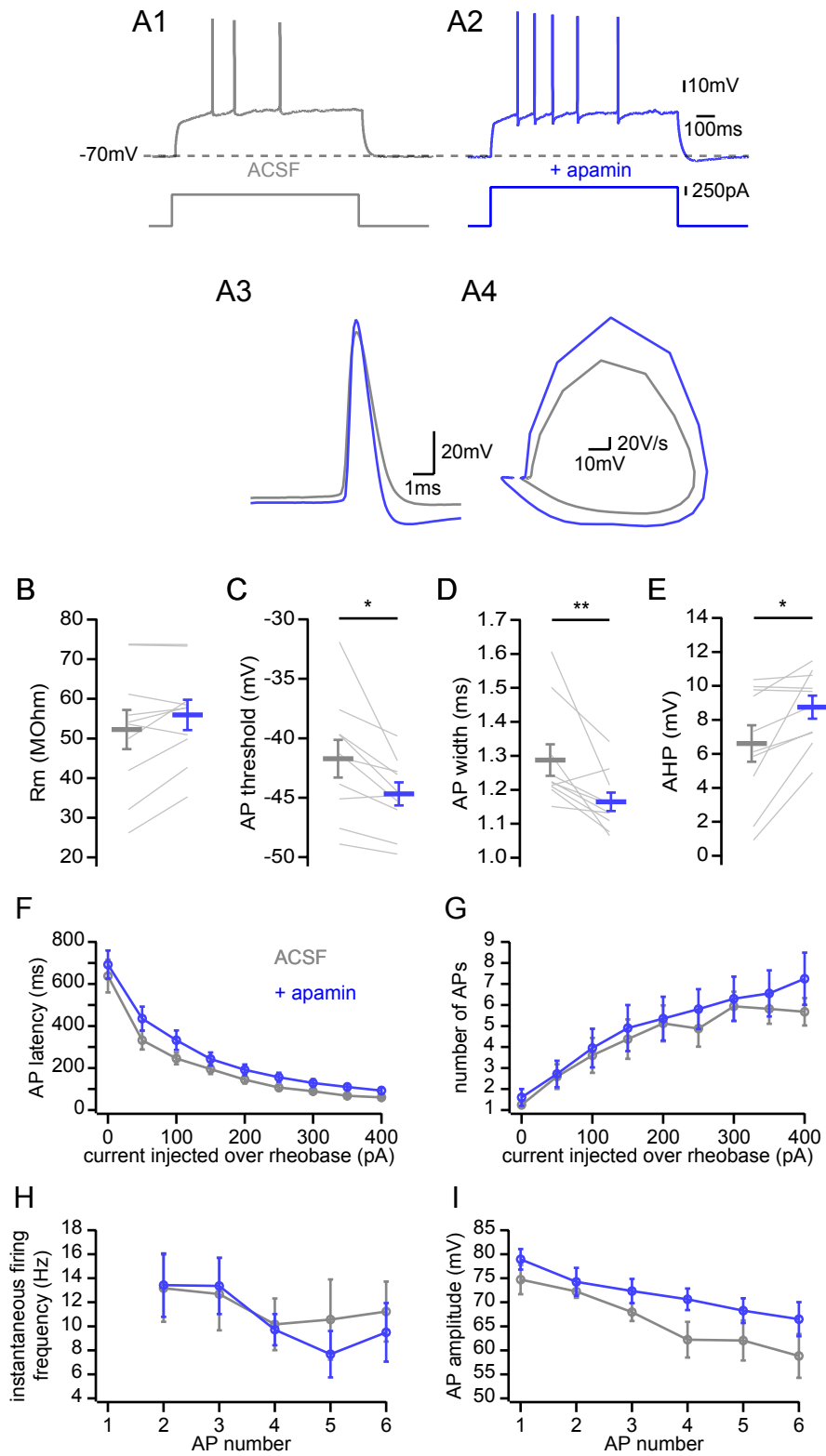


Figure 14.

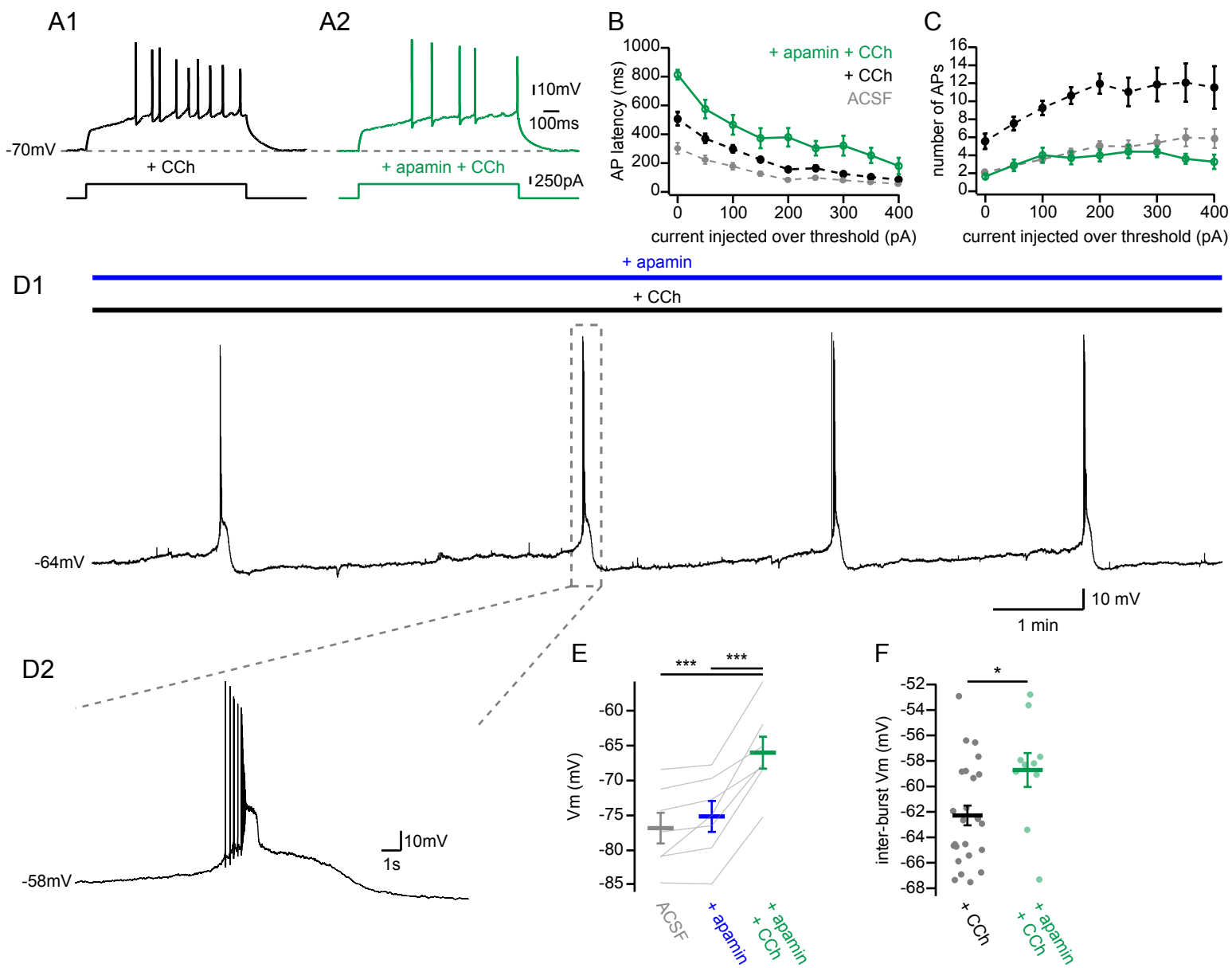


Figure 15.