

1 **Acetylcholine prioritises direct synaptic inputs from entorhinal cortex to**
2 **CA1 by differential modulation of feedforward inhibitory circuits**

6 Jon Palacios-Filardo¹, Matt Udakis¹, Giles A. Brown^{2\$}, Benjamin G. Tehan^{2\$}, Miles S.
7 Congreve², Pradeep J. Nathan^{2,3}, Alastair J.H. Brown² and Jack R. Mellor^{1*}

9 ¹Center for Synaptic Plasticity, School of Physiology, Pharmacology and Neuroscience,
10 University of Bristol, University Walk, Bristol BS8 1TD, UK

11 ²Sosei Heptares, Steinmetz Building, Granta Park, Great Abingdon, Cambridge CB21 6DG,
12 UK

13 ³Department of Psychiatry, University of Cambridge, UK

14 \$Current address OMass Therapeutics Ltd, The Schrödinger Building, Heatley Road, Oxford
15 Science Park, Oxford OX4 4GE, UK

16 Correspondence: Jack.Mellor@Bristol.ac.uk

17 * Lead contact

19 Keywords: acetylcholine, hippocampus, CA3, CA1, entorhinal cortex.

21 **Acknowledgements**

22 We thank Paul Anastasiades and Paul Chadderton for critical input to previous versions of the
23 manuscript and all members of the Mellor group for discussion. We also thank Dr. Jürgen
24 Wess (NIH, NIDDK) for providing the M₃ receptor KO mice This work was supported by
25 Wellcome Trust, Biotechnology and Biological Sciences Research Council (BBSRC).

27 **Contributions**

28 Conceptualization, J.P-F. and J.R.M.; Methodology, J.P-F. and M.U.; Investigation, J.P-F.
29 and M.U; Provision of reagents, G.A.B., B.G.T., M.S.C., P.J.N. and A.J.H.B.; Visualization,
30 J.P-F., M.U. and J.R.M.; Writing – Original Draft, J.P-F., M.U., and J.R.M.; Writing –
31 Review & Editing, J.P-F., M.U., P.J.N., A.J.H.B. and J.R.M.; Funding Acquisition, J.R.M.;
32 Supervision, J.R.M.

34 **Declaration of interests**

35 The authors declare no competing interests.

36 **Abstract**

37 Acetylcholine release in the hippocampus plays a central role in the formation of new
38 memory representations by facilitating synaptic plasticity. It is also proposed that memory
39 formation requires acetylcholine to enhance responses in CA1 to new sensory information
40 from entorhinal cortex whilst depressing inputs from previously encoded representations in
41 CA3, but this influential theory has not been directly tested. Here, we show that excitatory
42 inputs from entorhinal cortex and CA3 are depressed equally by synaptic release of
43 acetylcholine in CA1. However, greater depression of feedforward inhibition from entorhinal
44 cortex results in an overall enhancement of excitatory-inhibitory balance and CA1 activation.
45 Underpinning the prioritisation of entorhinal inputs, entorhinal and CA3 pathways engage
46 distinct feedforward interneuron subpopulations and depression is mediated differentially by
47 presynaptic muscarinic M₃ and M₄ receptors respectively. These mechanisms enable
48 acetylcholine to prioritise novel information inputs to CA1 during memory formation and
49 suggest selective muscarinic targets for therapeutic intervention.

50 Introduction

51 Cognitive processing in the brain must continuously adapt to changing environmental
52 situations. However, the stability of physical connectivity within neuronal networks, at least
53 over relatively short timescales (< min), means that the brain requires systems that can enact
54 rapid functional network reconfigurations. Release of neuromodulator transmitters via long-
55 range projections fulfils the requirements for functional reconfiguration (Marder, 2012) and
56 occurs in response to situations that demand behavioural or cognitive adaptation (Dayan,
57 2012). But the mechanisms by which neuromodulators such as acetylcholine reconfigure
58 neuronal networks remain largely unknown.

59 The widespread release of acetylcholine within the brain is historically associated with
60 arousal and attention (Hasselmo and Sarter, 2011; Micheau and Marighetto, 2011; Robbins,
61 1997; Teles-Grilo Ruivo and Mellor, 2013). More recently it has also been found to be
62 associated with unexpected rewards or punishments (Hangya and Kepecs, 2015; Teles-Grilo
63 Ruivo et al., 2017) signalling the need to update existing representations with new salient
64 information. To achieve this acetylcholine must reconfigure neural networks in two key
65 ways: (i) open a window for encoding new memories or updating existing ones, and (ii)
66 prioritise new sensory information for incorporation into memory ensembles (Hasselmo,
67 2006; Palacios-Filardo and Mellor, 2019). Acetylcholine facilitates the induction of synaptic
68 plasticity thereby opening a window for the creation of memory ensembles (Buchanan et al.,
69 2010; Dennis et al., 2016; Hasselmo et al., 1995; Isaac et al., 2009; Mitsushima et al., 2013;
70 Shinoe et al., 2005) and it increases the output gain from primary sensory cortices enhancing
71 signal-to-noise for new sensory information (Eggermann et al., 2014; Fu et al., 2014; Letzkus
72 et al., 2011). It is also proposed to prioritise sensory inputs from the neocortex into memory
73 ensembles within the hippocampus (Dannenberg et al., 2017; Hasselmo, 2006; Hasselmo and
74 Schnell, 1994; Hasselmo et al., 1995) but this critical component of the mechanism by which
75 acetylcholine gates the updating of memory representations has yet to be tested in detail.

76 The hippocampus is a hub for the encoding, updating and retrieval of episodic memories,
77 enabling events to be placed into a context. Individual items of information from the
78 neocortex are thought to be sparsely encoded and separated by strong lateral inhibition in the
79 dentate gyrus before being assembled into larger memory representations within the recurrent
80 CA3 network (Hasselmo, 2006; Prince et al., 2016). These memory representations are then
81 transferred via the Schaffer collateral (SC) pathway to CA1 which also receives new sensory
82 information directly from the entorhinal cortex layer III pyramidal neurons via the
83 temporoammonic (TA) pathway enabling CA1 to compare and integrate the new information
84 (Ahmed and Mehta, 2009; Eichenbaum, 2017; Takahashi and Magee, 2009; Witter, 1993). It
85 is therefore predicted that acetylcholine enhances the relative weights of TA inputs to CA1
86 over SC inputs during memory formation.

87 Perhaps counter-intuitively, acetylcholine inhibits both TA and SC glutamatergic excitatory
88 transmission in CA1. In the SC pathway this occurs via presynaptic muscarinic M₄ receptors
89 but the identity of the receptors mediating depression at the TA pathway is unclear (Dasari
90 and Gulleedge, 2011; Goswamee and McQuiston, 2019; Thorn et al., 2017). The anatomically
91 segregated targeting of TA and SC inputs to distal and more proximal dendritic locations on

92 CA1 pyramidal neurons respectively (Witter, 1993) together with muscarinic receptor
93 specificity provide potential mechanisms for differential sensitivity to acetylcholine and
94 therefore altering the relative weights of synaptic input. However, the evidence for this is
95 equivocal with exogenously applied cholinergic agonists indicating that SC transmission is
96 more sensitive to cholinergic modulation than TA transmission (Hasselmo and Schnell, 1994)
97 but the reverse reported for endogenous synaptically released acetylcholine (Goswamee and
98 McQuiston, 2019).

99 An alternative mechanism by which acetylcholine might rebalance the relative weight s of SC
100 and TA inputs is the modulation of the intrinsic and synaptic properties of hippocampal
101 GABAergic interneurons (Cea-del Rio et al., 2011; Cea-del Rio et al., 2010; Leao et al.,
102 2012; Szabo et al., 2010) which have a profound impact on CA1 pyramidal neuron input
103 integration rules and subsequent output (Leao et al., 2012; Milstein et al., 2015). Feedforward
104 interneurons in the SC pathway are primarily perisomatic targeting basket cells expressing
105 parvalbumin (PV) or cholecystokinin (CCK) (Basu et al., 2013; Freund and Katona, 2007;
106 Glickfeld and Scanziani, 2006; Klausberger and Somogyi, 2008; Milstein et al., 2015) whose
107 inhibition is strongly regulated by acetylcholine (Cea-del Rio et al., 2011; Szabo et al., 2010)
108 whereas the mediators of feedforward inhibition in the TA pathway are primarily CCK or
109 neuropeptide Y (NPY) expressing interneurons (Basu et al., 2013; Klausberger and Somogyi,
110 2008; Milstein et al., 2015) that are also potentially regulated by acetylcholine (Cea-del Rio
111 et al., 2010; Raza et al., 2017). Moreover, feedback inhibition via oriens lacunosum
112 moleculare (OLM) interneurons, which specifically target the same distal dendritic regions as
113 the TA pathway, are directly excited by acetylcholine (Leao et al., 2012; Pouille and
114 Scanziani, 2004). This indicates that cholinergic modulation of inhibition within the
115 hippocampal circuit strongly dictates excitatory input integration and CA1 output, but the
116 integrated effect of acetylcholine on the hippocampal network and its input-output function
117 has not been investigated.

118 In this study we tested the hypothesis that acetylcholine release in the hippocampus
119 prioritises new sensory input to CA1 via the TA pathway over internal representations via the
120 SC pathway. We find that endogenous synaptically released acetylcholine depresses SC and
121 TA excitatory inputs equally but that feedforward inhibition in the TA pathway is more
122 sensitive to cholinergic modulation. This produces an increase in excitatory-inhibitory ratio
123 selectively for the TA pathway driven by differential regulation of interneuron
124 subpopulations and distinct muscarinic receptor subtypes. We therefore provide a mechanism
125 by which acetylcholine dynamically prioritises sensory information direct from entorhinal
126 cortex over internal representations held in CA3.

127

128

129 **Results**

130 **Endogenous acetylcholine release modulates synaptic inputs to CA1.**

131 To enable selective activation of endogenous acetylcholine release we expressed the light-
132 activated cation channel channelrhodopsin-2 (ChR2) in a cre-dependent manner using mice
133 that express cre recombinase under control of the promoter for Choline AcetylTransferase
134 (ChAT-cre) crossed with mice expressing cre-dependent ChR2 (ChAT-ChR2 mice;
135 methods). Immunohistochemistry confirmed that ChR2 was expressed in cholinergic cells
136 within the medial septum (Figure 1A-B) whose axon fibers densely innervated the dorsal
137 hippocampus (Figure 1C) in agreement with the previously described anatomy (Teles-Grilo
138 Ruivo and Mellor, 2013). Whole-cell patch clamp recordings from medial septal neurons
139 expressing ChR2 confirmed they fired action potentials in response to 5ms of 470nm light up
140 to a maximum frequency of ~25Hz (Figure 1B). We also confirmed that light stimulation in
141 hippocampal slices resulted in acetylcholine release. Recordings from interneurons located in
142 stratum oriens revealed fast synaptic responses to light stimulation mediated by nicotinic
143 receptors (Figure 1D) consistent with activation of cholinergic axons and endogenous release
144 of acetylcholine (Leao et al., 2012). In these recordings and further recordings from CA1
145 pyramidal cells we saw no inhibitory postsynaptic currents that might be caused by light-
146 evoked co-release of GABA or glutamate from either local or long-range ChAT expressing
147 neurons (Figure 1E) (Takacs et al., 2018; Yi et al., 2015).

148 To selectively activate the Schaffer collateral and temporoammonic pathways into CA1
149 stimulating electrodes were placed within the two axon pathways in dorsal hippocampal
150 slices. This enabled independent stimulation of each pathway and the engagement of both the
151 direct excitatory inputs and disynaptic feedforward inhibitory inputs without activating direct
152 inhibitory inputs, demonstrated by the blockade of inhibitory inputs by NBQX (20 μ M)
153 (Figure S1A-B). We also pharmacologically confirmed the identity of the TA input by
154 application of the mGluR group II/III agonist DCG-IV (3 μ M) that selectively inhibits
155 glutamate release from temporoammonic pathway terminals (Ceolin et al., 2011) (Figure
156 S1C).

157 To test the effect of endogenous acetylcholine release on synaptic inputs to CA1,
158 hippocampal slices were stimulated with light at a frequency of 2 Hz for 5 minutes to evoke
159 physiologically maximal acetylcholine release (Jing et al., 2018). In the presence of the
160 GABA_A receptor antagonist picrotoxin, isolated SC and TA pathway excitatory postsynaptic
161 current (EPSC) amplitudes were depressed by very similar amounts (Figure 1F-G; SC
162 pathway $69 \pm 5\%$, n = 12 from 6 mice, p < 0.001; TA pathway $74 \pm 4\%$, n = 13 from 6
163 mice, p < 0.001) with a concomitant increase in the paired-pulse ratio (PPR) (Figure 1H; SC
164 pathway, $125 \pm 9\%$, p < 0.05; TA pathway, $118 \pm 5\%$, p < 0.01), indicating a presynaptic
165 locus of action. Application of nicotinic and muscarinic receptor antagonists atropine (25 μ M)
166 and mecamylamine (50 μ M) blocked the effects of endogenous acetylcholine release (Figure
167 1F-H; SC pathway $116 \pm 8\%$, n = 6 from 3 mice, p > 0.05; TA pathway $106 \pm 15\%$, n = 6
168 from 3 mice, p > 0.05). Therefore, contrary to our initial hypothesis (Dannenberg et al., 2017;
169 Goswamee and McQuiston, 2019; Hasselmo, 2006; Hasselmo and Schnell, 1994),

170 acetylcholine did not inhibit one pathway more than the other but instead depressed both
171 equally.

172 We next tested disynaptic feedforward inhibitory postsynaptic currents (IPSCs) in response to
173 stimulation of SC or TA pathways. The amplitude of evoked IPSCs was also reduced by
174 endogenous acetylcholine release (Figure 1I-J; SC pathway, $63 \pm 5\%$, n = 9 from 6 mice, p <
175 0.01; TA pathway, $77 \pm 5\%$, n = 10 from 6 mice, p < 0.01) but surprisingly IPSC PPR was
176 only increased in the SC pathway (Figure 1I,K; SC pathway, $150 \pm 14\%$, p < 0.05; TA
177 pathway, $112 \pm 7\%$, p > 0.05). Similar to EPSCs, the reduction in IPSCs was completely
178 blocked by muscarinic and nicotinic receptor antagonists (Figure 1I-K; SC pathway IPSC $122 \pm 6\%$ and
179 PPR $106 \pm 10\%$, n = 5 from 4 mice, p > 0.05; TA pathway IPSC $98 \pm 6\%$ and
180 PPR $99 \pm 6\%$, n = 6 from 4 mice, p > 0.05). The observation that IPSCs were depressed
181 equally in each pathway but PPR was increased in the SC pathway suggests that during
182 repetitive stimulation inhibitory drive will increase in the SC pathway relative to the TA
183 pathway. This predicts that although acetylcholine depresses excitatory synaptic transmission
184 in the TA and SC pathways equally, its overall effect on excitatory-inhibitory ratio favours
185 TA inputs during repetitive stimulation when the effects of acetylcholine on feedforward
186 inhibition are taken into account.

187

188 **Differential cholinergic modulation of excitatory-inhibitory ratio for Schaffer collateral
189 and temporoammonic inputs to CA1.**

190 To test whether excitatory-inhibitory balance was differentially altered between SC and TA
191 input pathways we recorded monosynaptic EPSCs and disynaptic feedforward IPSCs for SC
192 and TA pathways in the same CA1 pyramidal neuron (see methods; Figure 2A). 5
193 consecutive stimuli at 10 Hz were given alternately to SC then TA pathway to determine the
194 evolution of synaptic modulation by acetylcholine during a repetitive train of stimuli. In these
195 experiments we mimicked the release of endogenous acetylcholine with application of the
196 cholinergic receptor agonist carbachol (CCh), a non-hydrolysable analogue of acetylcholine
197 that is not selective between cholinergic receptor subtypes. Application of increasing
198 concentrations of CCh revealed that $10 \mu\text{M}$ CCh was required to induce depression for both
199 EPSCs and IPSCs in both SC and TA pathways similar to endogenous acetylcholine release
200 (Figure S2A; SC pathway EPSC, $35 \pm 6\%$, n = 20 from 11 mice; TA pathway EPSC, $50 \pm 5\%$,
201 n = 20 from 11 mice; SC pathway IPSC, $29 \pm 3\%$, n = 20 from 11 mice; TA pathway
202 IPSC, $40 \pm 4\%$, n = 20 from 11 mice), but at lower concentrations of CCh SC excitatory
203 synaptic transmission showed higher sensitivity to CCh than the TA pathway (Hasselmo and
204 Schnell, 1994) suggestive of different receptor affinities or signalling pathways regulating
205 presynaptic release (Figure S2A; CCh $1 \mu\text{M}$ at SC pathway, $52 \pm 6\%$, n = 9 from 4 mice; TA
206 pathway, $91 \pm 18\%$, n = 9 from 4 mice). The depression of EPSCs and IPSCs with $10 \mu\text{M}$
207 CCh occurred for all responses in both SC and TA pathways (Figure 2A and Figure S2B) but
208 the degree of depression was not consistent between pathways over the course of repetitive
209 stimulation. Cholinergic receptor activation enhanced synaptic facilitation and increased PPR
210 for excitatory and feedforward inhibitory connections in the SC pathway, while the TA
211 pathway only displayed a marked increase in PPR in excitatory but not feedforward

inhibitory inputs (Figure 2B and Figure S2B-E; 5th stimuli PPR change for SC EPSC, 197 ± 23 %, p < 0.01; SC IPSC, 188 ± 13 %, p < 0.001; TA EPSC, 170 ± 13 %, p < 0.001; TA IPSC, 120 ± 13 %, p > 0.05, n = 20 from 11 mice), supporting the initial results using endogenous acetylcholine release. Indeed, the close similarity in PPR increase for both excitatory and feedforward inhibitory transmission in the SC pathway ensured that the excitatory-inhibitory (E-I) ratio in the SC pathway did not change after cholinergic receptor activation for any stimuli within the train (Figure 2C; 5th stimuli on SC E-I ratio, 0.29 ± 0.05 and 0.41 ± 0.10, for baseline and CCh respectively, p > 0.05). Conversely, excitation-inhibition ratio in the TA pathway showed a marked increase after CCh application that evolved over the course of the train of stimuli (Figure 2C; 5th stimuli on TA E-I ratio, 0.34 ± 0.06 and 0.6 ± 0.10, for baseline and CCh respectively, p < 0.001). This meant that over the course of the train the TA input exerted relatively greater influence over the postsynaptic neuron compared to the SC input when cholinergic receptors were activated, as demonstrated by the comparison of excitation-inhibition ratio between the SC and TA pathways (Figure 2D; 5th stimuli on TA/SC E-I ratio, 1.28 ± 0.25 and 1.7 ± 0.25, for baseline and CCh respectively, p < 0.01). These data show that differential modulation of feedforward inhibition between SC and TA pathways by cholinergic receptor activation produces an increase in the relative strength of the TA input to CA1 pyramidal neurons. Furthermore, the data suggest that SC and TA pathways engage distinct local inhibitory interneuron populations with different overall short-term dynamic responses to acetylcholine.

232

233 Cholinergic modulation of interneuron recruitment to feedforward inhibitory synaptic 234 transmission.

Feedforward interneurons in the SC pathway are primarily perisomatic targeting basket cells expressing parvalbumin (PV⁺) or cholecystokinin (CCK⁺) whereas the mediators of feedforward inhibition in the TA pathway are likely dendritically targeting CCK⁺ or neuropeptide Y (NPY⁺) expressing interneurons (Basu et al., 2013; Freund and Katona, 2007; Glickfeld and Scanziani, 2006; Klausberger and Somogyi, 2008; Milstein et al., 2015). Analysis of our recordings revealed that feedforward SC IPSCs had faster decay kinetics than TA IPSCs (Figure 3A-C; SC IPSC decay tau, 43.0 ± 2.7 ms, n = 45 from 24 mice vs TA IPSC decay tau, 60.1 ± 3.4 ms, n = 92 from 36 mice, p < 0.005) in accordance with predictions that the more distal synaptic location of inhibitory inputs from TA feedforward interneurons and therefore increased dendritic filtering means that these IPSCs have slower kinetics (Milstein et al., 2015). GABAergic synapses from PV⁺ and NPY⁺, but not CCK⁺, interneurons onto CA1 pyramidal cells are depressed by μ-opioid receptors (Glickfeld et al., 2008; Gulyas et al., 2010; Krook-Magnuson et al., 2011). SC IPSCs were more sensitive to μ-opioid receptor agonist DAMGO (1 μM) than TA IPSCs (Figure 3D-E; IPSC 1st response peak after DAMGO, 51 ± 4 % and 69 ± 5 %, for SC and TA respectively, n = 11 from 4 mice, p < 0.05) indicating that in our experiments PV⁺ interneurons form a major component of feedforward inhibition in the SC pathway whereas CCK⁺ interneurons form the major component of feedforward inhibition in the TA pathway. There are also minor components from other interneuron subtypes, most likely CCK⁺ basket cells in the SC pathway and PV⁺

254 or NPY⁺ interneurons in the TA pathway (Basu et al., 2013; Freund and Katona, 2007;
255 Glickfeld and Scanziani, 2006; Klausberger and Somogyi, 2008; Milstein et al., 2015).
256 The engagement of different interneuron subtypes in feedforward inhibition in the SC and TA
257 pathways might explain the differential modulation of feedforward inhibition by
258 acetylcholine. Therefore, we investigated whether the output from these interneurons onto
259 CA1 pyramidal cells is modulated by acetylcholine and, if so, whether modulation evolves
260 differentially for the 2 inputs during a burst of responses. To test this we used mice
261 expressing ChR2 in PV⁺ or CCK⁺ interneurons (see methods) and gave a train of 5 light
262 stimuli at 10 Hz to the slices whilst recording IPSCs from pyramidal neurons at 0 mV in the
263 presence of NBQX and D-APV to avoid recording glutamatergic, disynaptic inhibitory inputs
264 or ChR2 currents (Figure S3). To test the sensitivity of PV⁺ and CCK⁺ synapses to
265 cholinergic modulation, CCh was bath applied to the slice whilst selectively evoking either
266 PV⁺ or CCK⁺ derived IPSCs (Figure 3F). PV⁺ evoked IPSCs displayed faster decay kinetics
267 to CCK⁺ evoked IPSCs supporting their perisomatic and dendritic synaptic locations
268 respectively (Figure 3G; PV⁺ decay kinetics, 14.9 ± 1.8 ms, n = 7 vs CCK⁺ decay kinetics,
269 21.9 ± 3.4 ms, n = 5, p < 0.05). Decay kinetics of optogenetically evoked IPSCs were faster
270 than disynaptically evoked feedforward IPSCs as predicted for inputs with greater synchrony.
271 CCh depressed IPSCs from both PV⁺ and CCK⁺ synapses indicating a direct cholinergic
272 modulation of these interneurons (Figure 3F,H; PV⁺ responses, 34.3 ± 6.0 %, n = 7, p <
273 0.005; CCK⁺ responses, 37.8 ± 4.8 %, n = 5, p < 0.005). Both synapses exhibited frequency-
274 dependent depression but CCh selectively increased PPR of PV⁺ but not CCK⁺ synapses
275 (Figure 3I-J; PV⁺ IPSC PPR, 136 ± 11 %, n = 7, p < 0.05; CCK⁺ IPSC PPR, 104 ± 4 %, n =
276 5, p > 0.05). The lack of effect of cholinergic receptor activation on PPR at CCK⁺ synapses
277 mirrors the lack of effect on PPR for feedforward inhibition in the TA pathway and confirms
278 that CCK⁺ interneurons are the major component of feedforward inhibition in the TA
279 pathway whereas PV⁺ interneurons and synapses that increase PPR form feedforward
280 inhibition in the SC pathway. The differential effect of acetylcholine at PV⁺ and CCK⁺
281 synapses provides a mechanism for the enhancement of TA pathway excitatory-inhibitory
282 ratio in comparison to SC pathway.

283

284 **Presynaptic muscarinic M₃ receptor modulation of TA pathway excitatory and** 285 **feedforward inhibitory synaptic transmission.**

286 The synaptic depression of Schaffer collateral inputs to CA1 by acetylcholine is characterised
287 genetically and pharmacologically to be mediated by muscarinic M₄ receptors (Dasari and
288 Gulledge, 2011; Thorn et al., 2017). This was confirmed by application of the dual
289 muscarinic M₄ and M₁ receptor agonist compond1 (1 μM; Figure S4), which selectively
290 depressed SC but not TA pathway excitatory inputs (Figure 4A-C; SC EPSC response, 63 ± 5
291 %, n = 17, from 8 mice, p < 0.001; TA EPSC response, 94 ± 5 %, n = 17, from 8 mice, p >
292 0.05). However, the identity of cholinergic receptors mediating the depression of TA inputs is
293 unclear. Therefore, we aimed to determine which cholinergic receptors modulate TA pathway
294 feedforward excitatory and inhibitory synaptic transmission onto CA1 pyramidal neurons.
295 TA pathway excitatory synaptic transmission was isolated by recording in the presence of

PTX and holding the membrane voltage at -65 mV (see methods; Figure 4D). Similar to previous results (Figures 1&2), TA EPSCs were depressed by application of 10 μ M CCh and PPR was increased (Figure 4E; EPSC response, $45 \pm 3\%$, n = 10 from 5 mice, p < 0.01; PPR, $129 \pm 8\%$, n = 10 from 5 mice, p < 0.05). These data suggest a presynaptic locus of action of cholinergic receptors. We next pharmacologically dissected which cholinergic receptor subtypes were involved. Application of the non-selective nicotinic receptor antagonist mecamylamine (25 μ M) had no effect on CCh depression of EPSCs (Figure 4F; $40.6 \pm 9.5\%$, n = 6 from 3 mice, p < 0.01) and PPR (Figure 4G; $124 \pm 8\%$, n = 6 from 3 mice, p < 0.05), while the non-selective muscarinic receptor antagonist atropine (10 μ M) blocked the decrease of EPSCs (Figure 4F; $91 \pm 4\%$, n = 6 from 3 mice, p > 0.05) and prevented the increase in PPR (Figure 4G; $105 \pm 4\%$, n = 6 from 23 mice, p > 0.05), suggesting a direct involvement of muscarinic receptors. Muscarinic M₁ receptor agonist GSK-5 (500 nM) (Dennis et al., 2016) did not replicate CCh depression of EPSCs and increase in PPR (Figure 4F-G; EPSCs, $91 \% \pm 4\%$, PPR $101 \pm 5\%$, n = 7 from 4 mice, p > 0.05) nor did the selective M₁ receptor antagonist, nitrocaramiphen (100 nM) prevent CCh induced depression and increase in PPR (Figure 4F-G; EPSC $51 \pm 4\%$, n = 6 from 4 mice, p < 0.01; PPR $124 \pm 6\%$, n = 6 from 4 mice, p < 0.05). The high density of muscarinic M₃ receptors localised to Stratum Lacunosum Moleculare where TA inputs synapse in CA1 (Levey et al., 1995) suggests a role for M₃ receptors modulating the TA pathway. Supporting a role for M₃ receptors, the selective M₃ receptor antagonist DAU5884 (1 μ M) (Gosens et al., 2004) prevented the EPSC depression and increase in PPR caused by CCh (Figure 4F-G; EPSC $105 \pm 11\%$, n = 6 from 4 mice, p > 0.05; PPR $101 \pm 6\%$, n = 6 from 4 mice, p > 0.05) suggesting that TA pathway synaptic transmission onto CA1 pyramidal neurons is modulated by presynaptically located muscarinic M₃ receptors.

To confirm the involvement of presynaptic muscarinic M₃ receptors, we tested the effects of CCh in M₃ receptor knock out mice (M₃ KO) (Yamada et al., 2001). Although TA evoked EPSCs recorded from M₃ KO slices were reduced by CCh with an associated increase in PPR (Figure 4H-I; EPSC, $61 \pm 6\%$, n = 8 from 4 mice, p < 0.001; PPR, $112 \pm 5\%$, p < 0.05), this CCh-induced depression was less than that recorded in WT slices (Figure 4H; WT EPSC vs M₃ KO EPSC, p < 0.05). This confirms the pharmacological data for presynaptic M₃ receptor involvement in the TA pathway but also suggests some compensation for M₃ receptor deletion within M₃ KO mice. The most likely subunit to compensate for M₃ deletion are M₁ receptors that are expressed in pyramidal cells and are also coupled to Gq signalling pathways. Therefore, to further explore possible compensatory mechanisms, we tested the selective muscarinic M₁ receptor agonist GSK-5 in the M₃ KO mice (Figure S5). M₁ receptor activation depolarises and increases spike rates in pyramidal neurons (Buchanan et al., 2010) thereby increasing spontaneous EPSCs. Application of GSK-5 increased spontaneous EPSC frequency in slices from both WT and M₃ KO mice (Figure S5A) but caused a selective decrease in TA EPSC and corresponding increase in PPR in the M₃ KO but not the WT (Figure S5B-C). This indicates that M₁ receptors partially replace the deleted M₃ receptors at presynaptic TA terminals in M₃ KO mice.

Feedforward synaptic inhibitory transmission in the TA pathway was isolated by holding the membrane voltage at 0 mV (see methods; Figure 4J). As previously described (Figures 1&2),

339 CCh depressed IPSCs without an effect on PPR (Figure 4K-L; IPSC, $48 \pm 6\%$, $n = 9$ from 4
340 mice, $p < 0.01$; PPR, $108 \pm 3\%$, $n = 9$ from 4 mice, $p > 0.05$). The pharmacological data
341 again supported a role for M₃ receptors. Nicotinic receptor antagonist mecamylamine (25
342 μM) did not prevent CCh-induced depression (Figure 4L; $31 \pm 6\%$, $n = 5$ from 3 mice, $p <$
343 0.01) but the muscarinic receptor antagonist atropine (10 μM) did (Figure 4L; $87 \pm 5\%$, $n = 6$
344 from 3 mice, $p > 0.05$), demonstrating that, as for excitatory synaptic transmission, inhibitory
345 inputs to CA1 pyramidal neurons are depressed by muscarinic receptor activation. Muscarinic
346 M₁ receptors did not alter TA IPSC as the agonist GSK-5 was unable to modulate inhibitory
347 synaptic transmission (Figure 4L; GSK-5 500 nM; $83 \pm 6\%$; $n = 4$ from 2 mice, $p > 0.05$)
348 and the M₁ receptor antagonist nitrocaramiphen was unable to block the CCh effect (Figure
349 4L; nitrocaramiphen 100 nM, $49 \pm 2\%$, $n = 4$ from 2 mice, $p < 0.01$). Similar to excitatory
350 transmission, muscarinic M₃ receptor antagonist (DAU5884 1 μM) blocked TA pathway
351 IPSC modulation by CCh (Figure 4L; $84 \pm 4\%$, $n = 8$ from 4 mice, $p > 0.05$). These results
352 show that M₃ muscarinic receptors are located at presynaptic TA terminals where they
353 depress release of glutamate onto CA1 pyramidal neurons and feedforward inhibition within
354 the TA pathway.

355

356 **Cholinergic disinhibition enhances CA1 output in response to temporoammonic but not**
357 **Schaffer collateral input.**

358 The modulation of hippocampal synaptic transmission and in particular the differential
359 regulation of excitatory-inhibitory balance of SC and TA synaptic pathways predicts that
360 acetylcholine prioritises CA1 response to inputs from entorhinal cortex via the TA pathway.
361 To test this prediction, we monitored spike generation in CA1 pyramidal neurons in response
362 to SC and TA pathway stimulation using trains of 10 stimuli at 10 Hz given to SC or TA
363 pathways. The stimulus intensities were set so that post synaptic potentials (PSPs) were
364 suprathreshold for action potential initiation on some but not all stimuli (P_{spike} ; see methods).
365 Application of 10 μM CCh depolarised CA1 pyramidal neurons (average depolarisation $5.3 \pm$
366 0.7 mV) so to dissociate the effects of CCh on membrane potential and synaptic inputs
367 current was initially injected to maintain membrane potential at baseline ($i \neq 0$) and assessed
368 changes in spike probability. Subsequently, the injected current was removed ($i = 0$) to
369 examine how cholinergic depolarisation affected spike probability. With membrane potential
370 maintained at baseline levels, CCh dramatically reduced the probability of spikes generated
371 by SC pathway stimulation (Figure 5A₁-C₁; P_{spike} baseline 0.59 ± 0.07 vs CCh $i \neq 0$ $0.14 \pm$
372 0.05, $n = 12$ from 5 mice, $p < 0.001$) and required more stimuli within a train and therefore a
373 longer delay to generate the first spike (Figure 5A₁-C₁; baseline, 298 ± 58 ms vs CCh $i \neq 0$,
374 775 ± 83 ms, $p < 0.001$). With current injection removed and membrane potential allowed to
375 depolarise, spike probability increased slightly but failed to return to baseline levels (Figure
376 5A₁-C₁; P_{spike} 0.33 ± 0.05 , $p < 0.05$ baseline versus CCh $i = 0$). In contrast, CCh application
377 had little effect on TA pathway driven spike probability and delay to the first spike when the
378 membrane potential was maintained at baseline levels (Figure 5A₂-C₂; P_{spike} baseline, $0.33 \pm$
379 0.06 vs CCh $i \neq 0$, 0.43 ± 0.08 , $n = 15$ from 10 mice, $p > 0.05$; delay to spike baseline, $397 \pm$
380 56 ms vs CCh $i \neq 0$, 483 ± 66 ms, $p > 0.05$). However, with current injection removed and

381 CA1 neurons allowed to depolarise spike probability increased and the delay to first spike
382 shortened (Figure 5A₂-C₂; P_{spike} 0.61 ± 0.06, p < 0.01 vs baseline; delay to spike 280 ms ± 27
383 ms, p < 0.05 vs baseline).

384 Since CCh or endogenous acetylcholine reduce excitatory synaptic inputs from the SC and
385 TA pathways equally (Figures 1&2), our data suggest the CCh-induced increase in spike
386 probability in response to TA pathway input is caused by a frequency-dependent depression
387 of feedforward inhibition, and therefore increase in excitatory-inhibitory balance, selectively
388 in the TA pathway (Figure 2). Indeed, a substantial hyperpolarising envelope driven by
389 inhibitory synaptic inputs was seen in spike probability recordings from both SC and TA
390 pathways and could be removed by application of a GABA_A receptor antagonist (picrotoxin,
391 50 µM) (Figure 6A-B; SC hyperpolarising envelope -2.45 ± 0.63 mVs, n = 15 from 5 mice
392 versus SC GABA_A antagonist -0.10 ± 0.66 mVs, n = 7 from 2 mice, p < 0.05; TA
393 hyperpolarising envelope -3.01 ± 0.46 mVs, n = 23 from 9 mice versus TA GABA_A
394 antagonist -1.19 ± 0.52 mVs, n = 7 from 2 mice, p < 0.05). To test the importance of CCh
395 effect on inhibition for prioritisation of TA inputs we next repeated spike probability
396 experiments in the presence of the GABA_A receptor antagonist. Under these experimental
397 conditions SC pathway behaved similarly, decreasing spike generation probability upon CCh
398 exposure when membrane potential was kept unaltered (Figure 6C₁-D₁; baseline, 0.7 ± 0.06
399 and CCh i ≠ 0, 0.21 ± 0.06, n = 9 from 3 mice, p < 0.01) and showed an increase during
400 depolarisation without reaching baseline levels (0.44 ± 0.07, p < 0.05 versus baseline), which
401 was correlated with delay to first spike (baseline, 318 ms ± 67 ms, CCh i ≠ 0, 673 ms ± 122
402 ms, CCh i = 0, 397 ms ± 92 ms, p < 0.05 baseline versus CCh i ≠ 0). In contrast, the TA
403 pathway, which increased P_{spike} after CCh when PSP included both excitatory and inhibitory
404 drive, yielded a similar spike probability outcome to SC pathway when inhibition was
405 blocked, decreasing spike probability whether membrane potential was depolarised or not
406 (Figure 6C₂-D₂; baseline, 0.58 ± 0.06; CCh i ≠ 0, 0.17 ± 0.05; CCh i = 0, 0.37 ± 0.08; n = 8
407 from 4 mice; p < 0.01 baseline versus CCh i ≠ 0 and p < 0.05 baseline versus CCh i = 0). This
408 was associated with increases in the delay to first spike (Figure 6C₂-D₂; baseline 246 ms ± 23
409 ms; CCh i ≠ 0, 631 ms ± 119 ms; CCh i = 0, 464 ms ± 119 ms; p < 0.05 baseline versus CCh
410 i ≠ 0).

411 Finally, we sought to confirm that endogenous release of acetylcholine in the hippocampus
412 also decreases the probability of SC evoked spikes and increases the probability of TA
413 evoked spikes. To test this, we reverted to optogenetic stimulation of cholinergic fibers in
414 mice expressing ChR2 in cholinergic neurons. This produced no change in membrane
415 potential and therefore required no current injection to maintain a constant resting potential.
416 After 5 minutes of 2 Hz light stimulation of acetylcholine release the probability of spiking in
417 response to SC pathway stimulation decreased (Figure 7A-C; SC P_{spike} baseline 0.61 ± 0.04
418 vs ACh release 0.44 ± 0.06, normalized SC decrease 0.77 ± 0.1, n = 13 from 8 mice, p <
419 0.05), while probability of spiking in response to TA pathway stimulation increased (Figure
420 7A-C; TA P_{spike} baseline 0.43 ± 0.05 vs ACh release 0.62 ± 0.05, normalized TA increase
421 1.71 ± 0.29, n = 14 from 7 mice, p < 0.005). This opposite modulation of SC and TA
422 pathways was striking in a subset of recordings made from both pathways in the same neuron
423 (P_{spike} SC vs TA, n = 11, p < 0.05). These changes were matched by an increase to the time

424 to first spike in the SC pathway and a decrease for the TA pathway (Figure 7D; SC
425 normalised time to first spike 1.77 ± 0.28 , $p < 0.05$ and TA 0.72 ± 0.05 , $p < 0.001$). The
426 effects of light evoked stimulation on CA1 spike probability and delay were completely
427 blocked by the inclusion of muscarinic and nicotinic antagonists (Figure 7A,C,D; SC
428 normalised P_{spike} 1.04 ± 0.1 , $n = 9$ from 4 mice, $p > 0.05$ & TA 0.82 ± 0.16 , $n = 6$ from 4
429 mice, $p > 0.05$; SC normalised time to first spike 0.86 ± 0.06 , $p > 0.05$ & TA 1.33 ± 0.17 , $p >$
430 0.05). Therefore, endogenous acetylcholine release down-regulates CA1 pyramidal neuron
431 responses to SC pathway and up-regulates responses to TA pathway. Altogether, our data
432 indicate that cholinergic receptor activation produces a decrease of spike output in response
433 to SC activity while enhancing output in response to TA activity via a differential effect on
434 feedforward inhibition to CA1.

435

436 Discussion

437 A long-standing and influential theory proposes that acetylcholine release in the hippocampus
438 prioritises novel sensory information input to enable incorporation into memory ensembles
439 (Hasselmo, 2006). This theory is based on computational modelling and the observation that
440 SC synaptic inputs are more sensitive than TA inputs to depression caused by exogenous
441 cholinergic agonists (Dannenberg et al., 2017; Hasselmo, 2006; Hasselmo and Schnell, 1994;
442 Hasselmo et al., 1995). In contrast, we show that excitatory synaptic transmission at SC and
443 TA inputs to CA1 are equally depressed by endogenous acetylcholine released in response to
444 optogenetic stimulation (Figure 1). Furthermore, in the absence of inhibition, we show that
445 this results in a dramatic reduction of spike output from CA1 in response to either SC or TA
446 input (Figure 6). However, when we considered the effects of acetylcholine on local
447 inhibitory networks as well as excitatory inputs, we find that acetylcholine depresses
448 feedforward inhibition in the TA pathway more than the SC pathway over the course of a
449 burst of stimuli (Figures 2&3). This results in an overall enhancement of spike output from
450 CA1 in response to TA input but not the SC input (Figures 5&7) supporting the hypothesis
451 that acetylcholine enhances responses to novel sensory information arriving via the TA
452 pathway.

453 The regulation of local inhibitory networks by acetylcholine is therefore central to
454 prioritisation of TA inputs by acetylcholine and differences in the regulation of synaptic
455 output from interneuron subtypes are a critical factor. Although the interneuron subtypes
456 engaged by the SC and TA pathways are a mixed population, the difference in IPSC kinetics
457 and sensitivity to the μ -opioid receptor agonist DAMGO in our recordings support previous
458 findings that PV⁺ cells form the majority of feedforward inhibition in the SC pathway
459 whereas CCK⁺ cells are the major contributors to feedforward inhibition in the TA pathway
460 (Basu et al., 2013; Freund and Katona, 2007; Glickfeld and Scanziani, 2006; Klausberger and
461 Somogyi, 2008; Milstein et al., 2015). Crucially, the synaptic output from PV⁺ and CCK⁺
462 interneurons is differentially regulated by acetylcholine. Whilst both outputs are depressed by
463 acetylcholine, the depression of CCK⁺ output is greater over the course of a burst of stimuli
464 showing enhanced depression for later responses in the burst, mirroring the effect of
465 acetylcholine on feedforward inhibition in the TA pathway. Acetylcholine does not cause a

466 greater depression for later responses in the burst for PV⁺ synaptic output and therefore
467 feedforward inhibition in the SC pathway is relatively greater over a burst of stimuli reducing
468 the impact of SC stimulation when acetylcholine is present. Interestingly, the excitability of
469 different interneuron subtypes is regulated by different cholinergic receptors with M₃
470 receptors in CCK⁺ interneurons, M₁ receptors in PV⁺ and NPY⁺ interneurons and nicotinic α2
471 receptors in OLM feedback interneurons (Cea-del Rio et al., 2011; Cea-del Rio et al., 2010;
472 Leao et al., 2012; Raza et al., 2017; Yi et al., 2014). This further supports the major
473 contribution of CCK⁺ interneurons in the TA pathway since the M₃ receptor antagonist
474 DAU5884 completely blocked the CCh-induced depression of feedforward inhibition in the
475 TA pathway.

476 The differential regulation of SC and TA pathways is mediated by selective expression of M₄
477 and M₃ receptors. The targeting of M₃ and M₄ receptors to presynaptic terminals of TA and
478 SC axons respectively fits with a broader picture of highly specific localisation of muscarinic
479 receptor subtypes to cellular and subcellular domains within the hippocampus that includes
480 the localisation of M₂ receptors to inhibitory presynaptic terminals of PV⁺ basket cells. This
481 agrees with the observed highly laminar localisation of M₃ receptors in the Stratum
482 Lacunosum Moleculare, M₄ receptors in Stratum Radiatum and M₂ receptors in the Stratum
483 Pyramidale ((Levey et al., 1995) but see (Goswamee and McQuiston, 2019)). At each
484 terminal, muscarinic receptors depress neurotransmitter release probability (Dasari and
485 Gullede, 2011; Levey et al., 1995; Szabo et al., 2010; Thorn et al., 2017) and we show that
486 this includes M₃ receptors targeted to presynaptic terminals of TA axons where they depress
487 release of glutamate. M₃ receptors are also expressed in CCK⁺ interneurons where they
488 increase excitability (Cea-del Rio et al., 2011; Cea-del Rio et al., 2010) and our data suggest
489 that M₃ receptors expressed in these cells can also regulate release of GABA at synapses onto
490 pyramidal cells (Figures 3&4). Given the importance of the TA input for synaptic plasticity
491 in the hippocampus (Bittner et al., 2015; Takahashi and Magee, 2009) it is expected that M₃
492 receptors play an important role in hippocampal-dependent learning. However, the evidence
493 from studies using mice with genetic deletion of M₃ receptors is somewhat equivocal (Poulin
494 et al., 2010; Yamada et al., 2001). A potential explanation lies in the compensation for
495 deletion of M₃ with expression of M₁ receptors (Figure S4) that couple to similar Gq-
496 mediated signalling pathways and it is interesting that knockin mutations of phosphorylation-
497 deficient M₃ receptors with potentially less compensation show greater effects on learning
498 and memory (Poulin et al., 2010). The compensation for M₃ deletion by M₁ receptors is
499 somewhat surprising since M₁ receptors are generally expressed widely in somatic and
500 dendritic cellular domains in pyramidal cells and interneurons where they regulate intrinsic
501 excitability leading to effects on synaptic plasticity and network oscillations (Atherton et al.,
502 2016; Betterton et al., 2017; Buchanan et al., 2010; Dennis et al., 2016; Fisahn et al., 2002;
503 Levey et al., 1995; Mitsushima et al., 2013; Shinoe et al., 2005; Tigaret et al., 2018) but are
504 not generally found in presynaptic terminals (Yamasaki et al., 2010).

505 Cholinergic neurons *in vivo* fire at frequencies ranging from 0.3 – 5 Hz with higher
506 frequencies recorded during waking activity (Hangya et al., 2015; Simon et al., 2006).
507 Responses to salient events such as positive or negative reinforcement have been
508 demonstrated (Hangya et al., 2015; Lovett-Barron et al., 2014; Teles-Grilo Ruivo et al.,

509 2017), but even in these conditions cholinergic firing rates do not increase dramatically but
510 rather activity across cholinergic neurons is synchronised (Hangya et al., 2015). Interestingly,
511 release of acetylcholine plateaus at firing rates around 2 Hz (Jing et al., 2018) indicating that
512 the dynamic range of acetylcholine release occurs at frequencies below 2 Hz. Therefore,
513 optogenetic stimulation that synchronises release at 2 Hz over extended time periods is likely
514 to be physiologically maximal. Cholinergic neurons are also reported to co-release glutamate
515 and more prominently GABA both from long-range projections and also local cholinergic
516 interneurons (Saunders et al., 2015; Takacs et al., 2018; Yi et al., 2015). However, we found
517 no evidence for glutamate or GABA release after optogenetic stimulation of cholinergic
518 fibres (Figure 1D). Therefore, under our experimental conditions, optogenetic stimulation of
519 cholinergic fibres at 2 Hz likely provides a maximally effective release of acetylcholine
520 without co-release of glutamate or GABA that was mimicked by exogenous application of 10
521 μM CCh.

522 Acetylcholine increases the output gain from primary sensory cortices enhancing signal-to-
523 noise for new sensory information and desynchronising the local cortical network by
524 reorganising inhibition to disinhibit pyramidal neurons (Eggermann et al., 2014; Fu et al.,
525 2014; Letzkus et al., 2011). A contrary situation is reported in the hippocampus where
526 cholinergic activation of dendritically targeting interneurons inhibits pyramidal neurons and
527 potentially gates CA1 output (Haam et al., 2018; Leao et al., 2012; Lovett-Barron et al.,
528 2014). Both of these mechanisms may be important for learning new representations,
529 however, neither of these situations addresses whether acetylcholine prioritises one set of
530 inputs over another. Here, we reveal a novel mechanism whereby acetylcholine alters the
531 short-term dynamics of information processing in CA1 by acting on two distinct muscarinic
532 receptor subtypes located in the SC and TA pathway. It will be interesting to discern in future
533 how these various mechanisms interact across different behavioural epochs.

534 Multiple compounds have been developed to selectively target M_1 and M_4 muscarinic
535 receptors for potential cognitive enhancement whereas M_2 and M_3 receptors have received
536 much less attention due to complications with peripheral effects on cardiac and enteric
537 function. The M_1/M_4 receptor dual agonist Xanomeline has cognitive enhancing and
538 antipsychotic efficacy in clinical trials (Bodick et al., 1997; Shekhar et al., 2008) and whilst it
539 is not clear whether M_1 or M_4 receptors are the key target, in separate studies selective M_1
540 agonists and M_4 agonists have been shown to have memory enhancing and/or antipsychotic
541 efficacy (Chan et al., 2008; Nathan et al., 2013) whereas deletion of M_1 receptors in mice
542 causes memory deficits (Anagnostaras et al., 2003). Our data provide a mechanism for the
543 actions of M_1/M_4 receptor dual agonists such as Xanomeline and Compound1 where
544 activation of M_1 receptors facilitates synaptic plasticity (Buchanan et al., 2010) and activation
545 of M_4 receptors prioritises new information to incorporate into memory. Our data also predict
546 that selective activation of M_3 receptors could potentially facilitate the consolidation of
547 memory by reducing interference from new information. Interestingly, the link that we
548 demonstrate between selective muscarinic receptor activation and distinct interneuron
549 subtypes suggests a mechanism to selectively target and regulate these interneuron
550 populations. This could have therapeutic value in disorders with disruption to specific
551 interneuron populations such as PV^+ neurons in schizophrenia (Lewis et al., 2005). Overall,

552 acetylcholine release in the hippocampus supports cognition and the identification of specific
553 roles for each muscarinic receptor subtype provides mechanisms to selectively modulate
554 individual aspects of acetylcholine's actions. The identification of M₃ receptors as regulators
555 of TA inputs in contrast to M₄ receptors acting on SC inputs provides a novel mechanism by
556 which specific targeting of these muscarinic receptors could represent a therapeutic strategy
557 to bias hippocampal processing and enhance cognitive flexibility.

558

559

560 **Methods**

561 **Animal Strains.** All experiments were performed using male mice. C57BL/6J (Charles
562 River) mice were used as the background strain. The generation of the M₃ receptor KO mice
563 has been described (Yamada et al., 2001). The M₃ KO mice used for this study had been
564 backcrossed for 10 times onto the C57BL/6NTac background. Cre reporter allele mice (The
565 Jackson Laboratory) were used to tag specific neuronal populations: Cholinergic neurons
566 (Chat-IRES-Cre; Stock No. 006410), parvalbumin interneurons (B6 PV^{CRE}; Stock No.
567 017320) and cholecystokinin interneurons (CCK-IRES-Cre; Stock No. 012706).
568 Homozygous cre reporter mice were crossed with homozygous Ai32 mice (B6.Cg-
569 Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J; Stock No. 024109) to generate
570 litters of heterozygous offspring expressing ChR2.

571 **Slice preparations.** All animal procedures were performed in accordance with Home Office
572 guidelines as stated in the United Kingdom Animals (Scientific Procedures) Act 1986 and EU
573 Directive 2010/63/EU 2010 and experimental protocols were approved by the British
574 National Committee for Ethics in Animal Research.

575 Brain slices were prepared from P30-40 male mice. Following cervical dislocation and
576 decapitation, brains were removed and sliced in ice-cold sucrose solution containing (in
577 mM): 252 sucrose, 10 glucose, 26.2 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 5 MgCl₂ and 1
578 CaCl₂ saturated with 95% O₂ and 5% CO₂. Parasagittal slices 350 µm thick were cut using a
579 VT1200 (Leica) vibratome. Slices were transferred to warm (32 °C) aCSF for 30 minutes
580 containing (in mM): 119 NaCl, 10 glucose, 26.2 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 1.3
581 MgSO₄ and 2.5 CaCl₂ saturated with 95% O₂ and 5% CO₂ and then kept at room
582 temperature until use.

583 **Electrophysiology.** Whole-cell patch clamp recordings were made from hippocampal CA1
584 pyramidal neurons visualised under infrared differential interface contrast on SliceScope Pro
585 6000 system (Scientifica). Slices were continually perfused with aCSF at 4-5 ml/min. Patch
586 electrodes (4-7 MΩ resistance) were pulled from borosilicate glass capillaries (Harvard
587 Apparatus) using a PC-87 Micropipette puller (Sutter Instrument). Recording pipettes were
588 filled with either voltage-clamp internal solution (in mM: 117 CsMeSO₃, 9 NaCl, 10 HEPES,
589 10 TEA, 2 MgATP, 0.3 NaGTP, 1 QX-314, 0.3 EGTA at pH 7.3 and 290 mOsm) or current-
590 clamp internal solution (in mM: 135 K-Gluconate, 10 HEPES, 7 glucose, 8 NaCl, 2MgATP,
591 0.3 NaGTP, 0.2 EGTA at pH 7.3 and 290 mOsm). Electrophysiological recordings were
592 made with an Axoclamp 200B (Molecular Devices) filtered at 5 kHz and digitized at 10 kHz
593 using a CED micro 1401 MKII board and Signal5 acquisition software (Cambridge
594 Electronic Design). Series and input resistances were monitored by applying a 20 pA and 500
595 ms square pulse. Experiments were discarded if the change in series resistance was
596 >25%. Membrane potentials were not corrected for junction
597 potentials.

598 **Dual pathway (SC and TA) stimulation.** Bipolar stimulating electrodes were placed in CA3 to
599 stimulate SC fibres and in the Stratum Lacunosum Moleculare (SLM) of subiculum to
600 stimulate TA fibres. Synaptic responses were evoked alternately in either pathway at 15 sec
601 intervals. Monosynaptic EPSCs were recorded either at -65 mV membrane potential in the

602 presence of GABA_A receptor blocker picrotoxin (50 μM) or in control aCSF at the
603 experimentally determined reversal potential for GABA_A receptors (-60 mV). Disynaptic
604 IPSCs were recorded in control aCSF at experimentally determined reversal potential for
605 AMPA receptors (0 mV). NBQX (20 μM) was applied at the end of experiments to ascertain
606 the contribution of direct stimulation of local interneurons to IPSCs and only responses which
607 showed > 70% reduction in IPSCs were used for analysis. In the experiments specified, SC
608 and TA pathway EPSCs and IPSCs were recorded from the same CA1 pyramidal neuron to
609 calculate excitation-inhibition ratio (E-I ratio). EPSC and IPSC contributions were measured
610 as charge transferred by calculating the area of each synaptic response in pC and the ratio of
611 EPSC and IPSC charge for each response determined the E-I ratio. PPRs were calculated by
612 normalising the amplitude of each response to the first response. TA over SC E-I ratio was
613 calculated for each cell before averaging across cells.

614 *Current clamp experiments* were performed at resting membrane voltage (-61.3 ± 3.5 mV).
615 TA and SC pathways were stimulated at intervals of 20 s with trains of 10 stimuli at 10 Hz.
616 Stimulation intensities were set to generate target spike probabilities between 30-70 %. Spike
617 probability was calculated as the number of spikes/number of stimuli. Time to first spike was
618 measured from the first stimulus in the train. Post synaptic potential (PSP) envelope was
619 measured by calculating the area under the curve generated by joining the points of maximum
620 hyperpolarisation in response to each stimulation as described previously (Chamberlain et al.,
621 2013). Carbachol (CCh 10 μM) -induced depolarisations were neutralised by current
622 injections to maintain a constant membrane voltage (i≠0). To investigate the impact of CCh
623 induced depolarisation, the injected current was removed (i=0).

624 **Optogenetic stimulation.** Blue light from a 470 nm LED was targeted to slices via a 469 nm
625 emission filter, a GFP dichroic mirror (Thorlabs) and the 4x (ChAT-Ai32) or 40x (PV-Ai32
626 or CCK-Ai32) microscope objective. 5 ms light pulses at 7-9 mW/mm² intensity were used
627 for all stimuli. Optogenetically-evoked IPSCs were recorded from pyramidal neurons at 0
628 mV membrane potential in the presence of the AMPA and NMDA receptor antagonists
629 NBQX (10μM) and DAPV (50μM).

630 **Confocal imaging.** Recorded slices were permeabilized with 0.1% Triton X-100 (Sigma) and
631 incubated with Alexa avidin (488 nm or 594 nm; ThermoFisher). CA1 pyramidal neurons
632 from Chat-Ai32 mice were labelled with Alexa-594 and test proximity to cholinergic axons
633 using Chat-Ai32 YFP fluorescence.

634 **Statistical analysis.** Experimental unit was defined as cell for all conditions and only one cell
635 recorded from each slice. Cell and animal numbers are reported for all experiments. All data
636 were plotted as the mean ± SEM. Where comparisons between two conditions were made
637 paired or unpaired two-tailed Student's t-tests were applied as appropriate. For comparisons
638 between more than 2 conditions one-way repeated measures ANOVA tests with Bonferroni
639 post hoc correction were used. The level of significance was set to 0.05 and p values are
640 shown as follows: * P < 0.05; ** P < 0.01; *** P < 0.001. Experiments on WT and M₃ KO
641 mice were performed blind to genotype.

642 **Reagents.** Carbachol (CCh), NBQX, DCG-IV, D-APV, picrotoxin, atropine, mecamylamine,
643 nitrocaramiphen, DAU-5884 were purchased from Tocris (UK). GSK-5 was synthetized in-

644 house at Eli Lilly and Co. Stock solutions of these compounds were made by dissolving in
645 water. The selective muscarinic M₁ & M₄ receptor agonist Compound1 was synthetized in
646 house at Sosei Heptares and dissolved in DMSO for stock solution. The purity of the final
647 compounds was determined by HPLC or LC/MS analysis to be >95%. Additional
648 experimental details relating to the synthesis of Compound1 and associated structures is
649 described in detail in WO2015/118342 which relates to the invention of agonists of the
650 muscarinic M₁ receptor and/or M₄ receptor and which are useful in the treatment of
651 muscarinic M₁/M₄ receptor mediated diseases.

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851 neurochemical characteristics, and capacity for cholinergic modulation. *Front Synaptic
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- 853
- 854

855 **Figure legends.**

856 **Figure 1. Endogenous release of acetylcholine reduces excitatory and inhibitory**
857 **synaptic inputs to CA1 pyramidal neurons.**

858 **A**, Coronal section illustration of medial septum (MS, yellow) and its projections to dorsal
859 hippocampus including Schaffer collateral (green) and temporoammonic (purple) inputs to
860 CA1 from CA3 and entorhinal cortex (EC) respectively.

861 **B**, Immunofluorescence of cholinergic neurons in medial septum filled with neurobiotin
862 (blue) expressing ChR2-YFP protein (left) and light evoked stimulation (blue dot, 10ms) of
863 cholinergic neuron, which reliably elicits action potentials at frequencies < 25 Hz (right).

864 **C**, Immunofluorescence of CA1 area of the hippocampus highlighting a CA1 pyramidal
865 neuron filled with neurobiotin (blue) and surrounding cholinergic axons (yellow). Nuclei
866 stained with DAPI (light blue) and location of Schaffer collateral (SC) and temporoammonic
867 (TA) axons illustrated in green and purple respectively.

868 **D**, Light-evoked stimulation of cholinergic axons (blue dot) elicits fast synaptic responses in
869 Stratum Oriens interneurons recorded at -60 mV that are sensitive to atropine (25 μ M) and
870 mecamylamine (50 μ M) but not picrotoxin (PTX, 50 μ M), NBQX (20 μ M) or D-APV (25
871 μ M).

872 **E**, No response to light-evoked stimulation of cholinergic axons (blue dot) was seen in CA1
873 pyramidal neurons recorded at 0 mV or -60 mV in contrast to electrical stimulation (black
874 line).

875 **F**, SC (green) and TA (purple) evoked EPSCs in CA1 pyramidal neurons are reversibly
876 depressed by endogenous release of acetylcholine evoked by 5 minutes light stimulation at 2
877 Hz (F_1). The depression of EPSCs is blocked by application of cholinergic antagonists
878 atropine (25 μ M) and mecamylamine (50 μ M) (F_2).

879 **G-H**, Acetylcholine release depressed SC and TA pathway evoked EPSCs (G) and increased
880 paired-pulse ratio (H, PPR).

881 **I**, Feedforward disynaptic IPSCs evoked by stimulation of SC and TA pathways are
882 depressed by light evoked acetylcholine release (I_1). The depression of IPSCs is blocked by
883 application of cholinergic antagonists atropine and mecamylamine (I_2).

884 **J-K**, Effects of acetylcholine release on SC and TA pathway evoked IPSC response (J) and
885 PPR (K).

886 Data are mean \pm SEM; Two-tailed paired Student's T-test *** p < 0.001 ** p < 0.01 * p <
887 0.05; g-h and j-k inter group comparison one-way ANOVA with post hoc Bonferroni
888 correction.

889

890 **Figure 2. Cholinergic receptor activation enhances excitatory-inhibitory balance for**
891 **temporoammonic synaptic inputs relative to Schaffer collateral inputs.**

892 **A**, Middle, schematic representation of the experimental approach incorporating
893 simultaneous recording of excitatory ($V_h = -60$ mV) and feedforward inhibitory ($V_h = 0$ mV)
894 synaptic inputs from Schaffer collateral (SC) and temporoammonic (TA) input pathways to
895 CA1 pyramidal neuron (bottom). Example traces for EPSCs and IPSCs in response to trains
896 of 5 stimuli at 10 Hz to SC (green, left) and TA (purple, right) pathways before and after
897 carbachol (CCh, 10 μ M) application.

898 **B**, Change in paired-pulse ratio (PPR) after CCh application for excitatory and inhibitory
899 responses to SC (B_1) and TA (B_2) pathway stimulation. PPR is measured compared to the
900 first response for each response in the train.

901 **C**, Comparison of synaptic Excitatory-Inhibitory (E-I) ratio before and after CCh application
902 measured by charge transfer at $V_h = -60$ mV and 0 mV for SC (C_1) and TA (C_2) input
903 pathways.

904 **D**, Comparison of synaptic E-I ratio between TA and SC input pathways before and after
905 CCh application. CCh enhanced the overall relative synaptic charge transfer from TA
906 pathway.

907 Data are mean \pm SEM; Two tailed Student's paired T-test *** $p < 0.001$ ** $p < 0.01$ * $p <$
908 0.05.

909

910 **Figure 3. Cholinergic modulation of inhibitory inputs from distinct feedforward**
911 **interneuron populations.**

912 **A**, Schematic representation of different feedforward interneuron populations engaged by
913 Schaffer collateral (SC) and temporoammonic (TA) pathways within CA1.

914 **B-C**, Disynaptic feedforward IPSCs (B) and distribution of decay kinetics (C) for Schaffer
915 collateral (SC, green) and temporoammonic (TA, purple) input pathways demonstrating
916 distinct populations of feedforward interneurons. Quantification of the IPSC tau decay (insert
917 C).

918 **D**, μ -opioid receptor agonist DAMGO (1 μ M) depression of disynaptic feedforward IPSCs
919 from SC and TA pathways.

920 **E**, IPSC decay kinetics and sensitivity to DAMGO correlate and distinguish SC from TA
921 evoked IPSCs.

922 **F**, Optogenetic activation of either PV (F1) or CCK (F2) interneurons at 10 Hz evoked a train
923 of IPSCs in CA1 pyramidal neurons. IPSCs from both interneurons are depressed by CCh (10
924 μ M).

925 **G-H**, IPSCs from PV interneurons display faster decay kinetics than IPSCs from CCK
926 interneurons (G) but CCh depressed the IPSC amplitudes of the first responses in the train by
927 a similar amount (H).

928 **I-J**, IPSCs from both PV and CCK interneurons demonstrated frequency-dependent
929 depression. Frequency-dependent depression was reduced after CCh application for PV (I₁-
930 J₁) but not CCK (I₂-J₂) evoked IPSCs.

931 Data are mean \pm SEM; Two tailed Student's paired T-test *** p < 0.001 ** p < 0.01 * p <
932 0.05.

933

934 **Figure 4. Muscarinic M₃ receptors modulate temporoammonic pathway EPSCs and**
935 **disynaptic IPSCs in CA1 pyramidal neurons.**

936 **A**, Schematic illustrating recording of pharmacologically isolated EPSCs from
937 temporoammonic (TA) and Schaffer collateral (SC) pathways.

938 **B-C**, The dual M₁ and M₄ and muscarinic receptor agonist Compounnd1 (1 μ M) depresses
939 evoked EPSCs (B) and increases paired pulse ratio (PPR) (C) for SC (green) but not TA
940 (purple) pathway.

941 **D**, Schematic illustrating recording of pharmacologically isolated EPSCs from
942 temporoammonic (TA) pathway.

943 **E**, CCh (10 μ M) reliably reduced evoked EPSC amplitudes.

944 **F-G**, Pharmacology of cholinergic depression of EPSCs. CCh-induced depression (F) is
945 prevented by application of muscarinic receptor antagonist atropine (Atrp, 10 μ M) or M₃
946 receptor antagonist DAU 5884 (DAU, 1 μ M) but not M₁ receptor antagonist Nitrocaramiphen
947 (NCP, 1 μ M) or nicotinic receptor antagonist mecamylamine (MEC, 25 μ M) and is not
948 replicated by M₁ receptor agonist GSK-5 (500 nM). PPR changes reflect conditions of
949 cholinergic-induced EPSC depression (G).

950 **H-I**, Comparison of the effects of CCh on TA pathway-evoked EPSCs in wild type (WT) and
951 M₃ receptor knockout mice (M₃ KO). EPSC depression (H) and PPR increase (I) by CCh
952 were reduced in slices from M₃ KOs in comparison to WT.

953 **J**, Schematic illustrating recording of disynaptic feedforward IPSCs from pyramidal neurons
954 at 0 mV in TA pathway.

955 **K**, CCh (10 μ M) reliably reduced evoked IPSC amplitudes.

956 **L**, Pharmacology of cholinergic depression of IPSCs. CCh-induced depression is prevented
957 by application of muscarinic receptor antagonist atropine or M₃ receptor antagonist DAU
958 5884 but not M₁ receptor antagonist Nitrocaramiphen or nicotinic receptor antagonist
959 mecamylamine and is not replicated by M₁ receptor agonist GSK-5.

960 Data are mean \pm SEM; Inter group comparison one-way ANOVA with post hoc Bonferroni
961 correction. Two tailed unpaired Student's T-test *** p < 0.001 ** p < 0.01 * p < 0.05.

962

963 **Figure 5. Cholinergic receptor activation enhances CA1 response to temporoammonic**
964 **over Schaffer collateral input.**

965 **A**, Responses in CA1 pyramidal neurons to 10 stimuli at 10 Hz given to Schaffer collateral
966 (SC, A₁) or temporoammonic (TA, A₂) input pathways. After application of CCh (10 μ M),
967 membrane potential (V_h) is initially held at baseline levels by injection of current (i \neq 0) and
968 then allowed to depolarise (i=0).

969 **B**, Heat maps depicting spike probability for 10 stimulation pulses from 10 cells for SC (B_1)
970 and TA (B_2) input pathways before and during CCh application.

971 **C**, Spike probability and time to first spike for SC (C_1) and TA (C_2) input pathways. Spike
972 probability decreased after CCh application in SC pathway but increased in TA pathway.

973 Data are mean \pm SEM; One-way ANOVA with repeated measures and post hoc Bonferroni
974 correction *** $p < 0.001$ *** $p < 0.01$ * $p < 0.05$.

975

976 **Figure 6. Cholinergic enhancement of CA1 responses to temporoammonic inputs is**
977 **mediated by inhibition.**

978 **A-B**, Inhibition of GABA_A receptors with picrotoxin (50 μ M) reduced the underlying
979 hyperpolarising envelope (red) in response to 10 stimuli at 10 Hz (D) and increased spike
980 probability for both SC (green) and TA (purple) input pathways to CA1 pyramidal neurons
981 (E).

982 **C-D**, In the presence of GABA_A receptor antagonist, CCh reduced spike probability and
983 increased time to spike in both SC (C_1 -D₁) and TA (C_2 -D₂) input pathways.

984 Data are mean \pm SEM; One-way ANOVA with repeated measures and post hoc Bonferroni
985 correction ** $p < 0.01$ * $p < 0.05$.

986

987 **Figure 7. Endogenous acetylcholine release enhances CA1 response to temporoammonic**
988 **over Schaffer collateral input.**

989 **A**, Modulation of SC (A_1) and TA (A_2) pathway spike generation by endogenous
990 optogenetically-evoked release of acetylcholine (2 Hz for 5 mins). Raster plots show
991 representative experiments where SC and TA pathway are stimulated alternately with trains
992 of 10 stimuli. Cholinergic receptor antagonists, atropine (25 μ M) and mecamylamide (50
993 μ M) prevented modulation of spike generation in SC and TA pathways by endogenous
994 acetylcholine release (A_{3-4}).

995 **B**, Time course of spike probability modulation on SC and TA pathway by endogenous
996 release of acetylcholine.

997 **C-D**, Quantification of spike probability (C) and time to first spike (D) on SC and TA
998 pathway either on naïve or in the presence of cholinergic antagonists.

999 Data are mean \pm SEM; Inter group comparison one-way ANOVA with post hoc Tukey's
1000 correction. Two tailed paired Student's T-test *** $p < 0.001$ * $p < 0.05$.

1001

1002 **Figure S1.**

1003 **A**, Independence of SC (green) and TA (purple) pathways was evaluated by the lack of
1004 facilitation of the second EPSC response when the alternate pathway was stimulated.

1005 **B**, Feedforward IPSCs from SC and TA pathways were recorded at 0 mV and confirmed to
1006 be disynaptic by sensitivity to NBQX.

1007 **C**, DCG-IV (3 μ M) blocked TA pathway but not SC pathway synaptic responses. Application
1008 of AMPA receptor antagonist (NBQX 20 μ M) blocked responses in both pathways.

1009 Data are mean \pm SEM; Two tailed unpaired Student's T-Test. ***P< 0.005.

1010

1011 **Figure S2.**

1012 **A**, Dose-response for CCh depression of EPSCs (A_1) and IPSCs (A_2) for SC (green) and TA
1013 (purple) pathways.

1014 **B**, Quantification of SC and TA EPSC (B_{1-2}) and IPSC (B_{3-4}) charge transfer for each
1015 response in the train illustrated in Figure 2A before and after CCh (10 μ M) application.

1016 **C**, EPSC (C_1) and IPSC (C_2) reduction by CCh for each of the 5 stimuli for SC (green) and
1017 TA (purple) pathways shown in Figure 2A.

1018 **D-E**, CCh reduced the depression index for SC (green) but not TA (purple) disynaptic
1019 feedforward IPSCs. Depression index is calculated as the amount of cumulative depression
1020 between the 2nd and 5th responses within the train of 5 responses.

1021 Data are mean \pm SEM; A compared via two tailed unpaired Student's T-test and E via one-
1022 way ANOVA with post hoc Bonferroni correction *** p < 0.001 ** p < 0.01.

1023

1024 **Figure S3.**

1025 **A**, ChR2 currents at different holding potentials recorded from a CCK⁺ and ChR2 expressing
1026 pyramidal neuron in response to 2ms light pulses in the presence of picrotoxin (50 μ M). At
1027 0mV (the reversal potential for ChR2) no ChR2 currents are observed.

1028 **B-C**, Light evoked GABAergic responses recorded from pyramidal neurons held at 0mV in
1029 the presence of NBQX and DAPV are abolished by picrotoxin (50 μ M).

1030

1031 **Figure S4.**

1032 Structure and in vitro pharmacological profile of Compound1. CHO-K1 cells stably
1033 expressing the human M₁-M₄ and rat M₄ receptors were used to determine the
1034 pharmacological profile of Compound1. ^a Calculated LogP value, LogD was measured at
1035 pH7.4. ^b Compound pEC50 values were measured using phosphor-ERK format (CisBio).
1036 Values reported as <4.7 were considered inactive and did not induce a >10% increase in the
1037 response at the highest concentration tested (30 μ M). ^c The maximum efficacy (Emax values)
1038 are expressed as a percentage of the response of a saturating concentration of acetylcholine
1039 (1 μ M) run in the same assay. ^d [3H]-NMS competition binding studies were used to define
1040 the affinity (pKi) for Compounnd1 at the human muscarinic M₂ receptor. ^e number of
1041 replicates. Data are the mean \pm S.E.M. Compound1 can be found within WO2015/118342

1042 which relates to the invention of agonists of the muscarinic M₁ receptor and/or M₄ receptor
1043 and which are useful in the treatment of muscarinic M₁/M₄ receptor mediated diseases.

1044

1045 **Figure S5.**

1046 **A**, Muscarinic M₁ receptor agonist (GSK-5, 500nM) produced an increase in the frequency of
1047 spontaneous excitatory events recorded from CA1 pyramidal neurons.

1048 **B-C**, GSK-5 caused a reduction of TA pathway EPSC (B) and an increase of PPR (C) in
1049 slices from M₃ KO mice but not in slices from WT mice.

1050 Data are mean ± SEM; Inter group comparison one-way ANOVA with post hoc Bonferroni
1051 correction. Two tailed paired Student's T-test *** p < 0.001 * p < 0.05.

Figure 1

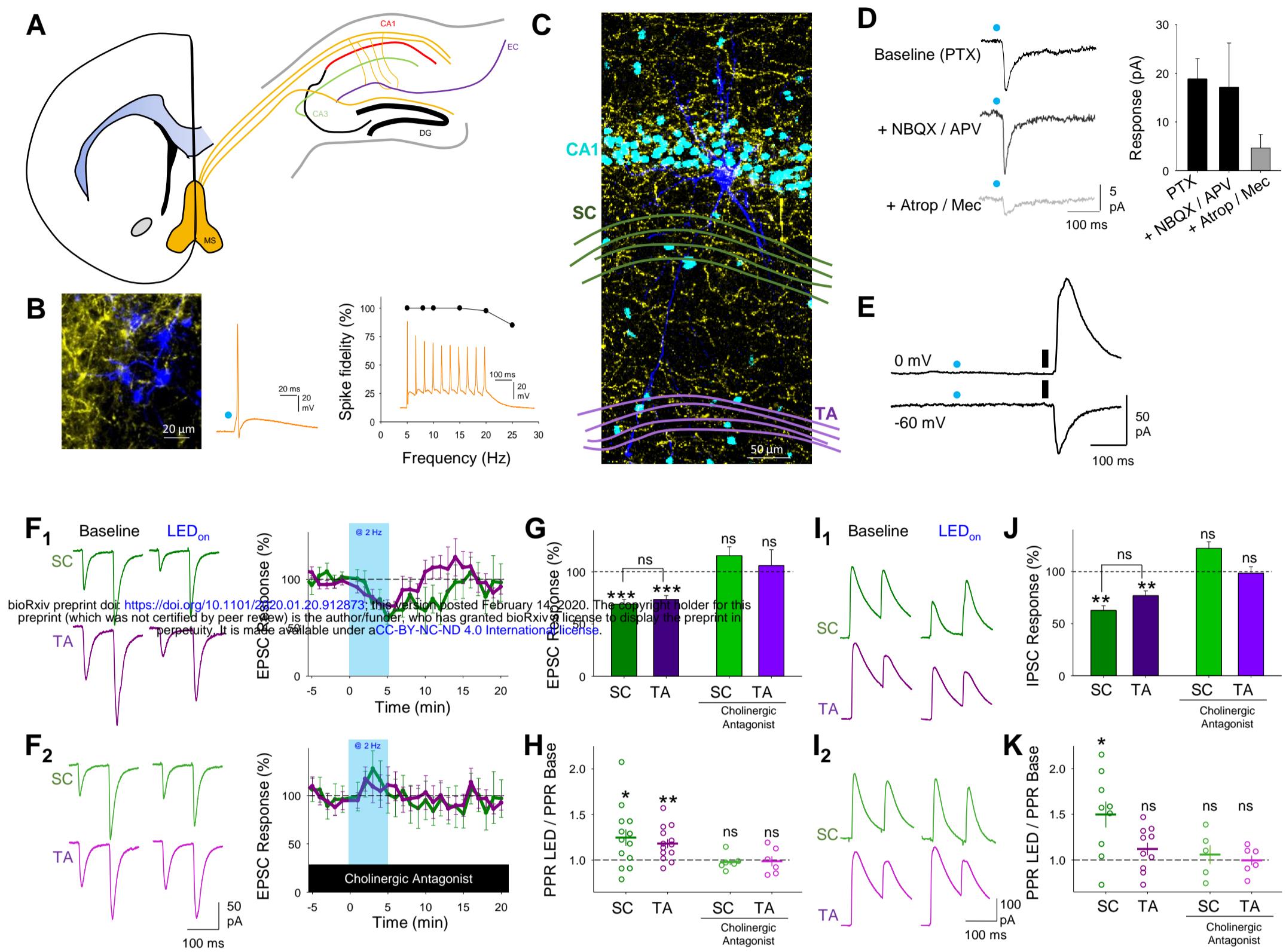


Figure 2

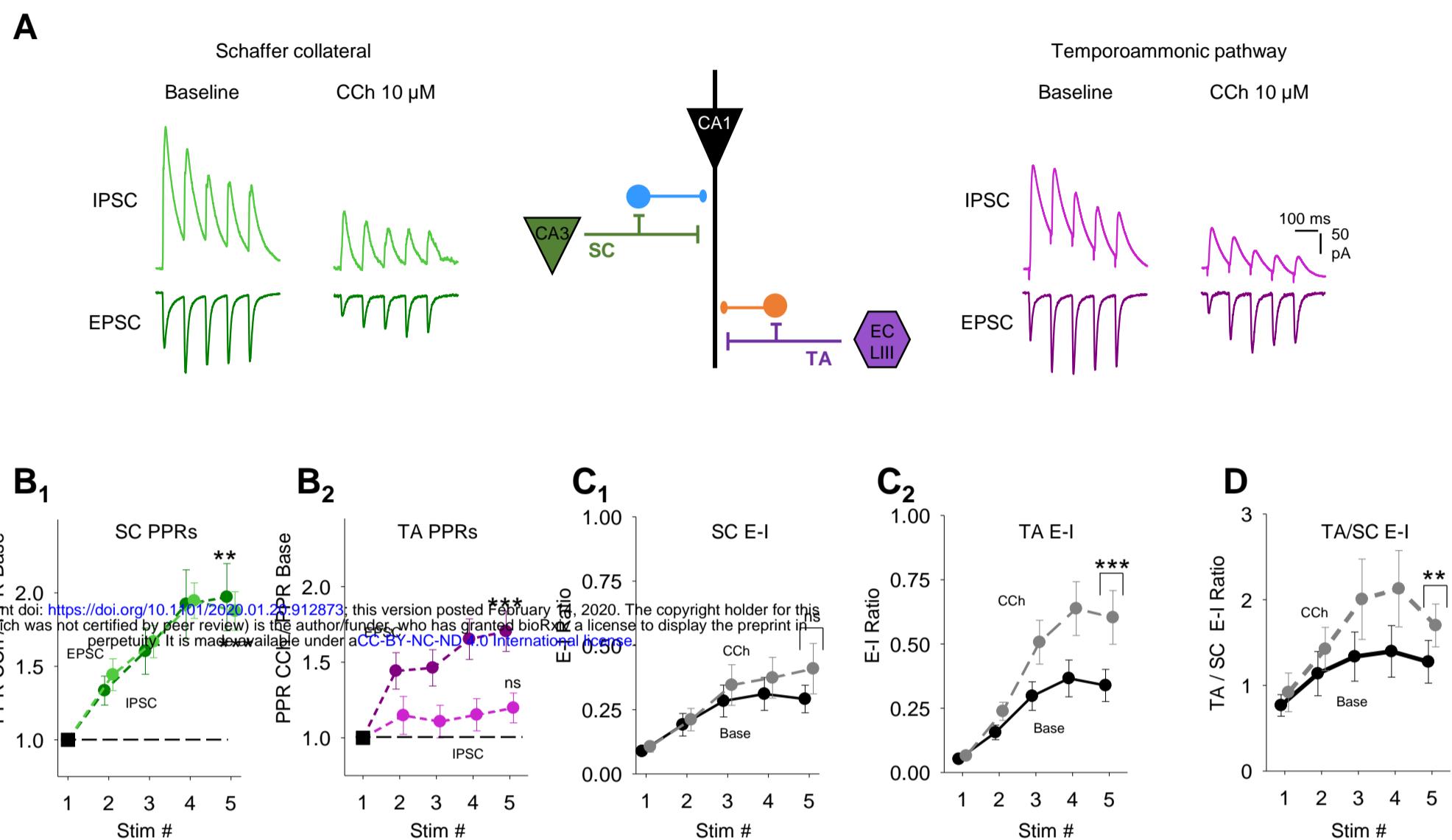


Figure 3

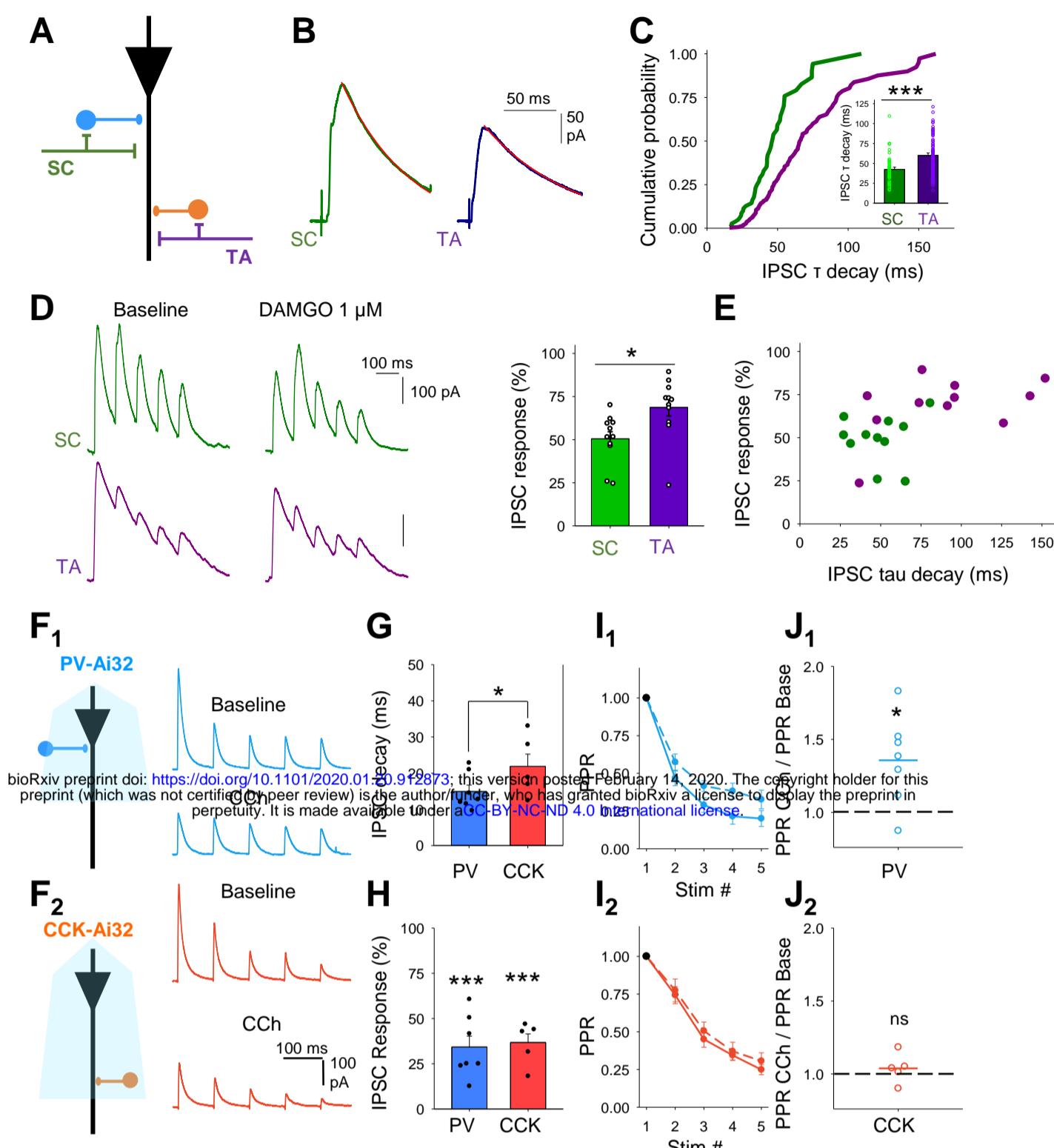


Figure 4

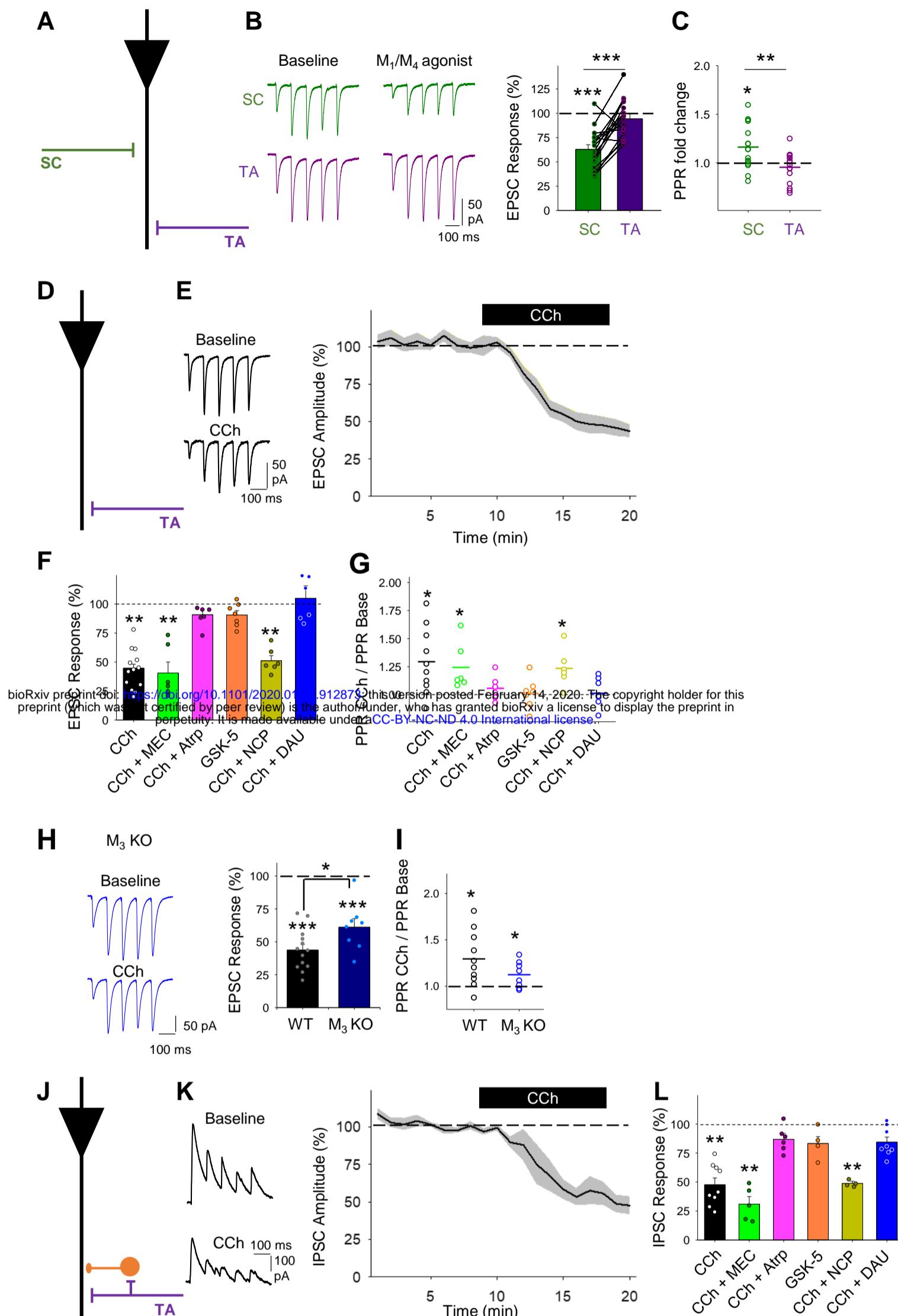


Figure 5

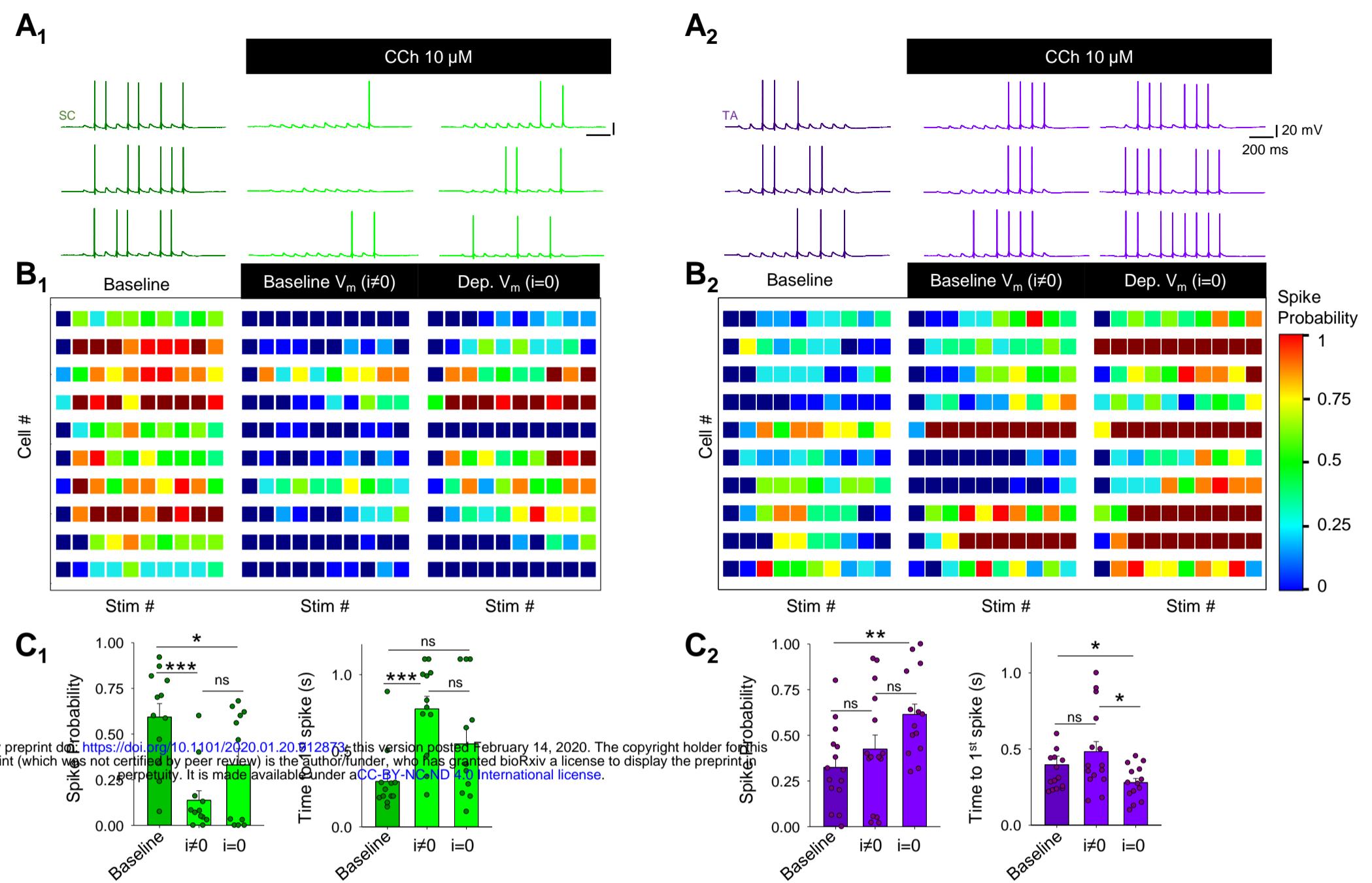
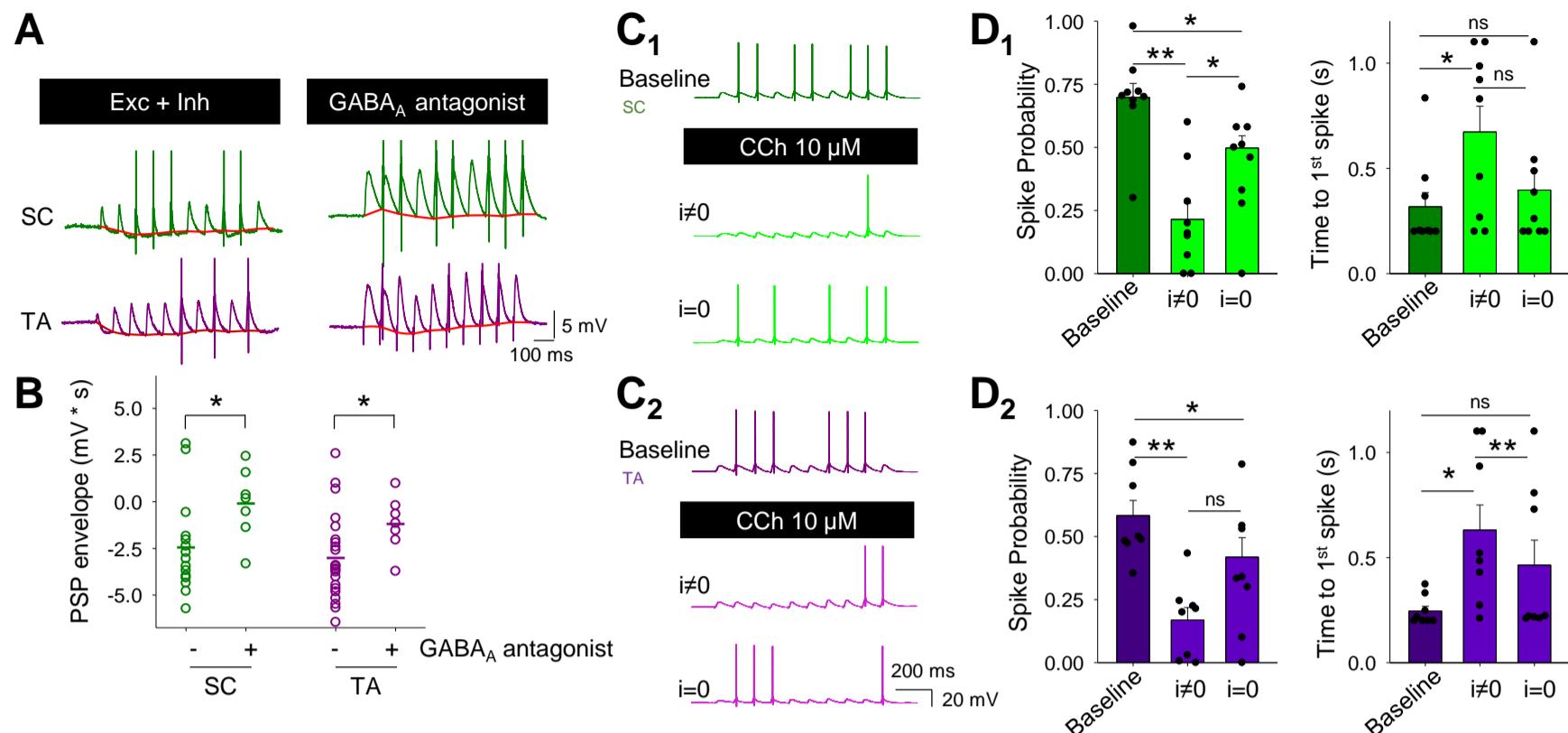
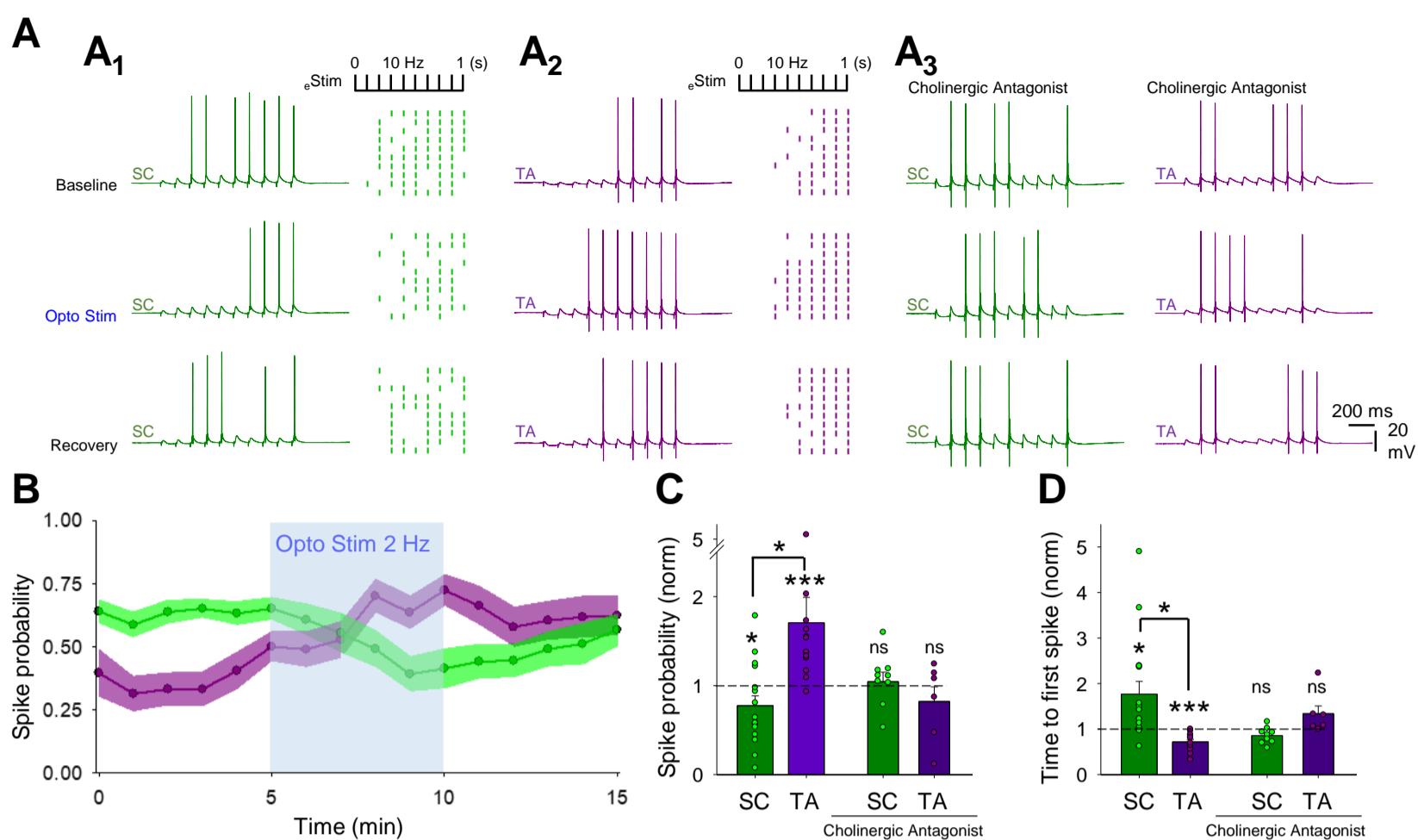


Figure 6



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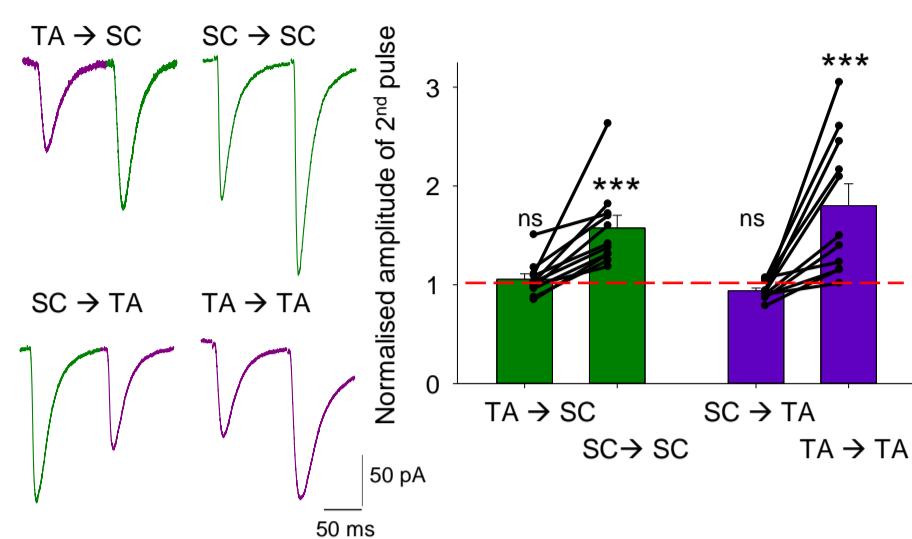
Figure 7



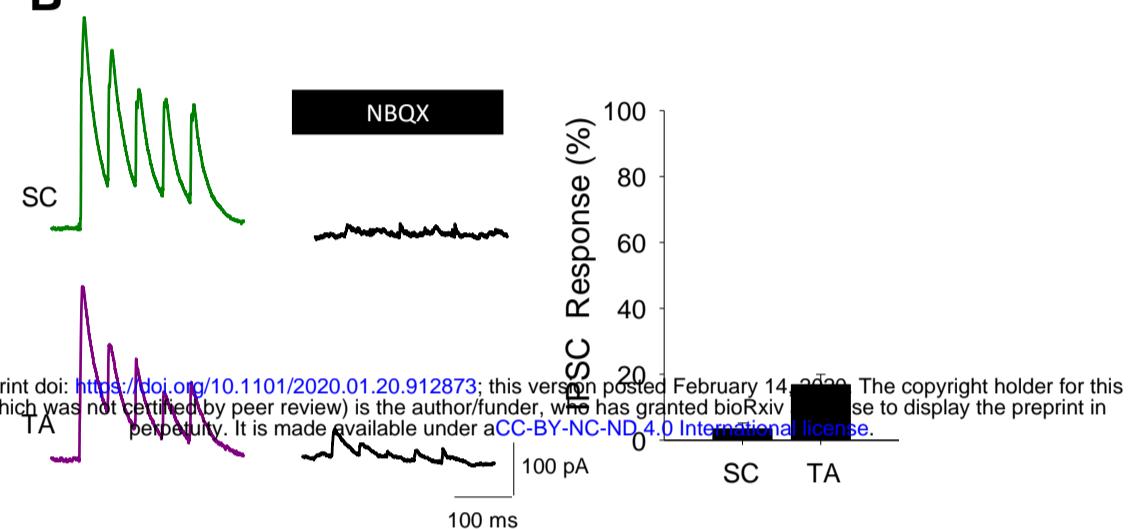
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Figure S1

A



B



C

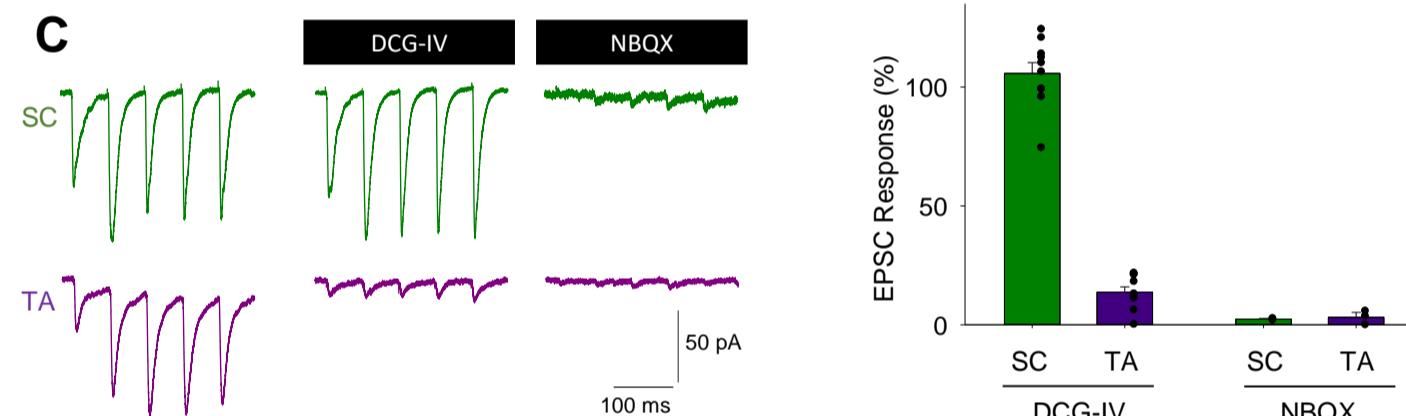


Figure S2

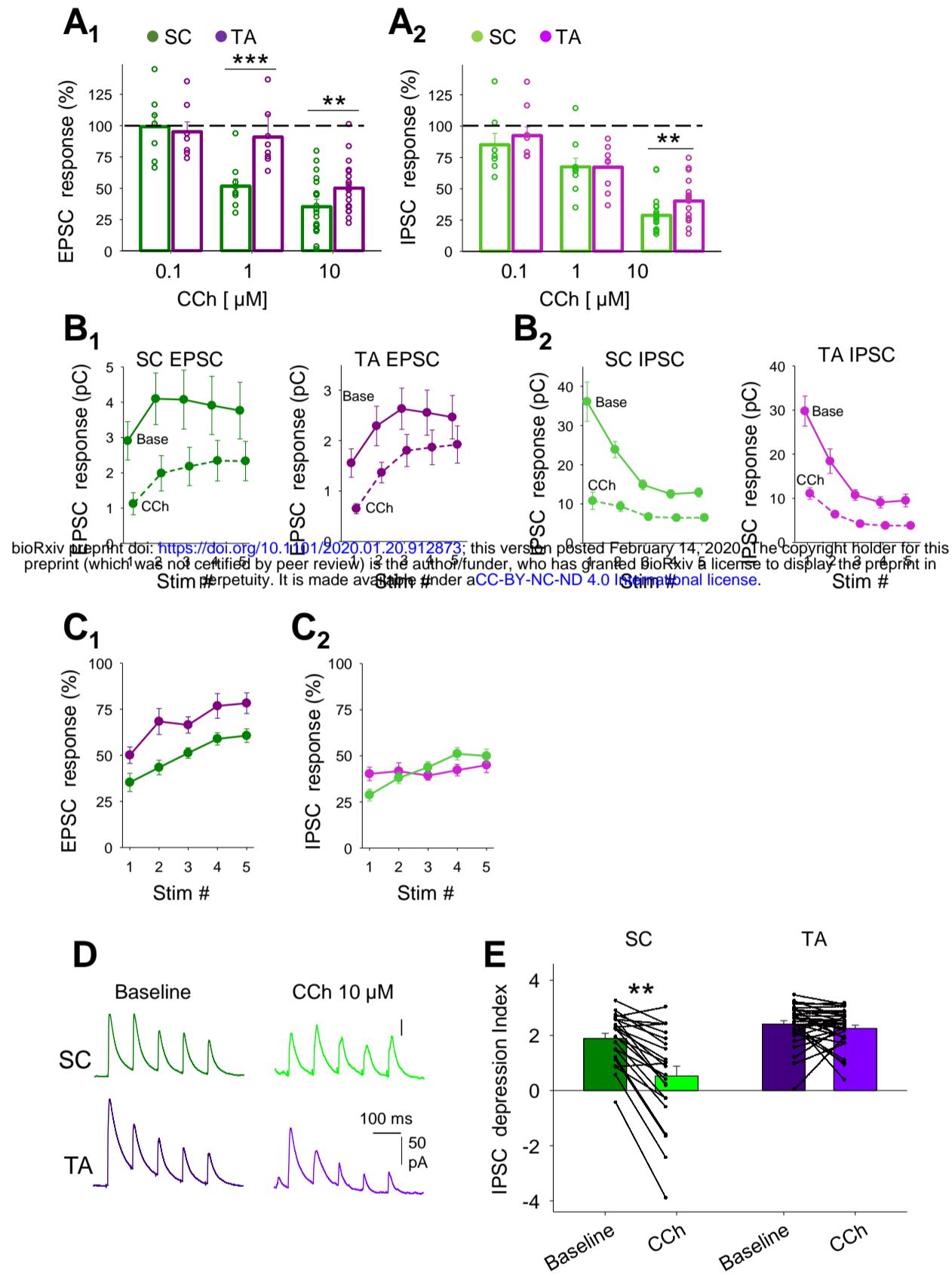
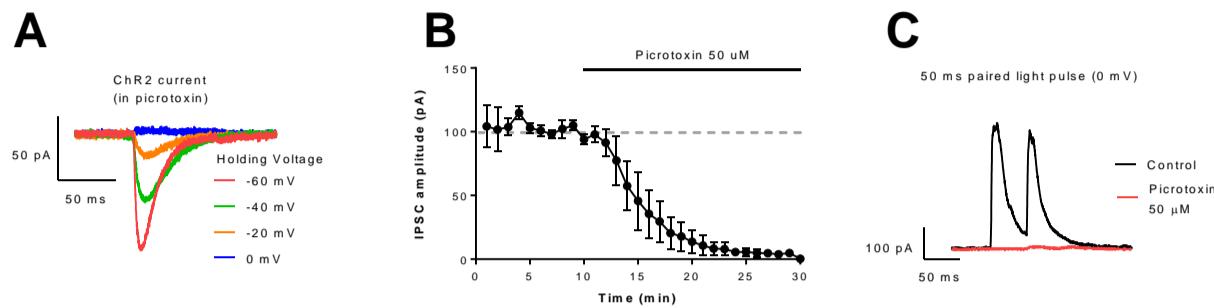


Figure S3



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Figure S4

Parameter	Value ^{b,c}	N ^e
MWt	332.44	
cLogP / LogD ^a	1.4 / 1.7	
hM ₁ pEC50 (Emax)	7.5 ± 0.33 (108)	16
hM ₂ pEC50 (Emax)	6.3 ± 0.97 (41)	5
hM ₃ pEC50 (Emax)	<4.7	3
hM ₄ pEC50 (Emax)	8.4 ± 0.25 (112)	16
rM ₄ pEC50 (Emax)	7.6 ± 0.14 (59)	5
hM ₂ pKi ^d	6.0 ± 0.23	4

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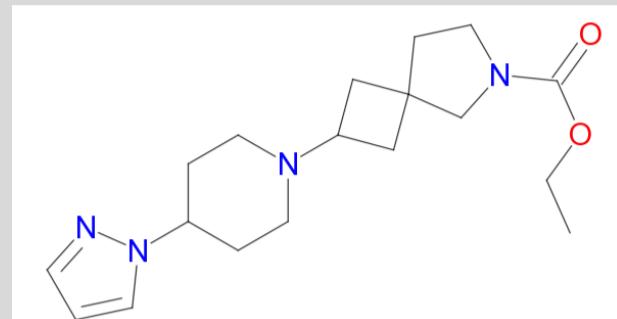
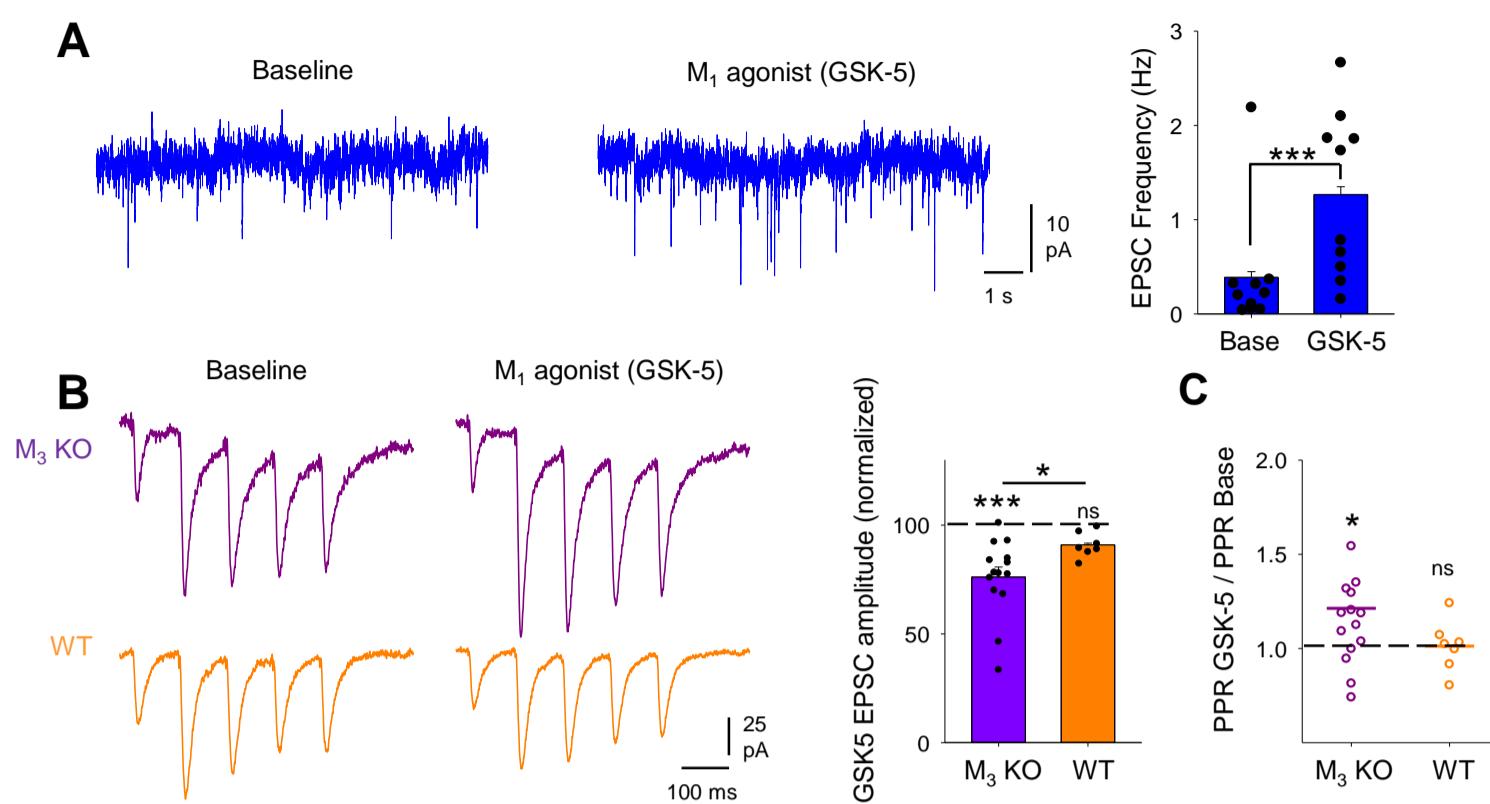


Figure S5



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