



# Serotonergic Regulation of Corticoamygdalar Neurons in the Mouse Prelimbic Cortex

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Neuromodulatory transmitters, such as serotonin (5-HT), selectively regulate the excitability of subpopulations of cortical projection neurons to gate cortical output to specific target regions. For instance, in the mouse prelimbic cortex, 5-HT selectively excites commissurally projecting (COM) intratelencephalic neurons via activation of 5-HT<sub>2A</sub> (2A) receptors, while simultaneously inhibiting, via 5-HT<sub>1A</sub> (1A) receptors, corticofugally projecting pyramidal neurons targeting the pons. Here we characterize the physiology, morphology, and serotonergic regulation of corticoamygdalar (CAm) projection neurons in the mouse prelimbic cortex. Layer 5 CAm neurons shared a number of physiological and morphological characteristics with COM neurons, including higher input resistances, smaller HCN-channel mediated responses, and sparser dendritic arbors than corticopontine neurons. Across cortical lamina, CAm neurons also resembled COM neurons in their serotonergic modulation; focally applied 5-HT (100  $\mu$ M; 1 s) generated 2A-receptor-mediated excitation, or 1A- and 2A-dependent biphasic responses, in ipsilaterally and contralaterally projecting CAm neurons. Serotonergic excitation depended on extrinsic excitatory drive, as 5-HT failed to depolarize CAm neurons from rest, but could enhance the number of action potentials generated by simulated barrages of synaptic input. Finally, using dual tracer injections, we identified double-labeled CAm/COM neurons that displayed primarily excitatory or biphasic responses to 5-HT. Overall, our findings reveal that prelimbic CAm neurons in layer 5 overlap, at least partially, with COM neurons, and that neurons projecting to either, or both targets, exhibit 2A-dependent serotonergic excitation. These results suggest that 5-HT, acting at 2A receptors, may promote cortical output to the amygdala.

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## INTRODUCTION

Output from the neocortex is organized into multiple information channels formed by subclasses of glutamatergic pyramidal neurons defined by their long-distance axonal projections to distinct cortical and subcortical targets. Cortical projection neurons are differentially regulated by a variety of neuromodulatory transmitters through cell-type-specific expression of receptors, signaling proteins, and ionic effectors. For instance, in the mouse prelimbic cortex, acetylcholine (ACh) and serotonin (5-HT) have opposing influences on the excitability of two broad classes of projection neurons: corticofugal neurons, including corticopontine (CPn) neurons, that project to

deep subcortical targets, and intratelencephalic neurons, including transhemispheric commissural (COM) neurons, that primarily target structures within the telencephalon. Acting at M1-subtype muscarinic receptors, ACh preferentially boosts the excitability of CPn neurons relative to COM neurons (Dembrow et al., 2010; Baker et al., 2018), while 5-HT inhibits CPn neurons via  $G_{i/o}$ -coupled 5-HT<sub>1A</sub> (1A) receptors, but promotes action potential (AP) generation in COM neurons via  $G_q$ -coupled 5-HT<sub>2A</sub> (2A) receptors (Avesar and Gullledge, 2012).

Corticoamygdalar (CAm) neurons in the prelimbic cortex that bilaterally target the basolateral amygdala (McDonald et al., 1996; Vertes, 2004; Gabbott et al., 2005) represent a less studied, but important, cortical projection that contributes to the expression of learned fear (Stevenson, 2011; Do Monte et al., 2016), and perhaps more generally to coping strategies in the face of external stressors (Varela et al., 2012). For instance, electrical stimulation of the prelimbic cortex promotes expression, and prevents extinction, of conditioned fear (Vidal-Gonzalez et al., 2006), while pharmacological inactivation of the prelimbic cortex impairs expression of conditioned freezing (Corcoran and Quirk, 2007; Sierra-Mercado et al., 2011; Almada et al., 2015). Indeed, unit activity (Burgos-Robles et al., 2009) and field potential oscillations (Dejean et al., 2016; Karalis et al., 2016) in the prelimbic cortex are correlated with animal freezing behavior in conditioned fear paradigms.

Accumulating evidence suggests that fear circuits may be regulated by 5-HT (for reviews, see Bauer, 2015; Bocchio et al., 2016). In rodents, fear conditioning is associated with 5-HT release in the medial prefrontal cortex (mPFC; Yoshioka et al., 1995; Hashimoto et al., 1999), and acute administration of selective serotonin reuptake inhibitors (SSRIs), which act to increase extracellular 5-HT levels, enhances the acquisition and expression of cued fear (Burghardt and Bauer, 2013; Ravinder et al., 2013). Studies targeting specific 5-HT receptor subtypes have found that activation of 1A receptors reduces, while 2A receptor stimulation enhances, conditioned fear responses. For instance, systemic injection of a 2A agonist (Zhang et al., 2013) enhances freezing in tests of cued and contextual fear memory in mice, while systemic activation of 1A receptors reduces freezing behavior in similar tests in rats (Inoue et al., 1996; Ohyama et al., 2016) or mice (Youn et al., 2009). Consistent with these findings, local activation of 1A receptors in the prelimbic cortex reduces conditioned fear responses and fear-potentiated startle (Ferreira and Nobre, 2014; Almada et al., 2015), whereas blockade of prelimbic 2A receptors impairs the expression of conditioned fear in a rat strain selectively bred to exhibit heightened anxiety (León et al., 2017). Together, these results suggest that serotonergic activation of 1A receptors in the prelimbic cortex may moderate fear responses, while engagement of 2A receptors may promote the expression of conditioned fear.

To better understand the physiology of CAm neurons, and the potential role of 5-HT in regulating cortical output to the amygdala, we used retrograde labeling to identify and record from CAm neurons in slices of mouse prelimbic cortex. Here we describe the physiological and morphological properties of these neurons, and their responsiveness to 5-HT, in the context of other previously studied prelimbic projection neuron subtypes. Our

results demonstrate that prelimbic CAm neurons are in many ways similar to, and overlap with, COM neurons, and suggest that 5-HT, acting on 2A receptors, may promote CAm output to the amygdala.

## MATERIALS AND METHODS

### Animals

Experiments utilized tissue from 8–12-week-old male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, United States) according to methods approved by the Institutional Animal Care and Use Committee of Dartmouth College. Animals had access to food and water *ad libitum* and were housed on a 12:12 h light:dark cycle.

### Retrograde Labeling

Red or green fluorescent beads (Retrobeads, Lumafuor Inc.) were injected unilaterally into the amygdala to label CAm projection neurons using age appropriate coordinates (Paxinos and Franklin, 2004). In some animals, an additional injection of Retrobeads was made into the ipsilateral mPFC to double-label CAm/COM dual-projection neurons. Animals were anesthetized throughout surgeries with vaporized isoflurane (~2%). Following craniotomy, a microsyringe was lowered into the brain region of interest, and 300 nL of undiluted Retrobead solution was injected over a 10 min period. In some experiments, a nanoject (Drummond scientific) was used to administer the microbeads instead of a microsyringe. Animals were allowed to recover from surgery for at least 72 h before use in electrophysiological experiments. Locations of dye injections were confirmed *post hoc* in coronal sections of the amygdala or mPFC.

### Slice Preparation

Following isoflurane anesthesia and decapitation, brains were quickly removed into artificial cerebral spinal fluid (ACSF) containing, in mM: 125 NaCl, 25 NaHCO<sub>3</sub>, 3 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 0.5 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, and 25 glucose, saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Coronal brain slices (250  $\mu$ m thick) of the mPFC or amygdala were cut using a Leica VT 1200 slicer and placed in a holding chamber filled with ACSF containing 2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>. Slices were stored at 35°C for ~45 min, and then kept at room temperature for up to 8 h before use in experiments.

### Electrophysiology

Slices were placed in a recording chamber on a fixed-stage Olympus BX51WI microscope and continuously perfused with oxygenated ACSF heated to 35–36°C. Epifluorescence illumination (470 or 530 nm LEDs) was used to identify CAm or CAm/COM neurons in the mPFC, and whole-cell current-clamp recordings were made from bead-labeled neurons using patch pipettes (5–7 M $\Omega$ ) filled with, in mM: 135 K-gluconate, 2 NaCl, 2 MgCl<sub>2</sub>, 10 HEPES, 3 Na<sub>2</sub>ATP, and 0.3 NaGTP (pH 7.2 with KOH). Data were acquired with Axograph software (Axograph Company) using BVC-700 amplifiers (Dagan Corporation) and ITC-18 digitizers

(HEKA Instruments). Membrane potentials were sampled at 25 kHz, filtered at 10 kHz, and corrected for a liquid junction potential of +12 mV. Hyperpolarization-activated cyclic-nucleotide gated (HCN)-channel-mediated rebound (i.e., “sag” potentials) were measured as the percent difference in the peak hyperpolarization and the steady state voltage (both relative to resting membrane potentials [RMPs]) in response to negative current injection set to hyperpolarize the neuron by approximately 20 mV.

5-HT (100  $\mu$ M) was dissolved in ACSF and loaded into patch pipettes placed  $\sim$ 50  $\mu$ m from targeted somata. Neurons were classified as 5-HT-inhibited, -excited, -biphasic, or -non-responsive based on their response to focal application of 5-HT, which was delivered for 1 s at  $\sim$ 15 PSI during periods of AP generation ( $\sim$ 5 Hz) evoked by somatic DC current injection. Serotonergic inhibition was quantified as the duration of cessation of AP generation, while excitatory responses were quantified as the percent increase in the mean instantaneous spike frequency (ISF; the inverse of the time interval between each AP) measured over the 500 ms following the peak post-5-HT ISF, relative to the mean pre-5-HT ISF baseline (10 s). Biphasic 5-HT responses were defined as responses having both brief inhibition (lasting at least 10 times the average baseline interspike interval) and a subsequent increase in ISF of at least 1 Hz above baseline levels. Mean “population” responses to 5-HT during DC-current-induced AP firing were made by resampling individual ISF plots at 2 Hz and then averaging those ISF plots across tested neurons. In some experiments, 5-HT receptors were selectively blocked with 1A (WAY 100635, 30 nM; Sigma-Aldrich) and/or 2A (MDL 11939, 500 nM; Tocris) receptor antagonists.

In some experiments, somatic current injections were used to simulate excitatory synaptic input, as previously described (Stephens et al., 2014). Because of intrinsic cell-to-cell variability in input resistance and excitability, for each neuron, the synaptic waveform was scaled in amplitude to evoke  $\sim$ 8 APs during baseline trials. The simulated synaptic current was then delivered 29 times at 3 s intervals, the exception being the sixth trial which was delayed 3 s due to application of 5-HT (100  $\mu$ M, 1 s).

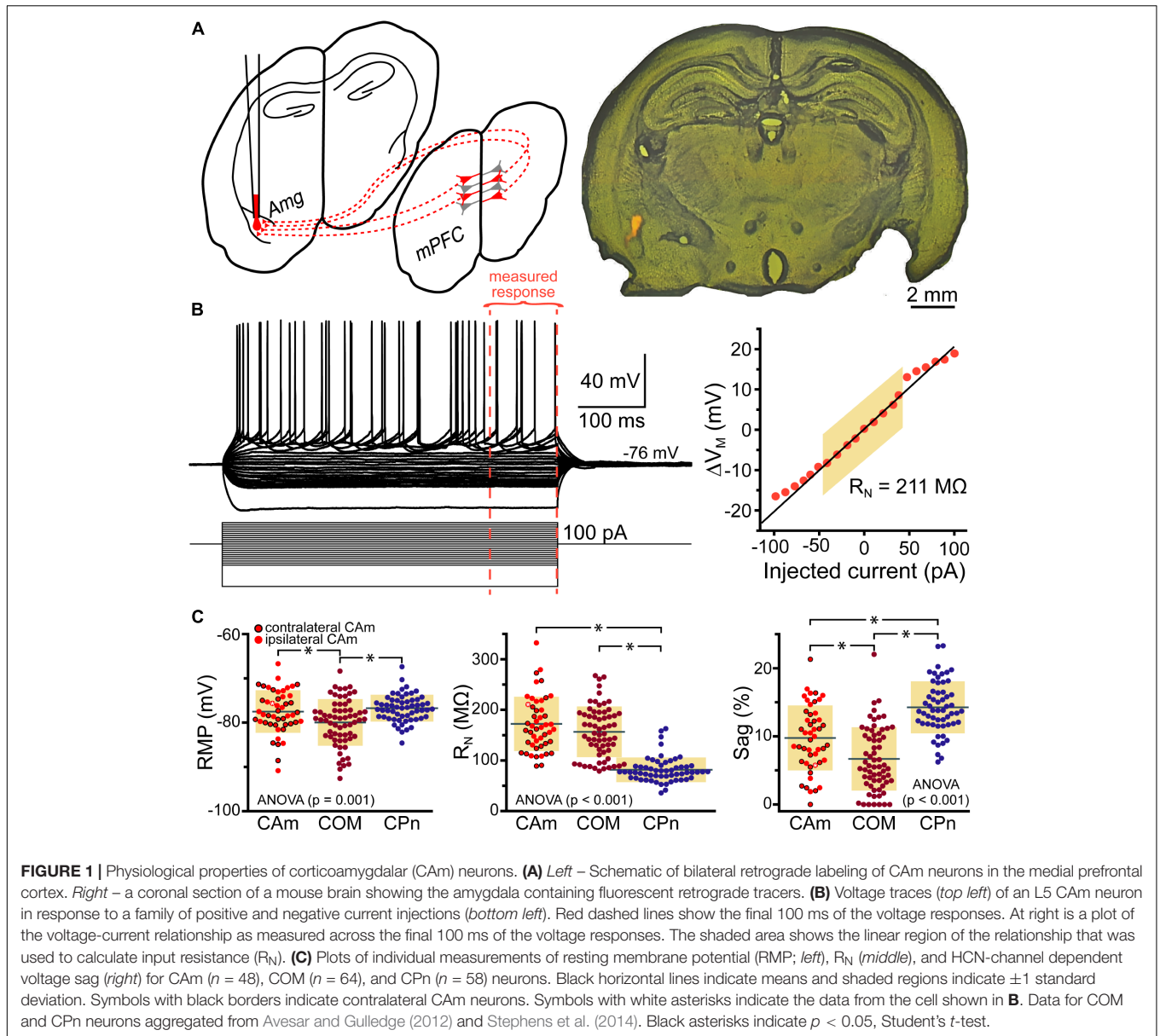
## Morphological Analyses and Single-Cell Imaging

In some experiments, biocytin (7 mg/ml; Tocris) was included in the patch-pipette solution. Following recordings, slices were placed in a phosphate buffered saline (PBS) solution containing 4% paraformaldehyde for 24 h. Slices were then rinsed three times for 15 min in PBS and placed in a PBS solution containing 0.25% Triton X-100 and avidin conjugated to either Alexa Fluor<sup>®</sup>-594 or Alexa Fluor<sup>®</sup>-488 (20  $\mu$ g/ml; Invitrogen) for 24 h. Slices were then rinsed with PBS three times for fifteen minutes each, dried, and mounted on slides in FluorSave (EMD Chemicals). Cells were imaged using a two-photon microscope (Bruker Corporation) or a Zeiss LSM 510 confocal microscope (Carl Zeiss). Data from COM and CPn neurons used for comparisons were previously published in Avesar and Gullledge (2012) and Stephens et al. (2014). Morphological measurements included somatic distance from the pia, maximum horizontal width of the apical tuft, the number of dendritic branch points in the tuft, and the number of primary and oblique dendrites. Tracings of neurons were made using NeuronJ from z-stack projections.

**TABLE 1** | Physiological properties of layer 5 neurons.

Grouping	N	RMP (mV)	$R_N$ (M $\Omega$ )	Sag (%)
CAM neurons	48	-77.5 $\pm$ 5.0	173 $\pm$ 55	9.8 $\pm$ 4.6
COM neurons*	64	-79.9 $\pm$ 5.3	157 $\pm$ 50	6.4 $\pm$ 4.5
CPn neurons*	58	-76.8 $\pm$ 3.0	81 $\pm$ 24	14.0 $\pm$ 4.2
CAM/COM neurons	14	-76.1 $\pm$ 5.7	203 $\pm$ 86	7.7 $\pm$ 3.9
<b>ANOVA (p-value)</b>		<b>0.004</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<b>CAM vs COM post-test (p-value)</b>		<b>0.039</b>	0.322	<b>&lt;0.001</b>
<b>CAM vs CPn post-test (p-value)</b>		0.729	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<b>CAM vs CAM/COM post-test (p-value)</b>		0.758	0.293	0.383
Female CAM	27	-77.4 $\pm$ 5.5	175 $\pm$ 63	9.9 $\pm$ 4.9
Male CAM	21	-77.6 $\pm$ 3.9	169 $\pm$ 40	9.5 $\pm$ 4.8
<b>Student's t-tests (p-value)</b>		0.933	0.689	0.802
Ipsilateral CAM	24	-76.7 $\pm$ 5.2	189 $\pm$ 53	10.4 $\pm$ 4.6
Contralateral CAM	24	-78.3 $\pm$ 4.4	156 $\pm$ 50	9.1 $\pm$ 5.0
<b>Student's t-tests (p-value)</b>		0.265	<b>0.031</b>	0.344
5-HT-Excited CAM	25	-77.6 $\pm$ 5.4	175 $\pm$ 55	9.0 $\pm$ 4.2
5-HT-Biphasic CAM	18	-77.4 $\pm$ 4.7	171 $\pm$ 56	11.0 $\pm$ 5.0
<b>Student's t-tests (p-value)</b>		0.869	0.778	0.180

Data presented as means  $\pm$  standard deviations. \*Data from Avesar and Gullledge (2012) and Stephens et al. (2014). P-values < 0.05 are in boldface type.



### Statistical Analyses

Unless otherwise noted, all data are presented as means  $\pm$  standard deviations. Comparisons across cell

groups utilized one-way ANOVAs with Šidák post-tests, while comparisons within groups was accomplished using two-tailed Student's  $t$ -tests (paired or unpaired), or repeated measures

**TABLE 2 |** Comparison of morphological properties of layer 5 projection neurons.

Grouping	<i>n</i>	Depth from pia ( $\mu\text{m}$ )	# Primary dendrites	# Oblique dendrites	Apical tuft width ( $\mu\text{m}$ )
CAm neurons	21	335 $\pm$ 78 <sup>†</sup>	7.1 $\pm$ 1.5	7.4 $\pm$ 4.1	289 $\pm$ 126
COM neurons*	10	332 $\pm$ 58	6.7 $\pm$ 2.4	4.6 $\pm$ 2.3	211 $\pm$ 95
CPn neurons*	10	405 $\pm$ 41	9.2 $\pm$ 2.8	13.3 $\pm$ 3.5	302 $\pm$ 96
<b>ANOVA (<i>p</i>-value)</b>		<b>0.026</b>	<b>0.018</b>	<b>&lt;0.001</b>	0.140
<b>CAm vs COM post-test (<i>p</i>-value)</b>		0.999	0.922	0.165	
<b>CAm vs CPn post-test (<i>p</i>-value)</b>		<b>0.043</b>	<b>0.030</b>	<b>0.002</b>	

Data presented as means  $\pm$  standard deviations. \*Data from Avesar and Gullledge (2012). <sup>†</sup> $n = 19$  for depth from pia. *P*-values  $< 0.05$  are in boldface type.



ANOVA, as appropriate. Comparisons of response proportions between groups utilized a Fisher's exact test. Significance was defined as  $p < 0.05$ .

## RESULTS

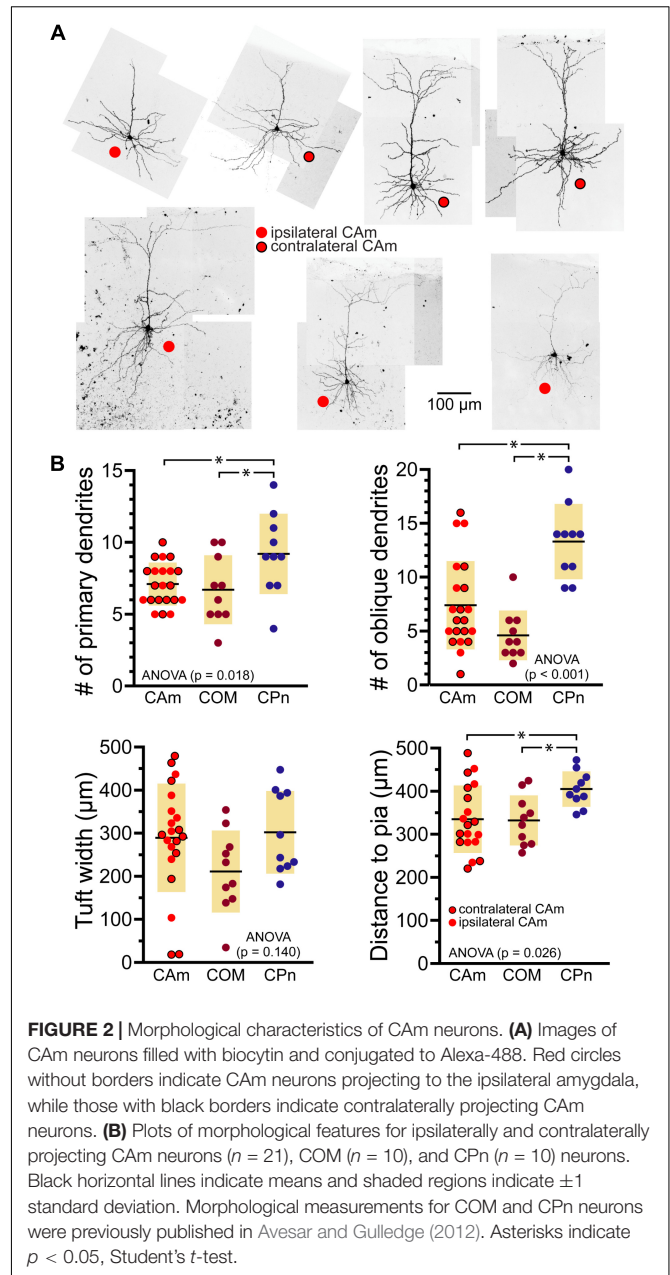
### Physiological and Morphological Characteristics of CA<sub>m</sub> Neurons

Retrograde-labeled pyramidal neurons projecting to either the ipsilateral (iCA<sub>m</sub> neurons;  $n = 24$ ) or contralateral (cCA<sub>m</sub> neurons;  $n = 24$ ) amygdala were targeted for whole-cell recordings in layer 5 of the mouse prelimbic cortex. For each neuron, measurements were made of RMP, input resistance ( $R_N$ ), and HCN-channel-mediated rebound "sag" potentials (% sag; see section "Materials and Methods"; **Table 1** and **Figure 1**). CA<sub>m</sub> neuron physiology was not sex-dependent (**Table 1**), and RMP and HCN responses were not dependent on the laterality of amygdalar projection (**Table 1**). However, ipsilaterally projecting CA<sub>m</sub> neurons had a significantly higher input resistance than contralateral CA<sub>m</sub> neurons ( $p = 0.031$ ; Student's  $t$ -test; **Table 1**). The physiological properties of CA<sub>m</sub> neurons, as a whole, were somewhat distinct from those previously observed in layer 5 COM and CPn neurons (Avesar and Gullledge, 2012; Stephens et al., 2014). While CA<sub>m</sub> neurons resembled COM neurons in having larger  $R_N$ s (at  $172 \pm 54 \text{ M}\Omega$ ;  $n = 48$ ;  $p < 0.001$ ; Student's  $t$ -test) and smaller HCN-dependent sag responses (at  $9.8 \pm 4.8\%$ ;  $p < 0.001$ ) than CPn neurons ( $n = 58$ ; **Table 1**), the RMPs of CA<sub>m</sub> neurons ( $-78 \pm 5 \text{ mV}$ ) were similar to those in CPn neurons ( $-77 \pm 3 \text{ mV}$ ;  $p = 0.73$ ), and more depolarized than those in COM neurons ( $-80 \pm 5 \text{ mV}$ ;  $n = 64$ ;  $p = 0.039$ ), likely reflecting their enhanced sag potentials relative to those of COM neurons ( $6.4 \pm 4.6\%$ ;  $p < 0.001$ ; see **Table 1** and **Figure 1C**).

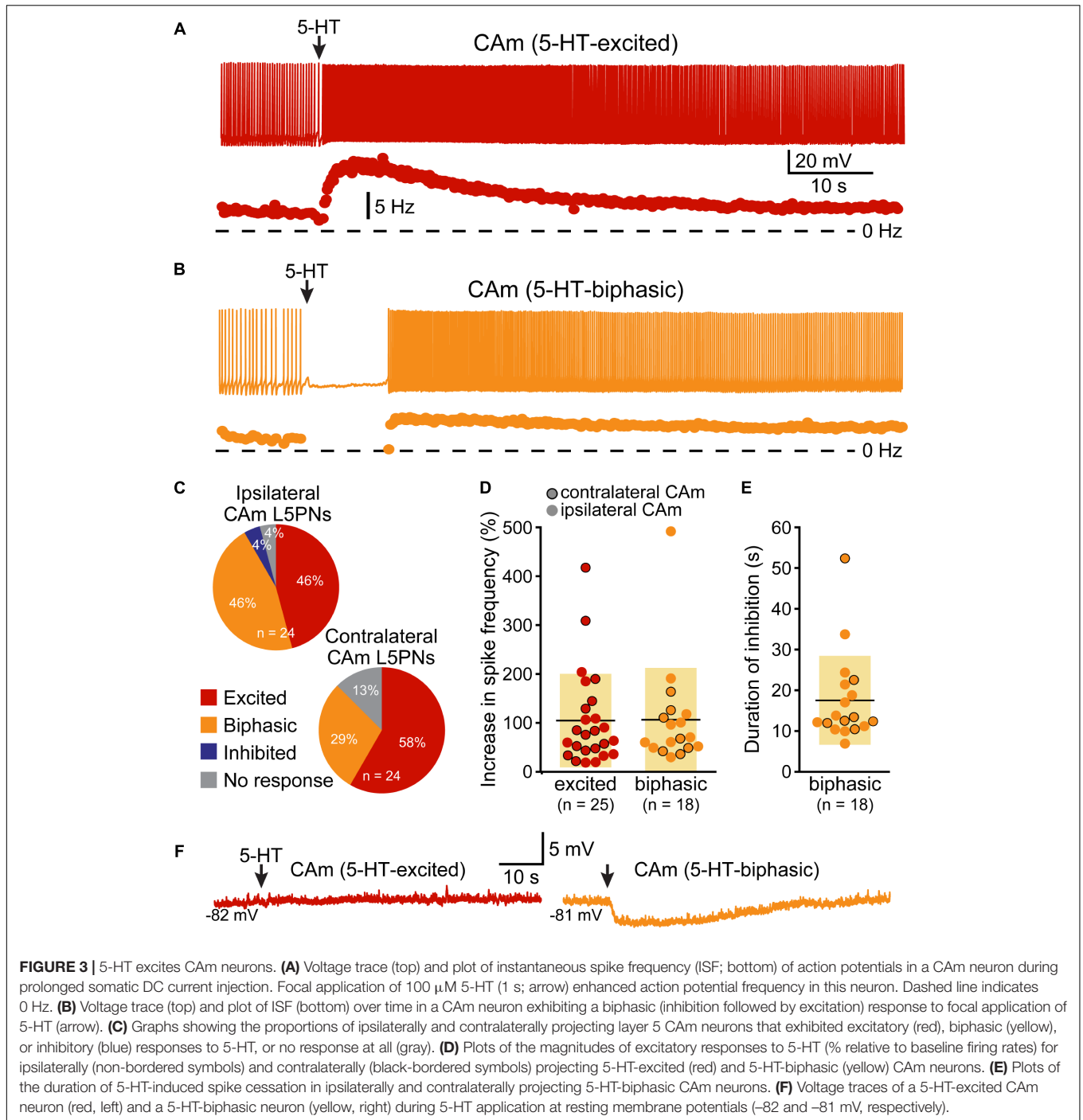
Morphologically, layer 5 CA<sub>m</sub> neurons ( $n = 21$ ) resembled COM, rather than CPn, neurons (**Figure 2**). Both CA<sub>m</sub> and COM neurons had fewer primary (means of  $7.1 \pm 1.5$  and  $6.7 \pm 2.4$ , respectively;  $p = 0.02$ ; ANOVA) and oblique (means of  $7.4 \pm 4.1$  and  $4.6 \pm 2.3$ , respectively;  $p < 0.001$ ; ANOVA) dendrites than did CPn neurons ( $n = 10$ ; **Table 2** and **Figure 2B**). While cells of all projection subtypes exhibited similar apical tuft widths (e.g.,  $289 \pm 126 \mu\text{m}$  for CA<sub>m</sub> neurons;  $p = 0.14$ , ANOVA), CA<sub>m</sub> and COM ( $n = 10$ ) neuron somata tended to be slightly more superficial (at  $339 \pm 18$  and  $332 \pm 18 \mu\text{m}$  from the pia;  $p = 0.029$  and  $0.010$ ; Šidák post-tests for CA<sub>m</sub> and COM neurons, respectively, vs CPn neurons) than the somata of CPn neurons ( $405 \pm 13 \mu\text{m}$ ; **Figure 2B** and **Table 2**). Thus, while CA<sub>m</sub> neurons were in some ways distinct from COM and CPn neurons (e.g., in their HCN-mediated responses), their overall physiological and morphological characteristics were more similar to COM neurons.

### CA<sub>m</sub> Neurons Are Excited by 5-HT via 2A Receptors

Corticoamygdalar neurons also resembled COM neurons in their responses to focal application of 5-HT during periods



of current-induced AP generation (**Figure 3** and **Table 3**). 5-HT enhanced the frequency of AP generation in 25 of 48 CA<sub>m</sub> neurons (52%), and generated biphasic responses in 18 CA<sub>m</sub> neurons (38%; **Figures 3A,B**). Four CA<sub>m</sub> neurons were non-responsive to 5-HT, and one CA<sub>m</sub> neuron exhibited a purely inhibitory response to 5-HT. The proportions of CA<sub>m</sub> excited and biphasic neurons were statistically similar in 24 iCA<sub>m</sub> and 24 cCA<sub>m</sub> neurons ( $p = 0.36$ ; Fisher's exact test; **Figure 3C**), suggesting that 5-HT influences CA<sub>m</sub> neurons independently of the laterality of their amygdalar projections. Indeed, the magnitudes of serotonergic excitation were similar between iCA<sub>m</sub> ( $n = 11$ ) and cCA<sub>m</sub> ( $n = 14$ ) 5-HT-excited neurons (mean excitations of  $84 \pm 63$  and  $121 \pm 115\%$ ,



respectively;  $p = 0.32$ ; Student's  $t$ -test). Excitatory responses in iCAM ( $n = 11$ ) and cCAM ( $n = 7$ ) 5-HT-biphasic neurons (mean excitations of  $120 \pm 131$  and  $85 \pm 49\%$ , respectively; **Figure 3D**) were also similar to each other ( $p = 0.44$ ) and to the magnitude of excitation observed across all 5-HT-excited CAM neurons ( $98 \pm 71\%$ ;  $n = 25$ ;  $p = 0.77$ , ANOVA). Further, serotonergic biphasic inhibitions in iCAM and cCAM neurons were of similar durations (at  $16 \pm 8$  and  $19 \pm 5$  ms, respectively;  $p = 0.64$ ; Student's  $t$ -test;

**Figure 3E**). These results suggest that 5-HT responses in CAM neurons are not dependent on projection laterality. Additionally, 5-HT responses in CAM neurons were not sex-dependent (**Table 3**).

5-HT responses in layer 5 CAM neurons were qualitatively and quantitatively similar (in both proportions and magnitudes) to 5-HT responses previously reported in COM neurons (see **Table 3**). Further, as previously reported in COM neurons (Stephens et al., 2014), serotonergic excitation of CAM neurons

required additional depolarizing drive, as 5-HT failed to depolarize 5-HT-excited CAM ( $n = 8$ ) or 5-HT-biphasic ( $n = 7$ ) neurons from RMPs (Figure 3F). In 5-HT-excited CAM neurons, the mean change in membrane potential relative to RMPs was  $+0.1 \pm 0.5$  mV ( $p = 0.54$ , paired Student's  $t$ -test). In 5-HT-biphasic CAM neurons, focal application of 5-HT at RMPs led to transient hyperpolarizations (of  $3.5 \pm 0.9$  mV;  $p < 0.001$ ) that lasted  $27 \pm 11$  s, but no subsequent depolarizations (mean peak membrane potential relative to RMPs was  $+0.4 \pm 1.1$  mV;  $p = 0.43$ ). Thus, serotonergic stimulation, on its own, was not sufficient to excite CAM neurons.

Serotonergic excitation of COM neurons is mediated by 2A receptors, whereas biphasic responses involve both 1A-mediated inhibition and 2A-driven excitation (Avesar and Gullledge, 2012; Stephens et al., 2014). To test whether 2A receptors also mediate serotonergic excitation of CAM neurons, we measured 5-HT responses in 5-HT-excited and 5-HT-biphasic CAM neurons before and after bath application of the selective 2A receptor antagonist MDL 11939 (500 nM). In both 5-HT-excited ( $n = 8$ ) and 5-HT-biphasic ( $n = 7$ ) CAM neurons, MDL blocked serotonergic excitation (Figure 4). In a subset of 5-HT-biphasic neurons ( $n = 3$ ), the inhibitory portion of biphasic responses was blocked by WAY 100635 (30 nM), a 1A antagonist (Figure 4C). These results confirm that serotonergic responses in prelimbic CAM neurons are mediated by the same receptor subtypes that gate serotonergic excitation and inhibition in COM and

CPn neurons (Avesar and Gullledge, 2012; Stephens et al., 2014).

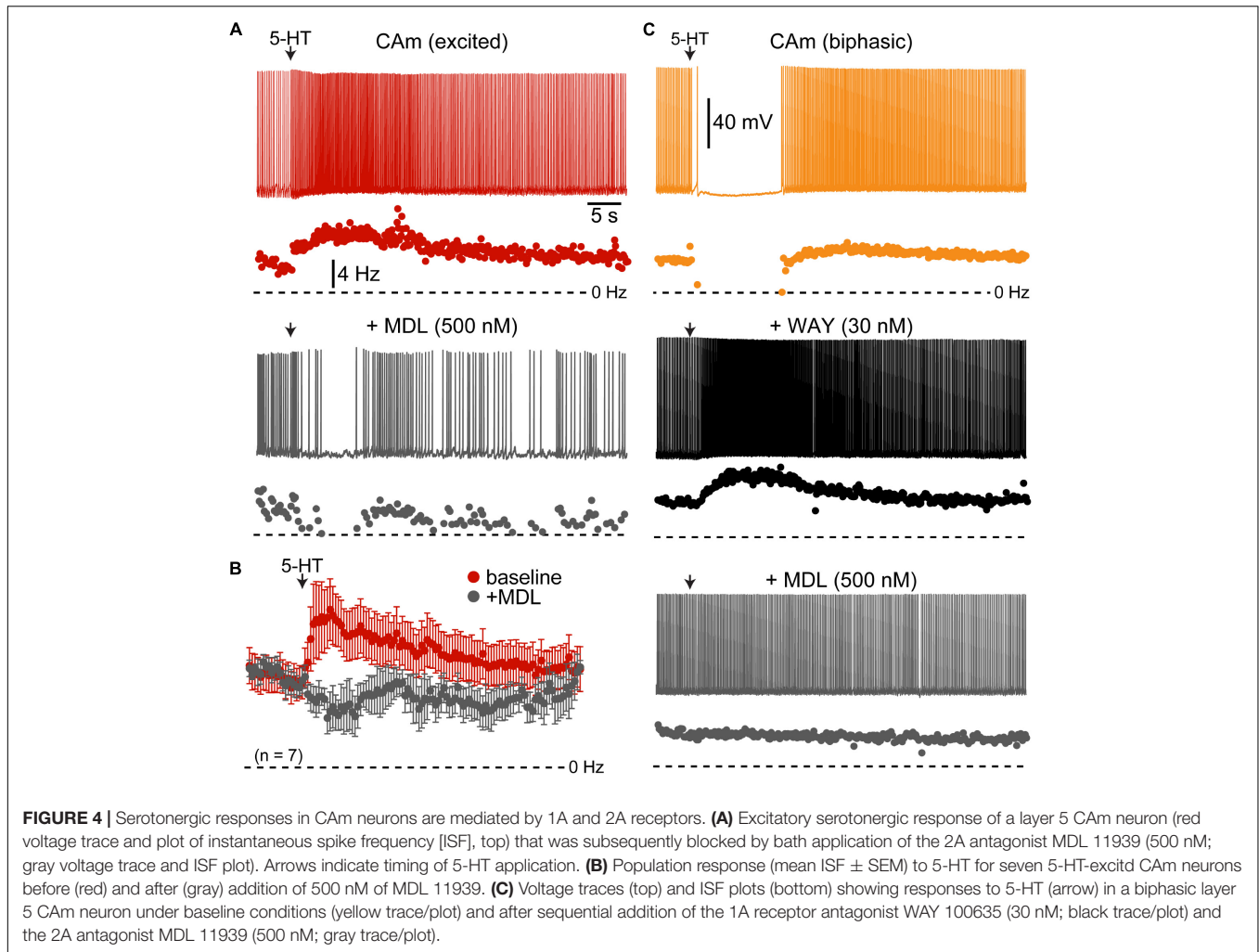
## 5-HT Enhances CAM Neuron Output to Simulated Synaptic Input

To test whether 5-HT can functionally enhance the output of CAM neurons driven by a more physiological stimulus, we repeatedly delivered a barrage of simulated synaptic current (see section "Materials and Methods") over 29 trials (3 s inter-trial intervals), and measured the number of APs generated in each trial, as well as the RMP just prior to the barrage (Figure 5). Following the initial five baseline trials, 5-HT was applied for 1 s before synaptic barrages were resumed for 24 additional trials. In 5-HT-excited CAM neurons ( $n = 14$ ), simulated synaptic barrages generated a mean of  $7.6 \pm 1.2$  APs during the five baseline trials. Following 5-HT application, there was an increase of  $2.4 \pm 1.9$  APs per trial, bringing the mean response to  $10.0 \pm 2.1$  APs across the subsequent five trials ( $p < 0.001$ ; repeated measures ANOVA; Figure 5B, red symbols). Conversely, 5-HT had little impact on AP output in biphasic CAM neurons (mean change of  $-1.6 \pm 4$  APs, relative to their baseline response of  $8.9 \pm 1.7$  APs;  $p = 0.31$ , Figure 5B, yellow symbols). Since 5-HT generated a small, but significant ( $p = 0.024$ ), depolarization of the RMP in 5-HT-excited CAM neurons (peaking with a mean of  $1.2 \pm 1.3$  mV on trial 11, about 6 s after 5-HT application,

**TABLE 3 |** Serotonergic responses in CAM neurons.

Grouping	<i>n</i>	5-HT response	Excitation (% increase in ISF)	Duration of inhibition (s)
L5 COM neurons*	43	Excited	$142 \pm 101$	NA
	21	Biphasic	$153 \pm 78$	$17 \pm 9$
L5 CAM neurons	25	Excited	$98 \pm 97$	NA
	18	Biphasic	$111 \pm 102$	$18 \pm 11$
L2/3 CAM neurons	7	Excited	$93 \pm 71$	NA
	6	Biphasic	$148 \pm 101$	$27 \pm 19$
L5 CAM/COM neurons	9	Excited	$54 \pm 34$	NA
	4	Biphasic	$70 \pm 45$	$16 \pm 7$
<b>ANOVA</b>			0.077	0.249
Ipsilateral L5 CAM	11	Excited	$84 \pm 63$	NA
Contralateral L5 CAM	14	Excited	$121 \pm 115$	NA
<b>Student's <i>t</i>-test (<i>p</i>-value)</b>			0.321	
Ipsilateral L5 CAM	11	Biphasic	$120 \pm 131$	$16 \pm 8$
Contralateral L5 CAM	7	Biphasic	$85 \pm 49$	$19 \pm 15$
<b>Student's <i>t</i>-tests (<i>p</i>-value)</b>			0.436	0.643
Female L5 CAM	15	Excited	$108 \pm 83$	NA
Male L5 CAM	10	Excited	$99 \pm 116$	NA
<b>Student's <i>t</i>-test (<i>p</i>-value)</b>			0.837	
Female L5 CAM	9	Biphasic	$120 \pm 142$	$20 \pm 15$
Male L5 CAM	9	Biphasic	$93 \pm 58$	$15 \pm 5$
<b>Student's <i>t</i>-tests (<i>p</i>-value)</b>			0.611	0.315

Data presented as means  $\pm$  standard deviations. \*Data from Avesar and Gullledge (2012) and Stephens et al. (2014). NA, not applicable.



and only after application of suprathreshold current input), in a subset of 5-HT-excited neurons ( $n = 8$ ) we tested whether depolarization alone could account for the additional APs produced after 5-HT application. In these control experiments, in which  $7.1 \pm 1.5$  spikes were generated in baseline trials, DC current was used to continuously depolarize neurons by  $1.8 \pm 0.7$  mV ( $p < 0.001$ ) during the remaining 24 trials. This depolarization alone failed to significantly increase the number of APs generated by the simulated synaptic input (mean of  $7.7 \pm 2.2$  APs;  $p = 0.12$ ). Together, these results confirm previous findings in COM neurons (Stephens et al., 2014), and suggest that 5-HT will preferentially enhance the output of 5-HT-excited CAM neurons that are receiving coincident suprathreshold synaptic drive.

### 5-HT Generates Biphasic and Excitatory Responses in L2/3 CAM Neurons

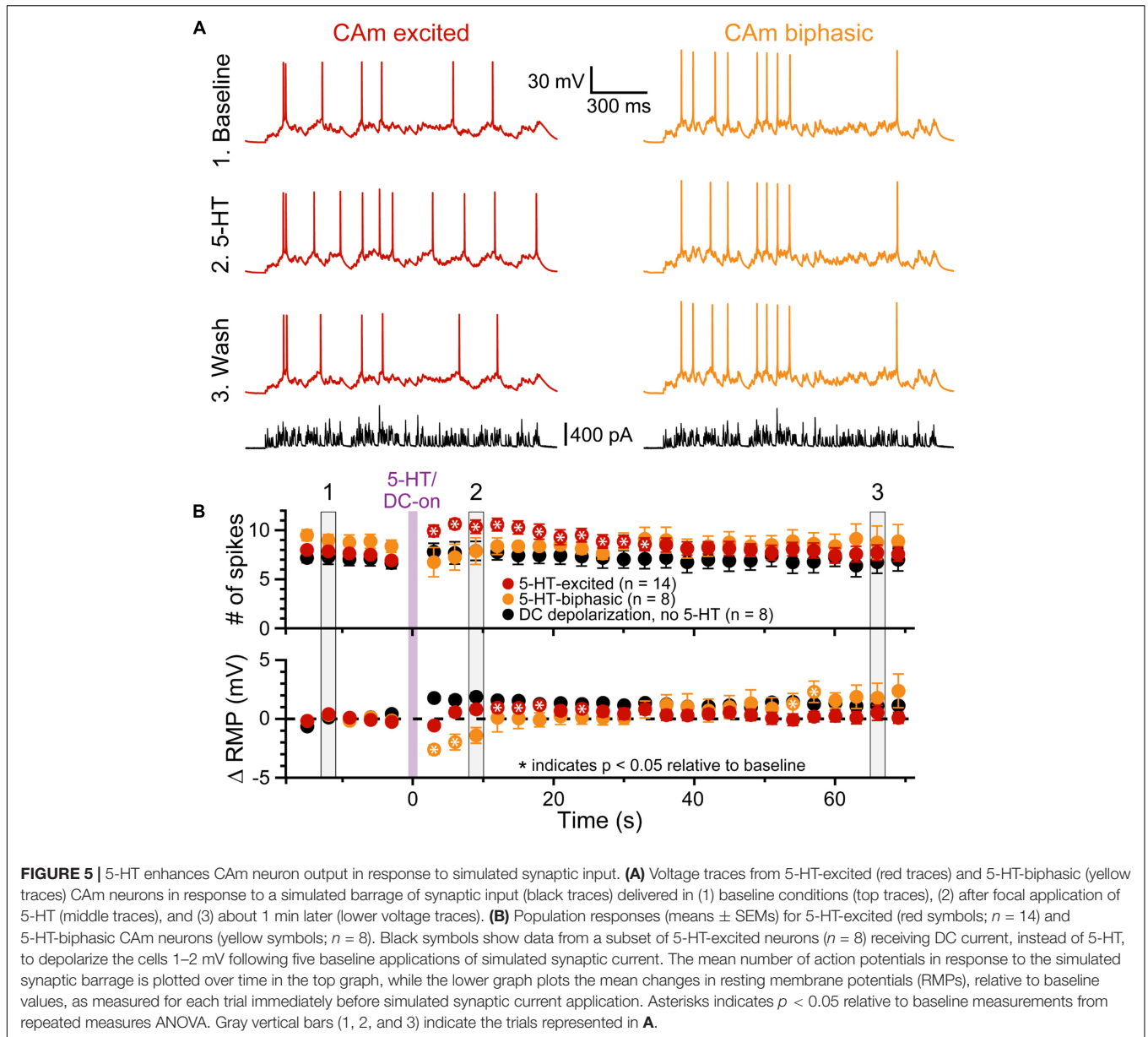
Although our study focuses on layer 5 pyramidal neurons, CAM neurons are present across cortical lamina (Little and Carter, 2013; Song et al., 2015; Cheriyan et al., 2016). To determine whether 5-HT regulates the excitability of superficial CAM

neurons, we recorded from Retrobead-labeled CAM neurons in layer 2/3 of the prelimbic cortex ( $n = 13$ ). Layer 2/3 CAM neurons had physiological properties comparable to those in layer 5, with mean RMPs of  $-77 \pm 7$  mV ( $p = 0.98$  vs 48 layer 5 CAM; Student's *t*-test), input resistances of  $208 \pm 82$  M $\Omega$  ( $p = 0.61$  vs layer 5 CAM), and sag potentials of  $7.3 \pm 3.8\%$  ( $p = 0.30$  vs layer 5 CAM). Layer 2/3 CAM neurons exhibited excitatory ( $n = 7$ ) or biphasic ( $n = 6$ ) responses to focally applied 5-HT (Figure 6 and Table 3) that occurred in proportions ( $p = 1.00$ ; Fisher's exact test) and magnitudes (mean increases above baseline ISF of  $93 \pm 71$  and  $148 \pm 101\%$  for 5-HT-excited and 5-HT-biphasic L2/3 CAM neurons, respectively;  $p = 0.22$  and  $0.91$ , when compared to layer 5 CAM neurons) consistent with those observed in layer 5 CAM and COM neurons. Thus, serotonergic regulation of CAM neuron excitability appears to be conserved across cortical layers in the prelimbic cortex.

### CAM Neurons Overlap With COM Neurons

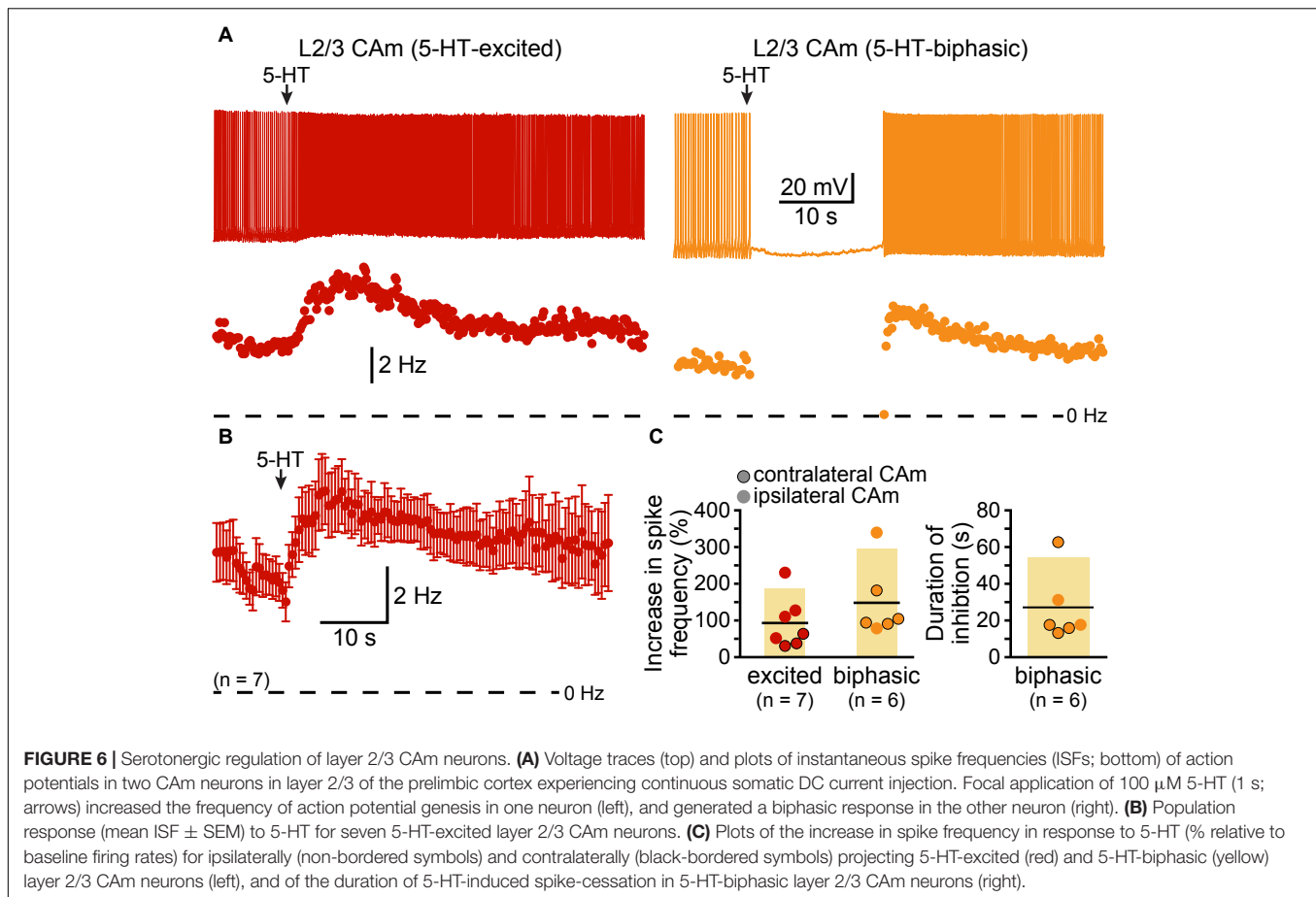
Based on their morphological and physiological similarities, we hypothesized that CAM and COM neurons may represent





overlapping populations of layer 5 neurons in the mPFC. We tested this hypothesis by injecting Retrobeads unilaterally into the contralateral amygdala and the mPFC to label CAm/COM dual projection neurons (**Figure 7A**). While CAm/COM neurons were much less numerous than single-labeled COM and CAm neurons, we recorded from 14 dual-labeled neurons, finding that the majority (64%,  $n = 9/14$ ) were excited by 5-HT, with an additional 29% ( $n = 4/14$ ) exhibiting biphasic responses to 5-HT. One CAm/COM double-labeled neuron (7%) exhibited a purely inhibitory response to 5-HT (**Figures 7B,C**). The magnitudes of excitatory responses in CAm/COM 5-HT-excited ( $54 \pm 34\%$ ) and 5-HT-biphasic ( $70 \pm 45\%$ ) neurons (**Figure 7D** and **Table 3**) were statistically similar to those observed in single-labeled 5-HT-excited CAm neurons ( $p = 0.13$  and  $0.51$ , for CAm/COM 5-HT-excited and 5-HT-biphasic neurons, respectively; Student's

$t$ -tests). Similarly, the durations of inhibitory responses in CAm/COM 5-HT-biphasic neurons ( $19 \pm 5$  s) were similar to those in single-labeled CAm neurons ( $p = 0.81$ ). The physiological properties of CAm/COM neurons were also similar to those observed in single-labeled CAm neurons (**Table 1**). These data demonstrate that, in the mouse prelimbic cortex, layer 5 CAm and COM neurons are overlapping neuron populations that share 2A-mediated serotonergic excitation. However, because retrograde labeling with beads does not label all projection neurons to a given target region, and has variable efficiency animal-to-animal, it was not possible to calculate the extent of CAm/COM overlap. Therefore, it remains possible that layer 5 CAm and COM cells largely represent a single, but diverse, population of intratelencephalic neurons.

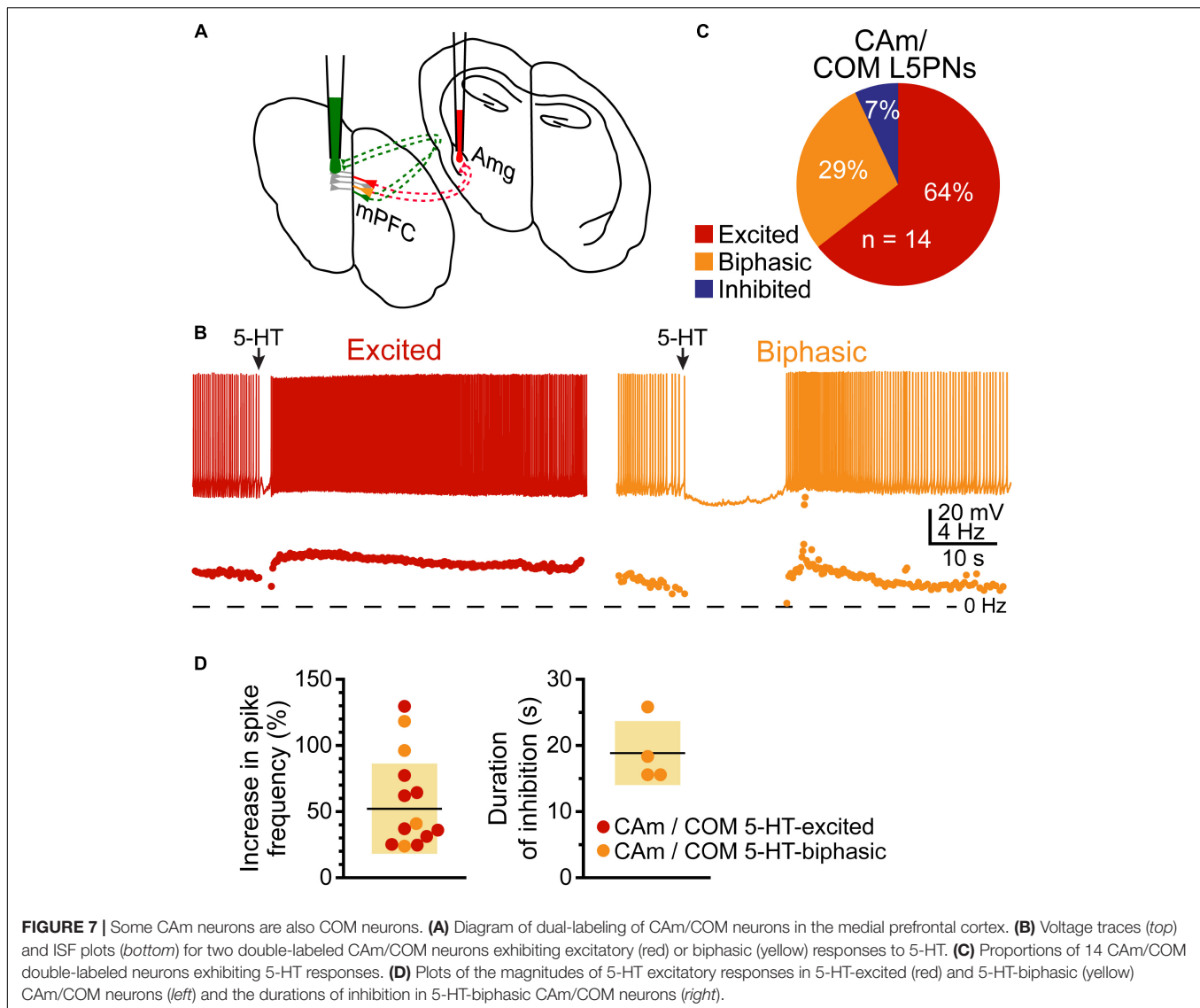


## DISCUSSION

Cortical projections to the amygdala convey top-down control of emotional responses, including fear behaviors (for review, see Courtin et al., 2013). Activity in the prelimbic cortex, likely conveyed to the amygdala via CAm neurons (Stevenson, 2011; Karalis et al., 2016), facilitates emotional responses to conditioned cues, most prominently freezing behavior (Corcoran and Quirk, 2007; Burgos-Robles et al., 2009; Dejean et al., 2016), but also active avoidance (Bravo-Rivera et al., 2014, 2015), suggesting a more generalized role of the prelimbic cortex in goal-directed behaviors and behavioral flexibility (for reviews, see Ragozzino, 2007; Euston et al., 2012; Orsini et al., 2015). Our findings demonstrate that layer 5 CAm neurons in the mouse prelimbic cortex share morphological and physiological properties with COM neurons. Like COM neurons, CAm neurons have sparser dendritic trees, higher input resistances, and less HCN-channel-mediated “sag” potentials than typically observed in neighboring corticofugal (e.g., CPn) neurons. Although the magnitude of HCN-mediated sag potentials in CAm neurons were somewhat larger (and RMPs more depolarized) than reported in COM neurons (Table 1 and Figure 1C), we identified double-labeled CAm/COM neurons following dual injections of red and green beads in the amygdala and contralateral cortex, suggesting that layer 5 CAm and COM

neuron populations overlap, at least partially, although the extent of such overlap was not determinable in our study. While we did not test for overlap among layer 2/3 CAm and COM neurons, our findings in layer 5 contrasts with the discrete, non-overlapping, ipsilateral CAm and COM populations reported in layer 2 of the mPFC in Swiss Webster mice (Little and Carter, 2013). This disparity may reflect differences in the laterality of CAm injections (ipsilateral vs contralateral), differences across animal strain, or functional diversity in CAm projections across cortical lamina. However, regardless of population overlap, our study, and that by Little and Carter (2013), agrees in finding that CAm and COM neurons are broadly similar in their physiological and morphological properties.

A main finding of our study is that CAm neurons also resemble COM neurons in their responsivity to 5-HT, whereby the vast majority of CAm neurons exhibit 2A-mediated responses, either in the form of purely excitatory responses to 5-HT (in 5-HT-excited neurons), or after transient 1A-mediated inhibition (in 5-HT-biphasic neurons). As with most other physiological measurements, serotonergic responses in CAm neurons were not laterality or sex-dependent (Table 3). Further, and as previously revealed in COM neurons (Stephens et al., 2014), 5-HT was unable to excite CAm neurons in the absence of additional excitatory drive, but was sufficient to promote AP generation in response to suprathreshold simulated synaptic



input in 5-HT-excited neurons. This suggests that 5-HT may act to promote cortical output to the amygdala by enhancing the activity of subsets of 2A-expressing CAM neurons that are simultaneously receiving strong excitatory input.

In COM neurons, serotonergic excitation is mediated by  $G_q$ -coupled 2A receptors that engage three postsynaptic ionic effectors to enhance cortical neuron excitability: inhibition of  $K_{V7}$  potassium channels and activation of two non-specific cation conductances (Stephens et al., 2018). Interestingly, these same ionic mechanisms underlie cholinergic excitation of CPn neurons in the prelimbic cortex (Baker et al., 2018), suggesting that they have a conserved role in  $G_q$ -mediated excitation across cortical neuron subtypes and neurotransmitter systems. On the other hand, serotonergic inhibition during biphasic responses results from  $G_{i/o}$ -coupled 1A receptors enhancing potassium conductances, including those mediated by G-protein-gated inwardly rectifying potassium channels (Andrade and Nicoll, 1987; Luscher et al., 1997). Using specific antagonists,

we confirmed that serotonergic excitation and inhibition of CAM neurons are mediated by the same 2A and 1A receptors (respectively) that gate serotonergic responses in COM neurons. While specific ionic mechanisms were not tested in this study, given the overlap in COM and CAM neuron populations, and the qualitative and quantitative similarities of their 5-HT responses, it would seem likely that 5-HT engages a similar set of mechanisms in both CAM and COM neurons.

## A Functional Role for Serotonergic Regulation of CAM Neurons

CAM neurons in the prelimbic cortex contribute to reciprocal CAM circuits that are engaged during the expression of conditioned fear (Stevenson, 2011; Karalis et al., 2016), and fear conditioning is associated with release of 5-HT into the prelimbic cortex (Hashimoto et al., 1999; Bland et al., 2003). Our results demonstrate that CAM neurons are responsive

to 5-HT, suggesting that 5-HT may influence the learning or expression of conditioned fear. Indeed, studies utilizing acute administration of SSRIs to globally increase extracellular 5-HT report enhanced acquisition and expression of cued (but not contextual) fear responses (for reviews, see Bauer, 2015; Bocchio et al., 2016). Further, pharmacological studies have revealed oppositional impact of 1A and 2A receptors in fear expression. For instance, systemic injection of a 2A receptor agonist enhances freezing in response to conditioned cues in rodents (Zhang et al., 2013), while 1A agonists delivered systemically (Inoue et al., 1996; Youn et al., 2009; Ohyama et al., 2016), or locally into the prelimbic cortex (Almada et al., 2015), reduce contextual freezing responses and fear-potentiated startle, respectively. Consistent with a role for 2A receptors in potentiating fear responses, systemic (Zhang et al., 2013) or local blockade of 2A receptors in the prelimbic cortex (León et al., 2017) can impair the expression of conditioned fear.

Since prelimbic CAM neurons exhibit 2A-dependent excitation, 5-HT could contribute to the enhanced functional connectivity observed between the prelimbic cortex and the amygdala during fear behaviors (Karalis et al., 2016). However, the net effect of 5-HT on prelimbic output is difficult to predict because, while 2A receptors may increase the excitability of CAM neurons, 5-HT also influences the excitability of cortical interneurons, and can act presynaptically to regulate glutamate and GABA release (for review, see Puig and Gullledge, 2011). Predictions of 5-HT effects on behavior are further complicated by potentially competing influences of CAM projections arising from different cortical areas (e.g., infralimbic vs prelimbic cortex; see, for instance, Vidal-Gonzalez et al., 2006), and from direct effects of 5-HT on the amygdala or other subcortical structures contributing to fear responses (Bocchio et al., 2016). However, future studies may surmount these limitations by employing genetically targeted approaches that allow for selective manipulation of specific neuron or 5-HT receptor subtypes in restricted brain regions (e.g., prelimbic cortex) at select

developmental time points. Such studies, carried out at a systems level, may reveal a role for 5-HT in facilitating not only fear behaviors, but also in regulating an extended intratelencephalic circuit encompassing COM and CAM neurons in both cerebral hemispheres, amygdalar neurons, CA1 neurons in the ventral hippocampus, and their collective overlapping efferents to the striatum (Cho et al., 2013; Heilbronner et al., 2016), to more broadly regulate emotional learning, decision making, and behavior (Vertes, 2006).

## ETHICS STATEMENT

All experiments were conducted according to methods approved by the Institutional Animal Care and Use Committee of Dartmouth College.

## AUTHOR CONTRIBUTIONS

DA and AG designed the research. DA and ES performed the research and analyzed the data. AG wrote the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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