Alcohol Dependence and Withdrawal Impair Serotonergic Regulation of GABA Transmission in the Rat Central Nucleus of the Amygdala

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Excessive serotonin (5-HT) signaling plays a critical role in the etiology of alcohol use disorder. The central nucleus of the amygdala (CeA) is a key player in alcohol-dependence associated behaviors. The CeA receives dense innervation from the dorsal raphe nucleus, the major source of 5-HT, and expresses 5-HT receptor subtypes (e.g., 5-HT_{2C} and 5-HT_{1A}) critically linked to alcohol use disorder. Notably, the role of 5-HT regulating rat CeA activity in alcohol dependence is poorly investigated. Here, we examined neuroadaptations of CeA 5-HT signaling in adult, male Sprague Dawley rats using an established model of alcohol dependence (chronic intermittent alcohol vapor exposure), *ex vivo* slice electrophysiology and ISH. 5-HT increased frequency of sIPSCs without affecting postsynaptic measures, suggesting increased CeA GABA release in naive rats. In dependent rats, this 5-HT-induced increase of GABA release was attenuated, suggesting blunted CeA 5-HT sensitivity, which partially recovered in protracted withdrawal (2 weeks). 5-HT increased vesicular GABA release in naive and dependent rats but had split effects (increase and decrease) after protracted withdrawal indicative of neuroadaptations of presynaptic 5-HT receptors. Accordingly, 5-HT abolished spontaneous neuronal firing in naive and dependent rats but had bidirectional effects in withdrawn. Alcohol dependence and protracted withdrawal did not alter either 5-HT_{1A}-mediated decrease of CeA GABA release or *Htr1a* expression but disrupted 5-HT_{2C}-signaling without affecting *Htr2c* expression. Collectively, our study provides detailed insights into modulation of CeA activity by the 5-HT system and unravels the vulnerability of the CeA 5-HT system to chronic alcohol and protracted withdrawal.

Key words: alcohol use disorder; central amygdala; electrophysiology; ethanol; GABA; serotonin

Significance Statement

Elevated GABA signaling in the central nucleus of the amygdala (CeA) underlies key behaviors associated with alcohol dependence. The CeA is reciprocally connected with the dorsal raphe nucleus, the main source of serotonin (5-HT) in the mammalian brain, and excessive 5-HT signaling is critically implicated in the etiology of alcohol use disorder. Our study, using a well-established rat model of alcohol dependence, *ex vivo* electrophysiology and ISH, provides mechanistic insights into how both chronic alcohol exposure and protracted withdrawal dysregulate 5-HT signaling in the CeA. Thus, our study further expands our understanding of CeA cellular mechanisms involved in the pathophysiology of alcohol dependence and withdrawal.

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Introduction

Alcohol use disorder (AUD) is a chronic, relapsing disease characterized by compulsive alcohol seeking and taking, loss of control in limiting alcohol intake, and the emergence of negative emotional states during absence from alcohol (Koob, 2008; Spanagel et al., 2014; Koob and Volkow, 2016). These states are regulated by specific neurochemical systems modulating neurocircuits involved in drug reward, negative affect, and executive control (Wise and Koob, 2014). Excessive serotonin (5-hydroxytryptamine [5-HT]) signaling has been implicated in the etiology of AUD and can elicit craving in abstinent patients (Krystal et al., 1996; Pettinati et al., 2003; Bonkale et al., 2006; Kranzler et

al., 2013; Marcinkiewicz et al., 2016). Specifically, polymorphisms in genes encoding for distinct components of the 5-HT system are either predisposing to the development of AUD or are linked to specific behaviors associated with AUD (Lappalainen et al., 1998; Feinn et al., 2005; Yohros et al., 2018). Accordingly, drugs modulating 5-HT signaling have been shown to decrease alcohol intake in both humans and animal models (Lê et al., 1996; Lejoyeux, 1996; Belmer et al., 2016; Dos Santos et al., 2018).

Chronic alcohol exposure alters serotonergic modulation of synaptic transmission and causes potential neuroadaptations in its receptor system (Belmer et al., 2016; Marcinkiewicz et al., 2016). In rodents, both acute alcohol and withdrawal increase excitability of dorsal raphe (DRN) neurons (Lowery-Gionta et al., 2015), thereby likely increasing 5-HT release into DRN-projecting areas. The DRN innervates all major players in the addiction cycle, including the central nucleus of the amygdala (CeA) (Vertes, 1991; Retson and Van Bockstaele, 2013; Linley et al., 2016). The CeA serves as an integrative hub mediating key aspects of negative emotional states associated with alcohol dependence, including anxiety-like behaviors (Gilpin et al., 2015). The CeA is a mainly GABAergic nucleus, particularly sensitive to both acute and chronic ethanol (Roberto et al., 2003, 2004, 2020). Thus, elevated CeA GABA transmission is a characteristic of alcohol dependence across species, including humans (Roberto et al., 2004; Herman et al., 2016; Augier et al., 2018; Jimenez et al., 2019). Importantly, acute alcohol administration enhances 5-HT release in the CeA (Yoshimoto et al., 2000).

The 5-HT receptor system comprises 14 different 5-HT-activated either inhibitory (5-HT₁ receptor subtypes) or excitatory (5-HT₂, 4, 6, and 7 receptor subtypes) receptors coupled to G-proteins and an excitatory ligand-gated ion channel (5-HT₃) (McCorvy and Roth, 2015). 5-HT receptor subtypes are abundantly expressed in the mammalian brain (Charnay and Léger, 2010), tightly regulating 5-HT signaling (Belmer et al., 2016). Multiple studies implicate a critical role of 5-HT_{1A} and 5-HT_{2C} signaling in the extended amygdala in addictive behaviors, including AUD (Müller et al., 2007; Marcinkiewicz et al., 2016). For instance, site-specific injections of 5-HT_{2C} antagonists into the nucleus accumbens shell block escalation of alcohol intake in rodents (Yoshimoto et al., 2012). Alcohol dependence elicits specific changes in 5HT_{2C} signaling conferring neuronal hyperexcitability in the ventral BNST (Marcinkiewicz et al., 2015) and increased amygdalar 5-HT_{2C} expression was observed in genetic models of alcohol preference (Pandey et al., 1996) corroborating a critical role for 5-HT_{2C} in excessive alcohol intake. Last, 5-HT induces anxiety-like behavior in the dorsal BNST via activation of 5-HT_{1A} (Garcia-Garcia et al., 2018).

However, despite strong serotonergic innervation of the CeA (Vertes, 1991; Retson and Van Bockstaele, 2013; Linley et al., 2016) accompanied by dense expression of 5-HT_{2C} and 5-HT_{1A} (Asan et al., 2013), little is known about 5-HT regulation of CeA activity under physiological (naïve) conditions and/or alcohol dependence and protracted withdrawal. Thus, we examined potential neuroadaptations of the CeA 5-HT system induced by alcohol dependence using a rat model of chronic intermittent vapor exposure, *ex vivo* slice electrophysiology, and ISH. Specifically, we studied 5-HT-modulation of CeA GABA_A receptor-mediated synaptic transmission, 5-HT effects on spontaneous neuronal firing, and expression of *Htr1a* and *Htr2c* mRNA accompanied by functional assessment of 5-HT_{1A} and 5-HT_{2C} signaling in naïve, dependent, and withdrawn rats.

Materials and Methods

All procedures were approved by the Scripps Research Institutional Animal Care and Use Committee and are in line with the National Institutes of Health *Guide for the care and use of laboratory animals*.

For this study, we used a total of 119 male Sprague Dawley rats (Charles River) weighing 225–250 g on arrival. Rats were group-housed (2 or 3 per cage) in standard plastic cages in a temperature- and humidity-controlled room and were maintained under a reverse 12 h/12 h light/dark cycle with *ad libitum* access to food and water.

Chronic intermittent alcohol exposure

To induce alcohol dependence, 57 rats were exposed to 14 h/day alcohol vapor (10 h air) using the standard chronic intermittent alcohol inhalation method in their home cages. Blood alcohol levels were determined 1 or 2 times per week from tail-blood samples (average blood alcohol level: 151 ± 7 mg/dl). Twenty-five dependent rats were subsequently subjected to protracted withdrawal for 2 weeks. Naïve controls were treated similarly, except that they were exposed to air only.

Slice electrophysiology

Preparation of acute brain slices and electrophysiological recordings were performed as previously described (Roberto et al., 2004; Tunstall et al., 2019; Khom et al., 2020). We decapitated deeply anesthetized rats (3–5% isoflurane anesthesia) and rapidly isolated their brains in ice-cold oxygenated high-sucrose cutting solution (composition in mM as follows: 206 sucrose, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 1.2 NaH₂PO₄, 26 NaHCO₃, 5 glucose, and 5 HEPES); 300- μ m-thick coronal slices containing the medial subdivision of CeA were cut using a VT 1000S vibratome (Leica Microsystems) and incubated for 30 min in warm (37°C), oxygenated aCSF (composition in mM as follows: 130 NaCl, 3.5 KCl, 2 CaCl₂, 1.25 NaH₂PO₄, 1.5 MgSO₄, 24 NaHCO₃, and 10 glucose) followed by another 30 min incubation at room temperature. Dependent rats were euthanized during the last hour of their daily alcohol vapor exposure. Slices were cut and incubated in ethanol-free solutions; thus, the slices from dependent animals underwent acute *in vitro* withdrawal (1–10 h).

We recorded from neurons located in the medial subdivision of the CeA using either whole-cell voltage clamp or cell-attached current-clamp mode. Neurons were visualized with infrared differential interference contrast optics using a 40 $\times$ water-immersion objective (Olympus BX51WI), and a CCD camera (EXi Aqua, QImaging). All recordings were performed in gap-free acquisition mode with a 10 kHz sampling rate and 10 kHz low-pass filtering using a MultiClamp700B amplifier, Digidata 1440A, and pClamp 10 software (Molecular Devices).

Patch pipettes were pulled from borosilicate glass (3–5 M Ω , King Precision) and filled with a KCl-based internal solution (composition in mM as follows: 135 KCl, 5 EGTA, 5 MgCl₂, 10 HEPES, 2 Mg-ATP, and 0.2 Na-GTP, pH 7.2–7.4, adjusted with 1 M KOH, 290–300 mOsm) for whole-cell recordings or a K-gluconate-based internal solution (composition in mM as follows: 145 K-gluconate, 0.5 EGTA, 2 MgCl₂, 10 HEPES, 2 Mg-ATP, and 0.2 Na-GTP, pH 7.2–7.4, adjusted with 1 M KOH, 300 mOsm) for cell-attached recordings. We pharmacologically isolated action potential (AP)-dependent GABA_A-receptor-mediated sIPSCs by adding blockers of glutamatergic transmission (20 μ M DNQX and 30 μ M DL AP-5) and GABA_B receptors (1 μ M CGP55845A) to the bath solution. To assess AP-independent GABA_A-receptor-mediated transmission (mIPSCs), we added 0.5 μ M TTX to block Na⁺ channels. Neurons were held at –60 mV. All data were obtained from neurons with an access resistance (R_a) further of ≤ 15 M Ω and a maximum change of R_a of $<20\%$ during the entire recording, as monitored by frequent 10 mV pulses. We recorded spontaneous AP firing of CeA neurons in cell-attached configuration in current-clamp mode in aCSF. Serotonin's effects on intrinsic membrane properties and excitability of CeA neurons were determined in aCSF using current-clamp mode and a step protocol comprising 500 ms hyperpolarizing and depolarizing steps (10 pA steps), starting from –120 pA as previously described (Roberts et al., 2019; Suárez et al., 2019).

Drugs

We purchased serotonin, WAY161503, SB2420840, 8-OH-DPAT, AP-5, CGP55845A, and DNQX from Tocris Bioscience, ethanol from Remet, and TTX from Biotium. Chemicals other than these were purchased from Sigma Millipore. Stock solutions of all drugs were prepared in either distilled water or DMSO and applied to the bath solution to achieve final concentrations. All drugs were applied by bath perfusion.

Tissue preparation for ISH

Male rats (3–4) from each treatment group (naive, dependent, and withdrawn) were anesthetized using isoflurane and perfused with ice-cold PBS/Z-fix (Fisher Scientific). Brains were dissected and immersion-fixed in Z-fix for 24 h at 4°C, cryoprotected in sterile 30% sucrose in PBS for 24–48 h at 4°C or until brains sank, flash frozen in prechilled isopentane on dry ice, and stored at –80°C. Brains were then sliced on a cryostat in 20- μ m-thick sections through the CeA, mounted on SuperFrost Plus slides (Fisher Scientific, 1255015), and stored at –80°C.

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We performed ISH using RNAscope fluorescent multiplex kit (ACD, 320850) under RNase-free conditions as previously described (Wolfe et al., 2019). First, a target retrieval pretreatment protocol was performed as outlined in the RNAscope pretreatment user manual (ACD, document #320535). Briefly, slides were submerged in target retrieval buffer (ACD, 322000) at 95–98°C for 10 min, immediately washed in distilled water, dehydrated in 100% ethanol (storage at –80°C if required), and lastly incubated in Protease IV for 20 min at 40°C. Next, the RNAscope Fluorescent Multiplex Reagent Kit User Manual (ACD, document #320293) was followed with a final step where slides were mounted with DAPI-containing Vectashield (Thermo Fisher Scientific, NC9029229). The probes used from ACD Biotechne were as follows: negative control (320751), *Htr2c* (469321-C2), and *Htr1a* (404801-C2). One or two images (on average, 2 slices/animal) were acquired using a Carl Zeiss LSM 780 laser scanning confocal microscope (40 $\times$ oil immersion, 1024 $\times$ 1024, tile scanning of CeA, 5 μ m z stacks), and all settings were maintained within experiments during image acquisition. Brightness/contrast and pixel dilation are the same for all representative images shown per figures. Quantification was performed blind to treatment groups using the imaging software Fiji (Schindelin et al., 2012). Nuclei were identified based on the nuclear stain DAPI by Fiji or by visual inspection when necessary for images of the CeA. Nuclei were considered positive for the probe of interest if corresponding fluorescent signal was present after background subtraction (determined by the negative control probe). The percent of positive nuclei to total nuclei was calculated for each image and normalized to the control/naive group to indicate relative values. Next, Fiji was used to measure the mean optical densitometry of the fluorescent signal of each probe after background subtraction per image and normalized to the control/naive group.

Data and statistical analysis

Electrophysiology. We analyzed frequencies, amplitudes, and rise and decay times of sIPSCs/mIPSCs semiautomatically using MiniAnalysis software (Synaptosoft). Each event (for both sIPSCs/mIPSCs, minimum amplitude was set to >5 pA) was visually confirmed. For analysis, we averaged sIPSC/mIPSC characteristics in 3 min bins. Drug effects were normalized to their own baseline (presented as control) before group analyses. We similarly analyzed cell-attached recordings semiautomatically using pClamp version 10 followed by a visual confirmation of each AP. Cells responding with an $\geq \pm 15\%$ change of sIPSC/mIPSC or firing rate were considered as drug-sensitive. Effects of serotonin on intrinsic

Figure 1. 5-HT increases AP-dependent and AP-independent CeA GABA release. Error bars indicate mean $\pm$ SEM of normalized effects of the indicated 5-HT concentrations on (A, B) sIPSC and (C, D) mIPSC characteristics compared to baseline control (dashed line). Error bars indicate mean $\pm$ SEM from 5 to 12 cells. * p < 0.05; ** p < 0.01; *** p < 0.001; compared with baseline control (dashed line) using one-sample t tests. Differences between effects of individual 5-HT concentrations versus the zero-drug condition were calculated using a one-way ANOVA followed by a Dunnett's *post hoc* mean comparison: # p < 0.05; ## p < 0.01; ### p < 0.001; #### p < 0.0001.

membrane properties and excitability were analyzed using the NeuroExpress software (version 19.4.09.) developed and provided by A. Szűcs.

ISH. All analyses were performed on raw images, and outliers detected by Grubb's test were removed. Analysis of cell counts and densitometry was performed per image and normalized to the naive group indicating relative changes.

All data in this study are represented as mean $\pm$ SEM. Statistical analysis and data graphing were performed using GraphPad Prism 8.0 (GraphPad). We set the criterion for statistical significance to p < 0.05 and used one-sample t tests to assess per se drug effects (i.e., statistical difference from control baseline before drug application) as in Figures 1A–D, 4B, C, E, F, 5B, 6E, F, and 7E, F and Table 1. Of note, in case of significantly different SDs between treatments (Bartlett's test), we performed median split analyses (see Figs. 4E, 5B). We used paired t tests to evaluate 5-HT effects on passive and active membrane properties (see Fig. 2; Table 2). Statistical differences between more than two treatment groups were calculated using one-way ANOVA with appropriate *post hoc* mean comparisons as in Figure 1A–D (Dunnett's), Figure 3B–E, G, J (Dunnett's), Figure 4B, C, E, F (Tukey's), Figure 5B (Tukey's), Figure 6B, C, E, F (Tukey's), and Figure 7B, C, F, G (Tukey's). Sample sizes for each in experiment are given in the figure legends whereby n denotes the total number of cells or images analyzed.

Results

5-HT strongly increases both AP-dependent and AP-independent CeA GABA release

Given the strong serotonergic innervation of the CeA (Vertes, 1991; Asan et al., 2013; Retson and Van Bockstaele, 2013; Linley et al., 2016) and the dense expression of 5-HT receptors in the CeA, we first examined 5-HT regulation of GABAergic synaptic transmission in the medial subdivision of the CeA, the major output region of the amygdalar complex. We tested different 5-HT concentrations (0.5–50 μ M) on pharmacologically isolated

Table 1. Summary of 5-HT effects on CeA sIPSC and mIPSC characteristics in naive rats^a

	Frequency	Amplitude	Rise time	Decay time
sIPSCs				
Control/0 μM 5-HT	95.4 $\pm$ 2.3 $t = 2.028$, $df = 4$ $p = 0.1125$ Control: 1.32 $\pm$ 0.36 Hz 5-HT:	104.8 $\pm$ 7.2 $t = 0.6715$, $df = 4$ $p = 0.5387$ Control: 74.0 $\pm$ 6.9 pA 5-HT:	100.5 $\pm$ 4.3 $t = 0.1068$, $df = 4$ $p = 0.9201$ Control: 2.45 $\pm$ 0.07 ms 5-HT:	99.1 $\pm$ 3.1 $t = 0.3029$, $df = 4$ $p = 0.7771$ Control: 6.56 $\pm$ 0.78 ms 5-HT:
0.5 μM 5-HT	1.23 $\pm$ 0.3 Hz 122.5 $\pm$ 39.7 $t = 0.5669$, $df = 4$ $p = 0.6011$ Control: 1.34 $\pm$ 0.56 Hz 5-HT:	76.4 $\pm$ 5.4 pA 115.7 $\pm$ 23.0 $t = 0.6816$, $df = 4$ $p = 0.5329$ Control: 52.1 $\pm$ 4.0 pA 5-HT:	2.46 $\pm$ 0.11 ms 92.8 $\pm$ 3.7 $t = 1.924$, $df = 4$ $p = 0.1267$ Control: 2.62 $\pm$ 0.19 ms 5-HT:	6.43 $\pm$ 0.62 ms 94.6 $\pm$ 13.1 $t = 0.4131$, $df = 4$ $p = 0.7007$ Control: 8.15 $\pm$ 1.38 ms 5-HT:
3 μM 5-HT	1.83 $\pm$ 1.70 Hz 268.0 $\pm$ 48.0 $t = 3.503$, $df = 6$ $p = 0.0128$ Control: 2.08 $\pm$ 0.53 Hz 5-HT:	59.7 $\pm$ 11.5 pA 102.4 $\pm$ 10.9 $t = 0.2203$, $df = 6$ $p = 0.8329$ Control: 59.4 $\pm$ 14.4 pA 5-HT:	2.42 $\pm$ 0.19 ms 101.8 $\pm$ 3.2 $t = 0.5688$, $df = 6$ $p = 0.5901$ Control: 2.57 $\pm$ 0.39 ms 5-HT:	7.71 $\pm$ 1.58 ms 95.6 $\pm$ 9.2 $t = 0.4739$, $df = 6$ $p = 0.6524$ Control: 7.0 $\pm$ 1.11 ms 5-HT:
12.5 μM 5-HT	4.91 $\pm$ 0.87 Hz 259.3 $\pm$ 30.7 $t = 5.183$, $df = 7$ $p = 0.0013$ Control: 2.13 $\pm$ 1.66 Hz 5-HT:	61.3 $\pm$ 8.2 pA 107.5 $\pm$ 13.7 $t = 0.5479$, $df = 7$ $p = 0.6008$ Control: 67.5 $\pm$ 9.2 pA 5-HT:	2.61 $\pm$ 0.15 ms 103.8 $\pm$ 2.2 $t = 1.751$, $df = 7$ $p = 0.1235$ Control: 2.51 $\pm$ 0.59 ms 5-HT:	6.82 $\pm$ 0.88 ms 99.4 $\pm$ 8.1 $t = 0.0739$, $df = 7$ $p = 0.9432$ Control: 7.03 $\pm$ 0.94 5-HT:
25 μM 5-HT	5.96 $\pm$ 1.96 Hz 458.8 $\pm$ 92.4 $t = 3.884$, $df = 4$ $p = 0.0178$ Control: 1.53 $\pm$ 0.69 Hz 5-HT:	74.3 $\pm$ 16.5 pA 105.6 $\pm$ 12.8 $t = 0.4352$, $df = 4$ $p = 0.6857$ Control: 65.8 $\pm$ 7.5 pA 5-HT:	2.53 $\pm$ 0.19 ms 104.4 $\pm$ 6.4 $t = 0.6858$, $df = 4$ $p = 0.5305$ Control: 2.61 $\pm$ 0.14 ms 5-HT:	6.73 $\pm$ 0.88 ms 106.5 $\pm$ 6.4 $t = 0.1650$, $df = 4$ $p = 0.5718$ Control: 6.69 $\pm$ 0.97 ms 5-HT:
	5.18 $\pm$ 1.53 Hz	70.7 $\pm$ 14.5 pA	2.69 $\pm$ 0.09 ms	6.94 $\pm$ 1.02 ms
mIPSCs				
Control/0 μM 5-HT	100.8 $\pm$ 3.3 $t = 0.2466$, $df = 4$ $p = 0.8173$ Control: 0.82 $\pm$ 0.18 Hz 5-HT:	101.8 $\pm$ 2.0 $t = 0.9056$, $df = 4$ $p = 0.4164$ Control: 63.8 $\pm$ 8.2 pA 5-HT:	100.2 $\pm$ 1.3 $t = 0.1815$, $df = 4$ $p = 0.8616$ Control: 2.59 $\pm$ 0.04 ms 5-HT:	103.7 $\pm$ 2.2 $t = 1.734$, $df = 4$ $p = 0.1579$ Control: 5.71 $\pm$ 0.80 ms 5-HT:
12.5 μM 5-HT	0.86 $\pm$ 0.16 Hz 122.3 $\pm$ 6.7 $t = 3.309$, $df = 7$ $p = 0.0130$ Control: 0.86 $\pm$ 0.21 Hz 5-HT:	67.4 $\pm$ 9.9 pA 103.2 $\pm$ 3.1 $t = 1.048$, $df = 7$ $p = 0.3295$ Control: 54.5 $\pm$ 4.1 pA 5-HT:	2.59 $\pm$ 0.08 ms 100.2 $\pm$ 2.2 $t = 0.07430$, $df = 7$ $p = 0.9428$ Control: 2.38 $\pm$ 0.14 ms 5-HT:	6.01 $\pm$ 0.83 ms 105.9 $\pm$ 7.0 $t = 0.8377$, $df = 7$ $p = 0.4299$ Control: 4.94 $\pm$ 0.70 ms 5-HT:
25 μM 5-HT	1.05 $\pm$ 0.25 Hz 135.8 $\pm$ 9.6 $t = 3.741$, $df = 8$ $p = 0.0057$ Control: 0.41 $\pm$ 0.08 Hz 5-HT:	56.3 $\pm$ 4.7 pA 87.8 $\pm$ 6.0 $t = 2.017$, $df = 8$ $p = 0.0784$ Control: 56.9 $\pm$ 2.7 pA 5-HT:	2.37 $\pm$ 0.10 ms 108.8 $\pm$ 4.9 $t = 1.802$, $df = 8$ $p = 0.1093$ Control: 2.05 $\pm$ 0.11 ms 5-HT:	4.86 $\pm$ 0.63 95.0 $\pm$ 8.7 $t = 0.5747$, $df = 8$ $p = 0.5813$ Control: 4.59 $\pm$ 0.63 ms 5-HT:
	0.52 $\pm$ 0.07 Hz	49.2 $\pm$ 2.9 pA	2.19 $\pm$ 0.08 ms	4.07 $\pm$ 0.40 ms

(Table continues.)

Table 1. Continued

	Frequency	Amplitude	Rise time	Decay time
50 μ M 5-HT	141.5 $\pm$ 5.8 $t = 7.147$, $df = 8$ $p < 0.0001$ Control: 0.40 $\pm$ 0.05 Hz 5-HT: 0.57 $\pm$ 0.09 Hz	95.6 $\pm$ 4.7 $t = 0.7319$, $df = 8$ $p = 0.4851$ Control: 59.3 $\pm$ 3.1 pA 5-HT: 56.9 $\pm$ 3.3 pA	98.8 $\pm$ 3.7 $t = 0.3115$, $df = 8$ $p = 0.7634$ Control: 2.3 $\pm$ 0.06 ms 5-HT: 2.3 $\pm$ 0.08 ms	96.3 $\pm$ 7.6 $t = 0.4842$, $df = 8$ $p = 0.6412$ Control: 5.15 $\pm$ 0.44 ms 5-HT: 4.88 $\pm$ 0.45 ms
100 μ M 5-HT	134.3 $\pm$ 9.2 $t = 3.709$, $df = 8$ $p = 0.0060$ Control: 0.40 $\pm$ 0.22 Hz 5-HT: 0.52 $\pm$ 0.08 Hz	100.7 $\pm$ 5.4 $t = 0.1219$, $df = 8$ $p = 0.9060$ Control: 54.7 $\pm$ 3.7 pA 5-HT: 54.2 $\pm$ 3.1 pA	103.4 $\pm$ 7.4 $t = 0.4673$, $df = 8$ $p = 0.6527$ Control: 2.37 $\pm$ 0.17 ms 5-HT: 2.37 $\pm$ 0.08 ms	102.4 $\pm$ 11.1 $t = 0.2189$, $df = 8$ $p = 0.8322$ Control: 4.72 $\pm$ 0.53 ms 5-HT: 4.83 $\pm$ 0.59 ms

^aData are mean $\pm$ SEM from raw values and normalized to baseline control, respectively, from at least 5 different cells from at least 4 different animals per group. Statistical significance from baseline was calculated using one-sample t test.

spontaneous GABA_A receptor-mediated inhibitory AP-dependent PSCs (sIPSCs) and AP-independent mIPSCs (12.5–100 μ M) in CeA neurons from naive rats. As shown in Figure 1A, we found that serotonin (5-HT) strongly and rapidly increased sIPSC frequency at concentrations ≥ 25 μ M compared with a zero-drug control condition (ANOVA, $F_{(5,33)} = 8.871$, $p < 0.0001$) without significantly affecting postsynaptic measures, including sIPSC amplitudes, and rise and decay times at any concentration tested (Fig. 1B), suggesting that 5-HT modulates CeA network-dependent GABA release but not postsynaptic GABA_A receptor function. 5-HT at concentrations ≥ 25 μ M also significantly increased mIPSC frequency, indicative of 5-HT enhancing vesicular GABA release (Fig. 1C) compared with a zero-drug control condition (ANOVA, $F_{(4,35)} = 3.117$, $p = 0.0271$). 5-HT did not significantly alter mIPSC amplitudes or kinetics at any tested concentration (Fig. 1D), corroborating that 5-HT effects on CeA GABA release occur at the presynaptic site. All data for 5-HT effects on sIPSC and mIPSC characteristics are summarized in Table 1.

Serotonin alters active and passive membrane properties of CeA neurons

Next, we examined the effect of serotonin on passive and active membrane properties of CeA neurons by comparing current–voltage relationships in aCSF control conditions and in the presence of 50 μ M 5-HT (Fig. 2A). We found that 5-HT significantly altered slopes of current–voltage relationships (control: 0.4630 ± 0.01944 vs 5-HT: 0.3820 ± 0.01290 , assessed by a simple linear regression, $F = 12.16$, degrees of freedom (DFd) = 570, $p = 0.0005$; Fig. 2B) and voltage sag slopes (control: -0.068 ± 0.005 vs 5-HT: -0.049 ± 0.004 , assessed by a simple linear regression: $F = 8.775$, degree of freedom = 475, $p = 0.0032$; Fig. 2C). In line, we found that 5-HT significantly decreased membrane resistance, indicative of increased membrane conductance (control: 658.7 ± 52.7 M Ω vs 5-HT: 549.7 ± 45.1 M Ω , $t = 2.737$, $df = 19$, $p = 0.0131$) and decreased AP threshold (control: -47.72 ± 0.78 mV vs 5-

Figure 2. 5-HT alters active and passive membrane properties of CeA neurons. **A**, Representative traces for the current–voltage relationship of a CeA neuron in the absence (left, black trace) and in the presence of 50 μ M 5-HT (right, orange trace) in response to a hyperpolarizing and depolarizing current step, respectively. Inset, Vertical bars represent the amplitude of the voltage sag. **B**, Current–voltage relationship and **C** current–voltage sag relationship in the absence (black circles) and in the presence of 50 μ M 5-HT (orange squares). Data are mean $\pm$ SEM from $n = 20$ neurons. Significant differences between control conditions and during 5-HT were assessed using paired t test: ** $p < 0.01$; *** $p < 0.001$.

HT: -50.17 ± 1.21 mV, $t = 3.110$, $df = 19$, $p = 0.0058$). Last, we also found that 5-HT altered the membrane time constant τ (control: 34.3 ± 2.6 vs 5-HT: 28.0 ± 2.2 , $t = 2.805$, $df = 19$, $p = 0.0113$). We did not observe any significant changes in capacitance, rheobase, AP amplitudes, AP half-width, AP after-hyperpolarization, or cumulative number of APs after application of 5-HT (all data are summarized in Table 2). Importantly, we did not observe any cell type-specific (low-threshold bursting, regular or late spiking neurons) (Chieng et al., 2006; Herman and Roberto, 2016) effects of 5-HT and thus pooled data from all recorded cells.

Table 2. Summary of passive and membrane properties of CeA neurons in the absence and presence of 50 μ M 5-HT^a

	Control	5-HT (50 μ M)
Membrane resistance	658.7 $\pm$ 52.7 $\text{M}\Omega$	549.7 $\pm$ 45.1 $\text{M}\Omega^*$
Capacitance	70.8 $\pm$ 3.1 pF	67.7 $\pm$ 2.9 pF
Time constant τ	34.3 $\pm$ 2.6	28.0 $\pm$ 2.2*
Voltage sag slope	0.4630 $\pm$ 0.01944	0.3820 $\pm$ 0.01290**
Rheobase	21.2 $\pm$ 2.8 pA	21.5 $\pm$ 3.7 pA
AP threshold	−47.72 $\pm$ 0.78 mV	−50.17 $\pm$ 1.21 mV**
AP amplitude	82.5 $\pm$ 1.4 mV	82.2 $\pm$ 1.7 mV
AP half-width	2.1 $\pm$ 0.1 ms	2.1 $\pm$ 0.1 ms
AP after-hyperpolarization	6.8 $\pm$ 0.6 mV	6.6 $\pm$ 0.6 mV
Cumulative AP number	2.9 $\pm$ 0.7	3.1 $\pm$ 0.8

^aData are mean $\pm$ SEM ($n = 20$). Membrane resistance was calculated from the hyperpolarizing steps of the current–voltage relationship. The time constant τ was calculated as an average determined from monoexponential fits of the initial parts of individual voltage responses elicited by negative current steps in current-clamp mode. The voltage sag was plotted against the level of the injected current, and the corresponding relationship was fitted using a linear function.

* $p < 0.05$; ** $p < 0.01$; calculated using paired t tests.

Alcohol dependence-induced heightened GABA release persists into protracted withdrawal

Elevated GABA_A receptor-mediated signaling in the CeA is a hallmark of alcohol dependence across species (Roberto et al., 2004; Herman et al., 2016; Augier et al., 2018; Jimenez et al., 2019). Here, in line with our previous studies (Roberto et al., 2004; Khom et al., 2020), we found that chronic ethanol exposure significantly increased sIPSC frequencies (ANOVA, $F_{(2,155)} = 8.767$, $p = 0.0002$; Fig. 3A) in the CeA of both dependent (1.93 ± 0.18 Hz, $n = 49$) and withdrawn (2.45 ± 0.38 Hz, $n = 36$) rats compared with naive controls (1.30 ± 0.10 Hz, $n = 78$; Fig. 3B), but did not significantly affect sIPSC amplitudes (Fig. 3C), rise times (Fig. 3E), or decay times (Fig. 3F). Moreover, alcohol dependence also significantly increased mIPSC frequencies (ANOVA, $F_{(2,97)} = 6.085$, $p = 0.032$; Fig. 3F) in CeA neurons from dependent (0.84 ± 0.12 Hz, $n = 26$; Fig. 3G) and withdrawn rats (0.77 ± 0.10 Hz, $n = 28$) compared with naive controls (0.52 ± 0.05 Hz, $n = 53$). We also found a significant effect of ethanol exposure on mIPSC amplitudes (ANOVA, $F_{(2,97)} = 3.328$, $p = 0.0397$; Fig. 3H), but a Tukey's *post hoc* mean comparison did not reveal significant differences between the groups. Furthermore, mIPSC rise times did not differ between groups, but chronic alcohol significantly prolonged mIPSC decay times (ANOVA, $F_{(2,97)} = 8.46$, $p = 0.0004$) in dependent (7.87 ± 0.86 ms, $n = 26$; Fig. 3I) and withdrawn (7.76 ± 0.63 ms, $n = 28$) rats compared with naive controls (5.46 ± 0.33 ms, $n = 53$; Fig. 3J), indicative of long-lasting presynaptic and postsynaptic neuroadaptations of CeA GABA signaling.

Alcohol dependence blunts 5-HT-induced modulation of CeA synaptic network activity

Recent studies indicate that chronic alcohol exposure increases excitatory transmission onto 5-HT projection neurons in the DRN (Lowery-Gionta et al., 2015), suggesting enhanced 5-HT release into brain areas connected to the DRN. Thus, we first determined how chronic alcohol exposure affects the 5-HT-induced increase of AP-dependent GABA transmission, reflecting the activity of the entire CeA neuronal network. Based on the robust and strong increase of synaptic GABA transmission induced by 5-HT (see Figure 1A) we again used 50 μ M 5-HT and found that chronic alcohol exposure blunted 5-HT regulation of CeA GABA signaling (Fig. 4A). Specifically, we found a main effect of chronic alcohol history on 5-HT-enhanced sIPSC frequencies (Fig. 4B; ANOVA, $F_{(2,31)} = 4.249$, $p = 0.0234$). A *post hoc* analysis (Tukey's) revealed that the 5-HT-induced increase of sIPSC frequency was significantly less pronounced in

dependent rats compared with naive controls (naive: $724.9 \pm 126.4\%$ vs dependent: $308.7 \pm 73.9\%$, $p = 0.0175$) indicative of reduced sensitivity of the CeA GABAergic synaptic network to regulation by 5-HT after chronic alcohol. To assess how long this dysregulation lasts, we tested the effects of 5-HT in CeA of withdrawn rats. Notably, the 5-HT-induced increase of sIPSC frequency in withdrawn rats did not differ from either naive or dependent rats, suggesting a transient effect, which might recover with longer alcohol withdrawal duration. Last, alcohol dependence did not significantly alter any 5-HT induced postsynaptic measures, including sIPSC amplitudes (ANOVA, $F_{(2,33)} = 0.4887$, $p = 0.6178$), rise times (ANOVA, $F_{(2,33)} = 0.7820$, $p = 0.4658$), or decay times (ANOVA, $F_{(2,33)} = 0.1306$, $p = 0.8780$) of sIPSCs as observed for naive rats (Fig. 4C).

Alcohol withdrawal, but not alcohol dependence, dysregulates 5-HT modulation of CeA AP-independent GABA release

Next, we determined whether chronic alcohol alters 5-HT modulation of AP-independent GABAergic synaptic transmission. As shown in Figure 4D, 5-HT (50 μ M) increased mIPSC frequency in dependent rats as in naive (naive: $141.5 \pm 5.8\%$, $t = 7.148$, $df = 8$, $p < 0.0001$ vs dependent: $140.0 \pm 9.7\%$, $t = 4.137$, $df = 10$, $p = 0.0020$; Fig. 4E), indicative of unaffected modulation of CeA vesicular GABA release by alcohol dependence. Interestingly, in CeA neurons from withdrawn rats, the overall effect of 5-HT on mIPSC frequency was not significantly different from baseline control. However, given significantly different SDs (Bartlett's test: 20.57 , $p = 0.0004$) between the treatment groups, a subsequent median split analysis revealed bidirectional 5-HT effects on mIPSC frequencies, such as 5-HT increased it in one subset ($160 \pm 23\%$, $t = 2.581$, $df = 5$, $p = 0.0494$) while decreasing it in the other subset (77 ± 4 , $t = 6.485$, $df = 4$, $p = 0.0029$). 5-HT did not alter postsynaptic mIPSC characteristics (amplitudes and kinetics) in any of the treatment groups (Fig. 4F), suggesting that neuroadaptations in the 5-HT system regulating CeA GABA transmission predominantly occur at the presynaptic site.

5-HT decreases spontaneous neuronal firing of CeA neurons

Our data indicate that 5-HT induces a pronounced GABA release in the CeA. Given the mainly GABAergic nature of the CeA, this increased GABAergic signaling in response to 5-HT might thus stem from an activation of intra-CeA GABAergic neurons. Thus, we next assessed the effect of 5-HT on spontaneous AP firing of CeA neurons. Importantly, spontaneous baseline firing did not significantly differ between naive (2.1 ± 0.2 Hz, $n = 9$, ANOVA, $F_{(2,27)} = 0.6718$, $p = 0.5191$) dependent (1.6 ± 0.2 Hz, $n = 11$) or withdrawn rats (2.4 ± 0.8 Hz, $n = 10$). As shown in Fig. 5A; 5-HT strongly reduced the neuronal firing rate of CeA neurons in naive ($-68.5 \pm 26\%$, $t = 2.648$, $df = 8$, $p = 0.0293$; Fig. 5B) and dependent ($-61 \pm 15.7\%$, $t = 3.915$, $df = 10$, $p = 0.0029$; Fig. 5B) rats, suggesting that 5-HT overall decreases CeA excitability stemming from increased GABA release onto CeA neurons. Interestingly, the overall firing rate of CeA neurons from withdrawn rats was not significantly changed in the presence of 5-HT (Fig. 5B). However, based on significantly different SDs (Bartlett's test: 15.33 , $p = 0.0041$), we performed a *post hoc* median split analysis revealing bidirectional 5-HT effects on AP firing, such as 5-HT increased it in one subset ($142 \pm 50\%$, $t = 2.824$, $df = 4$, $p = 0.0477$) and decreased it in the other subset ($-87 \pm 10\%$, $t = 8.484$, $df = 4$, $p = 0.0011$), suggesting that protracted withdrawal from chronic ethanol disrupts 5-HT-mediated inhibition of neuronal firing.

Figure 3. Chronic alcohol exposure and withdrawal increase CeA GABA release. **A**, Representative sIPSC recordings from CeA neurons from naïve, dependent (Dep), and withdrawn (WD) rats. Error bars indicate mean $\pm$ SEM of sIPSC (**B**) frequencies, (**C**) amplitudes, (**D**) rise times, and (**E**) decay times. **F**, Representative mIPSC recordings from naïve, dependent, and withdrawn rats. Error bars indicate mean $\pm$ SEM of mIPSC (**G**) frequencies, (**H**) amplitudes, (**I**) rise times, and (**J**) decay times. Differences between groups were calculated using one-way ANOVA ($^{\#}p < 0.05$; $^{##}p < 0.01$; $^{###}p < 0.001$) followed by a Dunnett's *post hoc* mean comparison ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

5-HT1A regulates vesicular CeA GABA release despite chronic alcohol exposure

5-HT1A signaling has been strongly implicated in alcohol dependence associated behaviors, and 5-HT1A receptors are abundantly expressed in the CeA (Asan et al., 2013) likely functioning as heteroreceptors reducing transmitter release (Starke et al., 1989; Nautiyal and Hen, 2017). Thus, we examined the consequences of chronic alcohol exposure on both CeA *Htr1a* mRNA expression and 5-HT1A-mediated regulation of GABAergic synaptic transmission. As shown in Figure 6A–C, ISH, performed using RNAscope (ACD Biotechnique) technology, revealed no difference in cells expressing *Htr1a* or density of *Htr1a* mRNA in the CeA between treatment groups (i.e., naïve, dependent, and withdrawn; percent cells expressing: ANOVA, $F_{(2,30)} = 0.3300$, $p = 0.7215$, densitometry: ANOVA, $F_{(2,28)} = 0.2289$, $p = 0.7968$). Moreover, we also found that selective 5-HT1A activation using 8-OH-DPAT (10 μ M) decreased CeA mIPSC frequencies to comparable extents in all groups (ANOVA, $F_{(8,69)} = 0.6091$, $p = 0.7972$; naïve: $60.3 \pm 5.5\%$, $t = 7.254$, $df = 7$, $p = 0.0002$; dependent: $69.6 \pm 5.5\%$, $t = 5.491$, $df = 8$, $p = 0.0006$; withdrawn: $63.8 \pm 6.1\%$, $t = 5.974$, $df = 8$, $p = 0.0003$; Fig. 6D,E), while neither mIPSC amplitudes nor kinetics were significantly altered (Fig. 6F). These data suggest a robust 5-HT1A-mediated regulatory role of CeA presynaptic GABA release, which is not compromised by alcohol dependence.

Alcohol dependence disrupts 5-HT2C signaling

5-HT2C plays a pivotal role in anxiety-related behaviors, as activation of amygdalar 5-HT2C induces anxiety-like behavior in rodents, while 5-HT2C antagonism results in anxiolysis (Campbell and Merchant, 2003; Overstreet et al., 2006; Règue et al., 2019). Thus, we determined whether alcohol dependence alters 5-HT2C expression and function. Importantly, we identified a comparable expression pattern of *Htr2c* mRNA in the CeA of all treatment groups (Fig. 7A–C; percent cells expressing: ANOVA, $F_{(2,34)} = 0.2137$, $p = 0.8087$; densitometry: ANOVA, $F_{(2,34)} = 0.4025$, $p = 0.6718$), indicating that alcohol dependence and withdrawal do not affect *Htr2c* mRNA expression. Our electrophysiological studies revealed that activating CeA 5-HT2C using the selective agonist WAY161503 (30 μ M) robustly increased both sIPSC ($171.0 \pm 17.87\%$, $t = 3.973$, $df = 8$; Fig. 7D, E) and mIPSC frequency ($144.4 \pm 6.2\%$, $t = 7.120$, $df = 3$, $p = 0.0057$) in naïve rats without affecting either sIPSC (Fig. 7F) or mIPSC amplitudes, rise times, or decay times. However, the 5-HT2C agonist did not increase sIPSC frequency in either dependent ($99.51 \pm 7.34\%$, $t = 0.06629$; $df = 11$, $p = 0.9483$) or in withdrawn rats ($93.88 \pm 5.97\%$, $t = 1.026$, $df = 11$, $p = 0.3268$; Fig. 7D,E). In agreement, we found that the selective 5-HT2C antagonist SB2420840 (100 nM) decreased sIPSC frequency only in naïve rats ($70.00 \pm 6.79\%$, $t = 4.417$, $df = 7$, $p = 0.0031$; Fig. 7D,F), revealing a basal regulation of the CeA activity by 5-HT2C.

Figure 4. Alcohol dependence and withdrawal dysregulate 5-HT regulation of CeA GABA transmission. **A**, Representative sIPSCs under baseline control conditions (left) and during superfusion of 5-HT ($50 \mu\text{M}$) from the indicated experimental groups. Bars represent the effects of 5-HT ($50 \mu\text{M}$) on sIPSC (**B**) frequency and (**C**) amplitude, and rise and decay times in CeA neurons from naïve, dependent, and withdrawn rats. **D**, Representative mIPSCs under baseline control conditions and during superfusion of 5-HT ($50 \mu\text{M}$) from the indicated experimental groups. Bars represent the effects of 5-HT ($50 \mu\text{M}$) on mIPSC (**E**) frequency and (**F**) amplitude, and rise and decay times in CeA neurons from the indicated groups. A median split analysis (shaded, green bars) for data on 5-HT effects on mIPSC frequencies in withdrawn rats (**E**, green bar) has been performed. Differences between experimental groups were calculated using a one-way ANOVA and Tukey's multiple comparisons test ($\#p < 0.05$; $\#\#p < 0.01$), and differences to baseline control conditions (dashed line) were calculated using one-sample *t* tests ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). Data for 5-HT effects ($50 \mu\text{M}$) on sIPSCs and mIPSCs, respectively, are taken from Figure 1A–D. Error bars indicate mean $\pm$ SEM from $n = 9$ to 13 cells.

Figure 5. 5-HT decreases spontaneous AP firing of CeA neurons from naïve and dependent rats but has split/mixed effects on spontaneous firing in withdrawn rats. **A**, Representative cell-attached recordings from CeA neurons under control conditions and during superfusion of $50 \mu\text{M}$ 5-HT from the indicated animal groups. **B**, Error bars indicate mean $\pm$ SEM, depicting the effect of 5-HT on neuronal firing compared with baseline from 9 to 11 neurons (dashed line). One-sample *t* test: $*p < 0.05$; $**p < 0.01$. A median split analysis was performed for data obtained from CeA neurons from withdrawn rats (green, shaded bars). Differences between groups were assessed using a one-way ANOVA with a Tukey's *post hoc* mean comparison: $\#\#p < 0.01$.

signaling. The 5-HT_{2C} antagonist did not alter sIPSC frequency in either dependent ($94.46 \pm 8.96\%$; $t = 0.6180$ $df = 7$, $p = 0.5561$) or withdrawn rats ($105.4 \pm 8.81\%$, $t = 0.6135$ $df = 7$, $p = 0.5590$; Fig. 7F) and did not affect sIPSC amplitudes or kinetics in any of the groups. Thus, although alcohol dependence and withdrawal did not alter *Htr2c* mRNA expression, they induced a profound and long-lasting change of 5-HT_{2C} function in the CeA. Specifically, CeA network-driven GABA transmission becomes insensitive to regulation by both 5-HT_{2C} agonism and

antagonism with alcohol dependence, and it does not recover in protracted withdrawal. In summary, our data implicate a basal regulation of CeA GABA signaling via 5-HT_{2C} in naïve, which is lost with alcohol dependence and protracted withdrawal.

Discussion

The CeA, the major output area of the amygdalar complex, is a key mediator of negative emotional states associated with alcohol dependence (Wise and Koob, 2014; Koob and Volkow, 2016; Roberto et al., 2020) and is highly sensitive to both chronic and acute alcohol (Roberto et al., 2003, 2004). Elevated GABA signaling in the CeA is considered a hallmark of alcohol dependence across species (Roberto et al., 2004; Herman and Roberto, 2016; Augier et al., 2018; Jimenez et al., 2019). Importantly, the CeA and the DRN are reciprocally connected (Vertes, 1991; Linley et al., 2016), and there is compelling evidence for heightened DRN activity following chronic alcohol exposure in mice (Lowery-Gionta et al., 2015). Thus, given dense expression of 5-HT receptors in the CeA (Asan et al., 2013), we here examined potential neuroadaptations of CeA 5-HT signaling induced by alcohol dependence and withdrawal using *ex vivo* slice electrophysiology and ISH.

Our study unravels a specific vulnerability of the CeA 5-HT system to both chronic alcohol exposure and protracted alcohol

withdrawal. Specifically, we identified distinct, long-lasting neuroadaptations of CeA 5-HT signaling associated with alcohol dependence as well as distinct neuroadaptations resulting from protracted alcohol withdrawal. Since the CeA is predominantly GABAergic, we first studied 5-HT modulation of the two main forms of pharmacologically isolated GABA_A receptor-mediated transmission: (1) AP-dependent GABA_A receptor-mediated transmission (sIPSCs) reflecting the activity of the entire synaptic CeA network; and (2) AP-independent monosynaptic transmission (mIPSCs) mediated via vesicular GABA release.

5-HT strongly increased CeA AP-dependent GABA transmission (sIPSCs) of naive rats, but its efficacy was significantly reduced in alcohol-dependent rats, suggesting that alcohol dependence dampens the sensitivity of the GABAergic synaptic network to exogenous 5-HT. Alternatively, this reduced efficacy of exogenous 5-HT might also reflect a saturated 5-HT receptor system in the CeA. In such a scenario, 5-HT receptors would be maximally activated by elevated levels of endogenous CeA 5-HT originating from increased DRN activity (Lowery-Gionta et al., 2015). Interestingly, 5-HT effects on CeA GABA transmission in withdrawn rats did not significantly differ from either naive or dependent rats, suggesting that the apparent loss of efficacy of exogenous 5-HT action is transient and might fully recover with more prolonged withdrawal. Our study also implicates that 5-HT regulates CeA activity mainly via presynaptic mechanisms. 5-HT increased vesicular GABA release in the CeA of naive, dependent, and withdrawn rats to a similar extent. However, in the CeA of withdrawn rats 5-HT also decreased GABA release in a subset of CeA neurons. Interestingly, we could not find a clear correlation between CeA cell-type (i.e., low-threshold, regular spiking and late spiking neurons) (Chieng et al., 2006) and divergent 5-HT effects (increase/decrease). Thus, given that changes of CeA activity in response to 5-HT likely originate from activation of both excitatory and inhibitory 5-HT receptor subtypes, the observed bidirectional modulation of vesicular CeA GABA release may result from neuroadaptations specifically induced by protracted withdrawal at the presynaptic site. These neuroadaptations could involve mechanisms, such as recruitment/upregulation of distinct inhibitory 5-HT1 subtypes in distinct circuits, downregulation of excitatory 5-HT subtypes, or alterations of intracellular signaling pathways, resulting in differential 5-HT regulation of vesicular GABA release.

Our studies also suggest that 5-HT increases the excitability of CeA neurons in naive rats indicated by flattened slopes of the current-voltage-relationships in the presence of 5-HT and a decreased AP threshold. In addition, 5-HT decreased membrane

Figure 6. Intact 5-HT_{1R} signaling in alcohol-dependent and withdrawn rats. **A**, Representative images of *Htr1a* (green) and DAPI (blue) in the CeA are shown for naive, dependent (Dep.), and withdrawn (WD) groups. Scale bar, 10 μm. Error bars indicate mean ± SEM of (**B**) the percent cells expressing *Htr1a* relative to naive in CeA for the indicated groups, and (**C**) optical density of *Htr1a* relative to the naive in the CeA for the indicated groups. **D**, Representative traces of mIPSC recordings under baseline control conditions and during superfusion of the 5-HT_{1A} agonist 8-OH-DPAT (20 μM) from the indicated experimental groups. Error bars indicate mean ± SEM of 8-OH-DPAT on mIPSC (**E**) frequency and (**F**) amplitude, and rise and decay times in CeA neurons from naive, dependent, and withdrawn rats compared with baseline from 8 or 9 cells/group. Statistically significant differences between groups were calculated using a one-way ANOVA (Tukey's *post hoc* mean comparison). Differences from baseline (dashed line) were assessed with one-sample *t* tests: ****p* < 0.001. n.s., not significant.

resistance, indicating elevated membrane conductance. Previous studies (e.g., in motoneurons) suggested that these excitatory 5-HT effects occur via activation of different 5-HT receptors that in turn modulate various ion channels (Perrier et al., 2013). Interestingly, 5-HT almost completely abolished spontaneous AP firing of CeA neurons in naive rats, suggesting that, despite increased neuronal excitability, the large 5-HT-induced GABA release inhibits firing. Importantly, we found a similar 5-HT regulation of AP firing in neurons from dependent rats but again bidirectional modulation of neuronal firing in alcohol withdrawn rats. Of note, despite marked elevation of inhibitory GABAergic signaling in dependent and withdrawn rats compared with naive controls (see also Roberto et al., 2004; Khom et al., 2020), we did not find any significant differences in spontaneous firing rates between the treatment groups. Hence, one might speculate that the elevated

Figure 7. Alcohol dependence and withdrawal disrupt CeA 5-HT_{2C} receptor signaling. **A**, Representative images of *Htr2c* (red) and DAPI (blue) in the CeA are shown for naive, dependent (Dep), and withdrawn (WD) groups. Scale bar, 10 μ m. Error bars indicate mean $\pm$ SEM of **(B)** the percent cells expressing *Htr2c* relative to naive in the CeA for the indicated groups, and **(C, D)** optical density of *Htr2c* relative to the naive in the CeA for the indicated groups. Representative sIPSC recordings under baseline control conditions and during superfusion of either the 5-HT_{2C} agonist (30 μ M WAY161503) **(E)** or the 5-HT_{2C} antagonist (100 nM SB242084) in the indicated experimental group. Error bars indicate mean $\pm$ SEM and depict changes of sIPSC frequencies, amplitudes, and current kinetics in the presence of **(F)** the 5-HT_{2C} agonist or **(G)** the 5-HT_{2C} antagonist compared with baseline. Differences between groups were calculated **(D)** using a one-way ANOVA (Tukey's *post hoc* mean comparison) and differences from baseline were assessed with one-sample *t* tests: ***p* < 0.01; ##*p* < 0.01. n.s., not significant.

CeA GABAergic tone characteristic to alcohol dependence stems from local and extra-CeA inputs.

Among the 14 different 5-HT-gated receptors (McCorvy and Roth, 2015), multiple studies identified 5-HT_{1A} and 5-HT_{2C} as key players in addictive behaviors, including AUD (Müller et al., 2007; Marcinkiewicz et al., 2016), and both are densely expressed in the CeA (Asan et al., 2013). It has been reported that 5-HT_{1A} mediates alcohol-induced aggressive behaviors in mice (Miczek et al., 1998), and selective 5-HT_{1A} agonists decrease alcohol drinking of monkeys (McKenzie-Quirk and Miczek, 2003) and alcohol preference in Wistar rats (Schreiber et al., 1993). In addition, *Htr1a* polymorphisms have also been recently associated with substance use disorder, including AUD (Donaldson et al., 2016) and increased susceptibility to depression and related mood disorders (Le François et al., 2008). 5-HT_{1A} receptors are abundantly expressed in the brain, including the CeA (Asan et al., 2013), serving as both autoreceptors and heteroreceptors (limiting either serotonin or other neurotransmitter release), and thus play a critical role in negative feedback (Starke et al., 1989; Nautiyal and Hen, 2017). Our study revealed that alcohol dependence and protracted alcohol withdrawal did not compromise CeA 5-HT_{1A} function or *Htr1a* expression. We report that a selective 5-HT_{1A} agonist decreased CeA GABA release in all treatment groups to similar extents, suggesting that 5-HT_{1A} signaling might be a homeostatic regulator of CeA synaptic transmission. Importantly, decreasing inhibitory inputs onto CeA neurons could also result in disinhibition; but given that elevated CeA GABA transmission mediates the negative emotional state of alcohol dependence (Roberto et al., 2004; Herman et al., 2016;

Augier et al., 2018; Jimenez et al., 2019), targeting 5-HT_{1A} to reduce CeA GABAergic tone could have therapeutic potential for the treatment of AUD.

Last, our study also identified long-lasting, dysregulated CeA 5-HT_{2C} signaling following alcohol dependence. Increased 5-HT_{2C} signaling in the BNST has been implicated in the etiology of AUD and associated affective and anxiety disorders (Marcinkiewicz et al., 2015). In addition, increased density of amygdalar 5-HT_{2C} has been found in genetic models of alcohol preference (Pandey et al., 1996). In line with this, the selective 5-HT_{2C} agonist lorcaserin reduced alcohol intake in alcohol-preferring rats, further corroborating the critical role of 5-HT_{2C} signaling in alcohol dependence (Rezvani et al., 2014). Here, we found that activation of the 5-HT_{2C} using a selective agonist robustly increased CeA GABA release likely via a presynaptic mechanism in naive rats. Conversely, the 5-HT_{2C} antagonist decreased CeA GABA release, indicative of endogenous 5-HT signaling via 5-HT_{2C} receptors regulating CeA synaptic activity. Notably, this regulatory function of CeA activity by the 5-HT_{2C} was abolished by chronic alcohol exposure, whereas *Htr2c* mRNA expression was not affected by either chronic alcohol exposure or subsequent protracted withdrawal. However, the ISH is limited in its abilities to detect subcellular localization of mRNA that may be trafficked to distal processes outside of the nucleus. Additionally, it is well accepted that mRNA levels do not necessarily reflect levels of translated protein, and protein localization and activity may also impact function/signaling without altering mRNA levels. Furthermore, there is growing evidence that *Htr2c* undergoes RNA editing in neuropsychiatric

disease, such as PTSD (Baratta et al., 2016), and such *Htr2c* alternative splicing can cause the resulting 5-HT_{2C} isoform to be constitutively active in the absence of ligands (Tanaka and Watanabe, 2019). Chronic ethanol vapor exposure in mice (C57BL/6) also increases levels of edited isoforms of *Htr2c* mRNA (Watanabe et al., 2014) in the nucleus accumbens, which is associated with increased ethanol drinking. Thus, it is tempting to speculate that similar changes of *Htr2c* might occur in the CeA. Alternatively, heightened DRN activity following chronic alcohol exposure (Lowery-Gionta et al., 2015) accompanied by potentially decreased amygdalar serotonin transporter levels (Mantere et al., 2002; Storvik et al., 2007, 2008) suggests increased 5-HT levels in the CeA. In such a scenario, CeA 5-HT_{2C} would be already saturated; therefore, application of an agonist would not result in further increases in GABA signaling.

Collectively, our study provides detailed insights into modulation of CeA activity by the 5-HT system and how alcohol dependence and protracted alcohol withdrawal dysregulate CeA 5-HT signaling at multiple levels. Notably, this is the first time we are reporting neuroadaptations in rat CeA 5-HT signaling originating from protracted alcohol withdrawal. Often, it is assumed that protracted withdrawal reverses maladaptive processes stemming from chronic drug exposure. However, AUD is not conceptualized as the mere result of repeated alcohol intake but as a complex disorder shaped by the negative emotional states associated with the absence of alcohol (Koob, 2013, 2015). Thus, it is conceivable that alcohol dependence-induced maladaptive processes last into protracted withdrawal, but it is also important to understand that protracted drug abstinence induces neuroadaptations in various neurochemical signaling systems. Hence, this study further expands our knowledge of CeA cellular mechanisms involved in the pathophysiology of alcohol dependence and withdrawal.

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