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Serotonin Attenuates Feedback Excitation onto O-LM Interneurons

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Abstract

The serotonergic system is a subcortical neuromodulatory center that controls cortical information processing in a state-dependent manner. In the hippocampus, serotonin (5-HT) is released by ascending serotonergic fibers from the midbrain raphe nuclei, thereby mediating numerous modulatory functions on various neuronal subtypes. Here, we focus on the neuromodulatory effects of 5-HT on GABAergic inhibitory oriens lacunosum-moleculare (O-LM) cells in the hippocampal area CA1 of the rat. These interneurons are thought to receive primarily local excitatory input and are, via their axonal projections to stratum lacunosum-moleculare, ideally suited to control entorhinal cortex input. We show that 5-HT reduces excitatory glutamatergic transmission onto O-LM interneurons. By means of paired recordings from synaptically connected CA1 pyramidal cells and O-LM interneurons we reveal that this synapse is modulated by 5-HT. Furthermore, we demonstrate that the reduction of glutamatergic transmission by serotonin is likely to be mediated via a decrease of calcium influx into presynaptic terminals of CA1 pyramidal cells. This modulation of excitatory synaptic transmission onto O-LM interneurons by 5-HT might be a mechanism to vary the activation of O-LM interneurons during ongoing network activity and serve as a brain state-dependent switch gating the efficiency of entorhinal cortex input to CA1 pyramidal neurons.

Key words: 5-HT, CA1, paired recordings, presynaptic neuromodulation

Introduction

The hippocampus receives a dense serotonergic innervation originating from the midbrain raphe nuclei, both from the Median raphe nucleus and to a lesser extend from the Dorsal raphe nucleus (Freund et al. 1990; Vertes 1991, 1999). Earlier studies on

the cellular neurophysiology of serotonergic signaling predominantly focused on the neuromodulation of intrinsic cellular properties and the neuromodulation of synaptic transmission on hippocampal pyramidal neurons. In these studies, multiple

important effects of 5-HT have been identified, mediated by a number of distinct pre- and/or postsynaptic receptors (Segal 1980; Andrade and Nicoll 1987; Barnes and Sharp 1999). In contrast, relatively few studies investigated the neuromodulatory effects of 5-HT on GABAergic inhibitory interneurons in the hippocampus (Varga et al. 2009; Winterer et al. 2011; Chittajallu et al. 2013). Complicated by the high diversity of interneurons it has not yet been possible to establish a clear picture of how serotonin acts on these neuronal subtypes (Parra et al. 1998).

GABAergic interneurons are recognized as key players in the synchronization of neuronal activity on various timescales and in the generation of oscillatory patterns in the brain. These different types of interneurons exhibit morphological, immunohistochemical, and electrophysiological characteristics and are differentially involved in oscillations at different frequencies (Maccaferri and Lacaille 2003; Maccaferri 2005; Somogyi and Klausberger 2005). One of the major classes of GABAergic interneurons in the stratum oriens of the CA1 subfield of the hippocampus are oriens lacunosum-moleculare (O-LM) interneurons. These interneurons fire correlated with hippocampal rhythms (Klausberger et al. 2003; Goldin et al. 2007; Varga et al. 2012; Pangalos et al. 2013; Katona et al. 2014) and have been hypothesized to coordinate cell assemblies (Tort et al. 2007). O-LM cells can be considered as classical feedback inhibitory neurons due to their predominantly local excitatory input from CA1 pyramidal cells. Their axonal projections impinge on distal apical dendrites of CA1 pyramidal cells as well as on local inhibitory interneurons targeting the proximal portions of CA1 pyramidal dendrites. In this respect O-LM interneurons are ideally suited to gate the activity in area CA1, where they are able to facilitate the input from the Schaffer collaterals via an indirect disinhibition of the proximal dendritic compartments while reducing the input of the temporoammonic (TA) pathway by inhibiting the distal apical dendrites of CA1 pyramidal neurons (Leão et al. 2012).

Here, we show that 5-HT reduces excitatory glutamatergic inputs onto O-LM interneurons and lowers their spike probability in area CA1 of the hippocampus. We identify one source of excitatory input that is modulated by 5-HT by paired recordings of synaptically connected CA1 pyramidal neurons and O-LM interneurons. Furthermore, our results indicate that this modulation is mediated by a decrease of calcium influx into presynaptic terminals of CA1 pyramidal cells.

Material and Methods

Ethics Statement

Animal husbandry and experimental intervention was performed according to the European Council Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes. All animal maintenance and experiments were performed in accordance with the guidelines of local authorities, Berlin (T0100/03).

Preparation

Hippocampal slices were prepared from Wistar rats (P16-24, both sexes) as previously described (Schmitz et al. 2003). In brief, the animals were anesthetized with isoflurane, decapitated and the brains were removed. Tissue blocks containing the subicular area and hippocampus were mounted on a Vibratome (Leica VT1200 S) in a chamber filled with ice-cold artificial cerebrospinal fluid, ACSF, containing (in mM): NaCl, 87; sucrose, 75; NaHCO$_3$, 26; KCl, 2.5; NaH$_2$PO4, 1.25; CaCl$_2$, 0.5; MgCl$_2$, 7; glucose, 25, saturated with 95% O$_2$, 5% CO$_2$, pH 7.4. Transverse slices were cut at 300 μm

thickness. The slices were taken from ventral to medial hippocampus. They were kept at 35°C for 30 min and then stored in a submerged chamber, where they were kept for 1–4 h before being transferred to the recording chamber. Another subset of slices was transferred directly after cutting to an interface chamber where they were stored at ∼32°C before being transferred to the recording chamber. The effect of serotonin was robust in both storage conditions.

In the recording chamber, slices were perfused with ACSF containing (in mM): NaCl, 119; NaHCO$_3$, 26; glucose, 10; KCl 2.5, CaCl$_2$, 2.5; MgCl$_2$ 1.3; NaH$_2$PO$_4$, 1 at a rate of 4–5 mL/min at 31–34°C. All ACSF was equilibrated with 95% O$_2$ and 5% CO$_2$.

Electrophysiology

Whole-cell recording electrodes were filled with (in mM): K-gluconate 120–135, HEPES 10, Mg-ATP 2, KCl 20, EGTA 0.5, Phosphocreatine 5 adjusted to 7.3 with KOH. For paired recordings, the electrode for the presynaptic CA1 pyramidal neuron was filled with (in mM): K-gluconate 105, HEPES 10, Na$_2$-ATP 2, Na$_2$-GTP 0.3, Mg-ATP 2, KCl 40, MgCl$_2$ 2, EGTA 0.1, Na$_2$-phosphocreatine 1, L-glutamate 0.1, adjusted to 7.3 with KOH. For staining and reconstruction of the recorded neurons, ∼0.25% biocytin was added to the intracellular solution. Depolarizing current steps of 1 s duration were applied to characterize the cells' discharge behavior.

Excitatory postsynaptic responses were evoked by electrical stimulation (100 μs at intervals of 50 ms) in *stratum oriens* of area CA1 via a broken patch-pipette (∼8 μm) filled with ACSF. Experiments were done in the presence of the GABA-A receptor antagonist gabazine (1 μM) and NBQX (100 nM) to prevent epileptiform activity and to minimize polysynaptic activity except where pairs or extracellular evoked spikes (Fig. 2D) were recorded. mEPSCs were recorded in the presence of tetrodotoxin (TTX) (1 μM) and gabazine (1 μM).

Peaks of extracellularly evoked spikes exhibited a delay of ∼4–8 ms to the stimulus. The interstimulus interval was set to 125 ms. In this set of experiments, the membrane potential of O-LM interneurons was kept constant at –60 mV.

Access resistances ranged between 9 and 31 MΩ (on average 14.2 ± 0.8 MΩ) for O-LM interneurons. They were continuously monitored during the recording and were not allowed to vary >30% during the course of the experiment. In current-clamp configuration, bridge balance compensation was used. In some experiments in voltage-clamp configuration slight changes in series resistance were compensated. Electrode resistances ranged from 3 to 5 MΩ.

Morphology of O-LM Interneurons and CA1 Pyramidal Neurons

After recording, slices were transferred into a fixative solution containing 4% paraformaldehyde and 0.2% saturated picric acid in 0.1 M phosphate buffer. To reveal the presynaptic axonal arborization and dendritic arbors in detail, the biocytin-filled cells were subsequently visualized with 3,3′-diaminobenzidine tetrahydrochloride (0.015%) using standard ABC kit (Vector) and reconstructed with the Neurolucida 3D reconstruction system (MicroBrightField, Inc., Williston, VT, USA).

Glutamate Uncaging

20 mL of 50 or 200 μM (MNI)-caged-L-glutamate (Tocris, Bristol, UK) were reperfused at 2.5–3.0 mL/min. For uncaging, we used a UV pulsed laser (Rapp Optoelektronik, Wedel, Germany) attached

with a 200 μm optical fiber coupled into the epifluorescence port of the microscope with an OSI-BX adapter (Rapp Optoelektronik, Wedel, Germany) and focused on the specimen by the objective lens. This yielded an illuminated circle of 20–50 μm. The duration of the laser flash was 2 ms. The laser power under the objective corresponding to the stimulus intensity level used was monitored using a photo diode array-based photodetector (PDA-K-60, Rapp Optoelectronics, Wedel, Germany) and did not change over time.

Glutamate was uncaged over the cell soma in the presence of the GABA-A receptor antagonist gabazine (1 μM) and NBQX (100 nM, to prevent epileptiform activity).

Fluorescence Measurements

The axonal fibers in CA1 *stratum oriens* were locally labeled with a pressure stream of the low-affinity calcium indicator magnesium green AM (Invitrogen, Molecular Probes) dissolved in 5% Pluronic for photodiode measurements (Breustedt et al. 2003). The indicator was injected into area CA1 stratum oriens, the filling pipette pointing toward the alveus (Fig. 6A). Recordings were started 40–90 min after slices were labeled. Axons were stimulated extracellularly (Fig. 6A) and epifluorescence was measured with a single photodiode from a spot a few hundred micrometers away from the loading site. The signals from the photodiode were digitized by data acquisition hardware (PCI-6036E National Instruments, Austin, TX) at 5 kHz. The fluorescence intensity was measured alternating every 30 s with and without stimulus and the change in fluorescence intensity (ΔF) relative to the initial baseline of fluorescence (F) was calculated. To exclude any postsynaptic contribution to the signal all recordings were performed in NBQX (20 μM) and D-AP5 (50 μM).

Data Analysis

Data were acquired and analyzed with Igor Pro software (WaveMetrics, Lake Oswego, OR), NeuroMatic and custom written MATLAB scripts (The MathWorks, Natick, MA).

Values in the text and the figures are expressed as mean ± standard error of the mean (SEM) unless indicated otherwise (as median and interquartile range, IQR). The nonparametric Wilcoxon rank test was used for statistical comparisons in sets of experimental data where normality could not be assumed. To compare the numbers of successfully evoked spikes in control versus fenfluramine, McNemar's test was used (Fig. 2D). For normal distributed sets of data a paired or unpaired Student's *T*-test was used. Differences were considered statistically significant if $P < 0.05$. All traces are averages of 5–10 sweeps unless otherwise stated. For display purposes some traces were low-pass filtered (<2 kHz) and a 50 Hz notch filter to remove line hum was applied if necessary. In paired recordings (Fig. 4C) successful synaptic transmission was counted as such if the postsynaptic amplitude was larger than 1.5 standard deviations of the baseline (in the absence of spontaneous events).

Drugs

5-Hydroxytryptamine creatine sulfate complex (5-HT), (*RS*)-*N*-ethyl-1-[3-(trifluormethyl)phenyl]propan-2-amin(fenfluramine) (both from Sigma), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*] quinoxaline-2,3-dione (NBQX), 4-[6-imino-3-(4-methoxyphenyl) pyridazin-1-yl] butanoic acid hydrobromide (SR95531, gabazine), D-(–)-2-amino-5-phosphonopentanoic acid (D-AP5), 4-Methoxy-7-nitroindolinyl-caged-L-glutamate (MNI-caged-L-glutamate), (4R,4aR,5R,6S,7S,8S,8aR,10S,12S)-2-azaniumylidene-4,6,8,12-tetra-

hydroxy-6-(hydroxymethyl)-2,3,4,4a,5,6,7,8-octahydro-1*H*-8a, 10-methano-5,7-(epoxymethanooxy) quinazolin-10-olate (TTX), 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-pyrrolo[3,2-b] pyridine hydrochloride (CP 94253 hydrochloride), (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT hydrobromide), (5′α,10α)-9,10-dihydro-12′-hydroxy-2′-(1-methylethyl)-5′-(phenyl-methyl)-ergotaman-3′,6′,18-trione mesylate (dihydroergocristine mesylate), (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4, 5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390 hydrochloride) (all from Tocris), Magnesium green AM (Invitrogen, Molecular Probes).

Results

O-LM interneurons were identified by their location in *stratum oriens*, the characteristic elongated shape of their somata and the horizontally oriented dendrites as well as their electrophysiological properties: the typical firing pattern in response to depolarizing current pulses and the prominent sag potential in response to hyperpolarizing current pulses (Fig. 1A, right, top). O-LM interneurons displayed a strong facilitation in response to consecutive synaptic stimuli (Fig. 1A, right bottom), as described previously (Ali and Thomson 1998; Losonczy et al. 2002; Biró et al. 2005). A subset of the recorded cells, identified by the above described criteria, were further validated by biocytin stainings in which we could identify the vertically projecting axons with an extensive arborization in stratum lacunosum-moleculare (Fig. 1A, left).

5-HT Reduces Glutamatergic Transmission on O-LM Interneurons

To study the excitatory synaptic transmission onto O-LM interneurons, cells were held in voltage-clamp mode at −60 mV. First, we examined the effect of 5-HT on the frequency of spontaneous excitatory postsynaptic currents (sEPSCs). The frequency decreased from 4.1 ± 0.6 Hz to 2.3 ± 0.3 Hz during application of 5-HT. This effect was fully reversible during washout: 5.8 ± 0.6 Hz ($n = 8$; control vs. 5-HT: $P = 0.0032$; 5-HT vs. wash: $P = 0.0003$, Student's *T*-test; Fig. 1B,C, left). The amplitude of sEPSCs did not change significantly (baseline: 21.4 ± 3.1 pA vs. 19.2 ± 1.6 pA during application of 5-HT, $P = 0.39$, paired Student's *T*-test, $n = 8$; recovery: 23.7 ± 3.0 pA, Fig. 1B,C, right).

Next, we examined the effect of 5-HT on stimulus-induced glutamatergic transmission in O-LM interneurons which was evoked by a stimulating electrode positioned at the border of the *alveus* and *stratum oriens* (Fig. 2A). 5-HT profoundly and reversibly reduced the amplitude of evoked excitatory postsynaptic currents (eEPSCs) by 50.86% (median, IQR: 28.16%, $n = 12$; Fig. 2B1,B2; baseline amplitude is significantly different from amplitude during application of 5-HT, $P = 0.0005$, paired sample Wilcoxon rank test). Upon application of 5-HT, we further observed a change in the holding current of the recorded cells: O-LM interneurons displayed a mean inward current of $−61.6 \pm 13.0$ pA ($n = 13$; data not shown) indicative of a postsynaptic expression of 5-HT receptors in O-LM interneurons (Lee et al. 1999; Chittajallu et al. 2013).

### Fenfluramine Mimics the Effect of 5-HT on Glutamatergic Transmission

To address the question whether physiological release of 5-HT from serotonergic fibers in the hippocampus induces similar effects, we used the compound fenfluramine, which is thought to provoke the release of serotonin: fenfluramine disrupts the
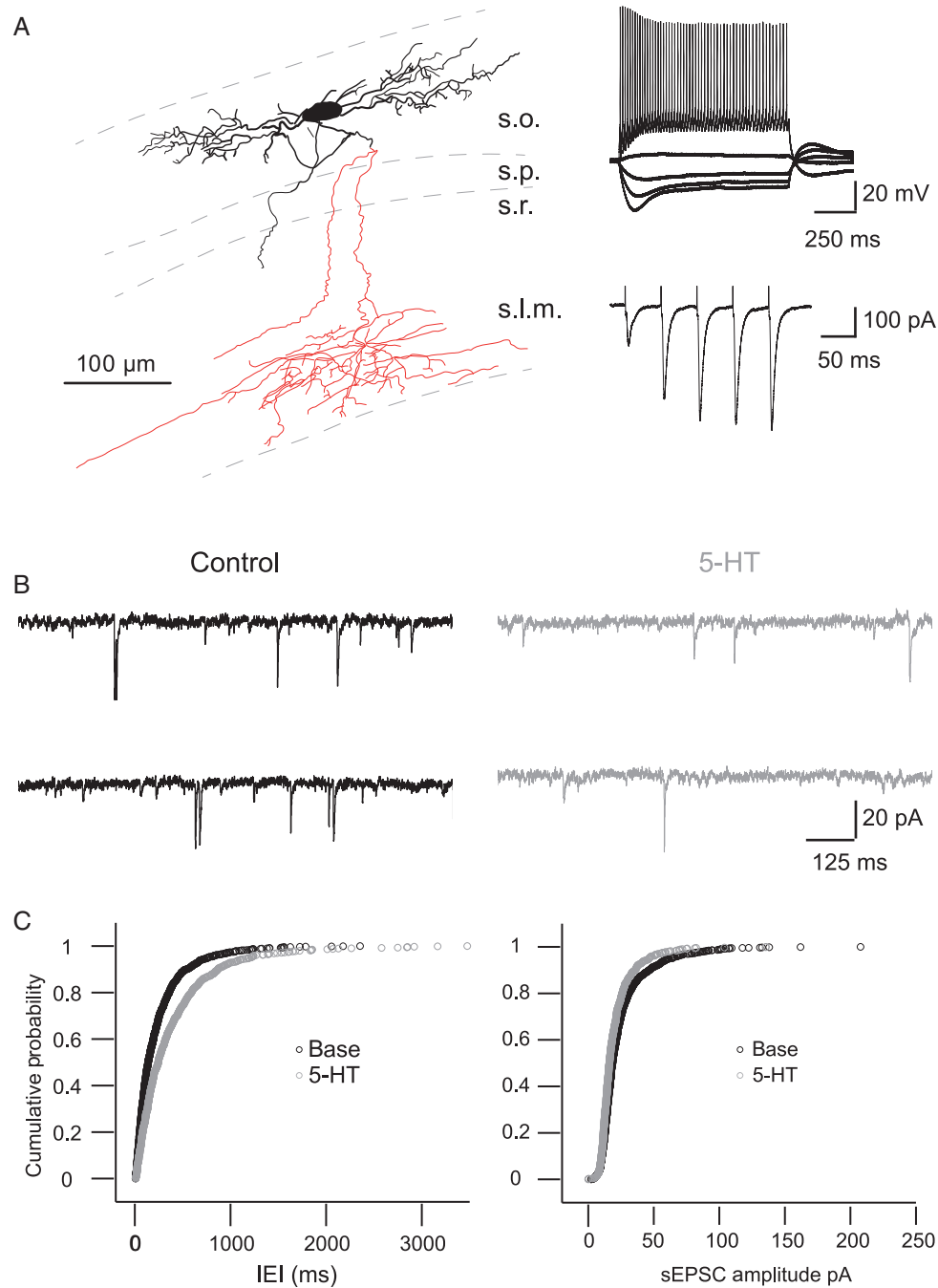
Figure 1. 5-HT inhibits spontaneous EPSCs in O-LM interneurons. (A) Left, reconstruction of an O-LM interneuron (dashed: outline of cell layers, black: cell body and dendrites, red: axon). Right top, typical voltage responses of an O-LM cell to de- and hyperpolarizing current pulses. Right bottom, strongly facilitating excitatory postsynaptic current amplitudes to consecutive extrasynaptic stimuli. (B) Example traces of spontaneous EPSCs under control conditions and in 10 μM 5-HT. (C) Cumulative probability of interevent intervals (IEI, left) and amplitude of spontaneous EPSCs (right) under control conditions, in 10 μM 5-HT and after wash (n = 8). s.l.m., *stratum lacunosom-moleculare*; s.o., *stratum oriens*, s.p., *stratum pyramidale*; s.r., *stratum radiatum*.

vesicular storage of 5-HT and consecutively reverses the serotonin transporter. As a result, the extracellular concentration of 5-HT is increased. In the following sets of experiments, we tested whether the inhibitory effect of serotonin on EPSCs could be mimicked by fenfluramine induced release of endogenous 5-HT from hippocampal serotonergic fibers. We observed a clear and reversible reduction of the EPSC amplitude by application of fenfluramine by 26.15%, median, IQR: 8.95% (amplitude before and during fenfluramine application is significantly different, paired

sample Wilcoxon rank test $P = 0.0156$, Fig. 2C1,C2, $n = 7$). Furthermore, we investigated the effect of fenfluramine on the spiking probability of O-LM interneurons. Spikes could be readily evoked by a theta stimulation protocol in which 5 brief current pulses with an interstimulus interval of 125 ms were delivered. The number of spikes elicited was either strongly reduced or all spikes were abolished under fenfluramine (number of successfully evoked spikes in control vs. fenfluramine: $P < 0.000001$, McNemar's test, Fig. 2D).
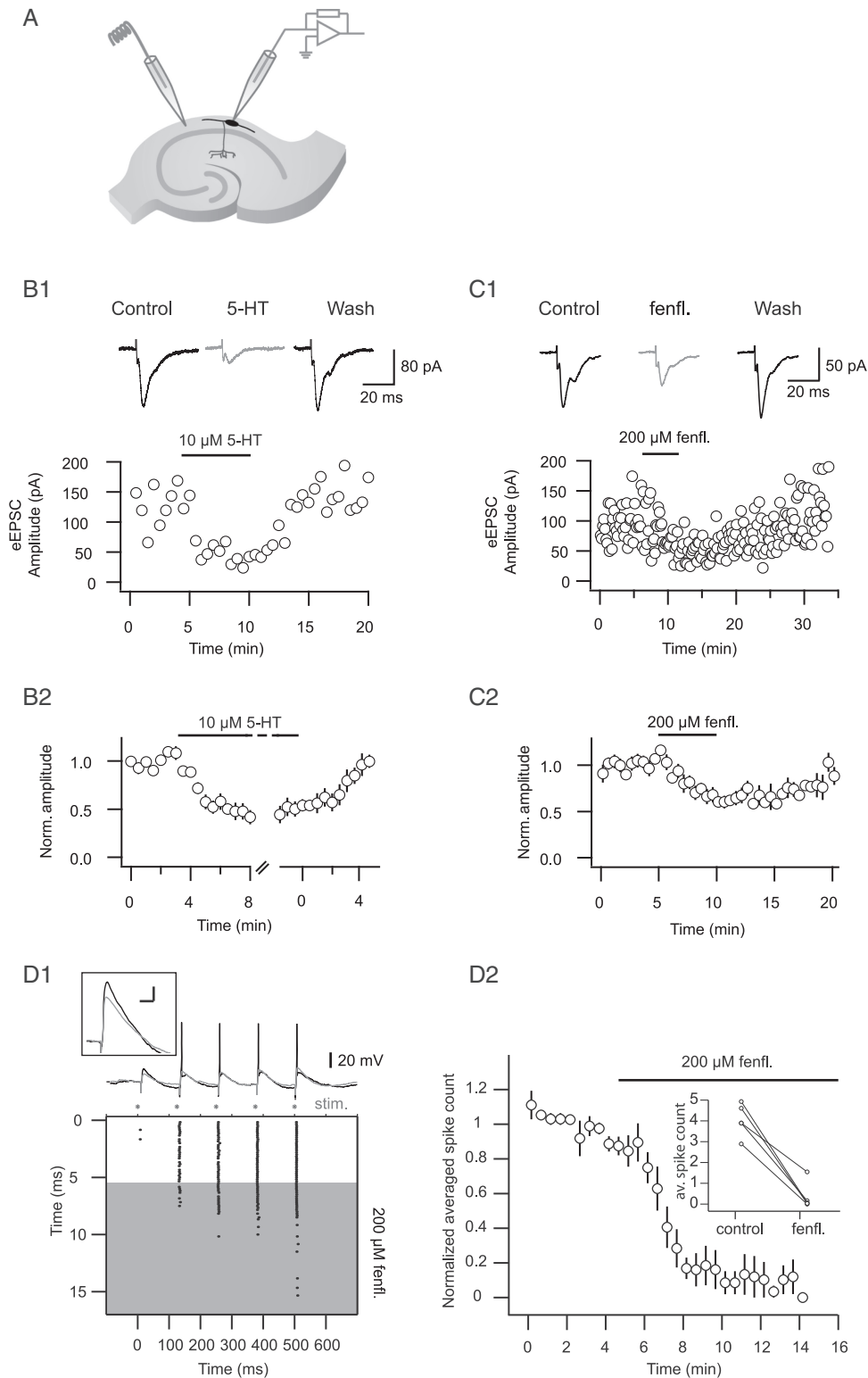
Figure 2. Bath-applied as well as endogenously released 5-HT decreases stimulus-evoked EPSCs. (A) Recording configuration with an extracellular stimulating electrode positioned at the border of the *alveus* and *stratum oriens*. (B1) Top, example traces of stimulus-evoked EPSCs. Bottom, time course of the EPSC amplitude before, during and after washout of 10 μM 5-HT. (B2) Summary of the time course of the normalized and binned EPSC amplitudes (*n* = 12). (C1) Top, example traces of stimulus-evoked EPSCs. Bottom, time course of the EPSC amplitude before, during and after washout of 200 μM fenfluramine. (C2) Summary of time course of the normalized and binned EPSC amplitudes (*n* = 7). (D) Endogenously released 5-HT reduces spiking probability. (D1) Example experiment where spikes were evoked in an O-LM interneuron by extracellular stimulation. Five pulses at theta frequency (interstimulus interval: 125 ms) were delivered. * Indicate stimulus time points. Top, example traces in control conditions (black) and in fenfluramine (200 μM, gray). (Inset) EPSP evoked by the first stimulus in control condition (black) and in fenfluramine (gray), average of 4 traces each, scale bar, *x*: 20 ms, *y*: 5 mV. Bottom, time course of experiment displayed as raster plot of spikes evoked in response to stimulation. Spikes were abolished shortly after washing in fenfluramine (gray area). (D2) Summary, spike count normalized to the summed and averaged spike counts of trials under control conditions. Inset, averaged spike counts under control conditions and in fenfluramine (*n* = 5).

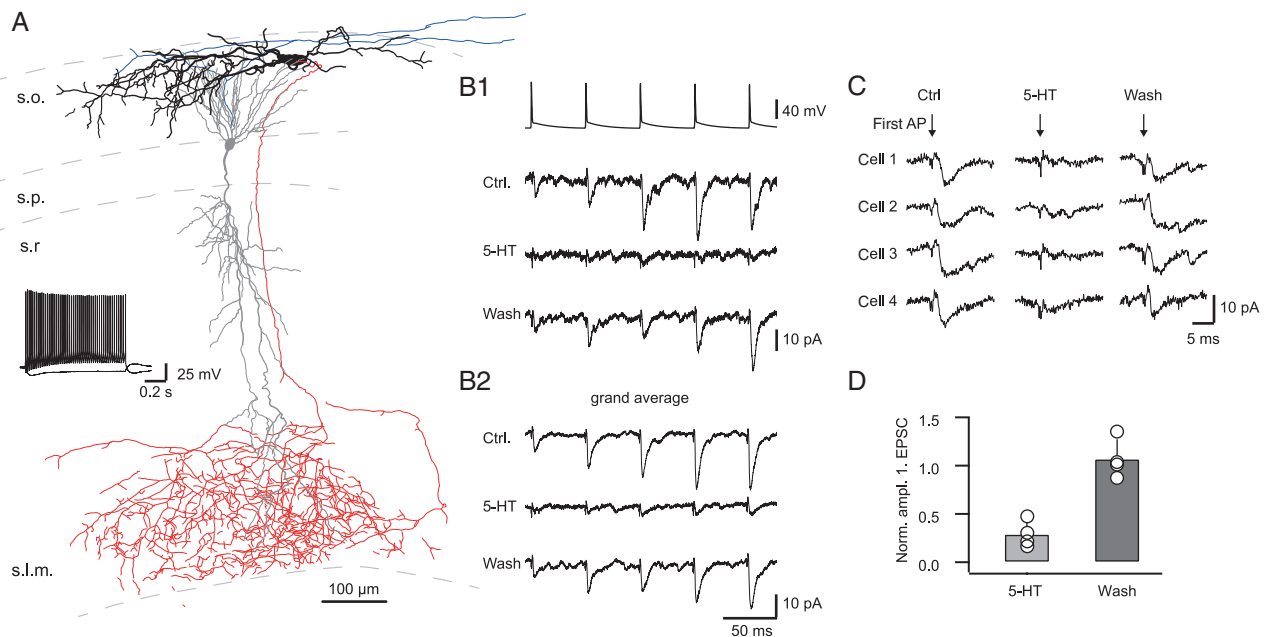## 5-HT Reduces Excitatory Synaptic Transmission at the CA1 Pyramidal Cell–O-LM Interneuron Synapse

O-LM interneurons are considered as classical feedback inhibitory interneurons as they receive excitatory input predominantly from recurrent CA1 pyramidal cell axons (Blasco-Ibáñez and Freund 1995). To test the assumption that 5-HT acts at the CA1 pyramidal–O-LM interneuron synapse, we performed paired recordings from synaptically connected CA1 pyramidal cells and O-LM interneurons. In this set of experiments, we tested 18 simultaneously recorded CA1 pyramidal cell–O-LM interneuron pairs, 5 of which were synaptically connected. Post hoc neuroanatomical analysis confirmed the cellular identities of O-LM and pyramidal neurons in all 5 synaptically connected paired recordings (Fig. 3A). O-LM interneurons displayed a strong facilitation of the postsynaptic amplitude in response to consecutive action potentials (APs) elicited in the presynaptic pyramidal cell (Fig. 3B1). The mean baseline amplitude of the first unitary EPSC (uEPSC) was 7.2 ± 0.4 pA (n = 4; one of the O-LM interneurons displayed a reliably detectable postsynaptic current only after the fourth presynaptic AP and was therefore not considered for analysis). After application of 5-HT, we observed a profound and fully reversible reduction in the amplitudes of all uEPSCs (Fig. 3B1,B2). 5-HT reduced the amplitude of the first uEPSC to 30.7 ± 6% (n = 4; Fig. 3C and D). Taken together, these results confirm the assumption that 5-HT reduces the excitatory drive from local CA1 collaterals onto O-LM interneurons.

## Presynaptic Modulation of Glutamatergic Transmission onto O-LM Interneurons

We sought to further characterize the mechanism underlying the observed modulation of glutamatergic inputs onto O-LM

interneurons in area CA1. We considered 3 possible scenarios to explain our findings: 1) The observed reduction in amplitude of EPSCs could be due to a modulation on the presynaptic site of excitatory terminals; 2) it could be mediated by modifications at the postsynaptic site, or 3) a combination of both scenarios could account for the effect of 5-HT. To study the location of 5-HT action, we first investigated the effect of 5-HT on miniature EPSCs (mEPSCs), recorded in the presence of the sodium channel blocker TTX. Under these conditions, neurotransmitter release upon spontaneous vesicle fusion can be tested independent of AP-mediated Ca^{2+} influx. There was no significant difference in the incidence or amplitude of mEPSCs after bath application of 5-HT (Fig. 4A, n = 10, frequency: control 2.4 ± 0.3 Hz vs. 1.9 ± 0.3 Hz in 5-HT (P = 0.15), amplitude: control: 24.3 ± 2.2 pA vs. 22.6 ± 2.1 pA in 5-HT, P = 0.191, paired Student's T-test).

To gain further insights into the site of serotonergic action, we performed single-photon laser stimulation of MNI-caged-L-glutamate. In this set of experiments, a constant amount of caged glutamate is uncaged by laser stimulation and therefore the presynaptic site is not involved. However, a decrease in the glutamate evoked EPSC amplitude by the application of 5-HT was not observed (Fig. 4B, n = 4). Furthermore, we analyzed the incidence of synaptic failures of the first and second EPSC in the synaptically connected paired recordings. We observed that the failure rate increased upon application of 5-HT (n = 4; failure rate of the first EPSC under control condition: 64.1 ± 5% vs. failure rate in 5-HT: 81.6 ± 3%, Fig. 4C. Failure rate of the second EPSC under control condition: 39.2 ± 3% vs. failure rate in 5-HT: 72.7 ± 7%). Summarizing these findings supports the conclusion that a postsynaptic mechanism is unlikely to be responsible for the reduced excitatory glutamatergic transmission onto O-LM

Figure 3. Serotonin reduces glutamatergic excitatory synaptic transmission at the CA1 pyramidal cell–O-LM interneuron synapse. (A) Reconstruction of a synaptically connected pyramidal cell–O-LM interneuron pair (dashed: outline of cell layers, black: cell body and dendrites of O-LM interneuron, red: axon of O-LM interneuron; gray: cell body and dendrites of pyramidal cell, blue: axon of pyramidal cell). Middle, left, voltage responses of the shown O-LM cell to de- and hyperpolarizing current pulses. (B1) Strongly facilitating unitary excitatory postsynaptic currents to consecutive APs, elicited in the presynaptic pyramidal cell. Below the postsynaptic response to consecutive APs after 5-HT application and after washout of 5-HT are shown. (B2) Grand average of the postsynaptic responses of 4 connected pairs in the indicated conditions. (C) Traces of uEPSCs evoked by the first presynaptic AP (time point indicated by arrow) in 4 cells under control conditions, after application of 10 µM 5-HT and after washout of 5-HT. (D) Summary of the reduction of the normalized first uEPSC amplitude by application of 5-HT and after washout of 5-HT (n = 4). s.l.m., *stratum lacunosom-moleculare*; s.o., *stratum oriens*, s.p., *stratum pyramidale*; s.r., *stratum radiatum*.
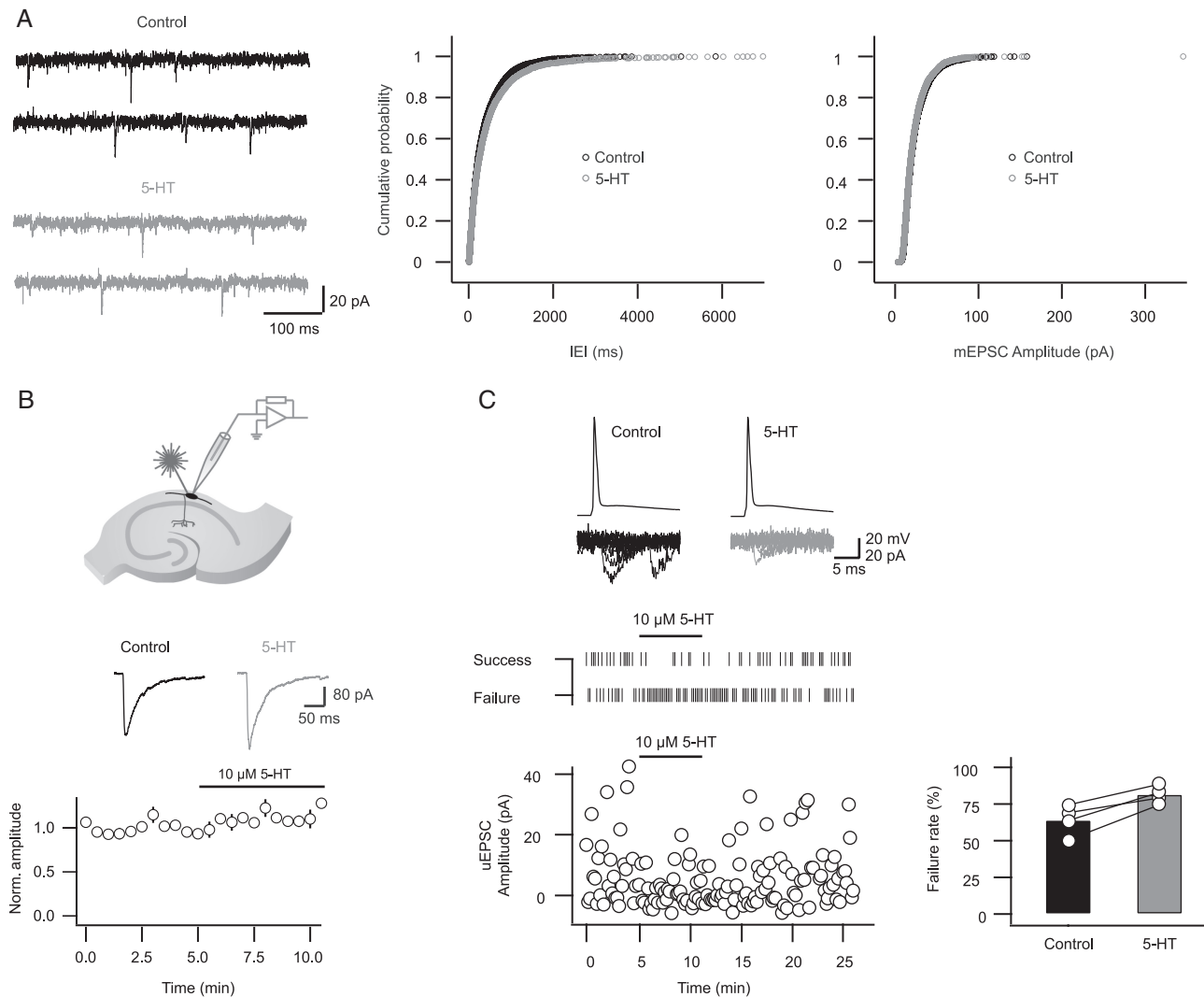
Figure 4. 5-HT acts most likely presynaptically. (A) Left, example traces of miniature EPSCs in control conditions (black) and in 10 μM 5-HT (gray). Right, cumulative probability of interevent intervals and amplitude of miniature EPSCs in control conditions and in 10 μM 5-HT ($n = 10$). (B) Top, recording configuration for glutamate uncaging. With a laser flash glutamate is uncaged in the immediate vicinity of the soma of an O-LM interneuron. Middle, example traces of the glutamate evoked current before (black) and after the application of 10 μM 5-HT (gray). Bottom, summary of the time course of the glutamate evoked current. Depicted is the normalized and binned peak amplitude. Note that the amplitude is not decreased after application of 5-HT ($n = 4$). (C) Left, example of connected pyramidal–O-LM pair, top, overlay of uEPSCs in control conditions and in 5-HT, middle, time course of successful synaptic transmission and failures. Bottom, time course of the uEPSC amplitude (as shown in top row) before, during and after washout of 10 μM 5-HT. Note that the failure rate increases during application of 5-HT. Right, summary of failure rate in synaptically connected paired recordings under control conditions and in 10 μM 5-HT ($n = 4$).

interneurons by serotonin application. Thus, we conclude that the mechanism is most likely presynaptic.

Mechanism of Action

After having localized the site of action, we aimed to identify the 5-HT receptor subtype mediating inhibition of glutamatergic transmission. At first, we confirmed with the unspecific 5-HT antagonist dihydroergocristine mesylate that the reduction in EPSC amplitude can indeed be blocked by antagonizing 5-HT receptors ($n = 7$, median of normalized amplitude in dihydroergocristine mesylate 0.84, IQR: 0.26, EPSC amplitude in dihydroergocristine mesylate vs. EPSC amplitude in dihydroergocristine mesylate and 5-HT: $P = 0.3$, paired Wilcoxon rank test, Fig. 5A). One candidate presynaptic receptor is the 5-HT_{1B} receptor that has been shown to modulate glutamatergic transmission of CA1 pyramidal cells (Winterer et al. 2011). However, application of the

5-HT_{1B} receptor agonist CP 94523 did not mimic the effect of 5-HT on evoked EPSCs (median of normalized amplitude in CP 94523 is 0.834, IQR: 0.28, EPSC amplitude in control vs. EPSC amplitude in CP 94523: $P = 0.15$, paired Wilcoxon rank test, Fig. 5B). Next, we investigated the possibility that presynaptic 5-HT_{1A} receptors might be responsible for mediating the reduction of glutamate release (Schmitz et al. 1995, 1999; Fink and Göthert 2007). Indeed, application of the 5-HT_{1A} agonist 8-OH-DPAT could, in part, mimic the effect of 5-HT on evoked EPSCs (median of normalized amplitude in 8-OH-DPAT: 0.76, IQR: 0.21, EPSC amplitude in control vs. EPSC amplitude in 8-OH-DPAT: $P = 0.016$, paired Wilcoxon rank test, Fig. 5C1). Furthermore, the 5-HT_{1A} receptor antagonist Way100635 reduced the action of 5-HT, when compared with control conditions (median of normalized amplitude: 0.78, IQR: 0.3, EPSC amplitude in Way100635 vs. EPSC amplitude in Way100635 and 5-HT: $P = 0.01$, paired Wilcoxon rank test, Fig. 5C2). We conclude that the presynaptic activation of 5-HT_{1A}
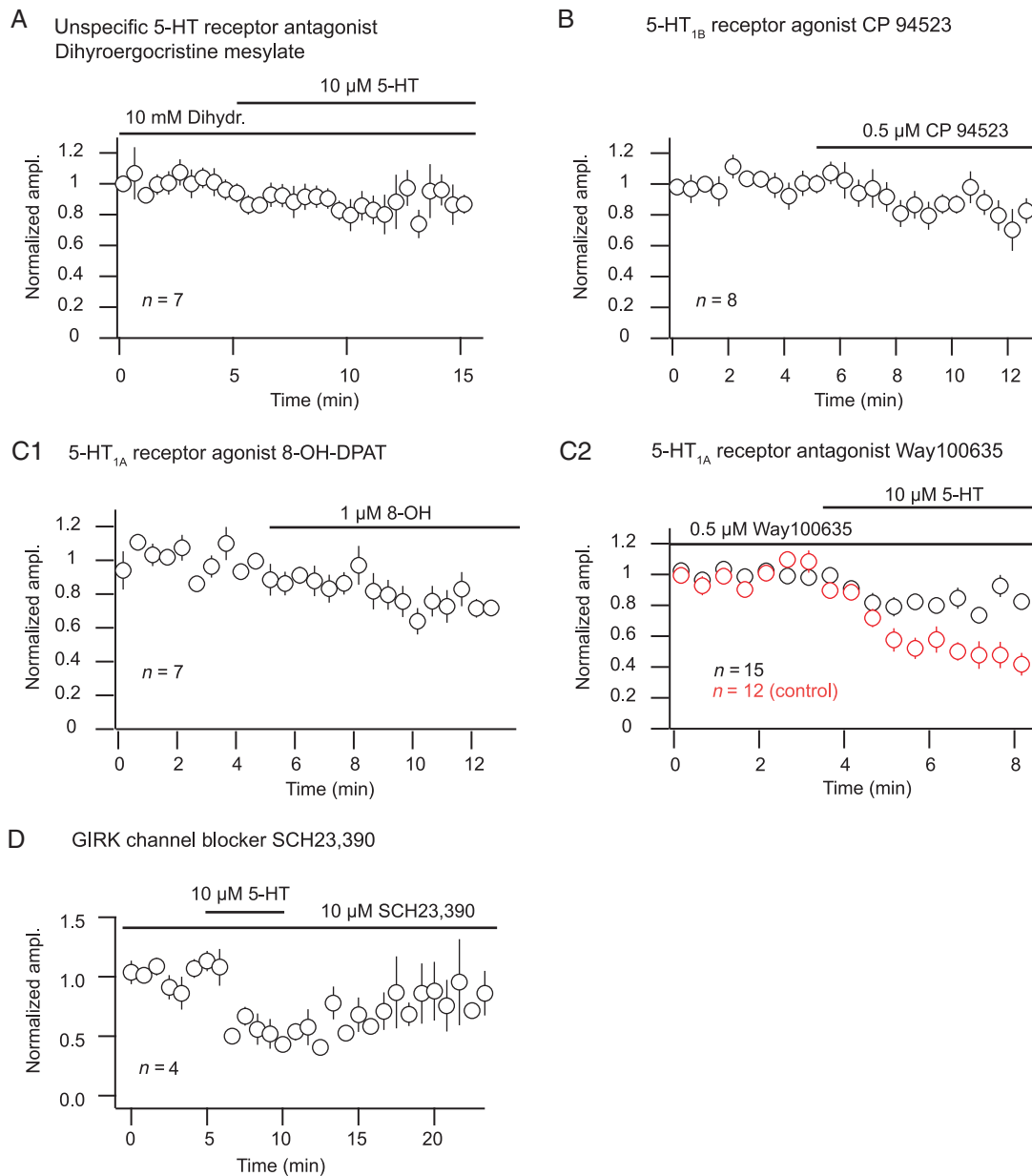
Figure 5. Effect of 5-HT receptor subtype (un)specific compounds on eEPSC amplitude. (A–D) Time course of averaged, normalized, and binned amplitude. The paired Wilcoxon sign rank test was used to compare amplitudes in the indicated conditions (tested on raw, not normalized amplitudes). (A) In the presence of the unspecific 5-HT receptor antagonist dihydroergocristine mesylate the effect of 5-HT on the EPSC amplitude is abolished. ($n = 7$, $P = 0.30$). (B) The 5-HT$_{1B}$ receptor agonist CP 94523 does not mimic the 5-HT effect. ($n = 8$, $P = 0.15$). (C1) The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT reduces eEPSC amplitudes ($n = 7$, $P = 0.02$). (C2) The 5-HT$_{1A}$ receptor antagonist WAY100635 partially blocks the 5-HT effect on eEPSC amplitude ($n = 15$, $P = 0.01$). For comparison the eEPSC amplitude in control conditions, that is, in the absence of the antagonist is shown ($n = 12$) (red, compare Fig. 2B, unpaired Wilcoxon test, $P = 0.0002$). (D) In the presence of the GIRK-channel blocker SCH23390 5-HT still reduces the amplitude of eEPSCs ($n = 4$). Also compare Figure 2B2.

receptors is partially responsible for the inhibition of glutamatergic transmission onto O-LM interneurons.

5-HT$_{1A}$ receptors might mediate the observed effect of 5-HT on glutamatergic transmission by hyperpolarizing the presynaptic pyramidal cell. This hyperpolarization is mediated by the opening of G-protein-gated inwardly rectifying K$^+$ channels (GIRK) (Andrade and Nicoll 1987; Segal et al. 1989; Schmitz et al. 1995). However, application of the GIRK-channel blocker SCH23390 did not prevent the reduction of excitatory synaptic transmission onto O-LM interneurons by 5-HT (mean inhibition of amplitude: 42.73% ± 9.7, $n = 4$, Fig. 5D).

5-HT receptor activation could target calcium channels via G-proteins (Mizutani et al. 2006) resulting in a reduced Ca^{2+} influx and thereby decreasing Ca^{2+}-dependent vesicle release. To test this hypothesis, we evaluated if 5-HT reduces Ca^{2+} influx into the presynaptic terminals of CA1 pyramidal cell axons that are predominantly contributing to the glutamatergic synaptic transmission onto O-LM interneurons. We adapted an optical recording method described previously (Regehr and Tank 1991; Breustedt et al. 2003), in which the presynaptic fibers in *stratum oriens* are labeled with the low-affinity fluorescent Ca^{2+} indicator dye magnesium green AM. These recordings were done in the
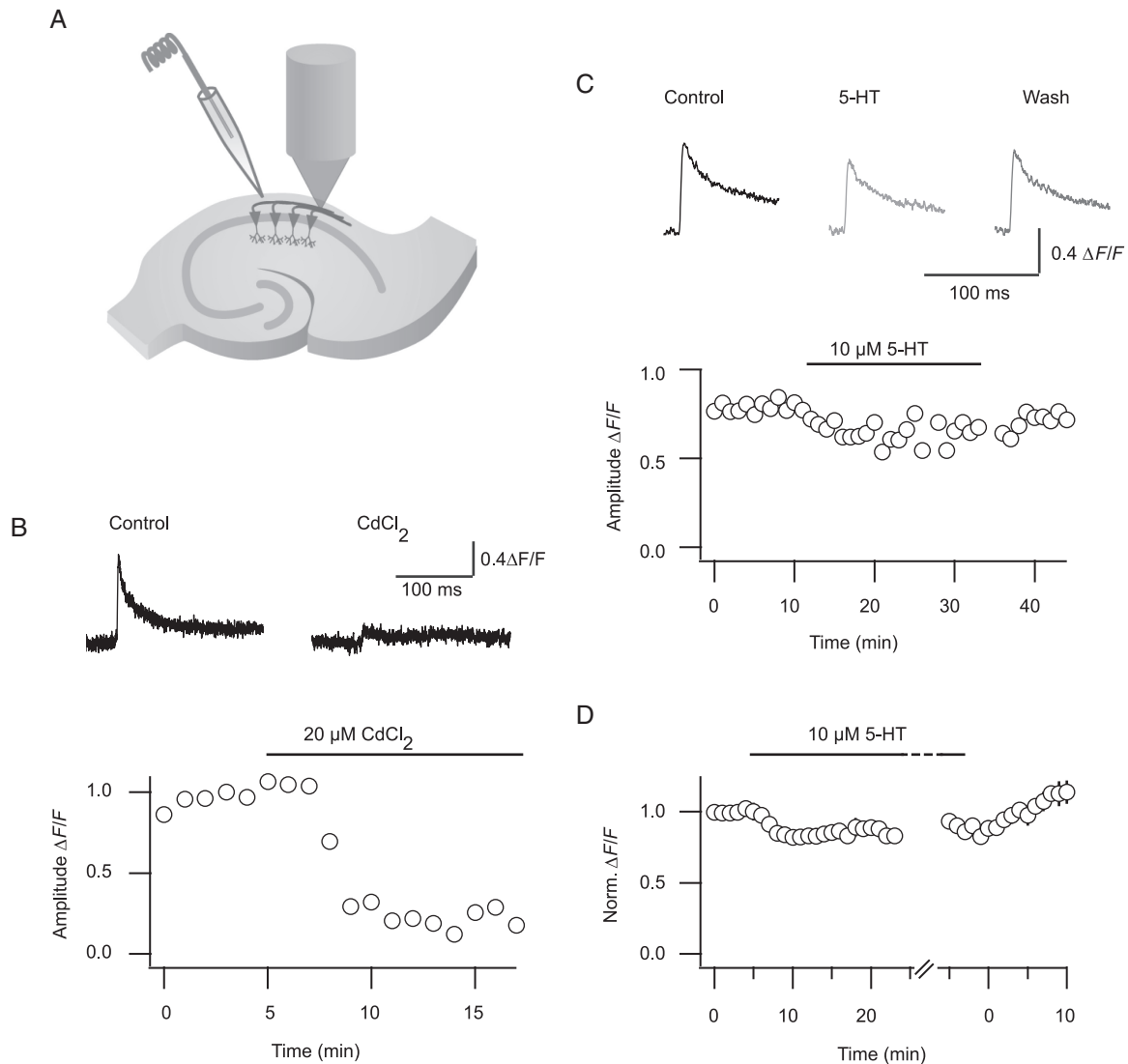
Figure 6. Decreased presynaptic calcium influx can account for the reduction of glutamatergic transmission by 5-HT. (A) Ca^{2+}-imaging recording configuration. Axonal fibers were filled with the Ca^{2+}-sensitive dye magnesium green AM (green) by bulk loading and stimulated with an extracellular electrode positioned at the border of the *alveus* and *stratum oriens*. The resulting Ca^{2+} transients were measured with a photodiode. (B) Top, example traces of the Ca^{2+} transients in control and in 20 μM $CdCl_2$. Bottom, time course of $\Delta F/F$ amplitude of the Ca^{2+} transients shown above. Note that the transient is abolished in the presence of the calcium channel blocker CdCl2. (C) Top, example traces of the calcium transient in control, in 10 μM 5-HT and after wash. Bottom, time course of $\Delta F/F$ amplitude of the above transients. (D) Summary of time course of $\Delta F/F$ amplitude ($n = 11$).

presence of NBQX and D-AP5 to exclude a postsynaptic contribution to the signal. AP driven Ca^{2+} influx into the presynaptic terminal was elicited by a single extracellular stimulation electrode positioned at the border of *stratum oriens* and *alveus*. The rise in presynaptic Ca^{2+} was quantified using the transient increase of the fluorescence signal (see Materials and Methods; Fig. 6A). To ensure that the detected signal was actually due to Ca^{2+} influx, we applied the unspecific Ca^{2+} channel blocker $CdCl_2$ which abolished the Ca^{2+} transient (Fig. 6B). We then tested the effect of 5-HT on the presynaptic Ca^{2+} transient and found that indeed 5-HT reversibly decreased the Ca^{2+} transient amplitude by $13.9 \pm 3.2\%$ on average ($n = 11$, amplitude in 5-HT is significantly different from control: $P = 0.006$, paired Student's T-test, Fig. 6C,D).

To further corroborate this finding, we tested whether a reduction in Ca^{2+} influx can account for the inhibition in glutamatergic transmission induced by serotonin. We therefore reduced

the extracellular Ca^{2+} concentration from 2.5 to 2.0 mM ($n = 5$, amplitude in 2.5 mM Ca^{2+} is significantly different from the amplitude in 2 mM Ca^{2+}: $P = 0.0022$, paired Student's T-test, Fig. 7A,C) and compared the reduction in amplitude of the calcium transient to the reduction observed under 5-HT: 5-HT application as well as lowering the extracellular Ca^{2+} concentration displayed a comparable amount of reduction of the Ca^{2+} transient ($13.9 \pm 3.2\%$ in 5-HT vs. $16.0 \pm 1.7\%$ in 2 mM Ca^{2+}, the reduction in amplitude observed in 5-HT and lowered Ca^{2+} concentration are not different: $P = 0.6731$, unpaired Student's T-test, Fig. 7A,C, left).

We hypothesized that if the reduced calcium influx into the presynaptic terminal is responsible for the inhibition of glutamatergic transmission, a reduction of the extracellular calcium concentration should also be able to mimic the 5-HT effect on EPSCs evoked by electrical stimulation. Indeed, reducing the available calcium by the same amount as in the imaging experiments (0.5 mM), resulted in a reduction of the amplitude of the eEPSCs
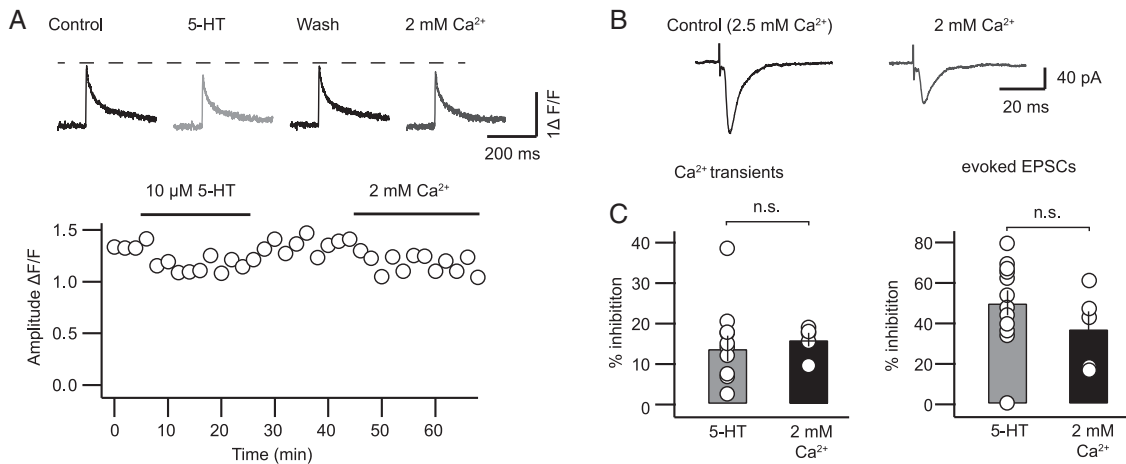
Figure 7. The 5-HT induced reduction of the presynaptic Ca^{2+} transient as well as the reduction of the glutamatergic synaptic transmission can be mimicked by a reduction of the extracellular Ca^{2+} concentration. (A) Top, example traces of Ca^{2+} signals in control conditions, after application of 10 µM 5-HT, after washout of 5-HT and after reduction of the extracellular Ca^{2+} concentration from 2.5 to 2 mM. Bottom, time course of the binned amplitude of the Ca^{2+} transient in control conditions, after application of 10 µM 5-HT, after washout and in 2 mM Ca^{2+}. (B) Example traces of stimulus-evoked EPSCs in control conditions and after reduction of the extracellular Ca^{2+} concentration from 2.5 to 2 mM. (C) Summaries of the effect of 5-HT and the reduced extracellular Ca^{2+} concentration on the amplitude of the Ca^{2+} transients and on the amplitude of stimulus-evoked EPSCs.

by $37.4 \pm 8.6\%$ ($n = 5$, Fig. 7B,C right), similar to the reduction we observed with 5-HT ($n = 12$; $50.1 \pm 6.1\%$, Fig. 7C, right, $P = 0.2676$, unpaired Student's *T*-test).

In summary, we could show that 5-HT inhibits excitatory synaptic transmission at the pyramidal cell–O-LM interneuron synapse in CA1. This effect most likely involves a decrease of calcium influx into the presynaptic terminal and is mediated by presynaptic 5-HT receptors.

Discussion

Here we show by means of electrophysiological recordings and Ca^{2+} measurements that 5-HT reversibly reduces excitatory glutamatergic synaptic transmission onto O-LM interneurons, which leads to a decrease in spiking probability of O-LM interneurons in area CA1 of the hippocampus. Our findings indicate that 5-HT decreases the Ca^{2+} influx into the presynaptic terminal of CA1 pyramidal cells and that this modulation is most likely responsible for the reduction in glutamatergic synaptic transmission at the pyramidal cell–O-LM interneuron synapse.

At first, we observed a reduction of the frequency of spontaneous EPSCs in O-LM interneurons by the application of 5-HT. Spontaneous EPSCs are generated either by Ca^{2+}-independent spontaneous fusion of vesicles with the presynaptic plasma membrane (mEPSCs) or by Ca^{2+}-dependent vesicular release in response to spontaneous APs. We found that the observed neuromodulation by 5-HT critically depends on presynaptic APs, as the decrease in the frequency of sEPSCs was lost after application of TTX. The finding that mEPSCs are not affected, neither in frequency nor in amplitude, could be in line with the following scenario: 5-HT could induce a hyperpolarization of the presynaptic pyramidal cell, mediated by the opening of GIRK channels by $5-HT_{1A}$ receptor activation (Andrade and Nicoll 1987; Segal et al. 1989; Schmitz et al. 1995). This would diminish the frequency of sEPSCs because of a decrease in the number of spontaneous spikes as has been shown in vivo (Richter-Levin and Segal 1992). This possibility seems to be unlikely because application of a GIRK-channel blocker could not prevent the reduction of excitatory synaptic transmission onto O-LM interneurons by 5-HT.

Another potential target of 5-HT might be astrocytes, which have been shown to react on 5-HT (Schipke et al. 2011), and in turn are able to modulate neurotransmission (Araque et al. 1999). However, the time course of reaction to 5-HT in astrocytes (Schipke et al. 2011) differs largely from the type of modulation described here.

The more likely site of action is an activation of 5-HT receptors at the axon terminals of the presynaptic cell, which leads to a decrease in Ca^{2+} influx; this mechanism would not affect the probability of spontaneous vesicle fusion, and is in line with our finding that 5-HT does not affect mEPSCs.

We next investigated the effects of 5-HT on EPSCs evoked by extracellular stimulation in *stratum oriens/alveus* where activation of axonal fibers from CA1 pyramidal cells is most likely. As excitatory connections on O-LM cells originate predominantly from local CA1 collaterals (Blasco-Ibáñez and Freund 1995), the 5-HT-mediated decrease in amplitude of stimulus-evoked currents is most likely the result of a depression of glutamatergic transmission from local CA1 pyramidal cells. We confirmed this assumption with paired recordings from synaptically connected CA1 pyramidal neurons and O-LM interneurons. In this set of experiments 5-HT mediated a robust increase of synaptic failures, that is, presynaptic AP initiation without successful synaptic transmission. The very low initial release probability at this synapse and its further reduction due to 5-HT prevented the analysis of changes in short-term facilitation upon 5-HT application. In a further set of experiments, we circumvented a possible serotonergic modulation of the presynaptic site by means of photolytically activating glutamate. In doing so, the amount of glutamate that activates postsynaptic glutamate receptors is kept constant. Under these experimental conditions, we found that 5-HT had no effect on the glutamate evoked response. Together, these observations are suggestive for a presynaptic mechanism mediating the decrease in glutamatergic transmission.

Presynaptic modulation of transmitter release can be mediated via a G-protein-mediated block of Ca^{2+} channels (Thomson 2000; Mizutani et al. 2006; but see Gerachshenko et al. 2009). As a consequence the Ca^{2+} influx into the presynaptic terminal is reduced. We were able to show that indeed application

of 5-HT leads to a decrease in presynaptic Ca^{2+} levels. Although our method does not allow specific loading of axon terminals on O-LM cells, they are likely to constitute a considerable fraction of the loaded fibers; hence, they contribute substantially to the measured Ca^{2+} transient. Since these recordings were performed under blockade of N-methyl-D-aspartate- and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate-receptors, a postsynaptic contribution to the measured Ca^{2+} signal can be excluded. The observed inhibition of Ca^{2+} influx upon 5-HT application was within the expected range since the relationship between Ca^{2+} influx and transmitter release is nonlinear (Mintz et al. 1995; Gundlfinger et al. 2007). Furthermore, we could show that a decrease in extracellular Ca^{2+} concentration is able to mimic the effects of 5-HT. This applies for the experiments where we probed the presynaptic Ca^{2+} influx into the presynaptic terminals by means of fluorescence measurements as well as in the experiments where we tested the stimulus-induced EPSCs in O-LM interneurons.

Under physiological conditions, 5-HT is released in the hippocampus by axons originating from serotonergic neurons in the midbrain raphe nuclei. With the aim of avoiding the exogenous application of serotonin by bath, we made use of fenfluramine to mimic physiological release of serotonin in the hippocampus. Indeed, we observed that also endogenously released serotonin is able to reduce the excitatory synaptic transmission onto O-LM interneurons. O-LM interneurons have been shown to be active during hippocampal theta oscillations (Klausberger et al. 2003; Katona et al. 2014). We aimed to mimic theta-timed input onto O-LM-interneurons by extracellular stimulation and could readily evoke spikes, suggesting that the low release probability can be overcome by an appropriate stimulus. Furthermore, the serotonergic modulation of glutamatergic transmission described here significantly reduces the spiking probability and therefore has an impact on the output of O-LM interneurons

Serotonergic neuromodulation of O-LM interneurons could have an important influence on the dynamics of hippocampal-entorhinal cortex interaction. Active O-LM cells are presumed to inhibit input from the entorhinal cortex via postsynaptic $GABA_A$ receptor activation in *stratum moleculare* and might in addition mediate a reduction of glutamate and GABA release by presynaptic $GABA_B$ receptors (Chalifoux and Carter 2011; Urban-Ciecko et al. 2015). Deactivation of O-LM cells is likely to strengthen entorhinal cortex input to CA1 via the TA pathway (Maccaferri and McBain 1995). Moreover, it has been shown recently that serotonin is able to induce a potentiation of the TA pathway–CA1 synapses (Cai et al. 2013). In this respect, serotonin is acting synergistically to increase the input via the TA pathway: 5-HT reduces the excitatory drive onto O-LM interneurons and consequently releases the target region of the entorhinal projections from inhibition. The finding that O-LM interneurons differentially modulate the input from CA3 and the entorhinal cortex onto hippocampal CA1 neurons (Leão et al. 2012) puts serotonergic neuromodulation of O-LM interneurons at center stage for switching the information flow from direct TA pathway inputs with sensory information to inputs with internal representations stored in CA3.

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Notes

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