Cerebral Cortex, December 2017;27: 5463–5476

doi: 10.1093/cercor/bhw312
Advance Access Publication Date: 12 October 2016
Original Article

ORIGINAL ARTICLE

Enduring Loss of Serotonergic Control of Orbitofrontal Cortex Function Following Contingent and Noncontingent Cocaine Exposure

Andrew M. Wright[1], Agustin Zapata[1], Michael H. Baumann[2], Joshua S. Elmore[2], Alexander F. Hoffman[1], and Carl R. Lupica[1]

[1]Electrophysiology Research Section, Cellular Neurobiology Branch, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, USA and [2]Designer Drug Research Unit, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, USA

*Address correspondence to Carl R. Lupica, Electrophysiology Research Section, Cellular Neurobiology Branch, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, US. Email: clupica@mail.nih.gov

Abstract

Clinical descriptions of cocaine addiction include compulsive drug seeking and maladaptive decision-making despite substantial aversive consequences. Research suggests that this may result from altered orbitofrontal cortex (OFC) function and its participation in outcome-based behavior. Clinical and animal studies also implicate serotonin in the regulation of OFC function in addiction and other neuropsychiatric disorders. Here we test the hypothesis that exposure to cocaine, through self-administration (CSA) or yoked-administration (CYA), alters the regulation of OFC function by 5-HT. Using whole-cell electrophysiology in brain slices from naïve rats we find that 5-HT_{1A} receptors generate hyperpolarizing outward currents in layer-V OFC pyramidal neurons, and that 5-HT_{2A} receptors increase glutamate release onto these cells. Following extended withdrawal from CSA or CYA, this 5-HT regulation of OFC activity is largely lost. In-situ hybridization of 5-HT receptor transcripts reveals that 5-HT_{1A} receptor mRNA is unaffected and 5-HT_{2A} receptor mRNA is significantly elevated after CSA or CYA. These results demonstrate that 5-HT control of OFC neurons is disrupted for extended periods following cocaine exposure. We hypothesize that this dysregulation of 5-HT signaling leads to enduring disruptions of OFC network activity that this is involved in impaired decision-making associated with cocaine addiction.

Key words: addiction, electrophysiology, prefrontal cortex, psychostimulants, self-administration

Introduction

The orbitofrontal cortex (OFC) is involved in associative learning (Jones and Mishkin 1972), through its regulation of "outcome-based" behaviors via the encoding of motivational values of environmental stimuli (Schoenbaum et al. 1998; Gallagher et al. 1999; Tremblay and Schultz 1999; Izquierdo and Murray 2004). The OFC also encodes changes in expectancies, leading to altered predictions regarding the consequences of behavior. This ability to encode the shifting environmental consequences of behavior is a hallmark of cognitive flexibility, which is essential to survival and considered a form of higher-order cortical processing (Schoenbaum and Esber 2010). Drug addiction is characterized by the inability to make appropriate decisions in the face of aversive outcomes and has been associated with dysregulated OFC function (Volkow and Fowler 2000; Bechara 2005; Schoenbaum and Shaham 2008; Lucantonio et al. 2012, 2014). In support of this, human cocaine addicts are seemingly unable to alter their behavior in the face of strong negative

consequences, and OFC metabolic activity is reduced in these individuals during extended withdrawal from cocaine (Volkow et al. 1993; Volkow and Fowler 2000). Moreover, animals exposed to cocaine show long-lasting impairments in OFC-dependent behavioral tasks, such as reversal learning (Jentsch et al. 2002; Schoenbaum et al. 2004; Calu et al. 2007), reinforcer devaluation (Schoenbaum and Setlow 2005), and Pavlovian over-expectation (Takahashi et al. 2013; Lucantonio et al. 2014). Importantly, the deficit in OFC-dependent cognition following cocaine self-administration (CSA) is thought to reflect an enduring loss of this structure's ability to encode outcome expectancies, and impaired learning based on these expectancies (Lucantonio et al. 2014).

Similar to other prefrontal cortical (PFCx) circuits, the OFC consists of both glutamatergic principal cells and GABAergic interneurons. The OFC receives sensory input from gustatory, olfactory, and visual sensory areas (Schoenbaum and Eichenbaum 1995; Ongur and Price 2000), and in addition to extensive connections with the mediodorsal thalamic nucleus (Krettek and Price 1977), the OFC also projects to various areas of the limbic system, including dorsomedial and ventral striatum (Ongur and Price 2000; Schilman et al. 2008), and the amygdala (Ongur and Price 2000; Rolls and Grabenhorst 2008). Thus, the OFC is positioned to integrate information from various sensory modalities with brain areas involved in emotional cognitive processing and decision-making (Rolls and Grabenhorst 2008). The OFC also receives dense serotonergic (5-HT) innervation from the dorsal raphe nucleus (DRN), and reciprocally connects with this area (Azmitia and Segal 1978; Hornung et al. 1990; Wilson and Molliver 1991a, 1991b; Goncalves et al. 2009; Vazquez-Borsetti et al. 2009; Roberts 2011; Chandler et al. 2013). Moreover, 5-HT is implicated as a key contributor in OFC-dependent behavioral flexibility, as well as in psychiatric illnesses that involve OFC function, such as obsessive compulsive disorder (OCD), impulsive-aggressive disorder (IAD), and depression (Roberts 2011). Consistent with this, 5-HT depletion produces deficits in OFC-dependent behavioral tasks requiring behavioral flexibility (Clarke et al. 2004; Roberts 2011; Izquierdo et al. 2012; West et al. 2013), and intra-OFC infusions of 5-HT receptor ligands modify OFC-dependent reversal learning (Boulougouris and Robbins 2010). Additionally, favorable therapeutic responses to 5-HT-directed pharmacotherapies in the treatment of OCD and IAD are associated with improved metabolic activity in the OFC (Saxena et al. 1999; New et al. 2004).

A possible link between cortical 5-HT and cocaine addiction is demonstrated by studies showing that 5-HT levels are diminished in frontal cortices following long-term withdrawal from cocaine (Egan et al. 1994), and that genetic deletion of the 5-HT transporter (SERT) can improve cognitive flexibility and prevent cocaine-induced deficits in reversal learning in rats (Nonkes et al. 2013). In addition, inhibition of cocaine seeking following CSA is associated with strong activation of both OFC and dorsal raphe 5-HT neurons, suggesting the involvement of these brain regions in a neural network mediating inhibitory control of relapse (Navailles et al. 2015).

Despite substantial behavioral and pharmacological data supporting a role for 5-HT in the control of OFC function in psychiatric disorders, and in psychostimulant addiction (Kish et al. 2009), few studies have examined the specific physiological function of 5-HT within the OFC, as well as the changes that occur in this system in drug addiction models. Here, we identify mechanisms through which 5-HT alters OFC neuron function, and describe a loss of 5-HT regulation of OFC activity in rats with a history of CSA, and those receiving noncontingent cocaine through yoked-administration (CYA).

Materials and Methods

All experimental procedures were approved by the Institutional Care and Use Committee of the National Institute on Drug Abuse Intramural Research Program (NIDA-IRP), National Institutes of Health (NIH) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals provided by the NIH and adopted by the NIDA-IRP.

Subjects

Male Long–Evans rats (Charles River Laboratories), aged 31–154 days at the time of electrophysiological recording, were housed 2–3 per cage for at least 1 week prior to experimental procedures in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). They were maintained in a temperature- and humidity-controlled environment under a reverse 12 h light/dark cycle with food and water available ad libitum. All rats included in the CSA or CYA groups were 70 days old at the time of surgery. For animals in CSA and CYA groups, 1 week following surgery, food was restricted to 15 g of standard rodent diet per day and made available after the daily self-administration sessions. Animals were food restricted until the end of self-administration training, after which ad-libitum feeding resumed. Behavioral experiments were conducted during the dark cycle.

Surgery

Rats were anesthetized with equithesin (1% pentobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol, 3 ml/kg, i.p.), and a silastic catheter (inner diameter, 0.020 inch; outer diameter, 0.037 inch; Dow Corning) was advanced 3.5 cm into the right jugular vein and secured with silk suture (5–0). The catheter terminated in a modified L-shaped 22G guide cannula (Plastics One) mounted on top of the skull with cranioplastic cement and secured with 3 stainless steel screws. Animals were given one injection of 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide (Meloxicam, 5 mg/mL, Boehringer/Ingelheim, Ingelheim, FRG) for postsurgical analgesia (0.2 mL, sc) and single housed for the remainder of the experiment. Catheters were flushed daily with 0.1 mL of heparinized saline to maintain patency. Animals were allowed to recover for at least 1 week after the surgery and before food restriction commenced. Catheters were flushed once daily (0.1 mL) with 120 mg/mL cefazolin until the end of operant training to forestall infection. Operant training started 2–3 days after the beginning of food restriction.

CSA Training

Self-administration training took place in standard rat operant chambers equipped with 2 retractable levers (Med Associates). Each chamber was located in a sound attenuating enclosure, equipped with a ventilation fan that provided background noise. Catheters were connected through a metal spring protected plastic tubing (Plastics One) via a liquid swivel (Instech) to a syringe mounted in an infusion pump (Med Associates) located outside the sound attenuating enclosure. Animals were trained to press one lever (designated as the active lever) for intravenous cocaine infusions under a fixed ratio 1 (FR1)

schedule. At the beginning of the session, the two levers were inserted into the chamber and a house light located on the wall opposite the levers was illuminated. Each lever press resulted in infusion of 0.75 mg/kg cocaine (2.25 mg/mL in sterile saline) accompanied by retraction of both levers, extinction of the house light and illumination of a cue light above the active lever for 30 s. Responses on the inactive lever had no consequences. To facilitate acquisition of the response, animals were allowed to self-administer during 6 h sessions until they received 40 infusions within a session. Most of the animals acquired self-administration within 1–2 sessions. After that, sessions were limited to 3 h or 75 infusions, whichever occurred first. CSA under the FR1 schedule continued for a total of 12 daily sessions. A separate group of yoked control animals was simultaneously run in a set of identical operant chambers in the same room. Each of the rats in the yoked group was paired to a rat in the self-administration group such that every lever press delivering a cocaine infusion in the self-administration group resulted in an infusion of cocaine for the yoked group. At the beginning of the session both levers were inserted and the house light was switched on. Additionally, infusions were never signaled by retraction of the levers nor cue lights for the yoked animals. Sessions for these rats terminated at the same time as the session for their self-administering counterpart by retracting both levers and switching off the house light. Thus, yoked controls were exposed to the same amount of cocaine as their self-administering counterparts, but they experienced cocaine as passive infusions. Following behavioral training, rats in the CSA and CYA groups remained single housed in their home cages in the colony room, with ad libitum food and water available, until the electrophysiology experiments.

Brain Slice Preparation

Animals were anesthetized with isoflurane and decapitated using a guillotine. Brains were extracted and transferred to an ice-cold *N*-methyl-D-glucamine (NMDG) cutting solution (in mM: NMDG, 93; KCl, 2.5; NaH_2PO_4, 1.2; $NaHCO_3$, 30; HEPES, 20; Glucose, 25; Ascorbic acid, 5; Sodium pyruvate, 3; $MgCl_2$, 10; $CaCl_2$, 0.5). The tissue was cut perpendicular to its longitudinal axis at 1.4 mm posterior to bregma using a razor blade, and then the cut surface glued to the stage of the vibrating tissue slicer (Leica VT1200S, Leica Biosystems). Five coronal sections (280 μm) were collected from each brain beginning at the appearance of the dorsolateral orbital cortex and the rhinal fissure (4.7 mm anterior to bregma), and ending at approximately 3.2 mm anterior to bregma. The slices were then hemisected and transferred to heated (34°C) NMDG for 5 min. The brain slices were then transferred to an oxygenated (95% O_2/5% CO_2) holding chamber filled with HEPES containing artificial cerebrospinal fluid (aCSF) (in mM: NaCl, 109; KCl, 4.5; NaH_2PO_4, 1.2; $NaHCO_3$, 35; HEPES, 20; Glucose, 11; Ascorbic acid, 0.4; $MgCl_2$, 1; $CaCl_2$, 2.5) at room temperature (22°C) for at least 30 min, and up to 7 h. For each animal, this procedure yielded 5 brain slices, and recordings were restricted to brain slices corresponding to these landmarks in all animals from all groups.

In Vitro Electrophysiology

Hemi-sectioned slices were transferred to a chamber (RC-26; Warner Instruments) continuously perfused (2 mL/min) with oxygenated aCSF (in mM: NaCl, 126; KCl, 4.5; NaH_2PO_4, 1.2; $NaHCO_3$, 26; Glucose, 11; $MgCl_2$, 1; $CaCl_2$, 2.5) containing picrotoxin (50 μM) using a peristaltic pump (Cole-Parmer). The temperature was maintained at 30–32°C using a solution heater (Warner Instruments). Multipolar neurons were identified in layer V of the lateral OFC (l-OFC) using an upright videomicroscope (BX51WI, Olympus) equipped with differential interference contrast imaging, and 900 nm infrared illumination. Recording electrodes were fabricated using borosilicate glass (Sutter Instruments, 1.5 mm O.D. × 0.86 mm i.d.) using a horizontal puller (P-97; Sutter Instruments) and filled with a potassium-based internal solution (in mM: K-gluconate, 140; KCl, 5; HEPES, 10; EGTA, 0.2; $MgCl_2$, 2; Mg-ATP, 4; Na_2-GTP, 0.3; Na_2-phosphocreatine, 10) neutralized to a pH of 7.2 using potassium hydroxide. Resistances were 3–7 MΩ. Whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices). Cells were voltage-clamped at −60 mV (with a calculated junction potential of −14 mV), and miniature excitatory postsynaptic currents (mEPSCs) and spontaneous EPSCs (sEPSCs) were sampled at 10 kHz using WinLTP software (WinLTP Ltd, Bristol, UK) and an A/D board (National Instruments, PCI-6251) housed in a personal computer. Hyperpolarizing voltage steps (−10 mV) were delivered via the recording electrode every 30 s to monitor whole-cell access and series resistance. Cells with an access change of more than 20% were excluded from analysis. Voltage ramps were performed from −64 to −144 mV over 320 ms (Yamamoto et al. 2014). Current clamp experiments were performed with depolarizing currents ranging from 100 to 600 pA. Serotonin (5-HT, 1 mM), prepared at low pH (~2) to prevent oxidation, was diluted 1:100 in aCSF flowing into the slice chamber (20 μL/min), to achieve a final bath concentration of 10 μM using a syringe pump (Model A-99, Razel Scientific Instruments). All other drugs were dissolved at their final concentration in aCSF and delivered using the peristaltic pump. Analyses of mEPSCs and sEPSCs were performed offline using the MiniAnalysis program (v 6.0.7, Synaptosoft, Inc.), and were visually inspected to verify this analysis.

Drugs

8-OH-DPAT hydrobromide ((±)-8-hydroxy-2-dipropylaminotetralin hydrobromide), WAY100635 maleate (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate), Ritanserin (6-[2-[4-[Bis(4-fluorophenyl)methylene]-1-piperidinyl] ethyl]-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidin-5-one), Sumatriptan succinate (3-[2-(dimethylamino)ethyl]-*N*-methyl-1*H*-indole-5-methanesulfonamide succinate), Picrotoxin, and serotonin were purchased from Sigma serotonin (5-HT), (3-(2-aminoethyl)-1*H*-indol-5-ol hydrochloride, Sigma), Tetrodotoxin, MDL100907 ((*R*)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol), and DOI ((−)-2,5-dimethoxy-4-iodoamphetamine hydrochloride) were purchased from Tocris, Cocaine was supplied through the NIDA Drug Supply Program.

Immunohistochemistry

Cells filled with an internal solution containing 0.2% biocytin were fixed immediately after recording in a phosphate-buffered saline (PBS) solution containing 4% paraformaldehyde (PF) and were kept refrigerated at 4°C for 24 h. Cells were then transferred into PBS and kept refrigerated at 4°C until used for staining. Cells were rinsed (3 × 10 min) in PBS, then rinsed with 0.3% Triton X-100/4% bovine serum albumin (BSA)/PBS for 2 h. Cells were then shielded from light and rinsed for an additional 2 h in 0.3% Triton X-100/4% BSA/PBS with the streptavadin fluorescent conjugate Alexa Fluor 488 at a dilution of 1:500. Cells were then rinsed (3 × 10 min) in PBS and slide mounted using

Permount® toluene solution (Fisher Scientific). Confocal imaging of immunofluorescence was performed on a Zeiss LSM510 microscope.

Tissue Preparation for Anatomical Studies

Eighteen rats (6 each in CSA, CYA, and naïve groups) were anesthetized with chloral hydrate (35 mg/100g) and perfused transcardially with 4% (W/V) PF in 0.1 M PBS, pH 7.3. Brains were left in 4% PF for 2 h at 4°C, rinsed with PBS and transferred sequentially to 12%, 14%, and 18% sucrose solutions in PBS. Coronal serial sections of 16 μm in thickness were then prepared.

Combined In-Situ Hybridization and Parvalbumin Immunolabeling

Coronal free-floating sections were processed as described previously (Wang and Morales 2008). Sections were incubated for 10 min in PBS containing 0.5% Triton X-100, rinsed 2 × 5 min with PBS, treated with 0.2 N HCl for 10 min, rinsed 2 × 5 min with PBS and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. Sections were then rinsed 2 × 5 min with PBS, and fixed with 4% PF for 10 min. Prior to hybridization and after a final rinse with PBS, the free-floating sections were incubated in hybridization buffer (50% formamide; 10% dextran sulfate; 5 × Denhardt's solution; 0.62 M NaCl; 50 mM DTT; 10 mM EDTA; 20 mM PIPES, pH 6.8; 0.2% SDS; 250 μg/mL salmon sperm DNA; 250 μg/mL tRNA) for 2 h at 55°C. Sections were hybridized for 16 h at 55°C in hybridization buffer containing [35S]- and [33P]-labeled single-stranded antisense of rat 5-HT_{1A} (nucleotides 1–1269, accession No. NM_012585) or 5-HT_{2A} (nucleotides 1–1566, accession No. NM_017254) receptor probes at 20×10^7 cpm/mL. Sections were treated with 4 μg/mL RNase A at 37°C for 1 h, washed with 1 × saline, sodium citrate solution (SSC), 50% formamide at 55°C for 1 h, and with 0.1 × SSC at 60°C for 1 h. After the last SSC wash, sections were rinsed with PBS and incubated for 1 h in PBS supplemented with 4% BSA and 0.3% Triton X-100. This was followed by the overnight incubation at 4°C with an antiparvalbumin goat antibody (Swant; 1:500). After rinsing 3 × 10 min in PBS, sections were processed with an ABC kit (Vector Laboratories). The material was incubated for 1 h at room temperature in a 1:200 dilution of the biotinylated secondary antibody, rinsed with PBS, and incubated with avidin-biotinylated horseradish peroxidase for 1 h. Sections were rinsed and the peroxidase reaction was then developed with 0.05% 3,3 diaminobenzidine-4 HCl and 0.03% H_2O_2. Free-floating sections were mounted on coated slides. Slides were dipped in Ilford K.5 nuclear tract emulsion (Polysciences, Inc.; 1:1 dilution in double distilled water) and exposed in the dark at 4°C for 4 weeks prior to development. Image analysis was performed using Fiji (Schindelin et al. 2012). Following background subtraction, a region of interest (0.08 × 0.072 cm) was drawn over the l-OFC, at the approximate locations of electrophysiological recordings, and the mean intensity was calculated for each section. Of note, 15–18 sections (240–288 μm total) were quantified for each subject, and averaged to yield a mean intensity value.

Results

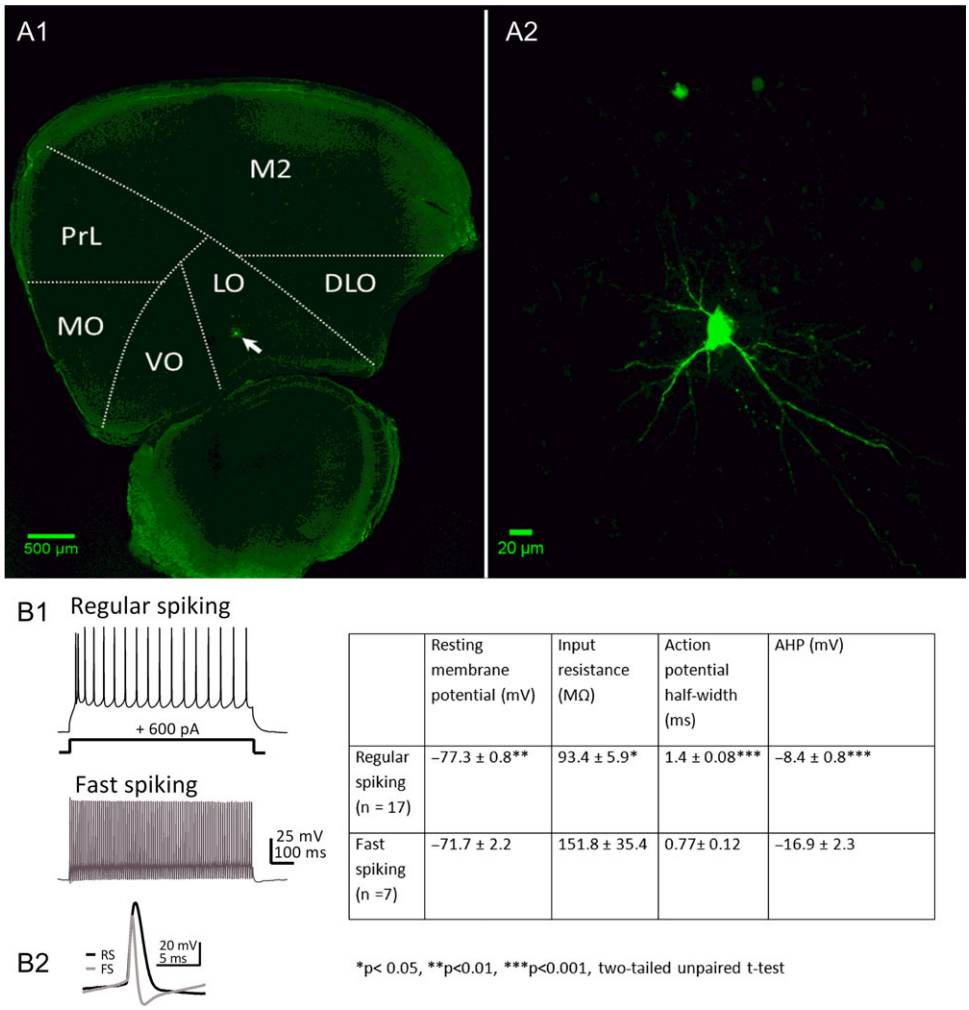
Whole-cell electrophysiological recordings were obtained from neurons located in the l-OFC in brain slices (Fig. 1A), and the cell's location was noted on a diagram of the coronal brain slice to ensure correspondence among brain slices from all experimental subjects. Injection of depolarizing voltage steps through the patch pipettes during current clamp recordings, obtained immediately after rupturing the membrane patch, revealed 2 groups of neurons distinguished by their electrophysiological properties. The first group demonstrated high rates of action potential discharge that was sustained throughout a 500 ms voltage step, brief action potential durations, and large afterhyperpolarizations (AHPs) (Fig. 1B). The second group of neurons demonstrated lower rates of firing, longer duration action potentials, and smaller amplitude AHPs. Based on prior work describing similar properties in several cortical regions, including the OFC (Badanich et al. 2013), these groups of neurons likely correspond to fast spiking (FS) and regular spiking (RS) cells, respectively. Previous work has also established that RS neurons comprise the largest group of cortical cells, also known as principal or pyramidal neurons, whereas FS cells likely represent GABAergic neurons (Badanich et al. 2013). As RS neurons represent the most frequently sampled population in behavioral OFC studies, our studies focused on these cells.

5-HT_{1A} Receptors Mediate Hyperpolarizing Outward Currents in OFC Neurons

The DRN sends a dense 5-HT projection to the OFC (Azmitia and Segal 1978; Hornung et al. 1990; Wilson and Molliver 1991a, 1991b). Our first experiments sought to determine the mechanisms through which 5-HT alters OFC neuron function. Application of 5-HT (10 μM) initiated an outward current that was associated with a decrease in input resistance (R_{in}) in approximately half of the recorded OFC neurons (Fig. 2A1–C2). To determine the identity of the current activated by 5-HT current-voltage (I/V) relationships were examined in voltage clamp, by slowly ramping the neuronal membrane potential from −64 to −144 mV in the absence and presence of 5-HT (Fig. 2C). These effects of 5-HT were compared to those of the $GABA_B$ receptor agonist baclofen (10 μM), which is also known to generate outward currents (Fig. 2B) and reduce input resistance via activation of a G-protein-coupled inwardly rectifying K^+ (GIRK) current mediated by Kir3.x ion channels. Although the effects of baclofen were larger than those of 5-HT, the agonists generated currents that were qualitatively similar with a reversal potential near that predicted by the Nernst equation for K^+ (ca. −90 mV; Fig. 2C). To identify the 5-HT receptor subtype mediating the outward currents, we performed pharmacological analyses with selective ligands. We found that the 5-HT_{1A} agonist, 7-(dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (8-OH-DPAT, 10 μM), generated outward currents in OFC neurons, and that the selective 5-HT_{1A} antagonist, WAY100635 (1 μM) prevented outward currents generated by 5-HT (Fig. 2D,E). Importantly, we also found that the 5-$HT2_{A/C}$ antagonist, ritanserin (4–8 μM), and the 5-HT_{2A} antagonist MDL100907 (500 nM) did not prevent outward currents generated by 5-HT, and that the 5-$HT_{1B/D}$ agonist, sumatriptan (1 μM) had no effect on OFC neurons (Fig. 2E). Together, these results suggest that the direct inhibition of OFC neurons occurs via the activation of 5-HT_{1A}-linked GIRK channels.

5-HT_{2A} Receptors Mediate Increased Glutamate Release in OFC Neurons

Initial experiments examined the effects of 5-HT (10 μM) on spontaneous, action potential-dependent EPSCs (sEPSCs) that were isolated from inhibitory synaptic currents via blockade of $GABA_A$ receptors. Serotonin increased the frequency of sEPSCs, and produced a small increase in the amplitudes of these synaptic currents (Fig. 3A). However, the kinetics of these



	Resting membrane potential (mV)	Input resistance (MΩ)	Action potential half-width (ms)	AHP (mV)
Regular spiking (n = 17)	−77.3 ± 0.8**	93.4 ± 5.9*	1.4 ± 0.08***	−8.4 ± 0.8***
Fast spiking (n =7)	−71.7 ± 2.2	151.8 ± 35.4	0.77± 0.12	−16.9 ± 2.3

*p< 0.05, **p<0.01, ***p<0.001, two-tailed unpaired t-test

Figure 1. Electrophysiological characterization of OFC neurons. (*A1*) Low-power fluorescent image of a brain slice containing the OFC. Regions are labeled according the atlas of Paxinos and Watson (1982) (M2, secondary motor cortex; PrL, prelimbic cortex; MO, medial orbital cortex; VO, ventral orbital cortex; LO, lateral orbital cortex; DLO, dorsolateral orbital cortex). Note the location of the biocytin-filled, Alexa Fluor-488 labeled neuron indicated by the arrow. (*A2*) High magnification, confocal image of the neuron indicated in *A1*. (*B1*) Table. Responses of two primary cell types to depolarizing current (600 pA) injection. Neurons were classified as either RS or FS based on the response to current injection. (*B2*) Single action potentials from each neuron type in *B1* are shown on an expanded time scale. Note the difference in action potential width and AHP size following the action potential. Parameters (mean ± SEM) of a subset of all recorded neurons are shown in the table at right. All subsequent figures reflect recordings only from RS neurons located within the LO region.

spontaneous synaptic currents were unaltered by 5-HT (Fig. 3*A2*). To determine whether action potential-independent mEPSCs were similarly altered by 5-HT, we measured these currents after application of the Na^+ channel blocker, tetrodotoxin (TTX, 500 nM). We found that mEPSCs were unaffected by 5-HT (Fig. 3*B*), suggesting that 5-HT likely increased action-potential-dependent glutamate release via depolarization of neurons presynaptic to the recorded cells. Consistent with this, 5-HT applied in the presence of WAY100635 produced inward currents in these OFC neurons (Fig. 6A). We next identified the 5-HT receptors involved in the facilitation of action potential-dependent glutamate release. Similar to 5-HT (Fig. 3), the 5-$HT_{2A/C}$ agonist (–)DOI (100–500 nM), significantly increased the frequency of sEPSCs, but did not alter their amplitudes (Fig. 4A). Inward currents were also observed in OFC neurons with (–)DOI application when 5-HT_{1A} receptors were blocked with WAY100635 (500 nM; Fig. 6B-C). Consistent with the effect of this agonist, 5-HT (10 µM) had no effect on sEPSCs in OFC slices that were treated with the 5-$HT_{2A/C}$ antagonist, ritanserin (8 µM; Fig. 4*B*). Similarly, pre-exposure of OFC slice to the selective 5-HT_{2A} antagonist, MDL100907 (500 nM), prevented both the facilitation of sEPSC frequency and inward currents following 5-HT (Figs. 4C, 6A). Together, these experiments suggest that 5-HT augments glutamate release onto l-OFC neurons via activation of 5-HT_{2A} receptors located on local circuit neurons within the OFC-containing brain slices.

Cocaine Exposure Alters 5-HT Signaling in the OFC

Animals exposed to cocaine exhibit impairments in OFC-dependent cognitive tasks (Jentsch et al. 2002; Schoenbaum et al. 2004; Schoenbaum and Setlow 2005; Calu et al. 2007; Takahashi et al. 2013; Lucantonio et al. 2014). Moreover, intact 5-HT signaling is necessary for OFC-dependent behavioral flexibility (Clarke et al. 2004; Roberts 2011; Izquierdo et al. 2012; West et al. 2013), OFC-dependent reversal learning, and outcome-based learning (Schoenbaum et al. 1998; Gallagher et al. 1999; Tremblay and Schultz 1999; Lucantonio et al. 2014).

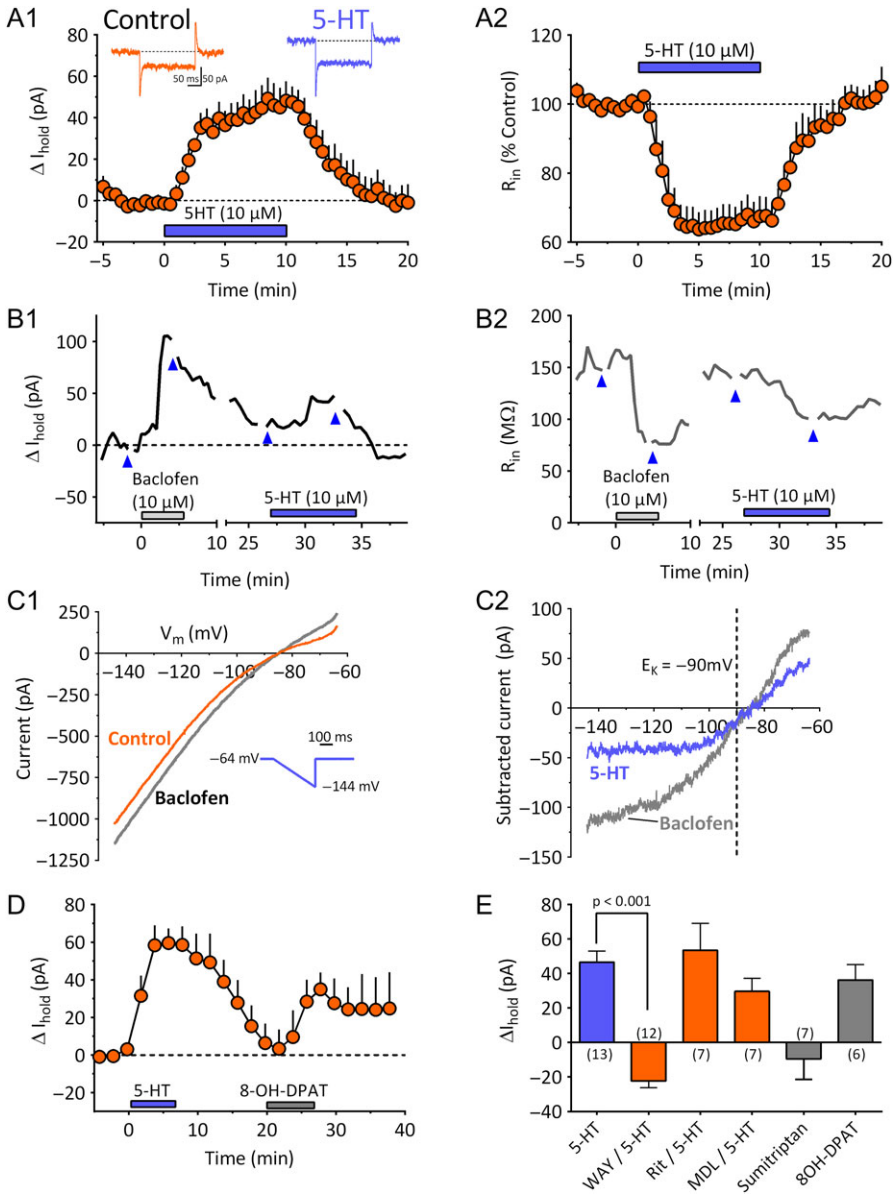


Figure 2. Postsynaptic effects of 5-HT in OFC neurons are mediated by 5-HT_{1A} receptors. (*A1*) Time course showing the effect of 5-HT (10 μM) on the amount of current required to voltage clamp OFC cells (n = 10 cells, 6 rats) at −60 mV (I_{hold}). (*A2*) Time course of the decrease in input resistance (R_{in}) during 5-HT application in the same group of OFC neurons shown in *A1*. The inset in A1 shows the current response to a voltage step of −10 mV from the −60 mV holding potential. Note the increase in step current and the outward shift in the baseline current. (*B1*) Time course showing the change in I_{hold} during application of either the $GABA_B$ receptor agonist baclofen or 5-HT in the same OFC neuron. (*B2*) Effects of baclofen and 5-HT on R_{in} in the same cell as shown in *B1*. The arrow heads indicate where voltage ramps were applied and current-voltage (I/V) plots were constructed, as shown in *C1* and *C2*. (*C1*) An I/V plot made, during the peak baclofen effect, by ramping the membrane voltage from −64 to −144 mV (inset). Note the increase in conductance caused by baclofen. (*C2*) Currents generated in OFC neurons during the peak effects of 5-HT or baclofen. Each current was obtained using the stepping protocol shown in *C1*, with the total current obtained during the predrug control period subtracted from that obtained during the peak drug effect. Note that although the subtracted current obtained during 5-HT application is smaller than that obtained during baclofen, both currents reverse near the predicted reversal potential for K^+, as determined by the Nernst equation. (*D*) Time course of the effect 5-HT and of the 5-HT_{1A} receptor agonist on I_{hold} in a group of OFC neurons (n = 6 cells, 5 rats). The absence of reversal of 8-OH-DPAT, compared to 5-HT, likely arises from the slow washout of this drug from the tissue, and the inability of the 5-HT transporter to facilitate its reversal. (*E*) Effects of 5-HT ligands on I_{hold} in OFC neurons. The effect of 5-HT (10 μM) on I_{hold} alone, and following application of the indicated 5-HT receptor antagonist is shown. Antagonists: (WAY) WAY100635 (1 μM), a 5-HT_{1A} antagonist, (Rit) Ritanserin (4–8 μM), a 5-HT_{2A} antagonist, (MDL) MDL100907 (500 nM), a 5-HT_{2A} antagonist. The effects of the 5-$HT_{1B/1D}$ agonist, sumatriptan (1 μM), and the 5-HT_{1A} agonist, 8-OH-DPAT (10 μM) are also shown. Note that both 5-HT and 8-OH-DPAT generated outward currents, and that only WAY antagonized the effects of 5-HT. This provides evidence that 5-HT_{1A} receptors mediate the inhibitory outward currents in OFC neurons. Each data point was obtained from 4 to 9 rats.

Therefore, to test the hypothesis that enduring alterations in 5-HT signaling contribute to impaired OFC function in cocaine addiction, we measured the effects of 5-HT on OFC neurons from CSA and yoked control (CYA) animals that had been withdrawn for 11–73 days (CSA = 49.3 ± 7.0, CYA = 46.3 ± 6.9, mean ± SEM, n = 32 rats). Effects of 5-HT in these groups were compared to those obtained in a naïve control group of animals.

Rats achieved stable rates of CSA by the third session of training (see Supplementary 1 A), maintaining 35–50 infusions

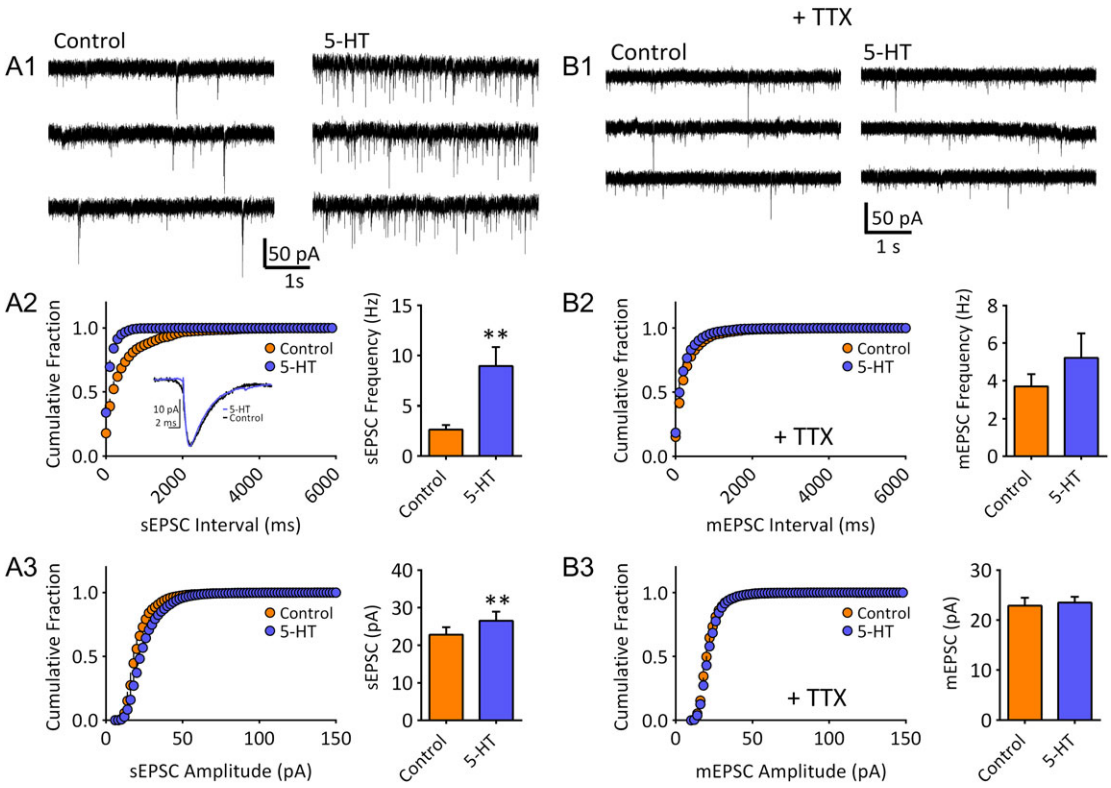


Figure 3. Serotonin increases action potential-dependent glutamatergic synaptic transmission in OFC neurons in brain slices from naïve rats. (*A1*) Individual traces from a single voltage-clamped OFC neuron showing an increase in action potential-dependent sEPSCs during 5-HT (10 μM) application. (*A2*) Left, mean cumulative inter-EPSC interval distribution showing a significant decrease in the interval between sEPSCs during 5-HT application ($n = 8$ neurons, 5 rats). The inset shows superimposed averaged sEPSCs collected during the control and 5-HT application periods, showing no change in sEPSC kinetics caused by 5-HT. Right, mean sEPSC frequency (sEPSC frequency = 1/inter-sEPSC interval) in the absence and presence of 5-HT (**$P < 0.01$, paired *t*-test). (*A3*) Left, mean cumulative amplitude distribution showing the effect of 5-HT ($n = 8$ neurons, 5 rats). There was a small increase in the mean sEPSC amplitude during 5-HT application, that is also observed in the mean sEPSC bar graph at right (**$P < 0.01$, paired *t*-test). (*B1*) Individual traces from an OFC neuron showing a lack of 5-HT (10 μM) effect on action potential-independent mEPSCs, recorded during blockade of Na^+ channels by TTX (500 nM). (*B2*) Mean cumulative inter-mEPSC interval distribution showing no effect of 5-HT on mEPSC interval with TTX application ($n = 7$ neurons, 5 rats). Right, absence of an effect of 5-HT on mean mEPSC frequency ($P > 0.05$, paired *t*-test). (*B3*) Absence of 5-HT effects on mean mEPSC amplitude cumulative distribution in the presence of TTX. Right, a similar lack of effect of 5-HT on mean mEPSC amplitude in TTX ($P > 0.05$, paired *t*-test). These data indicate that the effects of 5-HT on glutamate release are dependent upon action potentials.

per 2-h session, and receiving approximately 30–35 mg/kg cocaine from the 3rd to 12th sessions (see Supplementary 1B). No differences in resting membrane potential (RMP) were observed among OFC neurons obtained from naïve animals and those trained to self-administer cocaine (Fig. 5A). Similarly, the relationship between depolarizing current injected through whole-cell electrodes and the number of action potentials was not significantly different among OFC neurons from these groups, suggesting that neuronal excitability was unaltered by cocaine experience (Fig. 5B). A previous study has shown that exposure to cocaine alters signaling at several GPCRs (Hearing et al., 2013). Therefore, we examined the activation of postsynaptic $GABA_B$ receptors in OFC neurons from CSA and naïve animals. The $GABA_B$ agonist baclofen (10 μM) generated outward currents of similar magnitude in cells from both groups of rats (Fig. 5C), suggesting that GPCR function was not generally altered by cocaine experience. The effects of 5-HT were next measured in OFC neurons from naïve, CSA, and CYA rats. Consistent with studies described above (Fig. 2), 5-HT (10 μM) generated outward currents when its effects were averaged across all OFC cells from naïve rats (Fig. 5D,E, $n = 34$ neurons). This mean effect was obtained from a group of cells in which 44% demonstrated outward currents, and 56% demonstrated either no change or an inward change in I_{hold} (nonoutward currents; Fig. 5E,F). In contrast to OFC neurons obtained from naïve rats, neurons from either CSA ($n = 37$ neurons) or CYA ($n = 17$ neurons) rats exhibited small inward currents when the effects of 5-HT were averaged across all cells. Furthermore, the percentage of neurons demonstrating outward currents in response to 5-HT was lower in both CSA and CYA groups (14% and 18%, respectively; Fig. 5E,I), and the percentage of cells in these groups exhibiting either no response, or inward currents were higher than naïve controls (CSA = 86%, CYA = 82%; Fig. 5F). However, when present, the average magnitude of the outward current in response to 5-HT was similar in cells from naïve and CSA groups (Fig. 5G,H), despite the lower percentage of cells exhibiting this response in the CSA group. The differences in proportions of cells showing inward, or non-inward currents were statistically different among the 3 groups ($\chi^2(2) = 7.84$, $P = 0.02$), indicating that contingent or noncontingent exposure to cocaine significantly altered the proportion of cells expressing a postsynaptic 5-HT_{1A} receptor-mediated response to 5-HT.

Previous work in layer-V pyramidal neurons of the prelimbic or anterior cingulate subdivisions of the medial PFCx showed that inward currents can be activated by 5-HT in a subset of these cells, and that this was mediated by 5-HT_{2A} receptors (Beique et al. 2007). Moreover, this inward depolarizing current

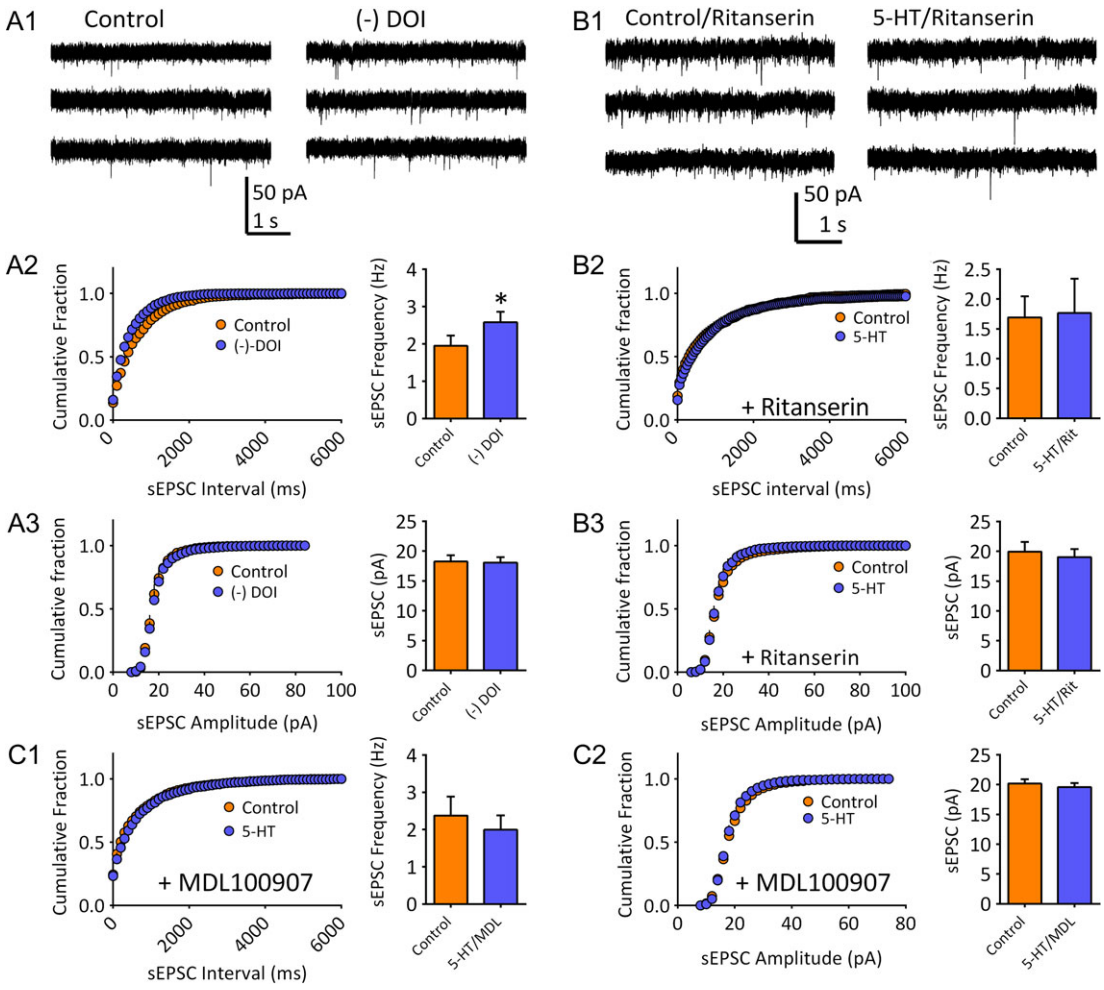


Figure 4. The effects of 5-HT on glutamate release are mediated by 5-HT$_{2A}$ receptors in OFC neurons (compare with effect of 5-HT shown in Fig. 3). (*A1*) Traces from a single OFC neuron showing an increase in sEPSCs with application of the 5-HT$_{2A/C}$ agonist (–)DOI (100–500 nM). (*A2*) (–)DOI (500 nM) increase in the frequency of sEPSCs, as seen by a decrease in the mean cumulative inter-sEPSC interval (left), and the mean frequency of sEPSCs (right, *$P < 0.05$, paired *t*-test, $n = 13$ neurons, 9 rats). (*A3*) (–)DOI did not significantly alter sEPSC amplitudes. (*B1*) Traces showing that pretreatment with the 5-HT$_{2A/C}$ antagonist, ritanserin (Rit, 8 μM), prevents the effect of 5-HT on sEPSCs (compare with effect of 5-HT in Fig. 3A). The data in *B2* and *B3* also show that 5-HT had no effect on sEPSC frequency or amplitude following Rit treatment ($n = 9$ cells, 5 rats). (*C1*, *C2*) Treatment with the 5-HT$_{2A}$ antagonist, MDL100907 (MDL, 500nM), prevents the presynaptic effect of 5-HT on sEPSCs in OFC neurons ($n = 10$ cells, 7 rats). Collectively, these data suggest that the effects of 5-HT on sEPSCs are mediated by presynaptic 5-HT$_{2A}$ receptors.

was proposed to mediate an increase in sEPSCs by 5-HT$_{2A}$ receptors in these cells via recurrent synapses (Beique et al. 2007). Since we also observed inward currents during 5-HT application, and 5-HT$_{2A}$-mediated increases in sEPSCs in layer-V OFC neurons, we sought to determine whether the inward currents are coupled to 5-HT$_{2A}$ receptors, and whether this is altered during cocaine withdrawal. In naïve brain slices pretreated with the 5-HT$_{1A}$ antagonist WAY100635 (1 μM, $n = 9$), we observed robust inward currents in layer-V OFC pyramidal neurons (Fig. 6A,C). Moreover, these 5-HT-mediated currents were not observed in a separate population of cells in naïve slices that had been pretreated with both WAY100635 and the 5-HT$_{2A}$ antagonist MDL100907 (500 nM, Fig. 6A,C; $n = 17$). Application of the 5-HT$_{2A}$ agonist DOI (500 nM) under these conditions also generated inward currents in a different group of OFC neurons (Fig. 6B,C; $n = 8$). Together, these data indicate that 5-HT$_{2A}$ receptors are located on OFC neurons and that they mediate excitatory inward currents in these cells. To determine whether the 5-HT-mediated inward currents are altered during cocaine withdrawal we compared them among naïve, CSA, and CYA groups (Fig. 6C). We found that 5-HT-mediated inward currents in naïve OFC neurons were significantly larger than those obtained from CSA and CYA rats (Fig. 6C; one-way ANOVA). However, the size of these inward currents did not differ between CSA and CYA groups (Fig. 6C). These data suggest that cocaine exposure decreases 5-HT$_{2A}$ receptor activation of inward currents in OFC neurons.

The effects of 5-HT on action potential-dependent sEPSCs were also compared in neurons from naïve, CSA, and CYA groups that were withdrawn from cocaine. A comparison of baseline sEPSCs from neurons in the naïve and CSA groups revealed no differences in the amplitudes or frequency of these events (Fig. 7A), suggesting that cocaine experience did not alter baseline action potential-dependent glutamate release in OFC. In contrast to the increase in sEPSC frequency caused by 5-HT (10 μM) acting via 5-HT$_{2A}$ receptors (Figs 3 and 4) in OFC neurons from naïve animals, 5-HT did not alter the mean frequency or amplitude of sEPSCs in OFC neurons obtained from CSA or CYA animals (Fig. 7B–D). Furthermore, the proportion of OFC neurons demonstrating no response to 5-HT was

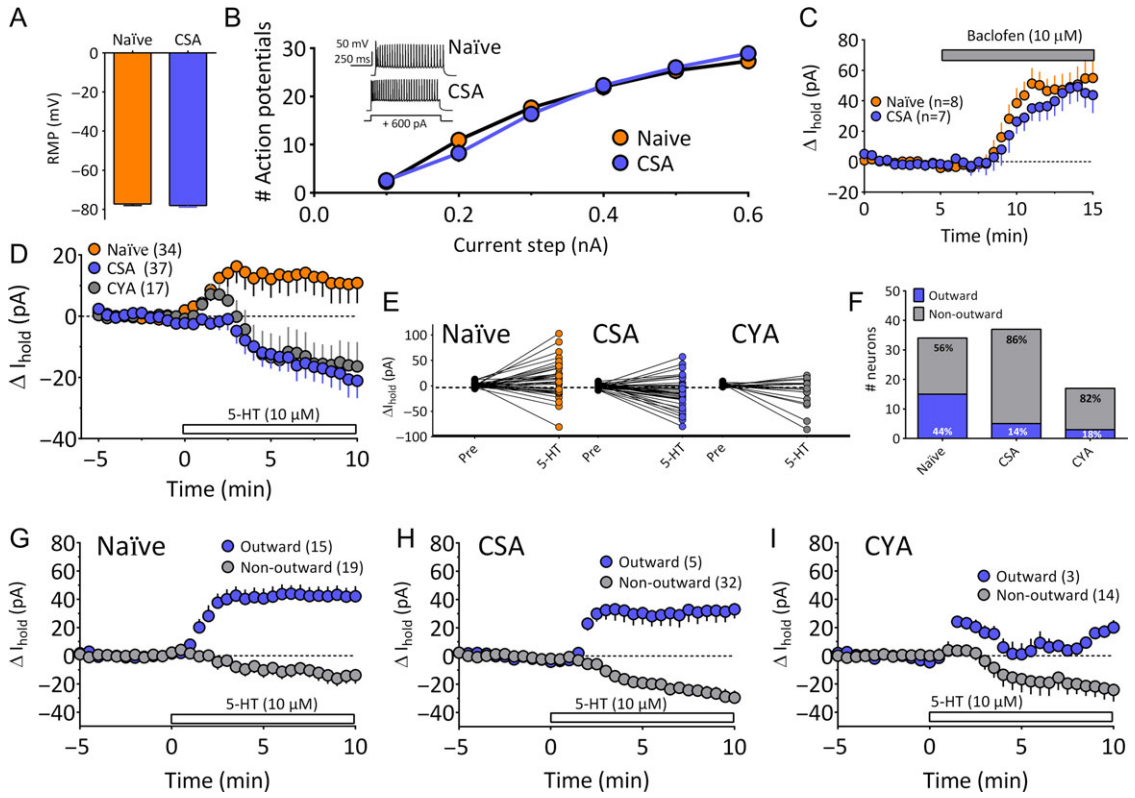


Figure 5. The postsynaptic response to 5-HT in OFC neurons is altered in rats exposed to cocaine via self-administration (CSA), or noncontingently, via yoked-administration (CYA). (*A*) CSA did not alter OFC neuron RMP, compared to neurons from naïve control rats (n = 19 cells in each group, 9 naïve, and 12 CSA rats). Means and standard errors are indicated for each group, though the error bars are much smaller than the mean RMPs. (*B*) CSA did not alter the excitability of OFC neurons, as determined by the relationship between the size of depolarizing current steps, injected through the patch pipette, and the number of action potentials generated by these steps ($F_{(1,29)}$ = 0.009075, P = 0.9248, 2-way repeated measures ANOVA). Inset shows representative membrane responses to depolarizing current injection (+600 pA) in single neurons obtained from naïve and CSA rats. Current steps were injected through the whole-cell electrode in cells recorded at RMP. (*C*) CSA (n = 7 neurons, 4 rats) did not alter the membrane current generated by activation of $GABA_B$ receptors by baclofen, compared to that observed in neurons from naïve rats (n = 8 neurons, 5 rats; $F_{(1,13)}$ = 0.004642, P = 0.9467, 2-way repeated measures ANOVA). (*D*) CSA and CYA alter the OFC neuron response to 5-HT. Mean time course of the effect of bath-applied 5-HT on membrane current in all neurons. In OFC neurons from naïve rats 5-HT generates outward currents, whereas following CSA or CYA the mean current response is inward. (*E*) The peak effect of 5-HT on membrane current is shown for each OFC neuron included in the data-set in panel *D* for naïve, CSA, and CYA groups. (*F*) Distribution of membrane current direction (outward or nonoutward) for all OFC neurons included in the analysis. Outward and nonoutward current responses were partitioned only when the change in current reached a threshold of ±2.5 standard deviations from the mean. Both CSA and CYA significantly reduced the proportion of neurons exhibiting outward currents, and increased the proportion showing nonoutward currents, in response to 5-HT, as compared to responses in cells from naïve rats (P < 0.01, Chi-square). (*G–I*) Mean time courses of membrane current response to 5-HT for all OFC neurons, sorted according to criteria defined in panel *F*. Note that although outward currents in response to 5-HT are observed in the CSA and CYA groups, the number of cells as a proportion of the total is smaller than in neurons from naïve rats. In (*D–I*) the number of cells is indicated in parentheses, and are obtained from 9 to 18 rats in each group.

significantly increased in the CSA and CYA groups compared to naïve controls ($\chi^2(2)$ = 12.7, P = 0.0017), suggesting that contingent and noncontingent cocaine exposure impairs serotonergic control of glutamate release in OFC following withdrawal.

5-HT_{1A} and 5-HT_{2A} Receptor In-Situ Hybridization

Our electrophysiological studies show that both 5-HT_{1A} and 5-HT_{2A} receptor-mediated responses were largely eliminated in the OFC of rats several weeks following CSA or CYA. To determine whether this cocaine experience altered mRNA for these receptors, in-situ hybridization was performed in the OFC of naïve, and in CSA, and CYA rats that were euthanized 45 days following the last cocaine experience (Fig. 8). Analysis of mRNA autoradiograms (Fig 8*A*,*B*) revealed that 5-HT_{1A} and 5-HT_{2A} receptor mRNA expression was affected differently by cocaine exposure, and this was confirmed with a 2-way ANOVA showing significant interaction between treatment group and receptor (2-way ANOVA, $F_{(2,15)}$ = 7.7, P = 0.005; Fig. 8*C*). Post-hoc analysis revealed that whereas 5-HT_{1A} mRNA expression was not significantly altered following CSA or CYA, 5-HT_{2A} mRNA was elevated in both groups, compared to naïve control rats (Sidak's test, P = 0.04, and P = 0.01, respectively; Fig. 8*C*). Therefore, there was a significant treatment effect on the ratio of 5-HT_{2A} to 5-HT_{1A} mRNA expression (one-way ANOVA, $F_{(2,15)}$ = 6.9, P = 0.02), and post-hoc analysis showed that these ratios were significantly elevated in OFC following CSA and CYA, compared to the naïve control group (P < 0.05, Holm-Sidak's test; Fig. 8*D*). Collectively, these data demonstrate that there was a significant elevation of 5-HT_{2A} mRNA at 45 days of withdrawal from CSA and CYA.

Discussion

These studies identify 2 cellular mechanisms through which 5-HT can influence OFC principal cell function. First, activation of postsynaptic 5-HT_{1A} receptors generates inhibitory outward currents in OFC neurons through the opening of GIRK (Kir3.x) channels. The second mechanism involves a 5 HT_{2A} receptor-mediated regulation of the probability of glutamate

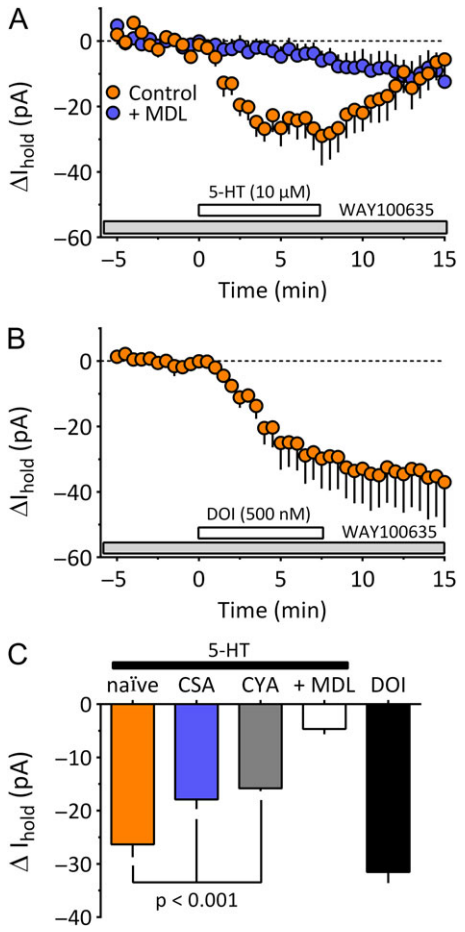


Figure 6. 5-HT$_{2A}$ receptor activation of depolarizing inward currents is reduced following CSA, or CYA in OFC neurons in vitro. (*A*) The inward current caused by 5-HT, during 5-HT$_{1A}$ receptor antagonism by WAY100635 (1 μM; n = 9 cells, 4 rats), is blocked by the 5-HT$_{2A}$ antagonist MDL100907 (MDL, 500 nM; n = 17 cells, 7 rats). (*B*) Time course of the inward current caused by the 5-HT$_{2A}$ agonist DOI during blockade of 5-HT$_{1A}$ receptors by WAY100635 (1 μM) in OFC neurons (n = 8 cells, 4 rats). The slower onset and lack of reversal of the effect of DOI likely represents the rate of partitioning into, and diffusion out of the brain slice. (*C*) Summary of magnitude of 5-HT$_{2A}$ receptor-mediated inward currents in OFC neurons obtained from naïve and cocaine-exposed rats. Shown are responses to 5-HT (10 μM) in cells from naïve rats, and from those withdrawn from CSA or CYA. Also, shown are the effects of the 5-HT$_{2A}$ antagonist MDL100907 on the 5-HT response, and the effect of the 5-HT$_{2A}$ agonist DOI in cells from naïve rats. Naïve, CSA, and CYA data are from cells shown in Fig. 5D. Note that 5-HT-mediated inward currents are significantly smaller following CSA or CYA (one-way ANOVA, $F_{4,20}$ = 183.9, P < 0.0001, P < 0.001, Tukey's multiple comparisons test).

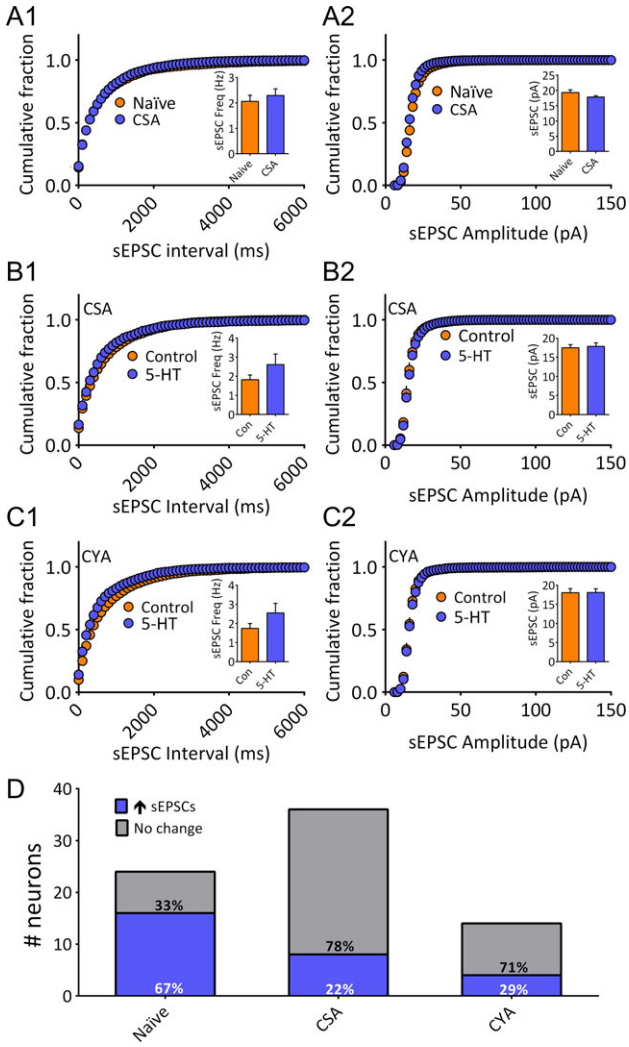


Figure 7. The presynaptic response to 5-HT in OFC neurons is altered in rats exposed to cocaine via self-administration (CSA), or noncontingently, via yoked-administration (CYA). (*A1*, *A2*) CSA does not alter baseline sEPSC frequencies or amplitudes compared to OFC neurons from naïve controls (naïve n = 34 cells, 19 rats, CSA n = 36 cells, 21 rats). (*B1*, *B2*) CSA prevents the 5-HT$_{2A}$ receptor-dependent increase in sEPSC frequency observed in OFC neurons from naïve rats (compare with Fig. 3A), and does not alter sEPSC amplitude (n = 36 cells, 18 rats). (*C1*, *C2*) CYA prevents the 5-HT$_{2A}$ receptor-dependent increase in sEPSC frequency observed in OFC neurons from naïve rats (compare with Fig. 3A), and does not alter sEPSC amplitude (n = 14 cells, 12 rats). (*D*) Proportions of OFC neurons showing increased sEPSC frequencies versus no change in frequencies for naïve, CSA, and CYA groups. The change in the proportion of OFC neurons showing increased sEPSCs upon 5-HT application was significant ($\chi^2(2)$ = 12.7, P = 0.0017). Inset bar graphs in *A*–*C* indicate mean sEPSC frequency and amplitude in each indicated group.

release onto OFC principal neurons that may be mediated by depolarization of OFC neurons forming recurrent collateral synapses on the recorded cells (Beique et al. 2007). We also show that exposure to cocaine, either through operant self-administration or passively through yoked administration, alters the ability of 5-HT to regulate OFC function. Specifically, the inhibition of OFC neurons by 5-HT$_{1A}$ receptors, and the increase in glutamate release by 5-HT$_{2A}$ receptors are largely lost following cocaine. Moreover, after cocaine, 5-HT$_{1A}$ receptors no longer initiate hyperpolarizing outward currents in OFC principal neurons, but rather, generate depolarizing inward currents. This switch from net inhibition to excitation by 5-HT may profoundly alter OFC network activity during cocaine abstinence. Similarly, the loss of 5-HT$_{2A}$ receptor-mediated increase in glutamate release would be expected to contribute to OFC dysregulation. As these changes are observed up to several weeks after withdrawal, they represent enduring alterations in function that may relate to long-term aspects of cocaine use, such as relapse and craving.

The changes in 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor-mediated signaling following cocaine experience occur without alterations in OFC pyramidal neuron RMP, or excitability. Moreover, disruption of 5-HT$_{1A}$ inhibition occurs in the absence of changes in GABA$_B$ receptor-mediated activation of GIRK, unlike a report in

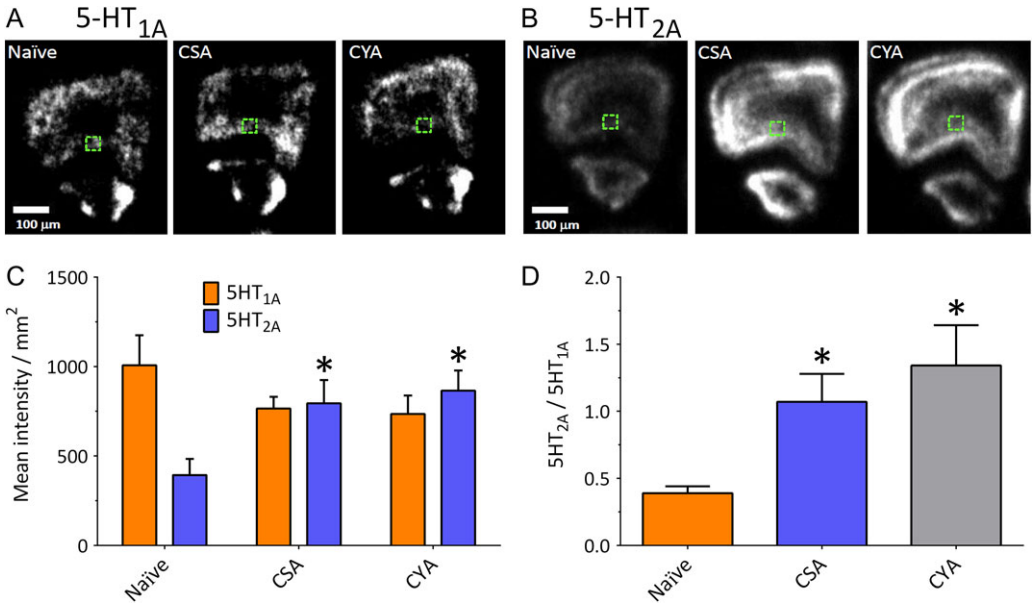


Figure 8. In-situ hybridization measurement of 5-HT_{1A}, and 5-HT_{2A} mRNA in rat OFC following either CSA, or CYA, compared to age-matched naïve rats (n = 6 rats per group, 45 days withdrawal). (*A*) Digital autoradiograms showing 5-HT_{1A} mRNA labeling in single coronal rat brain sections from rats in each group, at approximately 3.7 mm anterior to bregma (Paxinos and Watson 1982). (*B*) Digital autoradiograms showing 5-HT_{2A} mRNA labeling in coronal sections from rats in each group, at approximately 3.7 mm anterior to bregma. (*C*) Mean intensity of 5-HT_{1A} and 5-HT_{2A} probe radioactivity in the l-OFC for each group, measured at location shown with dashed green rectangle in *A* and *B* (*$P < 0.05$; Sidak's post-hoc test). (*D*) Ratio of 5-HT_{2A} to 5-HT_{1A} mRNA expression in the OFC. The relationship between 5-HT_{1A} and 5-HT_{2A} mRNA was significantly altered by both contingent and noncontingent exposure to the drug (*$P < 0.05$, Holm-Sidak post-hoc test).

which prelimbic cortex pyramidal neurons exhibited diminished $GABA_B$ receptor-mediated GIRK currents following repeated cocaine (Hearing et al. 2013). The 5-HT_{2A}-mediated increase in glutamate release observed in naïve OFC neurons is prevented by Na^+ channel block with TTX, and these receptors also generate excitatory currents in some OFC neurons. This suggests a local circuit effect of 5-HT in which glutamatergic neurons located in OFC are depolarized by 5-HT_{2A} receptors to modulate other OFC neurons. Consistent with this, inward currents were observed in layer-V OFC neurons with 5-HT or (–) DOI when 5-HT_{1A} receptors were blocked, and the 5-HT-induced inward currents were prevented by 5-HT_{2A} receptor antagonism. This 5-HT-mediated increase in glutamate release was lost, and there was a significant decrease in 5-HT-induced inward currents in OFC neurons following CSA or CYA. This suggests that a reduction in 5-HT_{2A} depolarization of these local circuit OFC neurons may be responsible for the absence of modulation of sEPSCs. Similar effects of 5-HT_{1A} and 5-HT_{2A} receptor activation in naïve animals have been described in pyramidal neurons in layer-V of prelimbic or anterior cingulate subdivisions of PFCx in brain slices from naïve rats and mice (Beique et al. 2007; Andrade 2011).

Although precise computational roles for these 5-HT-dependent mechanisms are not yet defined, the modulation of intrinsic recurrent excitatory networks by 5-HT_{2A} receptors and control of network input strength by 5-HT_{1A} receptors have been proposed for other PFCx areas (Andrade 2011). Based upon this, and our current findings, we hypothesize that activation of these receptors by 5-HT is integral to the proper function of the OFC and its involvement in cognitive and behavioral processes, and disruption of 5-HT receptor signaling by cocaine is central to the dysregulation of OFC-dependent behavior by this drug.

Our in-situ hybridization data indicate that the relationship between 5-HT_{1A} and 5-HT_{2A} receptor transcripts is altered by CSA and CYA. Specifically, in naïve rat OFC, 5-HT_{1A} mRNA levels are higher than those of 5-HT_{2A} receptors, and this difference is eliminated by cocaine experience. This change is accounted for by an increase in 5-HT_{2A} mRNA, with no significant change in 5-HT_{1A} mRNA. The lack of significant changes in 5-HT_{1A} mRNA expression implies that the loss of inhibitory function mediated by this receptor results from its post-translational modification, internalization, or reduced coupling to G-proteins. Moreover, as we show that activation of GIRK currents by $GABA_B$ receptors is unaltered by CSA, downregulation of these ion channels cannot explain the reduced 5-HT signaling. The increase in 5-HT_{2A} receptor mRNA following cocaine is particularly unexpected because modulation of glutamate release by this receptor was lost, and the inward current mediated by these receptors was significantly smaller following CSA or CYA. Therefore, the increase in 5-HT_{2A} mRNA represents an increase in 5-HT_{2A} receptor expression capacity that may or may not be associated with increased receptor protein expression. Related to this, a prior study detected no change in 5-HT_{2A} receptor protein expression in the PFCx 48 h after 7 days of experimenter-administered cocaine, but did observe an increase in 5-HT_{2A}-mediated phospholipase C, and extracellular regulated kinase activity (Franklin and Carrasco 2015). This study also demonstrated decreased phosphorylation of 5-HT_{2A} receptors following this cocaine regimen, as well as diminished G-protein receptor kinase-5 levels (Franklin and Carrasco 2015). Taken together, our results and those of this previous study suggest that 5-HT_{2A} receptor mRNA is increased and the expression of this receptor is unchanged following chronic cocaine. Therefore, it is likely that altered downstream signaling is responsible for the changes in physiological coupling that we observe.

It is well established that intact 5-HT function is necessary to support the behaviorally adaptive functions in which the OFC

participates, such as encoding outcome expectancies, response inhibition, and acquisition of conditioned reinforcement (Roberts 2011; Lucantonio et al. 2012). Moreover, a relationship between cocaine exposure, 5-HT function, and cognitive flexibility is found in a study in which genetic deletion of the SERT improves baseline reversal learning and prevents its impairment following CSA (Nonkes et al. 2013). Although we find no change in 5-HT levels or turnover in OFC 4 weeks following CSA (Supplementary Fig. 2), cocaine is an efficacious inhibitor of the SERT, and this leads acutely to increased extracellular levels of 5-HT (Hoffman et al. 1991; Torres et al. 2003). Therefore, this may cause altered 5-HT receptor function and dysregulation of 5-HT control of the OFC. However, in contrast to our data, another study shows that a 3–8-week administration of a selective-serotonin reuptake inhibitor desensitizes 5-HT_{1A} receptors, but spares 5-HT_{2A} receptor function in OFC (El Mansari and Blier 2005). The discrepancy between our results and these suggest that the deficit in OFC 5-HT signaling that we report may result from effects of cocaine that are more complex than intermittent SERT inhibition.

Increased metabolic activity in OFC is observed in human cocaine addicts in the early stages of cocaine withdrawal (Volkow et al. 1991), followed by a diminished metabolic response in latter stages (Volkow et al. 1993; Adinoff et al. 2001; Volkow et al. 2001; Kim et al. 2005). Moreover, both human (Ersche et al. 2008) and animal studies demonstrate that OFC-dependent reversal learning is impaired following chronic passive (Jentsch et al. 2002; Schoenbaum et al. 2002) or self-administered (Calu et al. 2007; Porter et al. 2011) psychostimulants. Additional work shows that cocaine exposure disrupts the ability of the OFC to encode outcome expectancies that are used to guide future behavior and support learning in rodents (Stalnaker et al. 2006; Lucantonio et al. 2012, 2014). A notable aspect of the impairments in OFC-dependent behavior in animal studies, and in OFC 5-HT signaling that we describe here is that these deficits persist for weeks to months after the last exposure to cocaine or other psychostimulants (Lucantonio et al. 2012). Collectively, this suggests that these changes in OFC function may relate to longer-lasting adaptations associated with addiction, and it is possible that impaired 5-HT signaling in the OFC may be critical to these behavioral changes. As the number of days of withdrawal in this study is broad, an important objective for future studies should be to more carefully define the temporal constraints of cocaine exposure and withdrawal on altered 5-HT signaling in OFC.

Clinical descriptions of psychostimulant addiction emphasize maladaptive decision-making and compulsive seeking of drug despite substantial aversive consequences, and it is hypothesized that this results at least partly from altered OFC function. In general, impairments in behavioral tasks involving the use of outcome expectancies and cognitive flexibility in animal models of addiction, or in human cocaine addicts, resemble those observed following damage of the OFC. Moreover, there is substantial clinical and preclinical literature supporting a role for 5-HT in regulating OFC activity in behavior, and addiction, and for its dysregulation in neuropsychiatric disorders. We propose that the loss of regulation of the OFC circuitry by 5-HT following cocaine experience represents an enduring change that may play a prominent role in the disrupted decision-making associated with addiction disorders.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

Funding

The National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program.

Notes

We would like to thank Dr Hui-Ling Wang and the NIDA-IRP Histology Core for performing the in-situ hybridization experiments, and Dr Geoffrey Schoenbaum for a critical reading of the manuscript. *Conflict of Interest*: None declared.

References

Adinoff B, Devous MD Sr., Best SM, George MS, Alexander D, Payne K. 2001. Limbic responsiveness to procaine in cocaine-addicted subjects. Am J Psychiatry. 158:390–398.

Andrade R. 2011. Serotonergic regulation of neuronal excitability in the prefrontal cortex. Neuropharmacology. 61: 382–386.

Azmitia EC, Segal M. 1978. An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. J Comp Neurol. 179:641–667.

Badanich KA, Mulholland PJ, Beckley JT, Trantham-Davidson H, Woodward JJ. 2013. Ethanol reduces neuronal excitability of lateral orbitofrontal cortex neurons via a glycine receptor dependent mechanism. Neuropsychopharmacology. 38:1176–1188.

Bechara A. 2005. Decision making, impulse control and loss of willpower to resist drugs: a neurocognitive perspective. Nat Neurosci. 8:1458–1463.

Beique JC, Imad M, Mladenovic L, Gingrich JA, Andrade R. 2007. Mechanism of the 5-hydroxytryptamine 2 A receptor-mediated facilitation of synaptic activity in prefrontal cortex. Proc Natl Acad Sci. 104:9870–9875.

Boulougouris V, Robbins TW. 2010. Enhancement of spatial reversal learning by 5-HT2C receptor antagonism is neuroanatomically specific. J Neurosci. 30:930–938.

Calu DJ, Stalnaker TA, Franz TM, Singh T, Shaham Y, Schoenbaum G. 2007. Withdrawal from cocaine self-administration produces long-lasting deficits in orbitofrontal-dependent reversal learning in rats. Learn Mem. 14:325–328.

Chandler DJ, Lamperski CS, Waterhouse BD. 2013. Identification and distribution of projections from monoaminergic and cholinergic nuclei to functionally differentiated subregions of prefrontal cortex. Brain Res. 1522:38–58.

Clarke HF, Dalley JW, Crofts HS, Robbins TW, Roberts AC. 2004. Cognitive inflexibility after prefrontal serotonin depletion. Science. 304:878–880.

Egan MF, Wing L, Li R, Kirch DG, Wyatt RJ. 1994. Effects of chronic cocaine treatment on rat brain: long-term reduction in frontal cortical serotonin. Biol Psychiatry. 36:637–640.

El Mansari M, Blier P. 2005. Responsiveness of 5-HT(1 A) and 5-HT2 receptors in the rat orbitofrontal cortex after long-term serotonin reuptake inhibition. J Psychiatry Neurosci. 30: 268–274.

Ersche K, Roiser J, Robbins T, Sahakian B. 2008. Chronic cocaine but not chronic amphetamine use is associated with perseverative responding in humans. Psychopharmacology (Berl). 197:421–431.

Franklin JM, Carrasco GA. 2015. Cocaine potentiates multiple 5-HT2A receptor signaling pathways and is associated with decreased phosphorylation of 5-HT2A receptors in vivo. J Mol Neurosci. 55:770–777.

Gallagher M, McMahan RW, Schoenbaum G. 1999. Orbitofrontal cortex and representation of incentive value in associative learning. J Neurosci. 19:6610–6614.

Goncalves L, Nogueira MI, Shammah-Lagnado SJ, Metzger M. 2009. Prefrontal afferents to the dorsal raphe nucleus in the rat. Brain Res Bull. 78:240–247.

Hearing M, Kotecki L, Marron-áFernandez-áde-áVelasco E, Fajardo-Serrano A, Chung H-á, Luj+ín R, Wickman K. 2013. Repeated cocaine weakens GABAB-Girk signaling in layer 5/6 pyramidal neurons in the prelimbic cortex. Neuron. 80: 159–170.

Hoffman BJ, Mezey E, Brownstein MJ. 1991. Cloning of a serotonin transporter affected by antidepressants. Science. 254: 579–580.

Hornung JP, Fritschy JM, Tork I. 1990. Distribution of two morphologically distinct subsets of serotoninergic axons in the cerebral cortex of the marmoset. J Comp Neurol. 297: 165–181.

Izquierdo A, Carlos K, Ostrander S, Rodriguez D, McCall-Craddolph A, Yagnik G, Zhou F. 2012. Impaired reward learning and intact motivation after serotonin depletion in rats. Behav Brain Res. 233:494–499.

Izquierdo A, Murray EA. 2004. Combined unilateral lesions of the amygdala and orbital prefrontal cortex impair affective processing in rhesus monkeys. J Neurophysiol. 91: 2023–2039.

Jentsch JD, Olausson P, De La Garza R II, Taylor JR. 2002. Impairments of reversal learning and response perseveration after repeated, intermittent cocaine administrations to monkeys. Neuropsychopharmacology. 26:183–190.

Jones B, Mishkin M. 1972. Limbic lesions and the problem of stimulus–reinforcement associations. Exp Neurol. 36: 362–377.

Kim SJ, Lyoo IK, Hwang J, Sung YH, Lee HY, Lee DS, Jeong DU, Renshaw PF. 2005. Frontal glucose hypometabolism in abstinent methamphetamine users. Neuropsychopharmacology. 30:1383–1391.

Kish SJ, Fitzmaurice PS, Boileau I, Schmunk GA, Ang LC, Furukawa Y, Chang LJ, Wickham DJ, Sherwin A, Tong J. 2009. Brain serotonin transporter in human methamphetamine users. Psychopharmacology (Berl). 202:649–661.

Krettek JE, Price JL. 1977. Projections from the amygdaloid complex to the cerebral cortex and thalamus in the rat and cat. J Comp Neurol. 172:687–722.

Lucantonio F, Stalnaker TA, Shaham Y, Niv Y, Schoenbaum G. 2012. The impact of orbitofrontal dysfunction on cocaine addiction. Nat Neurosci. 15:358–366.

Lucantonio F, Takahashi YK, Hoffman AF, Chang CY, Bali-Chaudhary S, Shaham Y, Lupica CR, Schoenbaum G. 2014. Orbitofrontal activation restores insight lost after cocaine use. Nat Neurosci. 17:1092–1099.

Navailles S, Guillem K, Vouillac-Mendoza C, Ahmed SH. 2015. Coordinated recruitment of cortical–subcortical circuits and ascending dopamine and serotonin neurons during inhibitory control of cocaine seeking in rats. Cereb Cortex. 25: 3167–3181.

New AS, Buchsbaum MS, Hazlett EA, Goodman M, Koenigsberg HW, Lo J, Iskander L, Newmark R, Brand J, O'Flynn K, et al. 2004. Fluoxetine increases relative metabolic rate in prefrontal cortex in impulsive aggression. Psychopharmacology (Berl). 176:451–458.

Nonkes LJP, Maes JHR, Homberg JR. 2013. Improved cognitive flexibility in serotonin transporter knockout rats is unchanged following chronic cocaine self-administration. Addict Biol. 18: 434–440.

Ongur D, Price JL. 2000. The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. Cereb Cortex. 10:206–219.

Paxinos G, Watson C. 1982. The rat brain in stereotaxic coordinates. New York: Academic Press Inc.

Porter JN, Olsen AS, Gurnsey K, Dugan BP, Jedema HP, Bradberry CW. 2011. Chronic cocaine self-administration in rhesus monkeys: impact on associative learning, cognitive control, and working memory. J Neurosci. 31:4926–4934.

Roberts AC. 2011. The importance of serotonin for orbitofrontal function. Biol Psychiatry. 69:1185–1191.

Rolls ET, Grabenhorst F. 2008. The orbitofrontal cortex and beyond: from affect to decision-making. Prog Neurobiol. 86: 216–244.

Saxena S, Brody AL, Maidment KM, Dunkin JJ, Colgan M, Alborzian S, Phelps ME, Baxter LR Jr. 1999. Localized orbitofrontal and subcortical metabolic changes and predictors of response to paroxetine treatment in obsessive-compulsive disorder. Neuropsychopharmacology. 21:683–693.

Schilman EA, Uylings HBM, Graaf YG-d, Joel D, Groenewegen HJ. 2008. The orbital cortex in rats topographically projects to central parts of the caudate–putamen complex. Neurosci Lett. 432:40–45.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. 2012. Fiji: an open-source platform for biological-image analysis. Nat Meth. 9:676–682.

Schoenbaum G, Chiba AA, Gallagher M. 1998. Orbitofrontal cortex and basolateral amygdala encode expected outcomes during learning. Nat Neurosci. 1:155–159.

Schoenbaum G, Eichenbaum H. 1995. Information coding in the rodent prefrontal cortex. II. Ensemble activity in orbitofrontal cortex. J Neurophysiol. 74:751–762.

Schoenbaum G, Esber GR. 2010. How do you (estimate you will) like them apples? Integration as a defining trait of orbitofrontal function. Curr Opin Neurobiol. 20:205–211.

Schoenbaum G, Nugent SL, Saddoris MP, Setlow B. 2002. Orbitofrontal lesions in rats impair reversal but not acquisition of go, no-go odor discriminations. Neuroreport. 13:885–890.

Schoenbaum G, Saddoris MP, Ramus SJ, Shaham Y, Setlow B. 2004. Cocaine-experienced rats exhibit learning deficits in a task sensitive to orbitofrontal cortex lesions. Eur J Neurosci. 19:1997–2002.

Schoenbaum G, Setlow B. 2005. Cocaine makes actions insensitive to outcomes but not extinction: implications for altered orbitofrontal-amygdalar function. Cereb Cortex. 15: 1162–1169.

Schoenbaum G, Shaham Y. 2008. The role of orbitofrontal cortex in drug addiction: a review of preclinical studies. Biol Psychiatry. 63:256–262.

Stalnaker TA, Roesch MR, Franz TM, Burke KA, Schoenbaum G. 2006. Abnormal associative encoding in orbitofrontal neurons in cocaine-experienced rats during decision-making. Eur J Neurosci. 24:2643–2653.

Takahashi YK, Chang CY, Lucantonio F, Haney RZ, Berg BA, Yau HJ, Bonci A, Schoenbaum G. 2013. Neural estimates of imagined outcomes in the orbitofrontal cortex drive behavior and learning. Neuron. 80:507–518.

Torres GE, Gainetdinov RR, Caron MG. 2003. Plasma membrane monoamine transporters: structure, regulation and function. Nat Rev Neurosci. 4(1):13–25.

Tremblay L, Schultz W. 1999. Relative reward preference in primate orbitofrontal cortex. Nature. 398:704–708.

Vazquez-Borsetti P, Cortes R, Artigas F. 2009. Pyramidal neurons in rat prefrontal cortex projecting to ventral tegmental area and dorsal raphe nucleus express 5-HT2A receptors. Cereb Cortex. 19:1678–1686.

Volkow ND, Chang L, Wang GJ, Fowler JS, Ding YS, Sedler M, Logan J, Franceschi D, Gatley J, Hitzemann R, et al. 2001. Low level of brain dopamine D2 receptors in methamphetamine abusers: association with metabolism in the orbitofrontal cortex. Am J Psychiatry. 158:2015–2021.

Volkow ND, Fowler JS. 2000. Addiction, a disease of compulsion and drive: involvement of the orbitofrontal cortex. Cereb Cortex. 10:318–325.

Volkow ND, Fowler JS, Wang GJ, Hitzemann R, Logan J, Schlyer DJ, Dewey SL, Wolf AP. 1993. Decreased dopamine D2 receptor availability is associated with reduced frontal metabolism in cocaine abusers. Synapse. 14:169–177.

Volkow ND, Fowler JS, Wolf AP, Hitzemann R, Dewey S, Bendriem B, Alpert R, Hoff A. 1991. Changes in brain glucose metabolism in cocaine dependence and withdrawal. Am J Psychiatry. 148:621–626.

Wang HL, Morales M. 2008. Corticotropin-releasing factor binding protein within the ventral tegmental area is expressed in a subset of dopaminergic neurons. J Comp Neurol. 509: 302–318.

West EA, Forcelli PA, McCue DL, Malkova L. 2013. Differential effects of serotonin-specific and excitotoxic lesions of OFC on conditioned reinforcer devaluation and extinction in rats. Behav Brain Res. 246:10–14.

Wilson MA, Molliver ME. 1991a. The organization of serotonergic projections to cerebral cortex in primates: regional distribution of axon terminals. Neuroscience. 44:537–553.

Wilson MA, Molliver ME. 1991b. The organization of serotonergic projections to cerebral cortex in primates: retrograde transport studies. Neuroscience. 44:555–570.

Yamamoto R, Hatano N, Sugai T, Kato N. 2014. Serotonin induces depolarization in lateral amygdala neurons by activation of TRPC-like current and inhibition of GIRK current depending on 5-HT2C receptor. Neuropharmacology. 82:49–58.