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Dorsal Raphe 5-HT_{2C} Receptor and GABA Networks Regulate Anxiety Produced by Cocaine Withdrawal

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Abstract

The serotonin system is intimately linked to both the mediation of anxiety and long-term effects of cocaine, potentially through interaction of inhibitory 5-HT_{2C} receptor and gamma-aminobutyric acid (GABA) networks. This study characterized the function of the dorsal raphe (DR) 5-HT_{2C} receptor and GABA network in anxiety produced by chronic cocaine withdrawal. C57BL/6 mice were injected with saline or cocaine (15 mg/kg) 3 times daily for 10 days, and tested on the elevated plus maze 30 minutes, 25 hours, or 7 days after the last injection. Cocaine-withdrawn mice showed heightened anxiety-like behavior at 25 hours of withdrawal, as compared to saline controls. Anxiety-like behavior was not different when mice were tested 30 minutes or 7 days after the last cocaine injection. Electrophysiology data revealed that serotonin cells from cocaine-withdrawn mice exhibited increased GABA inhibitory postsynaptic currents (IPSCs) in specific DR subregions dependent on withdrawal time (25 h or 7 d), an effect that was absent in cells from non-withdrawn mice (30 minutes after the last cocaine injection). Increased IPSC activity was restored to baseline levels following bath application of the 5-HT_{2C} receptor antagonist, SB 242084. In a separate cohort of cocaine-injected mice at 25 hours of withdrawal, both global and intra-DR blockade of 5-HT_{2C} receptors prior to elevated plus maze testing attenuated anxiety-like behavior. This study demonstrates that DR 5-HT_{2C} receptor blockade prevents anxiety-like behavior produced by cocaine withdrawal, potentially through attenuation of heightened GABA activity, supporting a role for the 5-HT_{2C} receptor in mediating anxiety produced by cocaine withdrawal.

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Introduction

functional mechanism for the production of cocaine withdrawal-induced anxiety, and suggests novel pharmacological targets for prevention of relapse.

Materials and Methods

Animals

Adult male C57Bl/6 mice (22–24 g at the start of the experiment) were obtained from Charles River, Inc. (Wilmington, MA) and housed 4 per cage. Mice were maintained on a 12 h light/dark cycle and provided food and water ad libitum. All testing was accomplished during the light phase. Animal use procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Temple University.

Drugs

Cocaine hydrochloride, generously provided by the NIDA drug supply program, was dissolved in 0.9% saline and administered intraperitoneally (i.p.) in a volume of 3 ml/kg body weight. The 5-HT_{2C}R antagonist 6-chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1*H*-indole-1-carboxamide dihydrochloride (SB 242084, Tocris Bioscience (Bristol, United Kingdom)) was dissolved in 0.9% saline and administered either i.p. (1mg/kg) in a volume of 3 ml/kg body weight or microinjected into the DR (0.5 µl of 50 mM solution; final dose of 25 nmol) via a chronic indwelling guide cannula.

Cocaine administration

Mice were injected with saline or cocaine (15 mg/kg) 3 times daily at 1 h intervals for 10 days to mimic a binge-like administration pattern (Unterwald et al., 1992; 2001). Mice were tested on the elevated plus maze either 30 minutes, 24 hours or 7 days after the last injection.

Guide cannula implantation

On day 8 of the 10 day cocaine or saline administration schedule, some cohorts of mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and stereotaxic surgery was performed to achieve implantation of a guide cannula into the midline DR. With the arm of the stereotaxic apparatus held secure at a 26 degree angle in order to avoid the cerebral aqueduct and damage to the midsagittal sinus situated above the DR, a stainless-steel 25 gauge guide cannula was aimed at the DR (anteroposterior, −4.3 mm; lateral, 1.8 mm; ventral 2.7 mm with respect to bregma) according to the coordinates in Paxinos and Watson (2001). Brains were collected after the experiments and cannulae placements were verified following injection of methylene blue dye (Tocris Bioscience (Bristol, United Kingdom)).

Elevated Plus Maze

The elevated plus maze test was used to assess anxiety-like behavior. The elevated plus maze (San Diego Instruments (San Diego, CA)) consists of four equal-sized runways (12" x 2") in the shape of a plus sign, and elevated 15.25 inches off the ground. Two of the arms are enclosed by solid walls (6 inches high, closed arms), whereas the other two arms have no

side walls (open arms). A ledge (1/8" high) is present around the perimeter of the open arms of the maze. The lighting in the test room was reduced to 15 lux. Mice were placed on the center of the elevated plus maze, and activity was video recorded for 10 minutes. The amount of time spent on the open arms is considered a measure of anxiety-like behavior, based on the rodent's natural aversion to open spaces versus the inclination to explore novel areas (Pellow et al., 1985). Studies have shown that confinement to the open arms leads to increases in stress or anxiety responses including freezing behavior, defecation, and increased plasma corticosterone levels (Pellow et al., 1985). Entry into an open arm was defined as the movement of the head, shoulders, and two front paws into the open arm area. Anxiety-like behavior was determined by calculating the amount of time each mouse spent on the open arms and reported as a percentage of the total time on the maze. In some cohorts, the selective 5-HT_{2C}R antagonist, SB 242084, (1 mg/kg i.p. or 25 nmol in 0.5 µl intra-DR) or saline was administered one hour prior to elevated plus maze testing. For intracranial drug infusions, an injector cannula was lowered into the dorsal raphe through the guide cannula. SB 242084 (25 nmol/0.5 µl) or saline (0.5 µl) was infused at a rate of 0.1 µl per minute, and the injector cannula was left in place for an additional 5 minutes before gradual removal. Data were analyzed using a two-way ANOVA with Bonferroni's post hoc analysis.

Open field test

The open field test was used to assess anxiety-like behavior. The open field is a white box (16" by 16") with center square (8" by 8"). The light in the room was maintained at around 450 lux. Mice were placed on the corner of the open field center square, and activity was video recorded for 10 minutes. The time spent in the center square of the open field as a percentage of total time was used as a measure of anxiety-like behavior based on the rodent's natural aversion to open spaces and inclination to remain near the perimeter of the open field arena; less time in the center is interpreted to indicate higher anxiety (Pruet and Belzung, 2003). Data were analyzed using an unpaired t-test.

Whole Cell Electrophysiology

Mice used for electrophysiology experiments underwent either chronic binge-pattern cocaine or saline administration for 10 days, and were tested for anxiety-like behavior on the elevated plus maze 30 minutes, 25 hours or 7 days following the last injection. Immediately following elevated plus maze testing, mice were rapidly decapitated and brains prepared for whole cell electrophysiology as described previously (Crawford et al., 2010; Kirby et al., 2008). Brains were immediately submerged in ice-cold artificial cerebrospinal fluid where sucrose (248 mM) was substituted for NaCl. DR slices (200 µm) were obtained using the Vibratome 3000 Plus (Vibratome (Bannockburn, IL)) and placed in ACSF ((in mM): 124 NaCl, 2.5 KCl, 2 NaH₂PO₄, 2.5 CaCl₂, 2 MgSO₄, 10 dextrose, and 26 NaHCO₃) at 35°C bubbled with 95% O₂/5% CO₂ for 1 h. Slices were then maintained in room temperature ACSF bubbled with 95% O₂/5% CO₂. Slices were transferred to a recording chamber (Warner Instruments (Hamden, CT)) and continuously perfused with ACSF at 1.5–2.0 ml/min at 32–34°C maintained by an in-line solution heater (TC-324; Warner Instruments (Hamden, CT)). One cell was recorded per brain slice. DR neurons were visualized using a Nikon E600 upright microscope (Optical Apparatus (Ardmore, PA)). The resistance of the

electrode was 4–8 M Ω when filled with an intracellular solution (in mM): 70 Kgluconate, 70 KCl, 2 NaCl, 4 EGTA, 10 HEPES, 4 MgATP, 0.3 Na₂GTP, 0.1% biocytin, pH 7.3).

Whole cell electrophysiology recordings were achieved in voltage-clamp mode ($V_m = -70$ mV) to record GABA_A inhibitory postsynaptic currents (IPSCs) from serotonin neurons in the DR with a Multiclamp 700B amplifier (Molecular Devices (Sunnyvale, CA)). Spontaneous IPSCs (sIPSCs) were recorded following bath application of the AMPA and kainate receptor antagonist, 6,7-dinitroquinoxaline-2,3 (1H,4H)-dione (DNQX; 20 μ M) in order to remove any residual glutamate excitatory activity from the recording. Miniature inhibitory post-synaptic currents (mIPSCs) were recorded for one minute in the presence of the DNQX (20 μ M) and the neurotoxin tetrodotoxin (TTX, 1 μ M) in order to isolate non-action potential-dependent IPSCs. In order to identify the role of the 5-HT_{2C}R in modulation of GABA activity, bath application of a 5-HT_{2C}R antagonist, SB 242084 (20 nM), was added and GABA_A IPSC activity was assessed over a 10 minute period. Cells in the lateral wing (lwDR), dorsomedial (dmDR) and ventromedial (vmDR) subregions of the DR were targeted. Current clamp mode was used in order to measure cell membrane and action potential characteristics prior to the measurement of synaptic activity.

MiniAnalysis software (Synaptosoft, Inc. (Decatur, GA)) was used to analyze GABA IPSC activity for frequency and amplitude. GABA IPSC frequency and amplitude measurements indicate levels of extracellular release of presynaptic GABA and GABA_A receptor number or occupancy, respectively (Katz, 1962). Statistical analyses were performed using a two-way ANOVA with treatment and time after the last injection as factors, with a Bonferroni's post hoc test when indicated. Differences were considered significant at $p < 0.05$. Linear regression analyses were performed in order to test the correlation between anxiety on the elevated plus maze and GABA IPSC frequency. GraphPad software was used to create figures and analyze data. Current clamp recordings of cell membrane and action potential characteristics were analyzed using Clampfit 9.0 (Molecular Devices (Sunnyvale, CA)). Tau, resistance, resting membrane potential (RMP), action potential (AP) threshold, activation gap, AP amplitude, AP duration, afterhyperpolarization (AHP) amplitude, and the time it takes for the AHP to depolarize to one-half its peak amplitude (AHP $t_{1/2}$) were measured directly from traces as previously described (Beck et al., 2004). Cell membrane and action potential characteristics were compared between treatment groups using a two-way ANOVA with treatment and subregion as factors, with a post hoc two-tailed t-test.

Immunohistochemistry – Serotonin cells

Post hoc immunohistochemistry was performed in order to confirm the serotonin phenotype of the recorded cells as previously described (Beck et al. 2004; Kirby et al. 2008). Briefly, cells were filled with biocytin during the recording through the recording electrode pipette. Tissue slices were post-fixed in 4% paraformaldehyde and stored at 4°C until further processing. Biocytin labeling was detected by processing tissue in a secondary antibody (1:200 Streptavidin Alexa Fluor 488 (Invitrogen (Grand Island, NY), S-11223), and serotonin expression was confirmed by labeling the rate-limiting enzyme in the serotonin synthesis pathway, tryptophan hydroxylase (TPH) (1:1000 primary rabbit TPH (Novus Biologicals (Littleton, CO), NBP1-61662) and 1:200 secondary goat anti-rabbit Alexa Fluor

Cy3 (Invitrogen (Grand Island, NY), T-30954). Sections were imaged by Leica DMIRE2 confocal microscope (Leica Microsystems (Exton, PA)).

Results

Acute withdrawal from repeated cocaine increased anxiety-like behaviors

Mice were tested on the elevated plus maze to assess anxiety-like behavior during active cocaine exposure (30 minutes following the last injection) or at 25 hours or 7 days of withdrawal. The data in Figure 1A represent the mean time spent on the open arms as a percentage of the total time on the elevated plus maze. Two-way ANOVA revealed a significant interaction between treatment and withdrawal time (interaction, $F_{(2,84)} = 4.518$, $p = 0.0137$; treatment, $F_{(1,84)} = 11.87$, $p = 0.0009$; withdrawal time, $F_{(2,84)} = 2.673$, $p = 0.0749$). Follow-up analysis with Bonferroni's post hoc analysis determined that mice withdrawn from cocaine for 25 hours spent significantly less time on the open arms as compared to saline controls ($p < 0.001$). Behaviors of mice on the elevated plus maze not undergoing cocaine withdrawal (30 minutes after last injection) were not different from that of control saline-injected mice. Likewise, no significant difference in open arm times was revealed between cocaine- and saline-injected mice at 7 days of withdrawal. Thus, a significant anxiety-like phenotype was found after 25 hours of cocaine withdrawal, but not during active cocaine exposure or after 7 days of cocaine withdrawal.

A second test for anxiety was used to verify the findings from the elevated plus maze. In agreement with the results from the plus maze, data from the open field test also indicated heightened anxiety-like behaviors during cocaine withdrawal. Open field testing demonstrated that at 25 hours of withdrawal, cocaine-treated mice spent significantly less time in the center of the open field arena compared with saline-injected controls (Figure 1B, unpaired t-test, $p < 0.05$), demonstrating heightened anxiety-like behavior during cocaine withdrawal.

Increased GABA spontaneous inhibitory postsynaptic currents (sIPSC) in DR serotonin cells from cocaine-withdrawn mice during acute (25 h) and protracted (7 d) withdrawal

GABA inhibitory postsynaptic currents were recorded from serotonin cells in the dorsomedial (dmDR), ventromedial (vmDR) and lateral wing (lwDR) subregions of the DR nucleus. Serotonin cells from cocaine-withdrawn mice demonstrated significantly higher GABA inhibitory tone in specific DR subregions dependent on time since the last cocaine injection. Figure 2 depicts comparisons of the mean sIPSC frequency and amplitude in the three subregions of the DR. Two-way ANOVA with Bonferroni's post hoc analysis revealed significantly heightened GABA sIPSC frequency in dmDR serotonin cells from mice at 25 hours of cocaine withdrawal (Figure 2A, $p < 0.001$), while no significant differences in sIPSC frequency were revealed at 30 minutes or 7 days after the last injection in the dmDR subregion (interaction, $F_{(2,51)} = 3.255$, $p = 0.0467$; treatment, $F_{(1,51)} = 9.263$, $p = 0.0037$, and withdrawal time $F_{(2,51)} = 3.317$, $p = 0.0442$). Analysis of GABA sIPSC amplitude from dmDR serotonin cells revealed no significant differences between cocaine-injected and saline control mice at either 30 minutes, 25 hours or 7 days after the last injection (interaction, $F_{(2,53)} = 0.1020$, $p = 0.9032$; treatment, $F_{(1,53)} = 0.04943$, $p = 0.8249$;

withdrawal time, $F_{(2,53)} = 6.605$, $p = 0.0027$). Although statistical significance was achieved for overall withdrawal time, Bonferroni's post hoc analyses were non-significant (Figure 2B).

Serotonin cells from the vmDR of mice at 25 hours of cocaine withdrawal demonstrated significantly higher GABA sIPSC frequency as compared to saline controls (Figure 2C, $p < 0.001$). No significant differences in sIPSC frequency were found at 30 minutes or 7 days after the last injection in the vmDR (interaction, $F_{(2,50)} = 4.663$, $p = 0.0139$; treatment, $F_{(1,50)} = 7.250$, $p = 0.0096$; withdrawal time $F_{(2,50)} = 6.324$, $p = 0.0036$). Two-way ANOVA revealed no significant differences in sIPSC amplitude in serotonin cells from the vmDR subregion of cocaine- versus saline-injected mice at 30 minutes, 25 hours, or 7 days following the last injection (interaction, $F_{(2,54)} = 0.5682$, $p = 0.5699$; treatment, $F_{(1,54)} = 1.245$, $p = 0.2695$; withdrawal time, $F_{(2,54)} = 2.064$, $p = 0.1369$).

Lateral wing DR neurons demonstrated significantly greater GABA sIPSC frequency at 7 days of cocaine withdrawal as compared to cells from saline controls (Figure 2E, $p < 0.01$). No significant differences in sIPSC frequencies between cocaine- and saline-injected mice were observed at 30 minutes or 25 hours following the last injection (interaction, $F_{(2,43)} = 3.036$, $p = 0.0584$; treatment, $F_{(1,43)} = 8.798$, $p = 0.0049$; withdrawal time, $F_{(2,43)} = 3.878$, $p = 0.0283$). No significant differences in GABA sIPSC amplitude were revealed at 30 minutes, 25 hours or 7 days after the last saline or cocaine injection (Figure 2F, interaction, $F_{(2,45)} = 0.8735$, $p = 0.4245$; treatment, $F_{(1,45)} = 0.3658$, $p = 0.5483$; withdrawal time, $F_{(2,45)} = 2.282$, $p = 0.1138$).

Figures 2G and H show representative traces comparing sIPSC frequencies and amplitudes of vmDR cells from saline- and cocaine-injected mice at 25 hours after the last injection. Figure 2I shows a representative biocytin-filled recorded cell double labeled for tryptophan hydroxylase. Taken together, these data demonstrate that at 25 hours of withdrawal, there was a significant increase in GABA sIPSC frequency in dmDR and vmDR serotonin cells from anxious, cocaine-withdrawn mice as compared to saline controls. Cells from cocaine-treated mice tested 7 days following the last injection showed significant increases in sIPSC frequency in the lwDR region, as compared to cells from saline controls. No significant differences were found in GABA sIPSC frequency or amplitude from serotonin cells between saline- and cocaine-treated mice at 30 minutes following the last injection.

Increased GABA miniature inhibitory postsynaptic currents (mIPSC) in DR serotonin cells in cocaine-withdrawn mice

Miniature IPSCs (mIPSCs) were recorded from serotonin cells in the dmDR, vmDR and lwDR subregions in order to compare activity of non-action potential evoked events of cells from cocaine-exposed mice versus saline controls. A significant treatment effect was revealed in mIPSC frequency from dmDR cells of cocaine-injected mice as compared to saline controls, but no significant interaction or withdrawal time effect was observed (Figure 3A, two-way ANOVA with Bonferroni's post hoc analysis, treatment, $F_{(1,34)} = 7.043$, $p = 0.0120$; interaction, $F_{(2,34)} = 0.9280$, $p = 0.4051$; withdrawal time, $F_{(2,34)} = 1.854$, $p = 0.1721$). There were no significant differences in the mIPSC amplitude of dmDR serotonin cells from cocaine- versus saline-injected mice at 30 minutes, 25 hours or 7 days after the

last injection (Figure 3B, interaction, $F_{(2,39)} = 0.1997$, $p = 0.8198$; treatment, $F_{(1,39)} = 1.090$, $p = 0.3028$; withdrawal time, $F_{(2,39)} = 1.120$, $p = 0.3365$).

In contrast with the dmDR, significantly higher mIPSC frequency in vmDR cells from mice withdrawn from cocaine for 25 hours versus saline controls was found (Figure 3C, $p < 0.001$; interaction, $F_{(2,37)} = 3.613$, $p = 0.0369$; treatment, $F_{(1,37)} = 9.212$, $p = 0.0044$; withdrawal time, $F_{(2,37)} = 9.890$, $p = 0.0004$). Post hoc analysis showed no significant differences in mIPSC frequency between cocaine and saline-injected mice at 30 minutes or 7 days following the last injection****. Serotonin cells in the vmDR region from cocaine-withdrawn mice at the 25 hour withdrawal time point exhibited significantly higher GABA mIPSC amplitude (Figure 3D, $p < 0.05$). No significant differences in vmDR mIPSC amplitudes between cocaine- and saline-injected mice were observed 30 minutes or 7 days following the last injection (interaction, $F_{(2,43)} = 4.553$, $p = 0.0161$; treatment, $F_{(1,43)} = 0.4928$, $p = 0.4865$; withdrawal time, $F_{(2,43)} = 0.2270$, $p = 0.7978$).

No significant differences in lwDR mIPSC frequencies were observed between saline- and cocaine-injected mice at 30 minutes, 25 hours or 7 days of withdrawal (Figure 3E, interaction, $F_{(2,35)} = 0.9608$, $p = 0.3924$; treatment, $F_{(1,35)} = 1.458$, $p = 0.2354$; withdrawal time, $F_{(2,35)} = 3.164$, $p = 0.0546$). Likewise, mIPSC amplitudes in lwDR cells were not different between saline- and cocaine-injected mice at 30 minutes, 25 hours or 7 days of withdrawal (Figure 3F, effect of interaction, $F_{(2,38)} = 0.4998$, $p = 0.6106$; effect of treatment, $F_{(1,38)} = 0.02084$, $p = 0.8860$; effect of withdrawal time, $F_{(2,28)} = 1.334$, $p = 0.2755$).

Figures 3G and H show representative traces comparing mIPSC frequencies and amplitudes of vmDR cells from saline- and cocaine-injected mice at 25 hours following the last injection. Figure 3I shows a representative biocytin-filled recorded cell double labeled positive for tryptophan hydroxylase. As depicted by the representative traces, significant increases in GABA mIPSC frequency and amplitude were evident in vmDR serotonin cells from cocaine-injected mice at 25 hours of withdrawal, as compared to cells from saline control mice.

Non-serotonin DR neurons from saline-injected and cocaine-withdrawn mice show no significant differences in IPSC frequency

GABA sIPSC and mIPSC frequencies were recorded in non-serotonin cells from saline- and cocaine-treated mice after 25 hours of withdrawal (data not shown). Two-way ANOVA revealed no significant differences in sIPSC frequency of non-serotonin cells from saline- and cocaine-injected mice at 25 hours of withdrawal (interaction, $F_{(2,9)} = 0.4085$, $p = 0.6764$; treatment, $F_{(1,9)} = 0.9404$, $p = 0.3575$; subregion, $F_{(2,9)} = 0.2271$, $p = 0.8012$). mIPSC frequencies of non-serotonin cells from cocaine-withdrawn mice at 25 hours of withdrawal were not different from those from saline controls (interaction, $F_{(2,6)} = 1.7$, $p = 0.2601$; treatment, $F_{(1,6)} = 0.2774$, $p = 0.6173$; subregion, $F_{(2,6)} = 0.1276$, $p = 0.8826$).

Correlation of GABA IPSC frequency and anxiety on the elevated plus maze

Linear regression analyses were performed in order to establish a relationship between IPSC frequency in cells from the dmDR, vmDR and lwDR subregions and anxiety-like behavior. Averages of IPSC frequencies from the cells of each mouse (both cocaine and saline) were

plotted against their open arm time on the elevated plus maze and used for statistical analysis. Linear regression analysis of vmDR cells revealed a significant negative correlation between both sIPSC ($r^2=0.3176$, $p=0.0003$) and mIPSC ($r^2=0.2973$, $p=0.0015$) frequency and time spent on the open arms of the elevated plus maze, such that lower time spent on the open arms of the elevated plus maze (i.e. more anxious) was correlated with higher sIPSC or mIPSC frequency (Figure 4 A and B). Analysis of dmDR cells did not reveal a significant correlation between either sIPSC ($r^2=0.07098$, $p=0.1218$) or mIPSC ($r^2=0.0045$, $p=0.7295$) frequency and behavior on the elevated plus maze. Likewise, analysis of lwDR cells did not reveal a significant correlation between either sIPSC ($r^2=0.1129$, $p=0.0646$) or mIPSC ($r^2=0.01907$, $p=0.0513$) frequency and time spent in the open arms of the elevated plus maze. These data reveal that heightened anxiety was associated with higher sIPSC and mIPSC frequencies specifically in the vmDR.

Bath application of SB 242084 reduces heightened GABA activity in cocaine-withdrawn mice

In order to investigate the participation of the 5-HT_{2C}R in altered DR GABA activity, a selective 5-HT_{2C}R antagonist, SB 242084 (20 nM), was added to the ACSF bath of the recorded cell in the DR, and changes in IPSC frequency were assessed. Regional distribution of 5-HT_{2C}Rs is uniform throughout the DR (Clemett et al., 2000; Serrats et al., 2005). As such, subregion differences in response to SB 242084 application were not observed (data not shown) and data presented represent cells from all subregions of the DR in sum. Cells from mice following 25 hours of cocaine withdrawal demonstrated heightened sIPSC frequency as compared to cells from saline controls (Figure 5A; two-way ANOVA, Bonferroni's post hoc analysis, sal/ACSF vs. coc/ACSF, ** $p < 0.01$) in agreement with the data presented in Figure 2A, C and E. This increase in sIPSC activity was restored to levels comparable to baseline upon bath application of SB 242084 (coc/ACSF vs. coc/SB 242084, # $p < 0.05$; interaction, $F_{(1,20)} = 3.658$, $p = 0.0702$; treatment, $F_{(1,20)} = 8.835$, $p = 0.0075$; SB 242084 bath application, $F_{(1,20)} = 6.358$, $p = 0.0203$).

In Figure 5B, data depicting mIPSC frequencies from cells of saline- and cocaine-injected mice at 25 hours of withdrawal are provided. No significant differences in mIPSC frequency were found between cells from saline controls and cocaine-withdrawn mice or between SB 242084 versus ACSF application (interaction, $F_{(1,34)} = 0.0962$, $p = 0.7583$; treatment, $F_{(1,34)} = 2.405$, $p = 0.1302$; effect of SB 242084 application, $F_{(1,34)} = 1.157$, $p = 0.2896$). Thus, bath application of SB 242084 normalized cocaine withdrawal-induced elevations in sIPSC frequency to baseline levels (Figure 5A), while not significantly affecting sIPSC or mIPSC frequencies of cells from saline-injected controls.

Cell membrane and action potential characteristics

Table 1 reports cell membrane characteristics of serotonin cells recorded from mice 25 hours after the last cocaine or saline injection. Two-way ANOVA with treatment and DR subregion main effects on the data presented in Table 1 revealed no significant differences in treatment, subregion or interaction on any parameter (Table 1; two-way ANOVA with post hoc two-tailed t-test; $p > 0.05$).

Action potential characteristics were recorded in current clamp mode to compare cell characteristics of serotonin cells from saline- and cocaine-injected mice 25 hours after the last injection (Table 2). No significant differences in action potential characteristics of afterhyperpolarization at $t_{1/2}$ or τ were found with two-way ANOVA analysis (Table 2; $p > 0.05$). There were significant subregion differences in action potential amplitude (interaction, $F_{(2,57)} = 0.5635$, $p = 0.5724$; treatment, $F_{(1,57)} = 0.7937$, $p = 0.3767$; subregion, $F_{(2,57)} = 3.685$, $p = 0.0313$) and afterhyperpolarization from the threshold (interaction, $F_{(2,57)} = 0.7741$, $p = 0.4659$; treatment, $F_{(1,57)} = 1.694$, $p = 0.1983$; subregion, $F_{(2,57)} = 3.296$, $p = 0.0442$), likely as a result of subregional differences in kinetics. Finally, a significant treatment effect was identified for action potential duration. A significantly lower action potential duration was found in vmDR neurons from cocaine-treated mice after 25 hours of withdrawal, as compared to vmDR cells from saline controls (interaction, $F_{(2,57)} = 2.380$, $p = 0.1017$; treatment, $F_{(1,57)} = 4.420$, $p = 0.0400$; subregion, $F_{(2,57)} = 0.1069$, $p = 0.8988$, Table 2; two-way ANOVA with post hoc two-tailed t-test, $*p = 0.0291$).

Involvement of the 5-HT_{2C}R in the regulation of anxiety produced by cocaine withdrawal

In order to establish a role for the 5-HT_{2C}R in regulating anxiety caused by withdrawal from repeated cocaine, mice received the 5-HT_{2C}R antagonist, SB 242084 (1 mg/kg, i.p.) or saline 24 hours following the last cocaine or saline injection, and were tested on the elevated plus maze for anxiety-like behavior one hour later. Mice withdrawn from cocaine for 25 hours demonstrated significantly heightened anxiety-like behavior on the elevated plus maze as compared to saline controls, similar to the data shown in Figure 1 (Figure 6A, sal/sal vs. coc/sal, $*** p < 0.001$). The heightened anxiety-like behavior in cocaine-withdrawn was attenuated upon pretreatment with SB 242084 one hour prior to testing on the elevated plus maze (coc/sal vs. coc/SB, $*** p < 0.001$; interaction, $F_{(1,34)} = 16.75$, $p = 0.0002$; treatment, $F_{(1,34)} = 5.398$, $p = 0.0263$; and pretreatment, $F_{(1,34)} = 4.872$, $p = 0.0341$). In addition, general locomotor activity on the elevated plus maze was analyzed by quantifying the total number of entries into both the open and closed arms. Total arm entries were not significantly different between SB 242084- and saline-injected mice (sal/SB vs. sal/sal, interaction, $F_{(1,35)} = 1.817$, $p = 0.1864$; pretreatment, $F_{(1,35)} = 1.336$, $p = 0.2555$; treatment, $F_{(1,35)} = 0.3497$, $p = 0.5581$, data not shown), indicating that SB 242084 did not increase general activity on the elevated plus maze.

5-HT_{2C}Rs located specifically in the DR were targeted in the second aspect of this study. Intra-DR microinjection of SB 242084 (25 nmol) one hour prior to elevated plus maze testing significantly attenuated anxiety-like behavior in cocaine-withdrawn mice at 25 hours of withdrawal (Figure 6B, coc/sal vs. coc/SB, $** p < 0.01$; effect of interaction, $F_{(1,25)} = 6.847$, $p = 0.0148$; effect of treatment, $F_{(1,25)} = 7.395$, $p = 0.0117$; effect of pretreatment, $F_{(1,25)} = 4.858$, $p = 0.0369$). These data provide evidence that 5-HT_{2C}Rs specifically in the DR nucleus regulate anxiety produced by withdrawal from repeated cocaine exposure. Both global and local DR blockade of 5-HT_{2C}Rs attenuated anxiety produced by cocaine withdrawal.

Schematic representation of the 5-HT_{2C}R and GABA negative feedback mechanism in the DR in the regulation of anxiety produced by cocaine withdrawal

Figure 7 represents a proposed mechanism of DR 5-HT_{2C}R and GABA activity during cocaine withdrawal, such that heightened GABA activity, which is 5-HT_{2C}R mediated, contributes to anxiety produced by cocaine withdrawal. This local DR 5-HT_{2C}R and GABA negative feedback mechanism potentially leads to an overall inhibition of serotonin neurotransmission during cocaine withdrawal.

Discussion

In the current study, elevated plus maze testing identified an anxiogenic phenotype in cocaine-injected mice at 24 hours of cocaine withdrawal, an effect that was absent at 30 minutes or 7 days after the last cocaine injection. Electrophysiology studies showed that DR serotonin neurons from anxious, cocaine-treated mice in acute 24 hour withdrawal demonstrated heightened sIPSC and mIPSC frequencies in the dmDR and vmDR. This effect was blocked in DR serotonin cells upon bath application of the 5-HT_{2C}R antagonist, SB 242084, and was absent in non-serotonin cells. Likewise, serotonin cells from cocaine-treated mice in acute withdrawal demonstrated heightened mIPSC amplitude in the vmDR subregion, as compared to cells from saline controls. Increased sIPSC and mIPSC frequency in various subregions during cocaine withdrawal indicates increased action potential-dependent and independent presynaptic GABA release. The heightened sIPSC amplitude evident in vmDR serotonin cells from cocaine-withdrawn mice likely represents greater occupancy of GABA_A receptors as a result of the increase in presynaptic GABA release. By recording mIPSC GABA events, an assessment of basal non-action potential-dependent GABA activity was achieved. Differences in mIPSC frequency and amplitude evident in serotonin cells from cocaine withdrawn mice indicate that cocaine withdrawal-induced alterations in GABA neurocircuitry is driven by local constitutive DR activity, as these changes are manifested in the slice preparation following blockade of action potentials. No differences in IPSC frequency or amplitude were found in cells from cocaine-injected mice that were not undergoing withdrawal and that did not show anxiety, suggesting that heightened DR GABA activity was a dysfunction specific to the stage of cocaine withdrawal, and was not associated with cocaine exposure itself.

After 7 days of cocaine withdrawal, anxiety-like behavior dissipated. This is in accordance with clinical reports showing that addicts commonly experience anxiety in the early but not prolonged phase of withdrawal (Gawin and Kleber, 1986). Preclinical studies also show no elevations in baseline anxiety-like behaviors at longer withdrawal times (Erb et al., 2010). An anxious phenotype following protracted abstinence from cocaine is evident only under conditions where the animal is exposed to a stressor or administered the stress hormone, corticotropin-releasing factor (Mantsch et al., 2008), or exposure to contextual cues previously associated with cocaine at the time of testing (Erb et al., 2006). Thus, the present results are in agreement with prior studies and indicate baseline elevations in anxiety-like behaviors are present during early withdrawal but are not evident at later withdrawal times in the absence of further activation.

There was an anatomical shift in heightened IPSC frequency in cells from mice at 7 days of cocaine withdrawal as compared with earlier time points. The lwDR subregion demonstrated significantly increased sIPSC frequency at 7 days of cocaine withdrawal, as compared to cells from saline controls. Cells in the lwDR have distinct physiology from those in the dmDR or vmDR DR with regard to anxiety mechanisms. A greater excitability and higher density of GABA neurons have been found in the lateral wings, as compared to the dmDR and vmDR subregions (Crawford et al., 2010). In addition, the lateral wing subregion demonstrates differential regulation of downstream structures. Afferents from the lwDR primarily innervate only subcortical regions, in particular the trigeminal somatosensory structures, whereas dmDR and vmDR afferents extend to both subcortical and cortical regions (Kirifides et al., 2001; Lowry et al., 2008; Muzerelle et al., 2014). The current study showed that during acute cocaine withdrawal, the vmDR and dmDR subregions demonstrated increased inhibitory tone, which potentially contributed to inhibition of downstream subcortical and cortical structures, such as the nucleus accumbens. The increased inhibitory GABA activity shifted after 7 days of withdrawal when it became evident in the lwDR subregion, perhaps producing dysregulation of afferents projecting to subcortical structures. The disparate neuronal populations and innervation of downstream structures of the three subregions may have implications for their differential involvement in anxiety mechanisms during cocaine withdrawal.

This investigation identified an important role for DR 5-HT_{2C}Rs in regulating anxiety produced by cocaine withdrawal. Specifically, blockade of 5-HT_{2C}Rs, via either systemic or intra-DR administration of 5-HT_{2C}R antagonist, SB 242084, attenuated expression of cocaine withdrawal-induced anxiety-like behavior. These findings are in accordance with studies that have identified a role for the 5-HT_{2C}R in the regulation of anxiety produced by other causes. Antagonists targeting the 5-HT_{2C}R produce an anxiolytic effect in an uncontrollable traumatic stress model (Christianson et al., 2010), and activation of 5-HT_{2C}Rs leads to enhanced anxiety in rats following single-prolonged stress (Harada et al., 2008). More specifically, these findings support a direct role for the 5-HT_{2C}R and GABA negative feedback mechanism in the DR in the regulation of anxiety-like behavior in mice (Spoida et al., 2014). It has been reported that SB 242084 can increase locomotor activity (Fletcher et al., 2009). However, in the present study, elevated plus maze total arm entries, which is an indicator of general locomotor activity, were not significantly different between SB 242084- and saline-injected mice, and SB 242084 did not increase open arm time in control mice not undergoing cocaine withdrawal. Thus, it is unlikely that the present results were confounded by a non-specific motor response.

The inhibitory effects of the 5-HT_{2C}R and GABA network in the DR found in the current study are in alignment with previous studies showing that 5-HT_{2C}R activity leads to an overall downregulation of both serotonin and dopamine neurotransmission. In the context of cocaine, the 5-HT_{2C}R has been identified as a regulator of cocaine-induced alterations in monoamine systems and behaviors associated with dopamine dysregulation (Liu and Cunningham, 2006). In the presence of cocaine, activation of 5-HT_{2C}Rs in other brain regions, namely the nucleus accumbens and ventral tegmental area, decreases mesolimbic dopamine neurotransmission (Navailles et al., 2007). The inhibitory modulation of the 5-HT_{2C}R on serotonin and dopamine neurotransmission is proposed to arise from a negative

feedback mechanism through GABA networks. 5-HT_{2C}Rs are expressed on GABA neurons (Boothman et al., 2006) and thus, 5-HT_{2C}R activity leads to heightened GABA activation in the DR and in serotonin projection areas (Liu et al., 2007; Quéree et al., 2009). In addition, firing rates of DR serotonin cells are decreased by 5-HT_{2C}R activation, an effect which is reversed upon application of a GABA_A receptor antagonist (Boothman et al., 2006). DR neurons project to mesolimbic regions involved in regulating addictive behaviors (De Deurwaerdère and Spampinato, 1999; Gervais and Rouillard, 2000). Therefore, it is likely that during cocaine withdrawal, DR serotonin dysregulation interferes with normal 5-HT_{2C}R signaling, leading to potentiated inhibition of local and long-range circuitry through GABA networks and anxiety.

This study supports the hypothesis that during cocaine withdrawal, the 5-HT_{2C}R - mediated alterations in GABA activity contribute to dysregulation of serotonin neurocircuitry and lead to anxiety. The increase in inhibitory tone of DR neurocircuitry during cocaine withdrawal is consistent with findings showing a deficiency of serotonin neurotransmission produced by cocaine withdrawal (Parsons et al., 1995). There is also a reduction in mesolimbic dopamine levels and brain stimulation reward thresholds that may be associated with anxiety produced by cocaine withdrawal (Markou and Koob, 1991, 1992). In light of evidence for a reduction of dopamine as well as serotonin during cocaine withdrawal paired with the inhibitory influence of the 5-HT_{2C}R on dopamine and serotonin neurocircuitry, it is likely that an integrated dysregulation of serotonin and dopamine neurotransmission through the 5-HT_{2C}R is responsible for producing anxiety during cocaine withdrawal. Future studies will aim to further address this link, and examine whether increased 5-HT_{2C}R activity is attributable to increased 5-HT_{2C}R expression and/or sensitivity.

This study identifies an important role for the 5-HT_{2C}R in the regulation of anxiety during cocaine withdrawal. A mechanistic description of neurochemical changes that contribute to anxiety during cocaine withdrawal was elucidated. These data are the first to show that blockade of 5-HT_{2C}Rs attenuated anxiety during cocaine withdrawal, specifically through DR 5-HT_{2C}Rs. Within the DR, the 5-HT_{2C}R participates in elevations in GABA activity in anxious, cocaine-withdrawn mice in a subregion-specific manner dependent on withdrawal time. Altered GABA function in the DR occurred in the vmDR and dmDR subregions at acute (25 hours) withdrawal, whereas the increased sIPSC frequency was evident only in the lwDR subregion during protracted (7 day) withdrawal.

The period of withdrawal is particularly important to address from a therapeutic standpoint, as this is when treatment intervention is clinically relevant. Current treatment strategies for the prevention of relapse in cocaine-dependent persons have not proven to be efficacious, and there are no FDA-approved pharmacotherapies for cocaine dependence. Therefore, it is critical to study the role of the serotonin system in cocaine withdrawal to understand the mechanisms underlying addictive behaviors, as well as the integrated neurocircuitry of the serotonin and dopamine systems associated with neural adaptations that lead to sustained drug use.

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Highlights

- Chronic cocaine produced an anxiogenic phenotype in mice at 24 hours of withdrawal.
- Dorsal raphe GABA activity increased in serotonin cells of cocaine withdrawn mice.
- 5-HT_{2C} receptor blockade restored GABA activity in cells of cocaine withdrawn mice.
- 5-HT_{2C} receptor blockade reduced anxiety behavior during cocaine withdrawal.

Figure 1.

A-B. Anxiety-like behaviors as measured by the elevated plus maze and open field test. (A) Elevated plus maze testing was conducted 30 minutes, 25 hours, or 7 days after the last injection of saline or cocaine. Cocaine-treated mice after 25 hours of withdrawal spent significantly less time on the open arms of the elevated plus maze, as compared to saline controls (**p<0.001), demonstrating an anxiogenic phenotype. Mice that were subjected to elevated plus maze testing 30 minutes or 7 days after the last cocaine injection did not demonstrate anxiety-like behavior on the elevated plus maze as compared to saline controls (n=10-23 mice/group). (B) Open field testing was performed 25 hours after the last injection of saline or cocaine. Cocaine-treated mice after 25 hours of withdrawal spent significantly less time in the center of the open field apparatus (*p<0.05), demonstrating an anxiogenic phenotype (n=8 mice/group).

Figure 2.

A-I. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in dorsal raphe serotonin neurons using whole cell patch-clamp electrophysiology techniques in vitro. (A) Serotonin neurons in the dmDR region from cocaine-treated mice demonstrated significantly heightened GABA_A sIPSC frequency after 25 hours of withdrawal (n= 6-17 cells/group, ***p<0.001), and no significant differences were observed at 30 minutes or 7 days of withdrawal (n=6-8 cells/group). (B) No significant differences in dmDR sIPSC amplitudes at 30 minutes, 25 hours or 7 days after the last injection were observed. (C) Serotonin cells from the vmDR aspect of the dorsal raphe exhibited heightened GABA_A sIPSC frequency at 25 hours of withdrawal (n=6-15, p<0.001), and no differences were observed in sIPSC frequency of vmDR cells at 30 minutes or 7 days after the last injection (n=6-8 cells/group). (D) No significant differences in sIPSC amplitude were observed in the vmDR subregion at 30 minutes, 25 hours or 7 days after the last saline or cocaine injection (n=6-17 cells/group). (E) Within the lateral wing subregion, serotonin neurons from cocaine withdrawn mice at 7 days of withdrawal exhibited significantly heightened sIPSC frequency as compared to cells from saline controls (n=6-13, p<0.01), while no differences were observed at 30 minutes or 25 hours after the last injection. (F) No significant differences were observed in sIPSC amplitudes of lwDR cells at 30 minutes, 25 hours or 7 days after the last injection. (G) Average amplitude traces for vmDR 25 hr cell data are shown. (H) Representative traces providing 1 minute bins of data are shown for vmDR 25 hr cell data. (I) Recorded cells were filled with biocytin during the whole cell electrophysiology recording and post-hoc

immunohistochemical analysis was performed to confirm serotonin expression in recorded cells (TPH = tryptophan hydroxylase).

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Figure 3.

A-I. Miniature inhibitory postsynaptic currents (mIPSCs) using TTX (1 μ M) were recorded in dorsal raphe serotonin neurons using whole cell patch-clamp in vitro electrophysiology techniques. (A and B) No significant differences were observed in mIPSC frequency (A) or amplitude (B) in dmDR cells at 30 minutes, 25 hours or 7 days after the last cocaine injection (n=5-12 cells/group). (C and D) Serotonin neurons from cocaine withdrawn mice demonstrated significantly heightened GABA_A mIPSC frequency (n= 5-10 cells/group, ***p<0.001) and amplitude (n=6-14 cells/group, p<0.05) in the ventromedial aspect of the dorsal raphe at 25 hours of withdrawal. (E and F) No differences were observed in mIPSC frequency or amplitude in lwDR cells from cocaine-treated mice at 30 minutes, 25 hours or 7 days following the last injection, as compared to cells from saline controls (n=7-12 cells/group). (G) Average amplitude traces are shown. (H) Representative traces providing 1 minute bins of data from vmDR cells are shown. (I) Recorded cells were filled with biocytin during the whole cell electrophysiology recording and post-hoc immunohistochemical analysis was performed to confirm serotonin expression in recorded cells (TPH = tryptophan hydroxylase).

Figure 4.

Linear regression analyses were performed to establish a correlation between either sIPSC or mIPSC frequency and time spent on the open arms of the elevated plus maze. Averages of single cell data from each mouse (both saline- and cocaine-injected) were used in analyses. There was a significant negative correlation in A) sIPSC (n=37 mice, $p<0.001$) and B) mIPSC (n=31 mice, $p<0.01$) frequencies of vmDR serotonin cells and the time spent on the open arms of the elevated plus maze.

Figure 5.

IPSC frequency of serotonin cells at 25 hours after repeated saline or cocaine: Effect of 5-HT_{2C} receptor blockade. (A) Serotonin cells from cocaine-withdrawn mice demonstrated significantly heightened sIPSC frequency as compared to saline controls (**p<0.01). Bath application of the 5-HT_{2C} receptor antagonist, SB 242084 (20 nM) significantly attenuated GABA_A sIPSC frequency in cells from anxious, cocaine-withdrawn mice (n=6 cells/group, #p<0.05). (B) An increase in mIPSC frequency was observed in cells from cocaine-withdrawn mice at 25 hours of withdrawal, as compared to saline controls, although no statistical significance was revealed. Bath application of SB 242084 had no significant effect on mIPSC frequency (n = 7-12 cells/group).

Figure 6.

5-HT_{2C} receptor regulation of anxiety during cocaine withdrawal was evaluated using the elevated plus maze. (A) Mice exposed to a chronic binge cocaine paradigm and 24 hours of withdrawal with a saline injection (i.p.) 1 hour prior to elevated plus maze testing spent significantly less time in the open arms than the saline-administered controls (n=8-13 mice/group, ***p<0.001). Administration of 1 mg/kg SB 242084 (i.p.) 1 hour prior to elevated plus maze testing blocked the expression of anxiety-like behavior in cocaine-withdrawn mice (***p<0.001). (B) Mice exposed to a chronic binge cocaine paradigm and 24 hours of withdrawal with a saline microinjection into the dorsal raphe prior to elevated plus maze testing spent significantly less time on the open arms than the saline-administered controls (n=6-8 mice/group, ***p<0.001). Intra-dorsal raphe administration of 25nmol SB 242084 1 hour prior to elevated plus maze testing prevented the expression of anxiety-like behavior in cocaine-withdrawn mice (**p<0.01). (C) Microinjection locations along the rostrocaudal gradient of the dorsal raphe (images from Paxinos and Watson, 1996). (D) Representative coronal section (40 μ) of the dorsal raphe with blue dye injected for confirmation of accurate injection site.

Figure 7.

Representative schematic illustrating the dorsal raphe 5-HT_{2C} receptor and GABA negative feedback mechanism. (A) Activation of 5-HT_{2C} receptors leads to stimulation of GABA release from GABA neurons. GABA then activates GABA_A receptors located on serotonin neurons, resulting in the influx of chloride ions and hyperpolarization of the cell, thus overall inhibition of serotonin release. (B) During cocaine withdrawal, this negative feedback mechanism is dysregulated and a heightened release of GABA through the 5-HT_{2C} receptor contributes to anxiety at this stage of addiction.

Table 1

Cell membrane characteristics in serotonin neurons from cocaine-withdrawn mice and saline controls. No significant differences were found between cocaine-treated mice at 25 hours of withdrawal and saline controls in tau, resistance, resting membrane potential (RMP), action potential threshold, or activation gap (n=10-20 cells/group).

	Treatment	Tau (ms)	Resistance (MOhms)	RMP (mV)	AP thresh (mV)	Activation gap (RMP-AP Threshold)
dmDR	Saline (n=17)	32.7±5.2	441.0±47.0	-56.1±3.5	-31.0±2.0	-26.5±3.6
	Cocaine (n=20)	31.0±4.0	402.5±54.5	-51.6±2.9	-32.7±1.4	-22.4±2.5
vmDR	Saline (n=13)	33.4±3.6	468.1±40.0	-54.0±3.1	-25.7±3.3	-29.9±4.7
	Cocaine (n=10)	32.3±6.0	387.3±36.3	-53.8±4.2	-29.8±3.1	-30.7±4.2
lwDR	Saline (n=11)	32.1±3.2	442.2±63.3	-53.3±5.0	-31.1±2.6	-25.0±4.8
	Cocaine (n=11)	29.3±6.3	503.5±80.2	-50.3±4.5	-31.7±2.3	-26.5±2.7
Two-way ANOVA analysis	Interaction	$F_{(2,76)} = 0.01$	$F_{(2,76)} = 0.8$	$F_{(2,76)} = 0.16$	$F_{(2,57)} = 0.24$	$F_{(2,57)} = 0.27$
		p = 0.99	p = 0.45	p = 0.85	p = 0.79	p = 0.77
	Treatment	$F_{(1,76)} = 0.21$	$F_{(1,76)} = 0.17$	$F_{(1,76)} = 0.64$	$F_{(1,57)} = 1.0$	$F_{(1,57)} = 0.03$
		p = 0.65	p = 0.68	p = 0.43	p = 0.31	p = 0.86
	Subregion	$F_{(2,76)} = 0.09$	$F_{(2,76)} = 0.47$	$F_{(2,76)} = 0.18$	$F_{(2,57)} = 1.5$	$F_{(2,57)} = 1.1$
		p = 0.91	p = 0.63	p = 0.83	p = 0.23	p = 0.34

Table 2

Action potential characteristics from serotonin neurons from cocaine-withdrawn mice and saline controls. No significant differences were found in action potential amplitude, after-hyperpolarization from threshold, AHP_{t1/2}, AHP tau (1/2). No differences were observed in action potential duration in the dmDR and lwDR cells; however, cells within the vmDR from mice withdrawn from cocaine for 25 hours had a significantly shorter action potential duration (n=6-16 cells/group, two-way ANOVA with post hoc two-tailed t-test, p<0.05).

	Treatment	AP amplitude (mV)	AP duration (ms)	AHP from thresh (mV)	AHP t1/2	AHP tau (t1/2)
dmDR	Saline (n=16)	52.2±3.4	2.6±0.1	-23.5±1.1	-12.8±1.0	40.1±4.1
	Cocaine (n=14)	55.1±3.3	2.3±0.3	-23.5±1.1	-11.7±0.5	41.8±3.7
vmDR	Saline (n=11)	45.9±4.9	3.18±0.4	-25.3±2.5	-8.8±3.2	34.2±5.0
	Cocaine (n=6)	54.3±4.2	*1.9±0.1	-26.7±1.3	-13.5±0.6	35.3±5.2
lwDR	Saline (n=9)	42.2±5.8	2.4±0.3	-19.2±2.3	-10.7±1.3	39.7±6.0
	Cocaine (n=7)	40.9±4.3	2.4±0.4	-23.5±2.1	-12.1±1.1	37.0±5.7
Two-way ANOVA analysis	Interaction	F _(2,57) = 0.56	F _(2,57) = 2.4	F _(2,76) = 0.77	F _(2,57) = 1.41	F _(2,57) = 0.11
		p = 0.57	p = 0.10	p = 0.47	p = 0.25	p = 0.9
	Treatment	F _(1,57) = 0.79	F _(1,57) = 4.42	F _(1,76) = 1.7	F _(1,57) = 1.38	F _(1,57) = 6.3e-005
		p = 0.38	p = 0.04	p = 0.2	p = 2.4	p = 0.99
	Subregion	F _(2,57) = 3.69	F _(2,57) = 0.11	F _(2,76) = 3.3	F _(2,57) = 0.22	F _(2,57) = 0.73
		p = 0.03	p = 0.9	p = 0.04	p = 0.80	p = 0.49