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Morphine history sensitizes postsynaptic GABA receptors on dorsal raphe serotonin neurons in a stress-induced relapse model in rats

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Abstract

The serotonin (5-hydroxytryptamine, 5-HT) system plays an important role in stress-related psychiatric disorders and substance abuse. Previous work has shown that the dorsal raphe nucleus (DR)-5-HT system is inhibited by swim stress via stimulation of GABA synaptic activity by the stress neurohormone corticotropin-releasing factor (CRF). Additionally, the DR 5-HT system is regulated by opioids. The present study tests the hypothesis that the DR 5-HT system regulates stress-induced opioid relapse. In the first experiment, electrophysiological recordings of GABA synaptic activity in 5-HT DR neurons were conducted in brain slices from Sprague-Dawley rats that were exposed to swim stress-induced reinstatement of previously extinguished morphine conditioned place preference (CPP). Behavioral data indicate that swim stress triggers reinstatement of morphine CPP. Electrophysiology data indicate that 5-HT neurons in the morphine-conditioned group exposed to stress had increased amplitude of inhibitory postsynaptic currents (IPSCs), which would indicate greater postsynaptic GABA receptor density and/or sensitivity, compared to saline-controls exposed to stress. In the second experiment, rats were exposed to either morphine or saline CPP and extinction, and then 5-HT DR neurons from both groups were examined for sensitivity to CRF *in vitro*. CRF induced a greater inward current in 5-HT neurons from morphine-conditioned subjects compared to saline-conditioned subjects. These data indicate that morphine history sensitizes 5-HT DR neurons to the GABAergic inhibitory effects of stress as well as to some of the effects of CRF. These mechanisms may sensitize subjects with a morphine history to the dysphoric effects of stressors and ultimately confer an enhanced vulnerability to stress-induced opioid relapse.

Keywords

opioid; reinstatement; swim stress; electrophysiology; 5-hydroxytryptamine; corticotropin-releasing factor

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There are no conflicts of interests.

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Introduction

It is well known clinically that drug relapse is a significant problem for recovering opioid addicts. In addition to re-exposure to drugs and exposure to a drug taking environment, stress is a particularly potent trigger of opioid relapse in both the clinic and in animal models of drug abuse (De Vries and Shippenberg, 2002; Self and Nestler, 1998; Shaham et al. 2003; de Wit H. and Stewart, 1981; de Wit H. and Stewart, 1983).

Investigations of the neurobiology of drug abuse and relapse have largely focused on traditional dopaminergic reward pathways (Koob and Volkow, 2010). While the serotonin (5-hydroxytryptamine, 5-HT) system has long been known to play an important role in stress and stress-related psychiatric disorders such as anxiety disorders and depression (for review see Charney et al. 1990b; Charney et al. 1990a; Meltzer, 1990), it has more recently been implicated in drug reward and drug abuse (Kirby et al. 2011; Filip et al. 2005; Hayes and Greenshaw, 2011; Kranz et al. 2010; Ciccocioppo, 1999). For example, genetic deletion of the dopamine transporter does not prevent mice from responding to the rewarding properties of cocaine as evidenced by self-administration (Rocha et al. 1998) and conditioned place-preference (CPP) models (Sora et al. 1998), indicating that non-dopaminergic mechanisms may also contribute to psychostimulant reward. In addition, double dopamine/5-HT transporter knockout mice fail to show cocaine CPP (Sora et al. 2001), indicating a role for 5-HT as well as dopamine in psychostimulant reward. The 5-HT system has also been shown to be involved in both euphoric effects of opioids (Carboni et al. 1989; Carboni et al. 1988; Carboni et al. 1989; Glick and Cox, 1977; Alaei et al. 2002) and the dysphoric responses that are part of the withdrawal syndrome (Cervo et al. 1983; Harris and Aston-Jones, 2001; Goeldner et al. 2011; Cervo et al. 1981). There is also a high degree of comorbidity of affective disorders such as major depression with opiate dependence (Brooner et al. 1997; Mason et al. 1998; Pani et al. 1997; Rounsaville et al. 1982; Woody et al. 1975). Indirectly, 5-HT circuits may also play a role in drug abuse via regulation of traditional dopaminergic reward pathways (Bardo, 1998; Kapur and Remington, 1996; Zahm, 1999). Ultimately, the 5-HT system may contribute to negative affective states which underlie vulnerability to both mood disorders as well as drug abuse, potentially via overlapping neural mechanisms.

Anatomically, the dorsal raphe nucleus (DR) contains the majority of 5-HT neurons projecting to the forebrain (Jacobs and Azmitia, 1992) and is known to be a target of both stress neurohormones such as corticotropin releasing factor (CRF) and opioids. CRF terminals densely innervate the DR (Kirby et al. 2000; Valentino et al. 2001) and the two known CRF receptors are also expressed at high levels (Chalmers et al. 1995; Lovenberg et al. 1995a; Day et al. 2004). The DR also expresses endogenous opioids (Martin-Schild et al. 1999; Neal, Jr. et al. 1999) and the major opioid receptors, μ , κ , and δ (Mansour et al. 1994a; Mansour et al. 1994; Mansour et al. 1995).

Additional evidence for regulation of the 5-HT DR system by opioids and stress comes from *in vivo* microdialysis and electrophysiology studies. For example, swim stress stimulates 5-HT neurotransmission in the striatum but inhibits 5-HT neurotransmission in several different brain regions including the lateral septum, amygdala and DR (Adell et al. 1997; Kirby et al. 1995; Kirby et al. 2000; Kirby et al. 2007; Price et al. 2002; Chou et al. 1995). Similarly CRF, at low and moderate doses, inhibits DR 5-HT neuronal activity and release via CRF-R1 receptors on GABA afferents and stimulates DR 5-HT neuronal activity at higher doses via CRF-R2 receptors on 5-HT neurons (Kirby et al. 2000; Kirby et al. 2008; Lowry et al. 2000; Price et al. 2002; Price et al. 1998; Pernar et al. 2004). Furthermore, swim stress-mediated inhibition of 5-HT release in the lateral septum is mediated by the

endogenous release of CRF and actions at its receptors within the DR (Price et al. 2002). In contrast to the inhibitory effects of CRF produced by low to moderate doses, acute administration of opioids such as morphine have excitatory effects on 5-HT DR neurotransmission (Tao and Auerbach, 1994; Tao and Auerbach, 2002a; Tao and Auerbach, 2002b). These stimulatory effects of morphine have been shown to be indirect, mediated by the inhibition of GABAergic afferents to 5-HT DR neurons (Jolas and Aghajanian, 1997; Tao and Auerbach, 2002a).

Based on the studies above indicating that stress and opioids converge on the DR to regulate 5-HT neurotransmission, we hypothesize that the 5-HT DR system plays a role in stress-induced opioid relapse. To test this hypothesis we used a morphine CPP model of opioid reward. In the first experiment we used forced swim stress to reinstate previously extinguished CPP, an established model of stress-induced drug relapse (Kreibich and Blendy, 2004; Ma et al. 2007). Next, we examined GABA synaptic activity in 5-HT DR neurons using whole-cell patch-clamp recordings in brain slices from these subjects. In the second experiment, in lieu of a stressor, we examined the sensitivity of DR neurons to *in vitro* application of the stress neurohormone CRF in brain slices from subjects exposed to morphine CPP followed by extinction. In summary, these experiments test the responses of DR neurons to interactions between opioid history and stressor/stress neurohormone exposure using an opioid relapse model.

Materials and Methods

Animals

Male Sprague-Dawley rats (Taconic Farms Germantown, NY) weighing 300–350 g were housed 2 per cage under standard temperature (20°C) and humidity (40%). Rats were kept under a 12 hour light/dark cycle (lights on at 7:00 AM). Food and water were provided *ad libitum*. Prior to the start of experiments, rats were handled for 5 min over 3–5 days. Animal protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Conditioned Place Preference

The CPP chambers consisted of 20H x 20W x 40L-cm custom-built Plexiglas boxes with a removable Plexiglas cover, divided into two distinct chambers, separated with a removable door. The chambers differ in wall design (1 cm vertical vs. horizontal black stripes on white background, 4 cm apart), floor texture (smooth black floor vs. wire mesh (1 cm squares) elevated 1 cm above a white floor) and cage top/lighting (black top vs. white top backlit with a small spotlight). All experimental work was performed during the light cycle under low lighting conditions. CPP chambers were designed so that animals would show no inherent preference for one side or another; all CPP experiments were conducted using an unbiased procedure.

For morphine-conditioned subjects, the conditioning phase consisted of 4 days (days 1–4) of subcutaneous injections of morphine (5 mg/kg) or saline given alternately in morning (9 am) and afternoon (3 pm) sessions (6 hours apart), each injection followed by immediate confinement of the rat to its drug-paired or saline-paired chamber for 45 min (Figure 1). For saline-conditioned controls, rats were given only saline and exposed to one side of the CPP box during the morning session and the other side during the afternoon session for 45 min/session over 4 days. Injections were counterbalanced for chamber and injection time of day. On the 5th day rats were tested for conditioning by placing them in the CPP chambers for 15 min with free access to both sides. Rats were videotaped in the conditioning test for later

subjective behavioral scoring by raters blind to the treatment group. Rats were considered to have entered a chamber when both front paws were inside the chamber. Amount of time spent in each chamber was recorded for the 15 min session. Since the focus of this study was on reinstatement, it was necessary to eliminate subjects with weak conditioning or extinction. Successful conditioned place preference was defined as time in drug-paired minus saline-paired chamber > 100 sec. A 100 sec criterion has been used previously as an effective discriminator of rats with stable morphine conditioned place preference (Herzig and Schmidt, 2004; Herzig and Schmidt, 2005). If the difference was below 100 sec then the rat was removed from the experiment.

The extinction phase consisted of 4 days (days 6–9), and had the same design as the conditioning phase (Figure 1) except that no morphine was administered. In the extinction test (day 10), rats were placed in the CPP chambers for 15 min with free access to both sides and videotaped for later behavioral analysis as described above. Successful extinction was defined as time in drug-paired minus saline-paired chamber < 100 sec. If the difference was greater than 100 sec then the rat was removed from the experiment.

Experiment 1—On day 11, morphine (N = 18) and saline treated rats (N = 17) that had undergone successful conditioning and extinction were exposed to a 6 min forced swim stress (placed in a swim tank that is 20 cm in diameter and filled with 21–22°C water to a depth of 30 cm) followed by a 20 min drying off period, and then placed in the CPP chambers for 15 min with free access to both sides (Figure 1). Animals were videotaped in the reinstatement test for later behavioral analysis as described above. Animals were sacrificed and brains prepared for electrophysiology within 30 min of the reinstatement test. Swim stress has been shown in previous studies to reinstate CPP in mice and rats under similar experimental conditions. Several studies employed a 6 min swim session (Kreibich and Blendy, 2004; Mantsch et al. 2010; Briand et al. 2010) and others a 10–15 min swim session (Redila and Chavkin, 2008; Ma et al. 2007). The majority of the studies employed room temperature water (20–25°C; Briand et al. 2010; Briand et al. 2010; Kreibich and Blendy, 2004; Ma et al. 2007; Mantsch et al. 2010) with one exception (30°C; Redila and Chavkin, 2008; Redila and Chavkin, 2008).

Experiment 2—In a separate group of subjects, on day 11, morphine (N = 12) and saline treated rats (N = 13) that had undergone successful conditioning, and extinction were sacrificed and brains prepared for electrophysiology to examine the sensitivity of 5-HT neurons in rats that had undergone extinction of CPP to CRF *in vitro* (Figure 1).

Electrophysiology

Brain slice preparation and electrophysiology were performed as described previously (Kirby et al. 2008). Subjects were sacrificed by rapid decapitation and brains were placed into ice-cold artificial cerebrospinal fluid (ACSF) in which sucrose (248 mM) was substituted for NaCl. Slices (200 μ m thick) were cut through the DR using a Vibratome 3000 Plus (Vibratome, Bannockburn, IL) and placed in ACSF ((in mM): 124 NaCl, 2.5 KCl, 2 NaH₂PO₄, 2 CaCl₂, 2 MgSO₄, 10 dextrose, and 26 NaHCO₃) with l-tryptophan (50 μ M) 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). Only 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 4–8M Ω when filled with an intracellular

solution ((in mM): 70 K-gluconate, 70 KCl, 2 NaCl, 4 EGTA, 10 HEPES, 4 MgATP, 0.3 Na₂GTP, 0.1% biocytin, pH 7.3).

Spontaneous inhibitory postsynaptic currents (IPSCs) recordings were made primarily in cells located in the ventromedial subdivision of the DR where 5-HT neurons are most densely populated. Whole-cell recordings were conducted in voltage-clamp mode ($V_m = -70$ mV) with a Multiclamp 700B (Molecular Devices, Sunnyvale, CA) or HEKA patch-clamp EPC-10 amplifier (HEKA Elektronik Lambrecht/Pfalz, Germany). Series resistance was monitored throughout the experiment. Signals were filtered at 1 kHz and digitized at 10 kHz. The liquid junction potential was ~9 mV between the pipette solution and the ACSF and was not subtracted from the data obtained.

The non-NMDA receptor antagonist 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione (DNQX; 20 μ M) was bath applied to isolate GABAergic IPSCs. Total spontaneous IPSCs (sIPSCs) were recorded in the absence and miniature spontaneous IPSCs (mIPSCs) in the presence of tetrodotoxin (TTX; 1 μ M) to block action potential-dependent events. To examine the CRF response (Experiment 2), 10 nM ovine CRF (generously provided by Dr. Jean Rivier, Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA) was added, followed by recording for 9 minutes. Ovine CRF is more selective for the CRF-R1 than CRF-R2 subtype (Lovenberg et al. 1995b). The 10 nM dose has been previously shown to have neurophysiological effects in brain slices (Liu et al. 2004; Nie et al. 2004; Kash and Winder, 2006; Tan et al. 2004). In addition, while more selective for CRF-R1, previous experiments in our lab used selective CRF receptor antagonists to show that the 10 nM dose of ovine CRF produces both CRF-R1-mediated (increased GABA synaptic activity) as well as CRF-R2-mediated (inward current) effects in 5-HT DR neurons (Kirby et al. 2008). Therefore this dose of ovine CRF is capable of engaging both CRF receptor subtypes in DR brain slices.

Immunohistochemistry

Following electrophysiology experiments, brain slices were postfixed in 0.4% paraformaldehyde and standard fluorescent immunohistochemical methods (Beck et al. 2004) were used to visualize the recorded biocytin-filled cell and tryptophan hydroxylase as a marker of 5-HT neurons. Mouse α tryptophan hydroxylase antibody (1:500; Sigma-Aldrich, St Louis, MO) was visualized using an Alexa 647-conjugated donkey anti-rabbit secondary antiserum (1:200; Molecular Probes, Eugene, OR). Biocytin was visualized using streptavidin-conjugated Alexa-488 (1:200; Molecular Probes). Sections were viewed and images captured by Leica DMIRE2 confocal microscope (Leica Microsystems, Exton, PA). No cell was included in the study if it could not be conclusively identified by immunohistochemistry.

Data Analysis

MiniAnalysis software (Synaptosoft, Decatur, GA) was used to analyze IPSC events on the basis of amplitude, rate of rise, duration, and area. Initially, noise analysis was conducted for each cell and amplitude detection thresholds set to exceed noise values. Events were automatically selected, analyzed for double peaks, and then visually inspected and confirmed. Event amplitude histograms were generated and compared to noise histograms to ensure that they did not overlap. Holding current was recorded and GABA synaptic activity analyzed for frequency, amplitude, rise time (calculated from 10 to 90% of peak amplitude) and decay time (calculated by averaging 200 randomly selected events and fitting a double-exponential function from 10 to 90% of the decay phase to generate an initial fast component and a subsequent slow component of the decay phase). All data are represented as mean $\pm$ SEM, except for rise time, which is represented as median $\pm$ SEM. For behavioral

testing the time spent on the drug paired side minus the unpaired side was analyzed using a two-way repeated measures ANOVA and post-hoc Tukey tests employing drug treatment (morphine vs. saline) and type of behavioral test (conditioning, extinction, and reinstatement) as the independent variables. Electrophysiological results from the forced swim stress-induced reinstatement rats were compared between morphine- and saline-conditioned groups via unpaired Student's t-test or Mann-Whitney Rank Sum test when appropriate. The amplitude of IPSC events in individual cells was also illustrated as a cumulative probability graph for amplitude in saline versus morphine-conditioned subjects and compared by the Kolmogorov–Smirnov test. Electrophysiological results from CRF treated sections were analyzed with a two-way repeated measures ANOVA and post-hoc Tukey tests employing conditioning treatment (morphine vs. saline) and drug (ACSF vs. CRF) as the independent variables. The relationship between behavioral responses during the CPP paradigm and subsequent electrophysiological measures in DR neurons was examined with Pearson product-moment correlation. All statistical analysis was done using SigmaStat 3.1 (Systat Software Inc., Chicago IL). A probability of $p < 0.05$ was considered significant.

Results

Behavior

CPP was used to evaluate drug reward during conditioning, extinction, and forced swim stress-induced reinstatement prior to electrophysiology (Figure 2). Because the focus of this study was on stress-induced reinstatement, animals that did not reach the success criteria of the conditioning or extinction tests were removed from this study. The success rate of morphine conditioning was 67% in Experiment 1 and 62% in Experiment 2. The success rate of extinction of morphine conditioning was 66% in Experiment 1 and 64% in Experiment 2. The success rates of the conditioning and extinction phases are comparable (Bruchas et al. 2011; Land et al. 2009; Blander et al. 1984) and in some cases exceed (Redila and Chavkin, 2008; Katz and Gormezano, 1979; Sherman et al. 1980; Rossi and Reid, 1976) the rates published in earlier rodent CPP studies. No animals were removed from the stress-induced reinstatement group in order to determine the relationship between the strength of reinstatement and electrophysiological measures as well as to determine the impact of stress exposure on electrophysiological measures. There was a significant main effect of drug treatment ($F_{(1, 33)} = 5.639$, $p < 0.05$) and a significant interaction between drug and behavioral test ($F_{(2, 66)} = 3.507$, $p < 0.05$) but no main effect of behavioral test. Post-hoc Tukey tests showed that in the conditioning phase, morphine-conditioned rats ($N = 18$) spent significantly more time on the drug-paired side when compared to saline controls ($N = 17$; $p < 0.05$). In the extinction phase there was no significant difference between morphine and saline-treated rats. Forced swim significantly increased time spent on the drug paired side in morphine treated rats when compared to saline controls (Figure 2, $p < 0.01$). Furthermore there was no significant difference within the saline-conditioned group between any of the test phases. In morphine-conditioned rats there was a significant difference between conditioning vs. extinction ($p < 0.01$) and extinction vs. forced swim stress-induced reinstatement ($p < 0.05$). There was no significant difference within the morphine treated rats between conditioning vs. forced swim stress-induced reinstatement (Figure 2). Similar behavioral results were seen with the second group of rats that underwent conditioning and extinction without forced swim stress-induced reinstatement (Supplementary Data, Table 1).

Electrophysiology

Experiment 1: GABA synaptic activity in DR neurons following forced swim stress-induced reinstatement—Twenty three 5-HT cells (Figure 3D) and 5 non-5-HT cells (Figure 4D) were recorded from 18 morphine conditioned rats; 15 5-HT cells and 4

non-5-HT cells were recorded from 17 saline-conditioned rats. 5-HT neurons ($N = 23$) from morphine-conditioned rats exposed to forced swim stress-induced reinstatement had a greater amplitude of sIPSCs ($t_{36} = 3.232$; $p < 0.01$) and mIPSCs ($t_{36} = 2.331$; $p < 0.05$) compared to 5-HT neurons ($N = 17$) from saline controls (Figure 3A, B, B', C, and C'). Cumulative probability graphs of sIPSC and mIPSC amplitude for an individual cell (Figure 3B'' and C'') illustrate a significant shift to the right in the morphine vs. saline-conditioned subjects (sIPSC: $Z = 3.42$, $p < 0.01$; mIPSC: $Z = 3.61$, $p < 0.01$). This increase in amplitude is an indication of postsynaptic receptor sensitization. There was no difference in sIPSC or mIPSC frequency in 5-HT neurons from morphine- vs. saline-conditioned rats following forced swim stress-induced reinstatement. There was no significant difference in non-5-HT neurons (morphine subjects: $N = 5$; saline subjects: $N = 4$; see Figure 4) from morphine- and saline-conditioned subjects in amplitude of sIPSCs ($t_7 = -0.00410$; n.s.) or mIPSCs ($t_7 = 1.203$; n.s.; Figure 4A, B, and C) or frequency of sIPSC ($t_7 = 1.152$; n.s.) or mIPSC ($t_7 = -0.187$; n.s.). Unlike in 5-HT neurons (see Figs. 3B'' and 3C'') cumulative probability graphs of sIPSC and mIPSC amplitude for an individual non-5-HT cell (Figure 4B' and C') illustrate no difference between morphine vs. saline-conditioned subjects (sIPSC: $Z = 0.75$, n.s.; mIPSC: $Z = 1.13$, n.s.). No significant differences between saline- and morphine-conditioned groups were seen in holding current or mIPSC kinetics (rise time, fast or slow decay) from 5-HT or non-5-HT neurons (Supplementary Data; Table 2A).

In order to examine the relationship between the behavioral reinstatement in the CPP protocol and the subsequent observed GABAergic sensitization of 5-HT DR neurons, data from saline- and morphine-conditioned subjects were pooled and correlational analyses performed. There was no correlation between preference for the drug-paired side during the stress-induced reinstatement tests and sIPSC or mIPSC amplitude. When morphine-conditioned and saline-conditioned subjects were examined separately, there were no significant correlations between stress-induced reinstatement and sIPSC or mIPSC amplitude (Supplementary Data; Table 3A).

Experiment 2: CRF responses of DR neurons following extinction—Eleven 5-HT cells and 5 non-5-HT cells were recorded from 12 morphine conditioned rats; 12 5-HT cells and 7 non-5-HT cells were recorded from 13 saline-conditioned rats. There was no difference in basal holding current in 5-HT neurons from morphine- and saline-conditioned subjects following extinction (Supplementary Data; Table 2A). 5-HT neurons ($N = 11$) from morphine-conditioned rats had a greater inward current response to CRF *in vitro*, which is a reflection of cellular depolarization, when compared to 5-HT neurons ($N = 12$) from saline controls (Figure 5A). There was a significant main effect of drug on inward current ($F_{(1, 21)} = 13.013$, $p < 0.01$) and a significant interaction between drug and conditioning treatment ($F_{(1, 21)} = 4.397$, $p < 0.05$) but no main effect of conditioning treatment. Post-hoc Tukey tests show that bath applied CRF produced a significant inward current response compared to vehicle treatment (ACSF) in 5-HT DR neurons from morphine-conditioned ($p < 0.01$) but not saline-conditioned subjects. This inward current response in the morphine group was significantly greater than the response in the saline group ($p < 0.01$). Saline-conditioned subjects had a slightly smaller CRF-inward current at 4.6 ± 2.7 pA and morphine-conditioned subjects had a significantly greater CRF-inward current than either of the other groups at 21.9 ± 3.1 pA. Given an input resistance of DR 5-HT neurons of 637 M Ω (Beck et al. 2004), the CRF-induced inward current response in morphine-conditioned subjects would indicate a 13.9 mV neuronal depolarization as compared to 2.9 mV in saline-conditioned controls. CRF had no effect on mIPSC frequency or amplitude in either treatment group (Figure 5B and C).

To examine the relationship between behavior during CPP and sensitivity to CRF, data from morphine-conditioned and saline-conditioned rats were pooled and correlational analyses

performed. There was no correlation between any of the behavioral responses during the CPP (conditioning or extinction) and CRF-R2-mediated inward current (Supplementary Data; Table 3B). However, when analyzed separately, morphine-conditioned animals showed a significant positive correlation between preference for the drug-paired chamber during the extinction trial and CRF-R2-mediated inward current ($R = 0.62$, $p < 0.05$; see Supplemental Data, Table 3B).

In contrast to the IPSC amplitude sensitization in 5-HT DR neurons from morphine-conditioned subjects following stress-induced reinstatement (Figure 3), there was no significant difference in basal sIPSC or mIPSC frequency or amplitude of 5-HT neurons between morphine- and saline-conditioned subjects following extinction with no stress exposure (Figure 5B and C). There was also no significant difference in basal IPSC frequency or amplitude between non-5-HT neurons from morphine ($N = 5$) and saline-conditioned subjects ($N = 7$) or in mIPSC kinetics in 5-HT or non-5-HT neurons from morphine vs. saline-conditioned subjects (Supplementary Data; Table 2B).

Discussion

Morphine history sensitized 5-HT DR neurons at a postsynaptic level to GABA synaptic activity following stress exposure. This sensitization was 1) not observed in 5-HT neurons from stress exposed saline controls, 2) not observed in 5-HT neurons from morphine-conditioned subjects that were not exposed to stress, and 3) not observed in non-5-HT neurons from morphine-conditioned subjects exposed to stress. Morphine history also sensitized 5-HT DR neurons to some of the effects of the stress neurohormone CRF. Specifically, this group exhibited augmented CRF-induced elevation of inward current compared to both saline-conditioned controls. These data show a unique response of 5-HT DR neurons to an interaction between opioid history and stress/stress hormones that may contribute to vulnerability to stress-induced opioid relapse.

Forced swim stress was able to reinstate opioid reward in morphine treated rats in a CPP model. The forced swim test has been used extensively as a behavioral screen for antidepressant efficacy (Porsolt et al. 1977b; Porsolt et al. 1977a; Porsolt et al. 1978). More recently the forced swim test has been used to model stress-induced cocaine relapse in mice (Kreibich and Blendy, 2004; Briand et al. 2010; Mantsch et al. 2010) and stress-induced morphine relapse in rats (Ma et al. 2007). Consistent with our behavioral data, Ma and colleagues (2007) also showed that forced swim induced opioid reinstatement of a similar magnitude to the initial CPP in morphine treated animals. These studies and our own indicate that the forced swim stress model can reliably reinstate opioid reward in rodents.

Forced swim-induced reinstatement increased IPSC amplitude of 5-HT neurons from morphine-conditioned rats compared to saline controls, which is an indication of greater postsynaptic GABA receptor sensitivity or receptor density on 5-HT neurons. This receptor sensitization would lead to greater inhibition of 5-HT neurons in the DR. Previous studies have shown that acute morphine increases 5-HT release through the inhibition of GABA in the DR (Jolas and Aghajanian, 1997; Jolas et al. 2000; Tao and Auerbach, 1994). Furthermore the increase in 5-HT from acute morphine adapts following chronic exposure (Jolas et al. 2000; Tao et al. 1998), and is inhibited during morphine withdrawal via an increase of GABA synaptic activity at 5-HT DR neurons (Jolas et al. 2000; Tao et al. 1998). The current study further shows adaptations of the interaction between GABA and 5-HT neurons during different phases of an opioid reward, extinction and relapse model, specifically indicating that 5-HT neurons are sensitized to GABA after stress-induced opioid reinstatement, which could lead to a decrease in 5-HT neurotransmission similar to that seen during withdrawal (Jolas et al. 2000; Tao et al. 1998; Goeldner et al. 2011).

Our results indicate that the 5-HT DR system demonstrates unique neurochemical adaptations following stress-induced opioid reinstatement. Previous studies have shown that 5-HT has a role in reinstatement of previously extinguished cocaine self-administration, and this was dependent on the 5-HT receptor involved (for review, see Filip et al. 2010). For example, whereas pharmacological blockade of 5-HT_{2A} receptors suppresses reinstatement elicited by priming doses of cocaine (Fletcher et al. 2002; Filip, 2005) or cocaine-associated cues (Filip, 2005; Burmeister et al. 2004; Nic Dhonnchadha et al. 2009), stimulation of 5-HT_{2C} receptors suppress cocaine- (Grottick et al. 2000; Neisewander and Acosta, 2007), cue- (Neisewander and Acosta, 2007; Burbassi and Cervo, 2008; Fletcher et al. 2008; Cunningham et al. 2011) and stress-induced cocaine reinstatement (Fletcher et al. 2008), indicating an oppositional effect of these two receptor subtypes on cocaine-seeking behavior. More global stimulation of the 5-HT system with 5-HT- selective reuptake inhibitors or 5-HT releasing agents suppresses both cue-elicited cocaine reinstatement (Baker et al. 2001; Burmeister et al. 2003) and also footshock-induced reinstatement of alcohol seeking behavior in rats (Le et al. 1999). Together these studies indicate that increased 5-HT, though dependent on the receptor subtype that is engaged under those conditions, can attenuate cue- and stress-induced drug reinstatement in animal models, and moreover imply that lower 5-HT levels may contribute to vulnerability to drug relapse in humans.

CRF applied *in vitro* increased the inward current of 5-HT neurons from rats with a morphine history compared to saline controls, without affecting mIPSC frequency or amplitude. Previous work by Kirby and colleagues (2008) using the same (10 nM) and higher doses of CRF (100 nM) in combination with CRF receptor subtype-selective antagonists, has shown in naïve subjects that CRF-induced increases in inward current of 5-HT neurons occur primarily through the CRF-R2 receptor whereas CRF-induced increases in mIPSC frequency and amplitude involve the CRF-R1 receptor (Kirby et al. 2008). An additional finding of the current study indicated a significant correlation between preference for the drug-paired chamber during the extinction trial and CRF-R2-mediated inward current in 5-HT DR neurons from morphine conditioned subjects. These data indicate that animals with the weakest extinction of morphine CPP (retaining some preference for the drug-paired side) have the greatest sensitivity to CRF-R2 receptor stimulation. Though these CRF-R2-sensitive subjects do not show stronger initial morphine CPP, it is possible that sensitization of CRF-R2 receptors in 5-HT DR neurons confers a more persistent morphine CPP that is resistant to extinction. The CRF-induced inward current was similar in magnitude between saline controls in the present study and naïve controls in the previous study (Kirby et al. 2008), it did not reach statistical significance in saline controls, likely due to reduced statistical power from the smaller number of subjects in the current study. In addition, the CRF-induced increase in mIPSC frequency and amplitude (naïve subjects; Kirby et al. 2008) was absent in 5-HT DR neurons from both morphine- and saline-conditioned subjects, potentially reflecting a desensitization of the CRF-R1 receptor in response to the stressors that are inherent to the CPP model (e.g. injections, handling). In support of this theory, a recent study showed that acute forced swim stress can alter CRF-R1 and CRF-R2 receptor distribution in 5-HT neurons in the DR (Waselus et al. 2009). Waselus and colleagues (2009) suggest that this receptor switch coincides with the switch from active behavioral coping to passive coping responses as seen during repeated forced swim tests. Future work will have to determine if this redistribution of CRF-R1 and -R2 receptors also occurs during the CPP paradigm. Nonetheless, sensitization of the CRF-R2 response in subjects with an opioid history may confer a predisposition to the adoption of passive coping strategies in response to stressors which may contribute to increased relapse vulnerability (Valentino et al. 2010).

A number of studies have shown that systemic or i.c.v.-administered CRF can induce stress-induced reinstatement in opioid-conditioned rats (Shaham et al. 1997; Shaham et al. 1998), and that stress or CRF-induced reinstatement can be attenuated by CRF receptor antagonists (Shaham et al. 1997; Shaham et al. 1998; Lu et al. 2000). Furthermore systemic administration of the select CRF-R1 receptor antagonist CP-154,526 was able to reduce footshock-induced cocaine or opioid reinstatement in rats (Shaham et al. 1998; Lu et al. 2000). While these earlier studies implicate a role for the CRF-R1 rather than the CRF-R2 receptor subtype in opioid reinstatement, a number of methodological differences between these experiments and the current study exist, leaving open potential contributions from both receptor subtypes. For example, Shaham and colleagues (1998) demonstrated CRF-R1-mediated stress-induced opioid reinstatement with a different drug and behavioral test: footshock-induced reinstatement of heroin self-administration. Lu and colleagues (2000) employed a morphine CPP model with significant methodological distinctions from the current procedure: a 6-day conditioning period followed by a 28-day home cage extinction period with reinstatement initiated by a footshock stressor.

CRF failed to induce a significant inward current in non-5-HT neurons from either saline treated or morphine treated rats that had undergone CPP followed by extinction. In contrast, previous work in our laboratory has shown that CRF induces a significant inward current in non-5-HT neurons in the DR from naïve untreated rats (Kirby et al. 2008). It is possible that CPP exposure itself alters CRF receptor expression or function on this population of non-5-HT neurons as described above, accounting for this difference in comparison to naïve animals. However, comparisons between these two studies are limited by the fact that the identity of these non-5-HT neurons and whether they represent the same or different populations in the two studies is unknown. It is possible that some of these non-5-HT neurons are GABAergic as previous work has shown that the DR contains a large population of GABA interneurons that express CRF-R1 and are innervated by CRF fibers (Roche et al. 2003; Day et al. 2004; Allers and Sharp, 2003). Furthermore these GABA interneurons have increased c-Fos expression following forced swim stress (Roche et al. 2003), indicating their engagement by this stressor. The role of this neuronal population in the DR in stress-induced opioid relapse will need to be the subject of future study.

Based on the current data as well as previous studies in other laboratories, Figure 6 describes a conceptual model of serotonergic involvement in mood dysfunction which may drive relapse during the opioid addiction cycle. Acute opioid administration results in euphoric mood, and the activation of the 5-HT system (shown in blue in Figure 6) via inhibition of GABA afferents (Jolas et al. 2000; Tao and Auerbach, 1994; Jolas and Aghajanian, 1997). Cessation of opioids results in dysphoric mood and the restoration of GABAergic inhibition of the 5-HT system (Jolas et al. 2000; Tao et al. 1998). Relapse can be conceptualized as a form of ‘self-medication’, normalizing both 5-HT dysregulation and mood dysfunction (Markou et al. 1998). In support of this model, a recent study has shown that mice exposed to chronic morphine followed by protracted abstinence exhibit behavioral symptoms of mood dysfunction that gradually replace the initial somatic symptoms of withdrawal (Goeldner et al. 2011). 5-HT levels were reduced in several forebrain structures and in the DR itself after acute withdrawal and remain low during protracted abstinence in the DR when compared to saline controls (Goeldner et al. 2011). Our data indicate that in subjects with an opioid history, stress sensitizes 5-HT neurons to GABAergic inhibition. We hypothesize that this sensitization inhibits the 5-HT system in a manner that mimics opioid withdrawal, resulting in dysphoric mood which then drives opioid relapse.

By contrast, our CRF findings are somewhat preliminary in nature and therefore not represented in this model (Figure 6). While we propose that the sensitized inward current response in morphine-exposed subjects is CRF-R2-mediated based on previous studies

(Kirby et al. 2008), this hypothesis needs to be confirmed using selective antagonists in future studies. An inward current would depolarize 5-HT neurons, an effect which would oppose the GABA-mediated hyperpolarization illustrated in the proposed model for opioid-exposed subjects (Figure 6). Ultimately, the relative contributions of GABA- and CRF-mediated regulation of 5-HT DR neurons in subjects with an opioid history and their net effects on 5-HT DR neuron excitability and forebrain 5-HT release will need to be determined *in vivo*.

In summary, this study shows that forced swim-induced reinstatement of previously extinguished morphine CPP sensitizes 5-HT neurons to GABAergic inhibition. This sensitization, specific to 5-HT DR neurons, is produced by the unique interactions between opioid history and stress and may lead to hypoactivity of the 5-HT system. Reductions in 5-HT neurotransmission have long been associated with negative affect and vulnerability to mood disorders (Charney et al. 1990a) and may also increase vulnerability to drug abuse, potentially via similar neural mechanisms and neuroanatomical substrates. We also demonstrated that opioid history sensitizes 5-HT DR neurons to some of the effects of the stress neurohormone CRF. Either or both of these adaptations of 5-HT DR neurons may contribute to enhanced relapse vulnerability in these subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental design time-line for morphine CPP paradigm with extinction and stress-induced reinstatement. Electrophysiology experiments in experiment 1 were conducted following the stress-induced reinstatement test on day 11. Electrophysiology experiments in experiment 2 were conducted on day 11 at the same time as in experiment 1 but without stress-induced reinstatement. Abbrev: M = morphine, S = saline, P = placement in CPP chamber (no injections).

Figure 2. Morphine conditioned place preference, extinction and swim stress-induced reinstatement in saline- or morphine-conditioned subjects. Data represent mean $\pm$ SEM from saline- (N = 17) and morphine-conditioned (N = 18) subjects. Asterisks indicate * $p < 0.05$ and ** $p < 0.01$ by post-hoc Tukey test.

Figure 3.

GABA synaptic activity is selectively sensitized in 5-HT DR neurons following swim stress-induced reinstatement of previously extinguished morphine CPP. sIPSC and mIPSC amplitude was greater in the 5-HT DR neurons from the morphine-conditioned group (N=23) than in saline controls (N=15) (A). Mean sIPSC and mIPSC amplitude ($\pm$ SEM) is shown in 5-HT neurons from the two groups (A) and in a representative 5-HT DR neuron (B and C). Representative traces are averaged from 200 randomly selected IPSC events. B' and C' show raw traces of sIPSCs (B') and mIPSCs (C'). B'' and C'' show a significant shift to the right of the cumulative probability graph of IPSC amplitude of 5-HT cells in the morphine group vs. saline controls by Kolmogorov-Smirnov test, $p < 0.01$. Immunohistochemical identification of the biocytin-filled recorded neuron (green) with TPH-IR (red) is shown in a 5-HT cell that is double-labeled (yellow) in the merged panel (D). Asterisks represent * $p < 0.05$ and ** $p < 0.01$ when compared to saline controls by unpaired Student's t-test.

Figure 4.

GABA synaptic activity is unchanged in non-5-HT DR neurons following swim stress-induced reinstatement of previously extinguished morphine CPP. There was no difference in sIPSC or mIPSC amplitude in non-5-HT DR neurons from morphine-conditioned subjects ($N=5$) compared to saline controls ($N=4$) (A). Mean sIPSC and mIPSC amplitude ($\pm$ SEM) is shown in 5-HT neurons from the two groups (A) and in a representative non-5-HT neuron (B and C). Representative traces are averaged from 200 randomly selected IPSC events. B' and C' show no shift of the cumulative probability graph of IPSC amplitude of 5-HT cells in the morphine group vs. saline controls. Immunohistochemical identification of the biocytin-filled recorded neuron (green) with TPH-IR (red) is shown in a non-5-HT neuron (D). Morphine and saline groups were compared by unpaired Student's t -test (A) or Kolmogorov-Smirnov test (B' and C').

Figure 5.

Following extinction of morphine CPP, the CRF-induced inward current response is sensitized in 5-HT DR neurons. This effect, previously shown in naïve subjects to be CRF-R2-mediated (Kirby et al, 2008), is present in the morphine (N = 11) but not saline group (N = 12) (A). In contrast, responses previously shown to involve the CRF-R1 receptor, elevation of mIPSC frequency and mIPSC amplitude (Kirby et al. 2008), are absent in both groups (B and C). Data indicate mean ± SEM. Asterisks indicate ** p < 0.01 by post-hoc Tukey test.

Figure 6.

Conceptual model of serotonergic involvement in mood dysfunction which drives relapse during the opioid addiction cycle. The 5-HT system is activated (denoted by a thick blue arrow) via disinhibition by acute opioids (Jolas et al. 2000; Tao and Auerbach, 1994; Jolas and Aghajanian, 1997), resulting in euphoric mood. Following cessation of the opioid and restoration of GABAergic inhibition, withdrawal is characterized by 5-HT inhibition (Jolas et al. 2000; Tao et al. 1998) (denoted by a thin blue arrow), resulting in dysphoric mood. Our data indicate that in subjects with an opioid history, stress sensitizes 5-HT neurons to GABAergic inhibition. We hypothesize that this sensitization inhibits the 5-HT system in a manner that mimics opioid withdrawal, resulting in dysphoric mood which then drives opioid relapse. Re-exposure to opioids during relapse normalizes both 5-HT dysregulation and mood dysfunction.