Protracted Withdrawal from Alcohol and Drugs of Abuse Impairs Long-Term Potentiation of Intrinsic Excitability in the Juxtacapsular Bed Nucleus of the Stria Terminalis

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Protracted Withdrawal from Alcohol and Drugs of Abuse Impairs Long-Term Potentiation of Intrinsic Excitability in the Juxtacapsular Bed Nucleus of the Stria Terminalis

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The juxtacapsular bed nucleus of the stria terminalis (jcBNST) is activated in response to basolateral amygdala (BLA) inputs through the stria terminalis and projects back to the anterior BLA and to the central nucleus of the amygdala. Here we show a form of long-term potentiation of the intrinsic excitability (LTP-IE) of jcBNST neurons in response to high-frequency stimulation of the stria terminalis. This LTP-IE, which was characterized by a decrease in the firing threshold and increased temporal fidelity of firing, was impaired during protracted withdrawal from self-administration of alcohol, cocaine, and heroin. Such impairment was graded and was more pronounced in rats that self-administered amounts of the drugs sufficient to maintain dependence. Dysregulation of the corticotropin-releasing factor (CRF) system has been implicated in manifestation of protracted withdrawal from dependent drug use. Administration of the selective corticotropin-releasing factor receptor 1 (CRF1) antagonist R121919 [2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo[1,5-a]pyrimidine], but not of the CRF2 antagonist astressin2-B, normalized jcBNST LTP-IE in animals with a history of alcohol dependence; repeated, but not acute, administration of CRF itself produced a decreased jcBNST LTP-IE. Thus, changes in the intrinsic properties of jcBNST neurons mediated by chronic activation of the CRF system may contribute to the persistent emotional dysregulation associated with protracted withdrawal.

Introduction

The bed nucleus of the stria terminalis (BNST) has been implicated in the reinforcing effects of alcohol and drugs of abuse (Epping-Jordan et al., 1998; Carboni et al., 2000; Delfs et al., 2000; Eiler et al., 2003; Macey et al., 2003; Dumont et al., 2005) and supports brain self-stimulation in animals with a genetic alcohol preference (Eiler et al., 2007). The BNST has also been shown to be involved in stress-induced reinstatement of drug-seeking behavior during protracted withdrawal (Erb and Stewart, 1999; Shaley et al., 2001; McFarland et al., 2004). Both drug withdrawal (Delfs et al., 2000; Olive et al., 2002; Erb et al., 2004) and stress (Stout et al., 2000) induce neurochemical changes in this brain region.

The juxtacapsular subdivision of the lateral BNST (jcBNST) is the only part of the BNST that receives dense glutamatergic projections from the basolateral nucleus of the amygdala (BLA) and, unlike other subregions of the lateral BNST, does not receive inputs from the central nucleus of the amygdala (CeA) (Lariviere-Sahd, 2004). In turn, the jcBNST projects back to the anterior BLA and to the CeA (Dong et al., 2000). The jcBNST also projects to the lateral hypothalamus, striatum, and nucleus accumbens, among other brain regions involved in the actions of drugs of abuse (Dong et al., 2000). The jcBNST, and the dorsolateral BNST in general, contain densely arrayed GABAergic neurons (Sun and Cassell, 1993; Veinante et al., 1997) and abundant corticotropin-releasing factor (CRF)-containing neurons (Sawchenko, 1987). Conversely, the jcBNST and the dorsolateral BNST in general are devoid of glutamatergic neurons (Hur and Zaborszky, 2005).

Neuronal activity can induce persistent modifications in the way a neuron reacts to subsequent inputs, both by changing synaptic efficacy and/or intrinsic excitability. The former has received greater attention in recent years, and it is generally ac-
cepted that changes in the strength of synaptic connections underlie memory storage and some of the maladaptive changes in reward processing induced by drugs of abuse (Thomas et al., 2000; Ungless et al., 2001; Melis et al., 2003; Malenka and Bear, 2004; Dumont et al., 2005). In many brain regions, neuronal activity has also been shown to induce long-term modifications of intrinsic neuronal excitability, i.e., the propensity of neurons to fire action potentials.

Long-term potentiation of the intrinsic excitability (LTP-IE) is a protracted increase in the probability of firing and/or in the efficacy of neuronal circuits (Turrigiano et al., 1994; Desai et al., 1999; Aizenman and Linden, 2000; Armano et al., 2000; Bekkers and Delaney, 2001; Ross and Soltész, 2001; Daoudal et al., 2002; Egorov et al., 2002; Debanne et al., 2003; Mahon et al., 2003; Smith and Otis, 2003; Cudmore and Turrigiano, 2004; Chen et al., 2007). Here we tested the hypothesis that a history of drug self-administration may induce long-lasting changes in the excitability of jcBNST neurons in animal models of alcohol, cocaine, and heroin self-administration. We observed a reduced capacity for synchronously increasing jcBNST neuronal activity during protracted withdrawal that could result in inefficient feedback inhibition to the CeA and increased emotional arousal. These effects were blocked by repeated administration of a CRF receptor 1 (CRF₁), but not CRF₂, receptor antagonists and could be mimicked by repeated, but not acute, administration of CRF.

Materials and Methods

Electrophysiological techniques. Acute brain slices were performed as described previously (Sanna et al., 2000, 2002b) with minor modifications. Briefly, coronal rat brain slices (350 μm) were collected from the rostral cerebrum of Wistar rats with a Leica VT1000E vibrating microtome, at the level shown in Figure 1A, in oxygenated artificial CSF (aCSF) [in mM: 130 NaCl, 3.5 KCl, 24 NaHCO₃, 1.25 NaH₂PO₄, 2.2 CaCl₂, 10 d-glucose, and 2 MgSO₄, pH 7.4]. Slices were preincubated in aCSF for 1 h at 32°C and then maintained at room temperature for at least 30 min before being transferred to a submerged recording chamber at 31°C. Extracellular field potentials were recorded with microelectrodes filled with aCSF (3–5 MΩ) using an Axoclamp 2B head-stage amplifier (Molecular Devices). Bipolar stimulating electrodes were placed in the stria terminalis (Fig. 1B). Constant-current pulses of 0.08 ms duration were used for stimulation. The amplitude of the second negative component of the field potential (Fig. 1C) was measured at its trough. Acquisition and analyses were performed with the LABVIEW software package (National Instruments). For field potential recordings, input–output (I/O) curves were performed in each slice, and test stimulation intensity was adjusted to obtain field potentials approximately one-third of the maximum amplitude. The high-frequency stimulation (HFS) paradigm consisted of five trains at 100 Hz for 1 s at 10 s intervals at the test stimulation intensity. For occlusion experiments, after bath application of GABAₐ...
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**Figure 2.** LTP-IE in the jcBNST. **A**, HFS of the stria terminalis induced only a transient EPSP potentiation that reverted to basal levels within 16 min. Stimuli artifacts were removed, and the asterisk indicates their location. **B**, Quantification of EPSP amplitude changes in cells of the jcBNST at the indicated times after HFS of the stria terminalis (*p < 0.05 from baseline; NS, *p* = 0.123). **C**, jcBNST neurons visualized by intracellular injection of biocytin after recording demonstrate a multipolar morphology (ic, internal capsula). **D**, HFS caused increased probability of firing in response to single stimuli (red line) applied to the stria terminals. Traces shown are 10 sweeps representing evoked responses before and 40 min after HFS. E, Threshold (arrowheads) for action potential generation in response to a depolarizing current pulse (500 ms, 0.07 nA) was shifted to more hyperpolarized membrane potentials for a protracted time after HFS (red trace). Inset, Such a shift also could be observed even in the absence of potentiation of EPSP after HFS (red trace), as in the representative neuron shown (same neuron as in D). F, The shift of the threshold for action potential generation after HFS was significant for over 40 min after HFS (**p < 0.01 from baseline, n = 14, at 10 min, t = 4.26; *p < 0.05 from baseline at 25 min, t = 2.72, and at 40 min, t = 6.66**). G, HFS-induced shift of the action potential threshold was prevented by a transient application for 20 min around the time of delivery of HFS (as in Fig. 1F) of either the NMDA inhibitor D-AP-5 (50 μM) or the mGluR inhibitor MPEP (10 μM) in the perfusion bath (t = −8.491, *p < 0.05 from control, n = 15; for D-AP-5, t = −1.376, n = 8, NS; and for MPEP, t = 1.044, n = 6, NS).

and GABAA inhibitors or 4-aminopyridine (4-AP), I/O curves were again determined, and stimulus intensity was adjusted as described to obtain one-third of maximum field potential amplitude before delivering the HFS. Intracellular recordings were made using sharp glass micropipettes filled with 2 M potassium acetate, pH 7.3 (180–200 MΩ), with an Axoclamp 2B head-stage amplifier in current-clamp mode. The threshold of the first action potential, evoked in response to single intracellular depolarizing pulses (500 ms at 15 s intervals) was measured at the sharp transition from prepotential to upstroke (Armano et al., 2000), as shown in Figure 2.E. Input resistance (Rin) was measured with sharp electrodes in current-clamp mode at the end of 350 ms hyperpolarizing current pulses of −0.2 nA. This current value is in the linear part of the I–V curve. The slope of the depolarizing prepotential was measured over the 10 ms immediately preceding the action potential upstroke and expressed in millivolts per millisecond. After 20 min of a stable baseline of threshold recording, HFS was delivered to the stria terminalis. For whole-cell recordings, slices of brain tissue containing the BNST were placed in a superfusion chamber mounted on an Olympus microscope stage and were viewed using infrared differential interference contrast optics and video microscopy. Whole-cell recordings were made using patch pipette with resistances of 3–6 MΩ filled with a solution containing the following (in mM): 120 KMeSO4, 10 KCl, 3 MgCl2, 10 HEPES, 10 phosphocreatine, 2 MgATP, and 0.2 GTP, pH 7.2 (osmolarity, 280–290 mOsm). Whole-cell access resistances were estimated from the peak current produced during voltage-clamp steps and were found to be 15–40 MΩ. Access and input resistances were monitored throughout the recordings to ensure stability. To isolate the α-dendrotoxin (α-DTX)-sensitive D-type K+ current (ID), the extracellular solution contained 0.5 μM TTX, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μM CdCl2, and 10 mM tetraethylammonium to block interfering K+ and inward currents. For analysis, only the current trace for the +40 mV step was used, and the average current amplitude between 50 and 100 ms after application of the voltage step was measured. For morphological analysis, 1–2 μg/ml biocytin (Sigma) was added to the intracellular solution. Immediately after recording, slices were fixed in 0.1 M PBS, pH 7.4, containing 4% paraformaldehyde and 0.1% picric acid for 24 h. Slices were then processed for biotinylated horseradish peroxidase conjugated to avidin (ABC-elite, Vectastain; Vector Laboratories).

**Alcohol self-administration.** Rats were trained to orally self-administer ethanol using a modification of the saccharin-fading procedure, as described previously (Roberts et al., 2000). After successful acquisition of operant responding, the animals were introduced to a free-choice task in which presses on one lever produced 0.1 ml of alcohol solution, whereas responses at the other lever resulted in delivery of an equal volume of water. Alcohol was alternated daily between the two levers. The criterion for stable baseline intake was ±20% across three consecutive 30 min sessions with a mean intake of >20 responses. Alcohol vapor chambers were used to induce dependence by intermittent exposure (14 h on/10 h off) to an air/ethanol mixture for a total of 4 weeks, which reliably produce alcohol dependence in rats (Gilpin et al., 2008). Intermittent alcohol exposure mimics human patterns of alcohol consumption (Slawecki et al., 1999; Sanna et al., 2002a; Repunte-Canonigo et al., 2007) and induces more rapid increases in self-administration than continuous exposure (O’Dell et al., 2004). Blood alcohol levels were measured with an oxygen-rate alcohol analyzer (Analox Instruments) and maintained at (mean ± SEM) 155 ± 15 mg%. 

**Intravenous self-administration.** Rats were prepared with chronic intravenous catheters as described previously (Ahmed et al., 2005; S. A. Chen et al., 2006). For cocaine self-administration, rats were allowed to self-administer cocaine on a fixed-ratio 1 (FR1) schedule (250 μg per injection in a volume of 0.1 ml delivered over 4 s). Each response on the lever resulted in a cocaine injection and was followed by a 20 s timeout period. Rats were allowed to self-administer cocaine either for 1 h short access (ShA) or 23 h (LgA) access on a fixed-ratio 1 schedule. Cocaine self-administration was prevented by intravenous administration of D-AP-5, an NMDA receptor antagonist, in a dose of 2 mg/kg per 0.1 ml of saline, 20 s before each self-administration response (ShA) (Slawecki et al., 1999; Sanna et al., 2002a; Repunte-Canonigo et al., 2007).

**Analysis of gene expression.** Target RNA was generated with the BioArray High Yield RNA Transcript Labeling kit (Enzo). Quality of total RNA was assessed using the NanoDrop ND-1000 spectrophotometer for quantification of small samples and the Agilent Bioanalyzer. The jcBNST
was dissected with minimal surrounding tissue from transilluminated vibratome-cut coronal rat brain slices (350 μm) kept cold but not frozen, as described by Cuello and Carson (1983). All BNST samples used for microarray and quantitative PCR (qPCR) were from animals in which the contralateral BNST was used for electrophysiological experiments. Similarly dissected jCBNST were used for Western blotting.

High density microarrays (Affymetrix RAE230A) were hybridized to target cRNA derived from double in vitro transcription as described previously (Sanna et al., 2005) with minor modifications and scanned according to the protocol of the manufacturer. Specifically, pools of three samples from alcohol-dependent animals and three matched control and three from cocaine lGal and three matched control were tested using RAE 230 Affymetrix arrays. Samples from each experimental group were pooled and run in duplicate. Signal intensities were scaled to a target intensity of 250 using the MAS 5.0 algorithm. Differentially expressed genes were obtained by the Affymetrix comparison analysis algorithm and t test analysis of GeneSpring 7.2-normalized expression values as described previously (Repunte-Canonigo et al., 2007).

Differentially expressed genes were validated by qPCR from individual animals with a history of dependence on the three drugs under study (alcohol, cocaine, and heroin) and matched controls. To this aim, we tested individual samples from a separate set of animals as previously done (Ahmed et al., 2005; Repunte-Canonigo et al., 2007). Primers were designed using the Beacon Designer Software (Premier Biosoft International). The iQ SYBR Green Supermix (Bio-Rad) was used in 25 μl reaction volume with an iQ5 Real-Time PCR Detection System (Bio-Rad) using 0.2 μm wells-thin-well PCR plates (Bio-Rad). Reaction steps were 95°C for 3 min, 40× (95°C for 10 s, 60°C for 20 s, 72°C for 20 s). For quantification, the relative standard curve method (Livak, 1997) was used as described previously (Ahmed et al., 2005; Repunte-Canonigo et al., 2007). β-Actin was used as a reference mRNA for normalization. Standard curves for both the target and reference genes were generated for each qPCR run with 1-log serial dilutions (1 to 10−5) of first-strand cDNAs from a pooled control sample by plotting cycle threshold (Ct) versus the log of input amount. qPCR standard curves had slopes in the range of −3.5 to −3.0. Linear correlation coefficients (R²) ranged from 0.98 to 0.99. Sample Ct values were interpolated using the following formula: (Ct−β/m), where β is the y-intercept of the standard curve line, and m is the slope of the standard curve line.

Western blotting was performed from individual animals as described previously (Sanna et al., 2000, 2002b) using a rabbit polyclonal specific for amino acids 417–499 of rat Kv1.2 (APC-010; Alomone Labs) at a dilution of 1:200; for control, β-actin was detected with a mouse monoclonal antibody (A1978; Sigma) at a dilution of 1:2000. Quantification of Western blot signal was done with the NIH Image software (http://rsb.info.nih.gov/nih-image).

Double in situ hybridization. Rats were perfused transcardially with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Brains were postfixed in 4% paraformaldehyde for 2 h at 4°C, rinsed with PB, and transferred sequentially to 12, 14, and 18% sucrose solutions in PB. Probes at 107 cpm/ml, together with the 600 ng mix of GAD65 and GAD67 sulfate, 5 μM digoxigenin-labeled antisense riboprobes. The antisense digoxigenin was subcutaneously at a dose of 20 mg/kg, three times in 20% hydroxypropyl β-cyclodextrin, pH 4.5, 1 h apart because of the extensive data available for this route of administration regarding doses sufficient to occupy brain CRF, receptors (Gutman et al., 2003) and to produce behavioral effects in models of ethanol or drug dependence (Sabino et al., 2006; Funk et al., 2007; Skelton et al., 2007a,b). The CRF2 antagonist (Rivier et al., 2002) was administered by intracerebroventricular injections (4 μg in 2 μl) as done previously (Cottone et al., 2007; Fekete et al., 2007), because as a peptide does not cross the blood–brain barrier, CRF was administered intracerebroventricularly at a dose of 1 μg as previously done (Izzo et al., 2005).

Data analysis. Student’s t test (paired or unpaired as appropriate) or one-way or two-way ANOVA was used to analyze the behavioral and electrophysiological data using StatView (SAS Institute) or Microsoft Excel. ANOVA was followed by Fisher’s LSD post hoc analysis. All results are expressed as mean ± SEM. Cutoff p values of <0.05 were considered to be statistically significant, and results are reported as either <0.05 or <0.01.

Results

LTP-IE in the jCBNST

In jCBNST brain slices from control rats, stimulation of the region of the stria terminals (Fig. 1A,B) that conveys glutamatergic inputs from the BLA (Dong et al., 2000) evoked a field potential characterized by two fast negative components followed by a more variable slow positive deflection (Sawada et al., 1980), as shown in Figure 1C. The second negative and slow positive components of the field potential were abolished by application of the AMPA receptor inhibitor CNQX either in the bath (20 μM) (Fig. 1C) or locally by diffusion of the inhibitor (25 mM) included in the recording electrode (Fig. 1D). Bath application of the glutamatergic NMDA receptor inhibitor D-2-amino-5-phosphonopentoic acid (D-AP-5) (50 μM) had no effect on the field potential (Fig. 1E). Blocking GABA A and GABA B receptors with bicuculline (30 μM) and SCH50911 [(2S)+(5,5-dimethyl-2-morpholineacetic acid) (20 μM), respectively, considerably increased the size of the second negative component of the field potential without abolishing the positive component or altering the field morphology (Fig. 1C). Together, these observations indicate that the second negative and positive components of the field potential are postsynaptic responses mediated by AMPA receptors and originate locally in the jCBNST, whereas the first negative component of the field potential represents the presynaptic volley, i.e., the spike activity of the afferent fibers.

Delivery to the stria terminalis of an HFS paradigm consisting of five trains at 100 Hz for 1 s at 10 s intervals resulted in protracted potentiation of the amplitude of the negative postsynaptic component of the field potential (184 ± 1.6% at 60 min after HFS; n = 9) (Fig. 1F,G). A transient bath application of either D-AP-5 (50 μM) or the mGluR5 inhibitor 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (10 μM) for 20 min around the time of delivery of HFS prevented potentiation of the field potential amplitude (110.9 ± 0.6%, n = 10 and 110.3 ± 1%, n = 5, 60 min after HFS for D-AP-5 and MPEP, respectively; not significantly different from baseline before delivery of HFS for
Protracted withdrawal from self-administration of alcohol, cocaine, and heroin impairs LTP-IE of jcBNST

To test the hypothesis that LTP-IE in the jcBNST would be altered in rats with a history of alcohol exposure sufficient to produce escalated self-administration, we first measured the amplitude of the field potentials, and we then performed intracellular measures of the threshold for action potential generation.

We used a validated animal model of escalated dependent alcohol intake induced by exposure to alcohol vapors (Roberts et al., 2000; O’Dell et al., 2004). Rats were trained to orally self-administer alcohol in a concurrent, two-lever, free-choice contingency using a modification of the sweet solution fading procedure. After acquisition of operant responding for ethanol, half of the rats were exposed to intermittent ethanol vapors for 4 weeks to induce dependence (dependent group), whereas the remaining rats were exposed to air in the same apparatus for the purpose of control (nondependent group). During protracted withdrawal (3–5 weeks after withdrawal), rats were again tested for operant responding for ethanol and used for electrophysiology experiments.~1–2 weeks after testing. The average number of presses for ethanol in the post-dependent group during protracted withdrawal was significantly increased compared with the nondependent group (83.25 ± 6.3 vs. 24.5 ± 7.4, p < 0.01) (Fig. 3A). As shown in Figure 3B, in dependent rats at 4–6 weeks after withdrawal, potentiation of the field potential, which as shown above reflects LTP-IE in the jcBNST, was significantly impaired at 60 min after HFS (104.9 ± 1.7%, n = 10, p < 0.05 from controls). Conversely, at 4–6 weeks after withdrawal, in nondependent control rats (161 ± 0.7%, n = 7), field potentials were not significantly different from controls at 60 min after HFS (183.6 ± 1.5%, n = 22). However, in dependent rats tested during acute withdrawal (3–6 h), potentiation of the field potential at 60 min after HFS (208.5 ± 1.9%, n = 7) was not significantly different from controls, whereas at 1–2 weeks after withdrawal, a significant impairment was observed in both dependent (127.4 ± 1.9%, n = 8, p < 0.05 from controls and dependents during acute withdrawal) and nondependent (129.7 ± 0.9%, n = 8) rats. Thus, alcohol dependence induces a protracted impairment of the capacity for plasticity in the jcBNST, whereas a transient impairment is seen after a history of nondependent alcohol intake.

To explore the generality of these findings, we investigated the induction of LTP-IE in rats with or without extended access to cocaine or heroin self-administration sufficient to produce dependence (Koob et al., 2004; Ahmed et al., 2005; R. Chen et al., 2006). Rats self-administering cocaine for 1 h per day (Sha) showed stable cocaine intake over time (Fig. 3C). Conversely, extended access to cocaine self-administration for 6 h per day (LgA) induced an increase in drug intake, previously correlated with a persistent decrease in brain reward function during withdrawal (Koob et al., 2004) (Fig. 3C). Similar to alcohol-postdependent rats, a strong impairment of potentiation of the field potential amplitude was observed in the jcBNST of LgA rats 1–2 weeks after cessation of cocaine self-administration (109.4 ± 3.3%, n = 8, 60 min after HSF), whereas Sha rats showed a level of potentiation (132.7 ± 5.6%, n = 10) that was intermediate between that of control and LgA rats (Fig. 3D). LTP-IE in LgA heroin self-administering rats 1–2 weeks after cessation of co-
caine self-administration was also significantly impaired (110.2 ± 5.9%, 60 min after HFS, n = 5) compared with controls (176.7 ± 9.8%, n = 10) and ShA rats (140 ± 5.2%, n = 10, p < 0.05 from both control and ShA rats) (Fig. 3 E, F). Again, ShA rats showed an intermediate level of potentiation impairment (p < 0.01 from controls). Thus, multiple drugs of abuse induced a common disruption of potentiation of the field potential in the jcBNST after delivery of HFS to the striatal terminals during protracted withdrawal.

The threshold for action potential generation in jcBNST neurons is regulated by the $I_f$ current

Consistent with the disruption of potentiation of the field potential in the jcBNST of rats with histories of dependence on alcohol or LgA intravenous self-administration of cocaine or heroin during protracted withdrawal, the HFS-induced shift toward hyperpolarization of the threshold for action potential generation that is required for the potentiation of the field potential in the jcBNST was not observed (Fig. 4A). The threshold for action potential in animals with a history of alcohol, cocaine, or heroin dependence before delivery of HFS (control, −50.9 ± 1.5 mV, n = 14; alcohol post-dependent, −50.7 ± 1.9 mV, n = 7; cocaine, −47.5 ± 1.7 mV, n = 9; heroin, −54.1 ± 1.9 mV, n = 7) did not differ from controls (two-tailed t test assuming equal variance: control vs alcohol post-dependent, t = 0.11, NS; control vs cocaine, t = 1.92, NS; control vs heroin, t = −1.25, NS), indicating that the observed lack of shift toward hyperpolarization in post-dependent animals reflects impaired capacity for LTP-IE rather than occlusion by a preexisting LTP-IE.

As shown in Figure 4B, using combined DNA microarray and qPCR, we observed increased expression of the potassium channel Kv1.2 in post-dependent rats at the time points indicated in Figure 3 (4–6 weeks for alcohol dependent; 1–2 weeks for cocaine and heroin LgA). Consistently, Kv1.2 protein was significantly increased in the jcBNST of rats with a history of alcohol dependence by Western blotting (Fig. 4C). Kv1.2 is a member of the Kv1 family of potassium channels implicated in mediating the slowly inactivating 4-aminopyridine- and α-DTX-sensitive D-type $K^+$ current (Storm et al., 2004). The α-DTX-sensitive D-type $K^+$ current is a slow inactivating transient current first identified by Storm (1988) in hippocampal CA1 pyramidal neurons by its ability to delay firing action potentials. The $I_h$ current is selectively blocked by α-DTX and by micromolar concentrations of 4-aminopyridine (Storm, 1988; Coetzee et al., 1999). The $I_h$ current is known to be regulated by metabotropic glutamate receptors (mGluRs) (Wu and Barish, 1999) and controls action potential threshold in various neuronal types (Storm, 1988; Strom, 1993; Coetzee et al., 1999; Bekkers and Delaney, 2001; Kröner et al., 2005; Guan et al., 2007). Consistent with these previous findings, bath application of α-DTX (1 μM) or 4-AP (40 μM) induced a long-lasting shift of the
threshold for action potential generation toward more hyperpolarized membrane potentials in jcBNST neurons (Fig. 4D, E). Delivery of HFS after application of 4-AP did not further shift the AP threshold (from $-59.1 \pm 2.4$ mV before HFS to $-60.9 \pm 2.8$ mV 40 min after HFS; $t = 0.21$, NS, $n = 9$) (Fig. 4E). A transient application (15 min) of 4-AP induced a protracted shift of the threshold for action potential generation toward hyperpolarization that was unchanged by previous application of inhibitors of synaptic transmission that were maintained for the duration of the recordings (Fig. 4E). This suggests that the persistent shift of the threshold for action potential generation induced by a transient application of 4-AP was attributable to inhibition of the postsynaptic $I_{D}$ current. A transient application (15 min) of the selective mGluR5 agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) (200 $\mu$m) also induced a protracted shift of the threshold for the action potential generation toward hyperpolarization (from $-51.9 \pm 2.6$ mV before 4-AP application to $-59.1 \pm 2.4$ mV 40 min after 4-AP application, $n = 9$, white bar labeled 4-AP) similarly to an LTP-IE-inducing HFS. Delivery of HFS in the presence of 4-AP (4-AP + HFS) did not further shift the action potential threshold ($t = 0.21$, NS, $n = 9$), suggesting that HFS acts at least in part by reducing $I_{D}$. A transient (15 min) application of 4-AP (40 $\mu$m) also induced a persistent shift of the threshold for action potential generation from baseline that was significant for over 40 min after washout (column marked 1 in the graph; $t = 3.379$, $n = 6$, $p = 0.010$, at 40 min after washout). In a separate set of cells, synaptic transmission was blocked by application of the AMPA inhibitor CNQX (10 $\mu$m), the NMDA inhibitor n-AP-5 (50 $\mu$m), the mGluR5 inhibitor MPEP (10 $\mu$m), the GABAA inhibitor bicuculline (30 $\mu$m), and the GABAB inhibitor SCH50911 (20 $\mu$m) 15 min before the transient (15 min) application of 4-AP (40 $\mu$m). Again, a persistent shift of the threshold for the action potential generation from baseline was observed that was significant for over 40 min after washout (column marked 2 in the graph; $t = 2.92, n = 6$, $p = 0.017$, at 40 min after washout). A persistent shift of the threshold for action potential generation was also induced by a transient (15 min) application of the specific mGluR5 agonist CHPG (200 $\mu$m) ($t = 6.292$, $n = 5$, $p = 0.002$, at 40 min after washout). F, Activation of mGluR5 by 200 $\mu$m CHPG reduced the whole-cell current by 13.2 $\pm$ 1.96% compared with control. Subsequent application of 1 $\mu$m $\alpha$-DTX to block the $I_{D}$ current significantly reduced the whole-cell current to 23.9 $\pm$ 3.73% compared with control ($t = 3.43$, $**p < 0.01$, $n = 8$). When $\alpha$-DTX was first applied, it reduced the whole-cell current by 22.7 $\pm$ 5.14%, and subsequent application of CHPG no longer had an effect on the whole-cell current (23.3 $\pm$ 5.18%, $t = 1.04$, NS, $n = 6$). Sample recordings are shown in G, G. Top panel shows the voltage protocol used in patch whole cell to obtain the records shown below and in I. Cells were held in voltage-clamp mode at $-70$ mV, and a 2000 ms pulse to $-100$ mV was applied so that a maximal amount of $I_{D}$ was available for activation during subsequent voltage steps. This was followed by a 100 ms pulse to $-40$ mV to inactivate the $A$-current. Voltage steps from $-500$ to $-40$ mV were applied for 500 ms, and these were used for analysis of $I_{D}$. Middle panel demonstrates $I_{D}$ by subtracting the recording made in the presence of $\alpha$-DTX (1 $\mu$m) from the control recording (Bekkers and Delaney, 2001). Bottom panel shows $I_{D}$ during application of CHPG (200 $\mu$m) in the same cell as the panel above. CHPG blocked 56.9 $\pm$ 4.8% ($n = 8$) of the $I_{D}$. H, CHPG had no effect on whole-cell current (left) and $I$-$V$ relationship (right) when applied after application of $\alpha$-DTX, indicating that CHPG did not affect currents other than the $I_{D}$. Blue traces and blue line (diamond) in the graph represent total $K^{+}$ whole-cell current in ACSF; red traces and red line (triangles) in the graph represent $K^{+}$ whole-cell current in the presence of $\alpha$-DTX; green traces and green line (squares) in the graph represent $K^{+}$ whole-cell current in the presence of $\alpha$-DTX and CHPG.
is attributable to the inhibition of a mGluR5-modulated postsynaptic I\textsubscript{D} current.

**Enhanced temporal fidelity of firing in jcbNST LTP-IE**

Using extracellular single-unit recordings in the jcbNST in response to stimulation of the stria terminals, we investigated the variability of the latency to the first spike or “jitter of the spike,” a measure of the temporal fidelity of firing (Pouille and Scanziani, 2001; Sourdet et al., 2003). In the jcbNST of control rats, the jitter of the spike showed a significant and protracted reduction after delivery of HFS of the stria terminals that lasted for up to 40 min and resulted in increased temporal fidelity of firing (Fig. 5A, B). In the jcbNST of alcohol-dependent rats, the HFS-induced reduction of jitter was only transient, returning to control levels by 30 min after HFS (Fig. 5C). Because extracellular recordings do not allow one to determine the cellular mechanism behind the reduced jitter of the spike that characterizes LTP-IE in the jcbNST, we performed concomitant measures of the prepotential slope and jitter in the same jcbNST neurons of normal rats by intracellular recordings. As shown in Figure 5D–F, we observed that HFS induced a significant increase in the slope of the depolarizing prepotential from 0.38 ± 0.04 to 0.98 ± 0.3 mV/ms 40 min after HFS (p < 0.01, n = 9) (Fig. 5E), which was associated with a significant decrease of the jitter of the spike (2.64 ± 0.6 ms before vs 1.56 ± 0.5 ms 40 min after HFS; p < 0.05, n = 9) (Fig. 5F). Changes in the latency to the first spike as well as in the depolarizing prepotentials may reflect either increases of inward currents, such as Na\textsuperscript{+} and/or Ca\textsuperscript{2\textsuperscript{+}}-mediated currents, or decreases of outward currents, like the K\textsuperscript{+}-mediated I\textsubscript{K} or I\textsubscript{D}(Nisenbaum et al., 1994; Griffin et al., 1996). Therefore, we tested whether bath application of the I\textsubscript{D} blocker 4-AP at the concentration of 40 \(\mu M\), which does not block the I\textsubscript{D}, could mimic both the increase of the slope of the prepotential and the decrease of the jitter. As shown in Figure 5, E and F, the slope of the prepotential of jcbNST neurons was significantly increased from 0.45 ± 0.048 to 1.05 ± 0.15 mV/ms (p < 0.01, n = 9) (Fig. 5E), and the jitter was decreased from 2.32 ± 0.3 to 0.78 ± 0.43 ms, 40 min after 4-AP (40 \(\mu M\); p < 0.01; n = 9) (Fig. 5F). Conversely, in rats with a history of alcohol, cocaine, or heroin dependence, the decrease of the jitter measured by intracellular recordings was not significantly changed at 40 min after HFS (Fig. 5G).

Thus, LTP-IE in the jcbNST is characterized by increased intrinsic excitability attributable to a decrease in the firing threshold and by increased temporal fidelity of firing, both of which depend on the K\textsuperscript{+}-mediated I\textsubscript{K} and that were not seen in animals with a history of alcohol, cocaine, or heroin dependence.

**The impairment of jcbNST LTP-IE in protracted abstinence is associated with activation of the CRF system**

The increased anxiety-like behavior and drinking observed during acute withdrawal and protracted abstinence have been attributed to increased CRF activity (Rasmussen et al., 2001; Valdez et al., 2002), including within the BNST (Olive et al., 2002). Drug-seeking behavior previously maintained by various drugs of abuse, including alcohol, cocaine, and heroin, can be reinstated by either exposure to stress or by central administration of CRF in...
Next, we investigated the potential role of CRF in the impairment of jcBNST LTP-IE during protracted withdrawal in animals with a history of alcohol dependence. Repeated subcutaneous administration of the selective CRF1 receptor antagonist R121919 (formerly referred to as NBI 30775) (Chen et al., 1996) restored normal jcBNST LTP-IE in alcohol post-dependent rats (190.7 ± 1.4%, n = 8, 60 min after HFS), whereas vehicle (aCSF-containing 10% DMSO) had no effect (111.6 ± 1.7%, n = 4) (Fig. 7A). Three intracerebroventricular injections (4 µg in 2 µl) of the selective CRF2 receptor antagonist astressin-2-B (A2-B) (Rivier et al., 2002) every 12 h was also ineffective (Fig. 7A). The dose of A2-B was based on previous work (Cottone et al., 2007; Fekete et al., 2007). Repeated intracerebroventricular administration of CRF, but not acute administration of CRF, induced a significant impairment of jcBNST LTP in drug-naive rats that resembled the post-dependent phenotype (Fig. 7B). Together, these results suggest that the CRF system may contribute to dependence-associated changes in the integration properties of jcBNST neurons that can be normalized by a CRF1 receptor antagonist and mimicked by repeated central CRF administration.

Discussion

In the present study, we have observed that a history of drug exposure sufficient to produce escalated self-administration produced impairment in a novel form of activity-dependent LTP-IE in jcBNST neurons. This form of plasticity was characterized by a decrease in the firing threshold and an increase in the temporal fidelity of firing of jcBNST neurons that were both long lasting. LTP-IE in the jcBNST was differentially altered in animal models of controlled intake of an abusable drug versus animal models of dependent use at time points during protracted withdrawal that are characterized by behavioral manifestations of a negative emotional state (Koob et al., 2004), such as increased anxiety-like behavior (Zorrilla et al., 2001; Valdez et al., 2002; Zhao et al., 2007; Sommer et al., 2008) and intracranial self-stimulation reward thresholds (Ahmed et al., 2002). Additionally, rats with a history of heroin self-administration also show increased stress-induced reinstatement of heroin-seeking behavior at the time point investigated here (Shalev et al., 2001). Although mounting evidence suggests that changes in the intrinsic membrane properties of neurons may result from exposure to drugs of abuse (Cooper et al., 2003; Dong et al., 2005; Nasif et al., 2005), changes in the capacity for plasticity of intrinsic excitability have not been shown previously as consequences of drug exposure.

Consistent with classic studies showing that potentiation of a CRF antagonist-reversible manner (Shaham et al., 2003). Therefore, we performed double in situ hybridization for GABA synthetic enzymes GAD65 and GAD67 and for CRF in the dorsal BNST. A large number of GAD-containing neurons (shown in purple) are present in the jcBNST and in the dorsolateral BNST in general. In situ hybridization signal for CRF (brown grains) was seen in the dorsolateral BNST, including the jcBNST, and were primarily colocalized with GAD65 and GAD67. LV, Lateral ventricle. B, Higher magnification of the area in the dotted box in A shows high level of colocalization of CRF with GAD65 and GAD67 in the midsection of the jcBNST. C, Higher magnification of the two neurons containing CRF and GAD65 and GAD67 signal marked by the arrows in B.
the population spike can occur independently of EPSP potentiation (Bliss and Lomo, 1973; Miles et al., 2005), the protracted decrease in the firing threshold and increase in the temporal fidelity of firing of jcBNST neurons observed in LTP-IE were accompanied only by a transient EPSP potentiation. In a previous study, an LTP of the field potential in the mouse dorsolateral BNST was interpreted as a form of synaptic potentiation (Weitlauf et al., 2004). The present data show that the LTP of the field potential in the jcBNST is an a population spike manifesting the summation of action potentials of jcBNST neurons and cast a new interpretation on the previous work.

The decrease in the firing threshold of jcBNST neurons observed in LTP-IE was found to be regulated by the $I_{D}$ current. The repertoire, expression, and regulation of K+ channels are believed to be key factors in neuronal integration (Marder and Goaillard, 2006). Previous studies indicate that changes in K+ channel expression levels can alter intrinsic neuronal excitability (Dong et al., 2006) and capacity for plasticity of intrinsic excitability (Wang et al., 2006). Loss of LTP-IE of jcBNST neurons during protracted abstinence from alcohol, cocaine, and heroin self-administration was associated with increased expression of the Kv1.2 K+ channel, a main contributor to the $I_{D}$ current (Shen et al., 2004). Because increased expression of Kv1.2 was correlated with changes in excitability in other neuronal systems (Tsaur et al., 1992), this observation provides a potential mechanism contributing to Kv1.2 regulation. Additionally, a key mechanism of regulation of Kv1.2 appears to be phosphorylation-dependent trafficking of a fraction of the channel (Nesti et al., 2004). If this mechanism mediates the activity-dependent inactivation of Kv1.2 induced by HFS in jcBNST neurons, an increased pool of Kv1.2 consequent to its increased expression may be sufficient to prevent its downregulation in LTP-IE. Activity- and NMDA-dependent inactivation of another Kv1 channel, Kv1.1, has also been demonstrated in hippocampal CA1 neurons (Raab-Graham et al., 2006).

LTP-IE in the jcBNST was also found to be characterized by increased temporal fidelity of firing of jcBNST neurons, which also depended on the $I_{D}$ current and was impaired in animals with a history of alcohol, cocaine, or heroin dependence. At the circuit level, computational theory predicts that increased temporal fidelity of neuronal firing translates into more efficient use of the capacity of neural connections (Singer, 1999). At the cellular level, the ability to modify the extent and fidelity in which synaptic potentials are translated into activity outputs is believed to be a central feature of the integration properties of neurons (Singer, 1999). Forms of plasticity of intrinsic excitability have been proposed to be adaptive mechanisms directed toward maintaining the homeostasis of network excitability in the CNS in response to increases in synaptic inputs (Turrigiano, 1999). The glutamatergic projection to the jcBNST originates in the posterior BLA (Dong et al., 2000). In turn, the jcBNST projects back to the anterior BLA and to the CeA. Its lack of cells expressing glutamatergic markers (Hur and Zaborszky, 2005) and abundance of GABAergic cell bodies (Fig. 6) (Sun and Cassell, 1993; Veinante et al., 1997) suggest that the jcBNST is likely to play an inhibitory role on the amygdala. Therefore, the ability of jcBNST neurons to increase their ensemble activity through increased firing rate and temporal fidelity of their firing could provide a more efficient inhibitory influence on the CeA output at times of greater BLA activation. The BLA is activated in humans during drug craving elicited by exposure to drug-related environmental cues (Bonson et al., 2002) and is involved in contextual reinstatement of drug-seeking behavior in experimental animals (Hayes et al., 2003). Thus, reduced capacity for LTP-IE and temporal fidelity of firing of jcBNST neurons could be a compensatory response to excessive activation of the BLA in dependent animals and could result in inadequate feedback inhibition of the CeA. Unrestrained CeA activation would be expected to result in increased emotional arousal through its various projection areas, including the paraventricular nucleus of the hypothalamus and the lateral hypothalamus that are believed to be key in the expression of "emotional memories" by interfacing central stress and autonomic systems (LeDoux, 1993). The lateral hypothalamus has been found to undergo structural changes during cocaine escalation (Ahmed et al., 2005). Loss of jcBNST LTP-IE also will directly affect target regions of the jcBNST itself and, indirectly, of other BNST nuclei that in part overlap with those of the CeA (Dong et al., 2000).

Symptoms of a negative emotional state that emerge during drug withdrawal are believed to be attributable to adaptive changes in the brain reward and stress neurocircuitry that develop during excessive drug intake and persist in a latent state after acute withdrawal (Koob et al., 1998). The alteration of jcBNST plasticity described here was not seen during acute withdrawal in alcohol-dependent rats but became manifest only during protracted withdrawal. Similarly, enhanced LTP in the BLA–CeA pathway was seen after 14 d but not 1 d withdrawal from a 7 d cocaine treatment (Fu et al., 2007). Together, these data suggest that, in addition to acute drug opponent-process adaptations (Koob et al., 1998), drug use also may produce delayed functional changes that are specific to protracted withdrawal. The latter may be especially significant for the neurobiology of vulnerability to relapse to drug-taking after the acute withdrawal syndrome has ceased. Spontaneous anxiety-like behavior in rats with a history of alcohol dependence has been found recently to increase during acute withdrawal, then transiently normalize, but then to resurface during protracted withdrawal and has been linked to changes in the extrahypothalamic CRF system (Zhao et al., 2007). Similarly, such anxiety-like states can be retriggered by mild stressors that do not affect animals without a history of dependence, and these stress-like responses are linked to the extrahypothalamic CRF system (Valdez et al., 2002). It is particularly noteworthy that, in protracted abstinence, the hypothalamic–pituitary–adrenal axis shows a blunted response, but the extrahypothalamic CRF system remains activated and may even sensitize (Koob and Kreek, 2007). Together, these observations support the hypothesis that distinct neurobiological mechanisms are recruited during early and protracted withdrawal and suggest that the alteration of the LTP-IE described may be specific to protracted withdrawal. In the rat, exposure to stressors reinstates drug-seeking behavior previously maintained by various drugs of abuse, including alcohol, cocaine, and heroin, and administration of CRF antagonists prevents reinstatement of drug-seeking behavior (Shaham et al., 2003). Extracellular CRF is increased during ethanol withdrawal in the BNST (Olive et al., 2002), which has been implicated in CRF-mediated anxiety-like behavior (Sahusque et al., 2006) and stress-induced reinstatement of drug-seeking behavior depends on extrahypothalamic CRF in the BNST (Erb and Stewart, 1999; Leri et al., 2002; McFarland et al., 2004). Together with our results, these findings support the hypothesis that extrahypothalamic CRF signaling may regulate neuronal circuits associated with protracted abstinence and stress-induced reinstatement.

In conclusion, our results show that the integration properties of jcBNST neurons are regulated by a novel form of neural plasticity characterized by changes in the intrinsic excitability and temporal fidelity of jcBNST neurons, which was altered in a CRF-
dependent manner during protracted withdrawal. These results suggest that CRF-mediated impairment of jcbNST neuronal plasticity are associated with the persistent emotional dysregulation after chronic drug exposure and provide a novel neurobiological target for the mechanisms by which stress and protracted abstinence symptoms perpetuate alcohol and drug dependence.

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