Cocaine and Mefloquine-induced Acute Effects in Ventral Tegmental Area Dopamine and GABA Neurons

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Cocaine and Mefloquine-induced Acute Effects in Ventral Tegmental Area Dopamine and GABA Neurons

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A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Doctor of Philosophy Neuroscience

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ABSTRACT

COCAINE AND MEFLOQUINE-INDUCED ACUTE EFFECTS IN VENTRAL TEGMENTAL AREA DOPAMINE AND GABA NEURONS

David W. Allison

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Doctor of Philosophy

The aim of the two studies presented here was to evaluate the effects of cocaine and mefloquine (MFQ) on γ-aminobutyric acid (GABA) and dopamine (DA) neurons in the ventral tegmental area (VTA). **Cocaine:** In vivo, lower doses of intravenous cocaine (0.25-0.5 mg/kg), or methamphetamine (METH), enhanced VTA GABA neuron firing rate via D2/D5 receptor activation. Higher cocaine doses (1.0-2.0 mg/kg) inhibited their firing rate. Cocaine and lidocaine inhibited the firing rate and spike discharges induced by stimulation of the internal capsule (ICPSDs) at dose levels 0.25-2 mg/kg (IC50 1.2 mg/kg), but neither DA nor METH reduced ICPSDs. In VTA GABA neurons in vitro, cocaine reduced (IC50 13 µM) current-evoked spikes and sodium currents in a use-dependent manner. In VTA DA neurons, cocaine reduced IPSCs (IC50 13 µM), increased IPSC paired-pulse facilitation, and decreased sIPSC frequency, without affecting mIPSC frequency or amplitude. These findings suggest cocaine reduces activity-dependent GABA release on DA neurons in the VTA, and that cocaine’s use-dependent blockade of VTA GABA neuron voltage-sensitive sodium channels (VSSCs) may synergize with its DAT inhibiting properties to enhance mesolimbic DA transmission implicated in cocaine reinforcement. **Mefloquine:** Mefloquine (MFQ) is an anti-malarial agent, Connexin-36 (Cx36) gap junction blocker, 5-HT3 antagonist, and calcium ionophore. Mounting evidence of a Cx36-mediated VTA GABA neuron syncytium suggests MFQ-related dysphoria may attribute to its gap junction blocking effects on VTA synaptic homeostasis. We observed that MFQ (25 µM) increased DA neuron spontaneous IPSC frequency 6 fold, and mIPSC 3 fold. Carbenoxolone (CBX, 100 µM) only increased sIPSC frequency 2 fold, and did not affect DA mIPSC frequency. Ondansetron did not mimic MFQ. Additionally, MFQ did not affect VTA DA evoked IPSC paired pulse ratio (PPR). However, Mefloquine did induce a 3.5 fold increase in bath-applied GABA current. Remarkably, MFQ did not affect VTA GABA neuron inhibition. At VTA DA neuron excitatory synapses MFQ increased sEPSC frequency in-part due to an increase in the AMPA/NMDA ratio. These finding suggest MFQ alters VTA synapses differentially depending on neuron and synapse type, and that these alterations appear to involve MFQ’s gap junction blocking and calcium ionophore actions.

Key words: GABA, VTA, dopamine, cocaine, mefloquine, 5HT3, mesolimbic, Connexin 36, gap junctions, electrical synapse, D2/D5.
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I begin as my life did, with my parents, Judge W. and Frieda H. Allison. They gave me the gift of life, love, and the desire to live up to my potential. What has appeared to be a good set of genes hasn’t hurt either.

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CHAPTER 1

INTRODUCTION

The rationale for the cocaine and mefloquine research presented here is predicated on the belief that advancement in the understanding of the brain mechanisms underlying the recreational use and abuse potential of cocaine and other drugs of abuse will pave the way for more effective treatment strategies that would save lives and resources throughout the world.

The mesolimbic dopamine system

The mesolimbic dopamine (DA) system (Figure 1) consists of projections from the ventral tegmental area (VTA) to structures associated with the limbic system, primarily the nucleus accumbens (NAcc). The NAcc (part of the ventral striatum) located in the ventral forebrain. The can be divided anatomically and by the input it receives into the nucleus accumbens core and the nucleus accumbens shell. This system has been implicated in the rewarding effects of drugs of abuse (J. R. Blackburn et al., 1986; R. A. Wise and M. A. Bozarth, 1987; G. F. Koob, 1992; R. A. Wise, 1996), (D. L. McKinzie et al., 1999; R. C. Pierce and V. Kumaresan, 2006). The VTA is a relatively amorphous midbrain structure that contains at least three neuron types: the primary type or DA neurons that project to the NAcc, the secondary type or γ-aminobutyric acid (GABA) neurons that may participate in local circuitry (acting to inhibit DA neurons) or project to other brain regions, and a population of glutamatergic neurons (T. Yamaguchi et al., 2007). The medial VTA running rostral to caudal seems hold the greatest concentration of DA neurons. These DA neurons project to shell region of the nucleus accumbens The shell portion of the nucleus accumbens appears to be more linked to drug reward than the core (S. Ikemoto, 2007). Many drugs of abuse act in both the VTA and the
NAcc. Most rats and mice will self-administer (SA) cocaine (David et al., 2004, Rodd et al.,
2005), ethanol (E) (Gatto et al., 1994, Rodd-Henricks et al., 2000), nicotine (Museo and Wise,
1981, Laviolette and van der Kooy, 2003), cannabinoids (Zangen et al., 2006), and opiates (M.
A. Bozarth and R. A. Wise, 1981; H. Welzl et al., 1989; V. David and P. Cazala, 1994; D. P.
Devine and R. A. Wise, 1994) into the VTA. Taken together, these data suggest that DA
neurons in the VTA that project to the shell of the NAcc, and the GABA neurons that may
inhibit these DA neurons locally in the VTA, play an important role in mediating addiction to
various drugs of abuse.

**GABAergic transmission: A non-dopamine-dependent pathway of addiction**

Due to the evidence pointing to DA’s involvement in most drugs of abuse, a “Dopamine
Hypothesis” for reward and addiction has developed on the tenet that DA might be crucial for all
drug reward. The emerging view, however, is that DA is crucial for the rewarding effects of the
psychomotor stimulants such as cocaine and methamphetamine, and is important, but perhaps
not crucial, for the rewarding effects of benzodiazepines, opiates, nicotine, cannabis and ethanol.
The notion that DA-dependent mechanisms are the final common pathway in the processes
mediating drug or natural reward is perhaps too restrictive. It has been theorized that a non-DA-
dependent pathway may function in tandem or independently of this DA-dependent pathway.
We have previously identified a homogeneous population of GABA neurons in the VTA that are
linked by Connexin 36 (Cx36) gap junctions (GJs) (S. H. Stobbs et al., 2004; D. W. Allison et
al., 2006), and form part of a larger syncytium of GABA neurons in the reticular formation (M.
B. Lassen et al., 2007). We theorize that given their anatomical location, and previously
demonstrated role in cocaine (J. H. Ye et al., 1999; W. X. Shi et al., 2004), ethanol (M. Melis et
al., 2002; S. H. Stobbs et al., 2004; J. W. Theile et al., 2009) and opiate reward (H. Vargas-Perez
et al., 2009) that VTA GABA neurons are a possible candidate for a non-DA-dependent addiction pathway.

**Plasticity and the process of addiction**

An important conceptual advance in understanding addiction is that the process of addiction shares striking similarities with natural reward plasticity or reward learning and memory. The newly emerging thought is that the basic mechanisms of reward and learning are hijacked by drugs of abuse. The two neurotransmitter systems hijacked by drugs of abuse are the DA system (previously summarized) and the glutamate (GLU) transmitter system, including their intracellular and genomic targets (A. E. Kelley, 2004). Briefly put, maladaptive changes such as drug-induced long-term potentiation (LTP) at GLU synapses on VTA DA neurons translate into the long-term cellular and molecular alterations that form the physiological basis, or substrates of addiction and addiction-related maladaptive behaviors (G. F. Koob and M. Le Moal, 1997; J. D. Berke and S. E. Hyman, 2000; S. E. Hyman and R. C. Malenka, 2001; A. E. Kelley and K. C. Berridge, 2002; S. E. Hyman et al., 2006). In addition to plasticity being exhibited in the GLUergic and DAergic transmitter systems, plasticity at GABAergic synapses has now been demonstrated in many brain areas including the VTA. Evidence suggests the forms of plasticity in the GABAergic transmitter system are similar to those forms expressed in the DA and GLU transmitter systems (for review F. S. Nugent and J. A. Kauer, 2008). The adaptability of the GABAergic transmitter system, with its unique characteristic of being partly regulated through gap junctions, could make it an especially vulnerable target for drugs of abuse. Dividing these systems into completely separate and distinct pathways however, is probably an oversimplification. Together they form an integrated system. The interconnectedness of these transmitter systems underscores the vulnerability of the brain as a
whole to pharmacological insult. So while we refer to separate DA-dependent and non-DA-dependent pathways for the sake of simplicity, they are interconnected, and share points of convergence and divergence. It is the unique characteristic of its individual parts, and the interconnected nature of the reward system that makes it such an important focus of addiction researchers.

Cocaine may act via a dopamine-dependent and non-dopamine-dependent pathway

Cocaine, known as a dopamine transporter (DAT) blocker and local anesthetic, is widely believed to exert its addictive influence via the DA-dependent pathway, by blocking DAT in the NAcc leading to an increase in synaptic DA. Little attention has been paid to the question of whether cocaine’s anesthetic properties contribute to its addictive liability as well. The few studies that have examined this question have been unable to conclusively demonstrate that lidocaine, an anesthetic similar to cocaine but without its DAT blocking properties, has any addictive liability. For this reason, many researchers have ignored the anesthetic properties of cocaine to the point of not controlling for this property in addiction-related studies. In the following study we examined the possibility that cocaine’s anesthetic properties may bridge the gap between the DA-dependent and non-DA-dependent pathways of addiction. This may occur through cocaine decreasing VTA GABA neuron inhibition of VTA DA neurons via its anesthetic actions which may lead to an effectual synergizing of the two properties to increase cocaine’s addictive liability.

Mefloquine as a pharmacological tool

Mefloquine (MFQ) is a selective Cx36 GJ blocker commonly used to study electrical synapses. MFQ is also used as an anti-malaria agent and reportedly has many adverse side effects in humans ranging in severity from mild dysphoria to severe psychotic episodes or
seizure. In addition to data demonstrating the MFQ blockade of gap junctions, recent studies have also observed non-specific MFQ effects not usually associated with gap junction blockade. For us, the most interesting of these effects are the increase in inhibitory and excitatory synaptic activity (S. J. Cruikshank et al., 2004; C. Zhou et al., 2006), which may stem in part from MFQ’s ability to disrupt intracellular calcium (G. S. Dow et al., 2003; D. Caridha et al., 2008). In spite of its many non-specific effects that raise questions regarding its suitability as an effective pharmacological tool to study gap junctions, we reasoned that some of the heretofore labeled “non-specific” effects of MFQ might actually be the result of blockade of electrical synapses. The study of these “non-specific” effects in the VTA may shed light on the physiological relevance of an electrically coupled network of VTA GABA neurons. Indeed, the research presented here appears to show that MFQ is the pharmacological discrimination tool needed to demonstrate the possible mechanism whereby Cx36 gap junctions facilitate VTA GABA neuron inhibition of mesolimbic DA transmission.

**Objectives**

The overall objectives of the two studies detailed in this dissertation were: 1) Evaluate the role of a specific class of VTA GABA neurons in mediating the rewarding properties of cocaine; 2) Determine the role Cx36 gap junction connected VTA GABA neurons play in regulating VTA DA neuron activity, and how MFQ affects VTA synaptic activity.
This diagram depicts the mesolimbic dopamine pathway. The VTA DA neuron and receptors are beige. The VTA GABA neurons, GABA(A) receptors, and inhibitory input are red. Excitatory input and glutamate receptors are green. The DA neuron projection target neuron located in the nucleus accumbens is blue. Connexin 36 gap junctions between VTA GABA neurons are the small purple lines. The dopamine transporters located presynaptically on DA neurons are black. The two studies described in this dissertation involve the circuitry in this diagram.
CHAPTER 2

Cocaine Disinhibits Dopamine Neurons in the Ventral Tegmental Area via Use-Dependent Blockade of GABA Neuron Voltage-Sensitive Sodium Channels

(The work presented in this chapter has been published under the same title in the European Journal of Neuroscience 2008 Nov; 28(10):2028-40. The research diagramed in Figures 1 and 2 of this chapter are not the work of the author, but have been included to maintain continuity and context.)

INTRODUCTION

The mesocorticolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc) has been implicated in motivated behaviors, various types of reward, and in the habit-forming actions of addictive drugs including cocaine (for review see (R. A. Wise, 2004)). The prevailing view is that cocaine’s locomotor and reinforcing properties (D. C. S. Roberts et al., 1980; G. F. Koob et al., 1994) are mediated primarily by enhancement of extracellular DA release (Y. L. Hurd et al., 1989; H. O. Pettit and J. B. Justice, Jr., 1989, 1991; R. A. Wise et al., 1995; S. E. Hemby et al., 1997) via inhibition of the DA transporter (DAT; (M. J. Kuhar et al., 1991; M. J. Kuhar, 1992; W. L. Woolverton and K. M. Johnson, 1992)). Cocaine-induced cellular and molecular reshaping of this system may contribute to learned reinforcement (for review see (S. Jones and A. Bonci, 2005)). The potency of psychostimulants as positive reinforcers being correlated to their DAT binding affinity (M. C. Ritz et al., 1987; J. Bergman et al., 1989; K. M. Wilcox et al., 1999), and cocaine’s high affinity for the DAT (IC$_{50}$ = 0.3-0.8 µM; (R. B. Rothman et al., 2001)), support this view.
Other high-affinity targets for cocaine include voltage-sensitive sodium channels (VSSCs; (F. H. Gawin and E. H. Ellinwood, Jr., 1988)). It is well established that local anesthetics, including cocaine, are use-dependent blockers of VSSCs (G. Strichartz, 1976; B. P. Bean et al., 1983; S. W. Postma and W. A. Catterall, 1984; M. E. O'Leary and M. Chahine, 2002). Although cocaine’s affinity for VSSCs is lower than that for monoamine transporters (IC$_{50}$=14-17 µM (M. C. Ritz et al., 1987; A. N. Gifford and K. M. Johnson, 1992)), peak brain (2-6 min) cocaine levels of 2, 6, 9, and 26 µM can be obtained from single intravenous reinforcing doses of 0.1, 0.25, 0.5, and 1 mg/kg, respectively (J. S. Fowler et al., 1998). Indeed, much higher levels of cocaine would be obtained by self-administration, given that response intervals at these doses are typically shorter than the elimination kinetics of cocaine (H. O. Pettit et al., 1990; H. T. Pan et al., 1991). The studies demonstrating acute and chronic cocaine-induced synaptic plasticity in rodent VTA DA neurons utilize a 15 mg/kg intraperitoneal dose (M. A. Ungless et al., 2001; Q. S. Liu et al., 2005), corresponding to peak brain concentrations of at least 15 and 25 µM, respectively (H. T. Pan et al., 1991). Recently, it has been hypothesized that the reinforcing properties of cocaine might involve combined or opposing effects at both the DAT and VSSCs (E. A. Kiyatkin and P. Leon Brown, 2006).

Since repeated high-dose cocaine exposure induces LTP in VTA DA neurons via a reduction of GABA-mediated inhibition, a possible role for VTA GABA neurons in cocaine induced plasticity has emerged (Q. S. Liu et al., 2005). We have identified a homogeneous population of GABA neurons in the VTA which may serve to inhibit DA neurons (S. C. Steffensen et al., 1998), and whose firing rate and afferent-evoked responses are enhanced by DA (S. H. Stobbs et al., 2004; M. B. Lassen et al., 2007). We hypothesized that cocaine would enhance GABA neuron firing rate and evoked discharges at low reinforcing doses due to its
DAT inhibiting properties, but at higher reinforcing doses its use-dependent VSSC blocking effect would inhibit VTA GABA neurons leading to DA neuron disinhibition.

**METHODS**

**Animal Subjects**

Rats were housed two to a cage from the time of weaning (P25), with *ad libitum* access to food and water. The room temperature was controlled (22-25 °C) and maintained on a reverse 12 hr light/dark cycle (OFF 08:00 hrs, ON 20:00 hrs). Animal care, maintenance and experimental procedures were in accordance with the Brigham Young University Animal Research Committee and met or exceeded National Institutes of Health guidelines for the care and use of laboratory animals.

**Single-unit Recordings in Anesthetized Rats**

Extracellular potentials in Isoflurane (1%) anesthetized adult 250-400 g male Wistar rats (Charles River Laboratory, Hollister, CA) were recorded by a single 3.0 M NaCl filled micropipette (1-3 MΩ; 1-2 µm inside diameter), cemented 10-20 µm distal to a 4-barrel micropipette (20-60 MΩ resistance), and amplified and filtered with a MultiClamp 700A programmable amplifier (Axon Instruments, Union City, CA). Microelectrode assemblies were oriented into the VTA [from bregma: 5.6-6.5 posterior (P), 0.5-1.0 lateral (L), 7.0-8.5 ventral (V)] with a piezoelectric inchworm microdrive (Burleigh, Fishers, NY). Single-unit activity was filtered at 0.3-10 kHz (-3dB) and displayed on Tektronix 2200 digital oscilloscopes. Square-wave constant current pulses (50-1000 µA; 0.15 msec duration; average frequency, 0.1Hz) were generated by an IsoFlex constant current isolation unit controlled by a MASTER-8 Pulse Generator (AMPI, Israel), or by computer. The internal capsule (IC; from bregma: -1.5 AP, 2.5-
3.0 ML, 5.0-6.5 V) was stimulated with insulated, bipolar stainless steel electrodes. Extracellularly recorded action potentials (min 5:1 signal-to-noise ratio) were discriminated with WPI-121 (Sarasota, Fl) spike analyzers and converted to computer-level pulses.

**Characterization of VTA GABA Neurons in vivo**

All neurons classified as VTA GABA neurons *in vivo* were located in the VTA, met the criteria established in previous studies for spike waveform characteristics and response to IC stimulation (S. C. Steffensen et al., 1998; S. H. Stobbs et al., 2004; D. W. Allison et al., 2006), and often were activated and spike-coupled by microelectrophoretic dopamine (DA; (S. H. Stobbs et al., 2004)). Presumed VTA GABA neurons were characterized by short-duration (<200 µsec; measured at half-peak amplitude of the spike), initially negative-going, non-bursting spikes, and were identified by the following IC stimulation criteria (S. C. Steffensen et al., 1998): Short latency (i.e., 2-5 msec) antidromic or orthodromic activation via single stimulation of the IC; and multiple spiking following high-frequency (10 pulses, 200 Hz) stimulation of the IC (ICPSDs; (S. C. Steffensen et al., 1998; S. H. Stobbs et al., 2004; D. W. Allison et al., 2006; M. B. Lassen et al., 2007)). In all studies, stimulation was performed at a level that produced 50% maximum VTA GABA neuron ICPSDs. This was accomplished by determining the current needed to produce the maximum number of ICPSDs at 200 Hz and 10 pulses, and then adjusting the stimulus intensity until 50% ICPSDs were achieved.

**Single-unit Recordings in vivo**

Single-unit potentials, discriminated spikes, and stimulation events *in vivo* were captured by National Instrument’s NB-MIO-16 digital I/O and counter/timer data acquisition boards (Austin, TX) and processed by customized National Instruments LabVIEW software in Macintosh-type computers. Potentials were digitized at 20 kHz and 12-bit voltage resolution.
For single-unit activity, all spikes were captured by computer and time stamped. Spontaneous firing rates were determined on- and off-line by calculating the number of events over a 5 min epoch, typically 5 min before and at specific intervals after drug injection. Peri-stimulus and interval-spike histograms were generated off-line using IGOR Pro (WaveMetrics, Lake Oswego, OR) analysis of the time-stamped data. The duration (msec) and extent (#events/bin) of post-stimulus permutation of ICPSDs was determined by rectangular integration at specific time points on the peri-stimulus spike histogram using IGOR Pro analysis software. The minimum bin width for peri-stimulus spike histograms was 1.0 msec and the number of bins was 1000. These parameters allow for detection of all phases of pre- and post-stimulus spike activity.

**Drug Preparation and Administration in vivo**

Cocaine hydrochloride, cocaine methiodide, DA, lidocaine hydrochloride, and methamphetamine hydrochloride were dissolved in 0.9% saline and administered intravenously through an indwelling jugular catheter. Given the transient duration of effect of cocaine and lidocaine on VTA GABA neuron firing rate and ICPSDs, dose-response studies were performed for these two drugs, as well as for cocaine methiodide, in the same rats with a 40 min interval between each dose and by randomizing the sequence of dose levels, while dose-response studies for amphetamine, whose effects on firing rate were more prolonged, were performed in separate rats. For in situ microelectrophoretic application of drugs in the VTA, DA (10 mM), eticlopride hydrochloride (1.0 mM), SCH23390 hydrochloride (1.0 mM), SKF38393 hydrochloride (1.0 mM), and quinpirole hydrochloride (1 mM) were dissolved in distilled water and iontophoresed by current injection (25-100 nA) which was regulated by Medical Systems BH-2 iontophoretic pump and balance unit modules. For systemic drug studies on VTA GABA neuron responses, all drugs including cocaine were administered intravenously through a jugular catheter. For
pharmacology studies on cocaine effects on VTA GABA neuron responses, drugs or saline were administered 10 min before cocaine. All drugs except cocaine were obtained from Sigma Chemical (St. Louis, MO). Cocaine was a gift from NIDA.

**Preparation of Brain Slices**

Wistar rats (P21 – 45) were anesthetized with Ketamine (60 mg/kg) and decapitated. The brains were quickly dissected and sectioned in ice-cold artificial cerebrospinal fluid (ACSF), bubbled with 95% O2 / 5% CO2. This cutting solution consisted of (in mM): 220 Sucrose, 3 KCl, 1.25 NaH2PO4, 25 NaH2CO3, 12 MgSO4, 10 Glucose, 0.2 CaCl2, and 0.4 Ketamine. VTA targeted horizontal slices (~200 µm thick) were immediately placed into an incubation chamber containing normal ACSF at 34-35°, bubbled with 95% O2 / 5% CO2 at 36° consisting of (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 12 glucose, 1.5 MgSO4, 2 CaCl2, pH 7.3, and allowed to incubate for at least 45 minutes prior to being transferred to a recording chamber. Once transferred to a recording chamber with continuous normal ACSF flow (2.0 ml/min) maintained at 34-35° throughout the experiment, the slices where then allowed to settle for an additional 15 to 30 minutes before recordings began. These incubation and settling periods allowed cells to recover and stabilize while ketamine was washed out of the tissue. Cells were visualized with either a Nikon Eclipse FN1 or E600FN microscope in the transmitted de Sénarmont Differential Interference Contrast (DIC) / infrared (IR) configuration.

**Whole-cell Recordings in vitro**

Electrodes pulled from borosilicate glass capillary tubes were filled with one of two types of pipette solutions. For IPSCs, the pipette solution consisted of (in mM): 128 KCl, 20 NaCl, 0.3 CaCl2, 1.2 MgCl2, 10 HEPES, 1 EGTA, 2 Mg-ATP, 0.25 Na-GTP and 4.5 QX314 (pH 7.3). For voltage waveform and current-evoked spiking experiments the pipette solution consisted of (in
mM): 115 K-Gluconate, 9 NaCl, 25 KCl, 10 HEPES, 0.2 EGTA, 1.2 MgCl₂, 3 Na-ATP, 1 Na-GTP, and had resistances of 2-4 MΩ. Series resistance (Rₐ) typically 10 to 20 MΩ, and input resistance (Rₘ) typically 300 to 400 MΩ, were continuously monitored with a 10 mV voltage step delivered at 0.1 Hz throughout each experiment and only experiments that maintained stable Rₐ and Rₘ (less than 15% change) were included in this study. IPSCs were filtered at 2 kHz while voltage waveform-generated currents and current-drive spikes were filtered at 6 kHz using an Axon Instruments Multiclamp 700A or 700B amplifier and digitized at 5-20 kHz, respectively, using an Axon 1440A digitizer, and collected and analyzed using pClamp10 and Igor Pro (Wavemetrics: Oswego, OR) software packages. Evoked and spontaneous IPSCs were recorded in the presence of 100 µM D-L 2-amino-5-phosphonopentanoic acid (APV), 30 µM 6-cyano-23-dihydroxy-7-nitro-quinoxaline (CNQX), and 100 nM eticlopride to block NMDA, AMPA, and DA D2-mediated synaptic currents (A. Bonci and J. T. Williams, 1997), respectively. Miniature IPSCs (mIPSCs) were isolated from all other spontaneous IPSCs by addition of 0.5 µM TTX. To evoke IPSCs, cells were stimulated at 0.1 Hz with a stainless steel-platinum/iridium concentric bipolar stimulating electrode placed ~100 µm rostral to the recording electrode. Evoked IPSCs were inward at the holding potential of -70 mV and were completely blocked by picrotoxin (100 µM). Evoked IPSC amplitudes were calculated by taking the difference between the 1.0 msec window around the peak and the 5.0 msec baseline window immediately preceding the stimulation artifact. Spontaneous IPSC activity amplitude and frequency were calculated the same for both sIPSCs and mIPSCs; the average amplitude or frequency during a 2 min period 8-10 min following drug were normalized to the average amplitude or frequency from a 2 min window prior to drug.

**Characterization of Neuron Types in vitro**
Traditionally, neurons have been classified as either primary (DAergic) and secondary (GABAergic) based on electrophysiological and pharmacological properties (A. A. Grace and S. P. Onn, 1989; S. W. Johnson and R. A. North, 1992; D. L. Cameron et al., 1997; M. A. Ungless et al., 2004). Despite extensive research, no single best electrophysiological characteristic has been identified to conclusively distinguish VTA DA neurons from other neurons in the VTA. The most widely accepted electrophysiological method to distinguish DA neurons from non-DA neurons has been the presence of a non-cation specific inward rectifying current (Ih). However, not all Ih(+) neurons stain positive for tyrosine hydroxylase (TH), a molecular marker specific to DA neurons, while Ih(-) cells appear to stain negative for TH (S. W. Johnson and R. A. North, 1992; D. W. Allison et al., 2006; E. B. Margolis et al., 2006). The combination of several electrophysiological characteristics, depending on experiment type, were used to distinguish putative DA from putative non-DA neurons in this study. Specifically: neurons that exhibited a modest non-cation specific inward rectifying current (Ih) in combination with spike accommodation, low input resistance, higher spike threshold, and shorter spike duration were assumed to be DA neurons. Neurons that exhibited a complete lack of Ih in combination with high input resistance, lack of spike accommodation, and low spike threshold were assumed to be non-dopaminergic (or putative GABA neurons) (S. W. Johnson and R. A. North, 1992; D. W. Allison et al., 2006; E. B. Margolis et al., 2006). A recent anatomical study has demonstrated the presence of glutamatergic neurons in the VTA and surrounding areas (T. Yamaguchi et al., 2007). However, this subpopulation of VTA cells remains uncharacterized and the highest concentration of vesicular transport-2 mRNA is in the rostral medial aspects of the VTA, with the lowest density in the parabrachial pigmental area (PBP). Most of the in vitro recordings in this study were conducted in the PBP in order to maintain uniformity with previous work. To
provide additional assurance that the characterization criteria described above effectively
discriminates VTA DA neurons from non-DA neurons (putative GABA neurons), we performed
a separate set of experiments to link these electrophysiological criteria to the presence or absence
of tyrosine hydroxylase (TH) mRNA, a marker for DA neurons. This was accomplished using
quantitative real-time PCR.

**Single-cell Quantitative RT-PCR**

Following electrophysiological characterization, putative VTA GABA neurons and
putative DA neurons in mature rats were aspirated under visual observation by application of
suction attached to the recording pipette, and were immediately added to a reverse transcription
(RT) reaction mixture. The iScript cDNA synthesis kit (Biorad) was used for a total volume of
10 µl per reaction. Reactions were run at 25°C for 10 min, 42°C for 60 min, and 95°C for 5 min
in a PTC-200 thermal cycler (MJ Research Inc., Watertown MA). Reactions were then stored at -
20°C until running the PCR. A preamplification round of multiplex PCR was performed by
adding iTAQ Supermix with ROX (Biorad) and a cocktail of primers to the completed RT
reaction, for a final volume of 50 µL. The reactions were held at 94°C for 30 seconds then cycled
20 times. Each cycle consisted of: 92°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30
seconds. One µl samples of the initial multiplex PCR were then used as substrate for each
reaction in the subsequent real-time quantitative PCR. Real-Time quantitative PCR using gene
specific primers with FAM-TAMRA TaqMan® probes (Applied Biosystems; TH plus primer:
CTTCCAGTACAAGCAGGTGAA, TH minus primer: AGCGTGACATATA-
CCTCCTTCCA, and TH probe: CCCCATGTGGAATACACAGCGGAAGAG; D2 plus primer:
CGCAGAAAAGCTCTCCCAGCAGA, D2 minus primer:
GACTGGTGAGATGTGGACATCACA and D2 probe: CCATTGTTCGGGTGTTTCA; 18s
plus primer: GTGCATGGCCGTTCTTAGTTG, minus 18s primer: GCCACTTGTCCTCTTAAGAAGTTG, and 18s probe: TGGAGCGATTTGTCTGGTTAATTCCGATAAC) were performed using the iTaq Supermix with ROX (Bio-Rad) with an iCycler IQ (Bio-Rad) real-time PCR System. Samples were amplified in triplicate, together with a negative control for each subunit (an ACSF-only aspiration taken from the brain slice recording chamber when the cells were aspirated). The amplification protocol was 50°C for 2 minutes, 95°C for 5 minutes, then 50 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. Cycle threshold (Ct) values were calculated automatically by the iCycler IQ software, with threshold values set between 5 and 20. Relative fold expression was calculated using the $2^{-\Delta\Delta CT}$ method as described in (K. J. Livak and T. D. Schmittgen, 2001).

**Voltage Waveform Command**

In order to mimic in VTA GABA neurons recorded *in vitro* the frequency and underlying ionic currents linked to high frequency spontaneous spiking and current-evoked postsynaptic discharges elicited from VTA GABA neurons recorded *in vivo* a “typical” train of high frequency current-evoked spikes was used as a “voltage waveform command.” The voltage waveform command was derived from a recording of current-evoked spikes obtained in current clamp mode with a spiking frequency of 172 Hz evoked at 250 pA. This voltage waveform command was used due to the length of time required to generate a voltage-clamp protocol from a recorded current-clamp waveform for each neuron (R. Llinas et al., 1982; M. T. Do and B. P. Bean, 2003; A. Enomoto et al., 2006). The resulting data obtained in voltage-clamp mode was a train of whole-current events with inward TTX-sensitive (0.5 µM) currents that followed the voltage waveform command closely. In order to subtract passive membrane and capacitative
currents from the ionic current trace acquired from the voltage waveform (baseline -55 mV) the same waveform was applied sub-threshold (-120 mV), and the resulting current trace was subtracted from the current trace acquired at -55 mV. Three measurements were then obtained from the remaining current trace: 1) The amplitude of the first inward current event; 2) The average of all, or total inward current; and 3) The average total inward current of the last third (25 current events), or plateau phase of the trace. Currents are presented as percent of control 8 minutes following cocaine application to the ACSF. Due to space clamp problems common to slice preparations, no measurements of inward current kinetics were made. The voltage waveform command protocol utilized in this study was designed to clamp frequency, while imitating natural spike dynamics. We found that when $R_a$ and $R_m$ remained stable, and the time between the applications of the voltage waveform command protocol was not less than 2 minutes, waveform-generated current amplitudes remained constant and exhibited little sign of run-down.

**Statistical Analyses**

For *in vivo* and *in vitro* studies, the results for control and drug treatment groups were derived from calculations performed on VTA GABA neuron spontaneous firing rate, ICPSDs, current-evoked spikes, sodium currents, DA neuron evoked and spontaneous IPSCs, and mean relative fold expression differences of TH and D2 mRNA. A paired two sample for means t test was performed to determine statistical significance for all measures. Welch’s correction was applied to RT PCR data due to unequal variances. All results, except for paired pulse, in text are presented as the mean ± SEM and the t distribution variance for a two-tailed level of confidence of 95%. Paired pulse results are presented as one-tailed 95% confidence interval. Results in Figures are presented as mean ± SEM; $P < 0.05$ was taken as indicating statistical significance.
RESULTS

Effects of Systemic Cocaine, Cocaine Methiodide, Dopamine, Lidocaine and Methamphetamine on VTA GABA Neuron Firing Rate in vivo

Fifty three VTA GABA neurons were tested in vivo for their sensitivity to intravenous administration of cocaine, cocaine methiodide, DA, lidocaine or methamphetamine. Initially, we hypothesized that reinforcing doses of cocaine would enhance VTA GABA neuron firing rate similar to microelectrophoretic DA (S. H. Stobbs et al., 2004), given its DAT inhibiting properties. However, unexpectedly, cocaine (0.25-2 mg/kg) had somewhat variable, but significant dose-dependent effects on firing rate. Cocaine significantly increased VTA GABA neuron firing at doses at or below 0.5 mg/kg and significantly decreased firing rate at doses above 0.5 mg/kg (Figure 1A,B, 0.25 mg/kg: +56 ± 6.1 %, P<0.01, t(2,7)=2.3; 0.5 mg/kg: +68 ± 6.9 %, P<0.01, t(2,9)=2.2; 1.0 mg/kg: -18 ± 8 %, P<0.05, t(2,24)=2.0; 2.0 mg/kg: -31 ± 7.2 %, P<0.05, t(2,7)=2.3; mean saline firing rate = 31.4 ± 2.84 Hz; n=25), compared to saline control. In order to determine if cocaine’s well-known peripheral cardiovascular pressor effects (C. W. Schindler et al., 1995) or temperature effects (E. A. Kiyatkin and P. Leon Brown, 2006) might have contributed to cocaine effects on VTA GABA neuron firing rate, we evaluated the effects of intravenous DA, which has well-known pressor effects, and cocaine methiodide, which does not cross the blood-brain barrier, but has cocaine’s peripheral temperature and blood pressure effects (D. A. Shriver and J. P. Long, 1971; E. A. Kiyatkin and P. Leon Brown, 2006).

Intravenous administration of DA (3 mg/kg) increased mean arterial pressure 223%, respiratory
rate, and induced piloerection, but had no effect on VTA GABA neuron firing rate (P>0.05, $t_{(2,4)}=2.7$; n=5). Intravenous administration of cocaine methiodide did not significantly alter firing rate at any dose level tested (Figure 1B; 0.25 mg/kg: -1 ± 9 %, P>0.24, $t_{(2,4)}=2.7$; 0.5 mg/kg: -2.2 ± 1.3 %, P>0.05, $t_{(2,4)}=2.7$; 1.0 mg/kg: +5.5 ± 4.5 %, P>0.05, $t_{(2,4)}=2.7$; 2.0 mg/kg: -1.3 ± 1.3 %, P>0.05, $t_{(2,4)}=2.7$; mean saline firing rate = 26.3 ± 4.73; n=5), compared to saline control. Intravenous administration of lidocaine, a VSSC blocker with negligible DAT activity, significantly decreased VTA GABA neuron firing rate at all doses tested (Figure 1B; 0.25 mg/kg: -18 ± 5.8 %, P<0.05, $t_{(2,8)}=2.3$; 0.5 mg/kg: -26%, P<0.05, $t_{(2,6)}=2.4$; 1.0 mg/kg: -32 ± 6 %, P<0.05, $t_{(2,8)}=2.3$; 2.0 mg/kg: -38 ± 5.8 %, P<0.01, $t_{(2,6)}=2.4$; mean saline firing rate = 26.3 ± 4.73 Hz; n=9), compared to saline control. Intravenous administration of methamphetamine, a DAT inhibitor with negligible VSSC activity, significantly increased VTA GABA neuron firing rate at all dose levels tested (Figure 1B; 0.25 mg/kg: +45 ± 11.6 %, P<0.05, $t_{(2,4)}=2.7$; 0.5 mg/kg: +92 ± 22 %, P<0.05, $t_{(2,4)}=2.7$; 1.0 mg/kg: +140 ± 32 %, P<0.05, $t_{(2,4)}=2.7$; 2.0 mg/kg: +176 ± 28 %, P<0.05, $t_{(2,4)}=2.7$; mean saline firing rate = 22.2 ± 3.93 Hz; n=9), compared to saline control.

**Dopamine Pharmacology of Cocaine Effects on VTA GABA Neuron Firing Rate in vivo**

Of the forty eight VTA GABA neurons tested in vivo for sensitivity to microelectrophoretically applied DA, 46% of them were activated by DA (S. H. Stobbs et al., 2004; M. B. Lassen et al., 2007). We evaluated the DA pharmacology of 22 of these neurons. Microelectrophoretic application of DA (Figure 1C; + 50 nA; P<0.001, $t_{(2,21)}=5.0$; n=22), or the D2 agonist quinpirole (+50 nA; P<0.001, $t_{(2,14)}=4.31$; n=15; data not shown) significantly increased the firing rate of VTA GABA neurons 162 % and 140 %, compared to saline control (mean saline firing rate = 27.3 ± 4.1 Hz; + 50 nA ejection current). Neither the D1 agonist SKF38393 nor the D1 antagonist SCH23390 (+ 50 nA; P>0.05, n=4) significantly altered the
firing rate of VTA GABA neurons, compared to saline control (data not shown). In situ microelectrophoretic administration of eticlopride blocked DA activation of firing rate, and iv administration of eticlopride (1.0 mg/kg) blocked low-dose (0.5 mg/kg) iv cocaine activation, but not higher dose iv cocaine inhibition, of VTA GABA neuron firing rate P<0.05; n=6; Figure 1C).

Effects of Systemic Cocaine, Cocaine Methiodide, Dopamine, Lidocaine and Methamphetamine on VTA GABA Neuron Evoked Spikes in vivo

Brief, high frequency stimulation of the internal capsule (IC) results in multiple post-stimulus VTA GABA neuron spike discharges (ICPSDs; Figure 2A; (S. C. Steffensen et al., 1998)). We studied the effects of intravenous cocaine on ICPSDs obtained in 53 VTA GABA neurons and compared its effects to cocaine methiodide, DA, lidocaine, and methamphetamine. Figure 2A shows the effects of 1.0 mg/kg cocaine on ICPSDs obtained from a representative VTA GABA neuron. While saline had no effect, this dose of cocaine markedly reduced VTA GABA neuron ICPSDs approximately 50-60 % 1 min after injection. The time course of cocaine inhibition at the 1.0 mg/kg dose was characterized by rapid and pronounced inhibition at 1 min with recovery in 30 min (Figure 2B; n=5). The effects of cocaine on VTA GABA neuron ICPSDs was tested at dose levels 0.25-2.0 mg/kg (Figure 2C), and significantly decreased ICPSDs at doses at or above 0.25 mg/kg (0.25 mg/kg: -16.7 ± 2.7%, P<0.005, t(2.5)=2.5; 0.5 mg/kg: -27.5 ± 1.9%, P<0.002, t(2.5)=2.5; 1.0 mg/kg: -42.8 ± 2.7 %, P<0.002, t(2,5)=2.5; 2.0 mg/kg: -55.3 ± 5.3 %, P<0.0004, t(2,5)=2.5; mean 50% maximum saline ICPSDs = 128.5 ± 30.7; n=6 each), compared to saline control. The IC50 for cocaine on ICPSDs was approximately 1.2 mg/kg, based on linear interpolation from the dose-response curve (r²=0.86). As with firing rate, in order to determine if cocaine’s well-known cardiovascular pressor effects or temperature
effects might contribute to cocaine reduction of VTA GABA neuron ICPSDs, we evaluated the
effects of intravenous DA and cocaine methiodide. Intravenous administration of DA (3 mg/kg)
had no effect on VTA GABA neuron ICPSDs (P>0.05, n=4). Similarly, intravenous
administration of cocaine methiodide did not significantly affect ICPSDs at any dose level tested
(Figure 2C; 0.25 mg/kg: -2 ± 2 %, P<0.24, t(2,5)=2.5; 0.5 mg/kg: -11 ± 2.7 % P<0.18, t(2,5)=2.5;
1.0 mg/kg: -6 ± 1.7 %, P<0.105, t(2,5)=2.5; 2.0 mg/kg: -10.1 ± 3.5 %, P<0.11, t(2,5)=2.5; mean
50% maximum saline ICPSDs = 121.5 ± 4.2; n=6), compared to saline control. Since local DA
markedly enhanced firing rate (Figure 1; (S. H. Stobbs et al., 2004)), we evaluated the effects of
local application of DA on ICPSDs. Compared to saline control, microelectrophoretic
application of DA (+ 50 nA) did not significantly alter ICPSDs (P>0.5; mean 50% maximum
saline ICPSDs = 60.6 ± 4.4; n=21 each). Intravenous administration of lidocaine markedly
decreased ICPSDs at all doses tested (Figure 2C; 0.25 mg/kg: -18.3 ± 2.4 %, P<0.005, t(2,5)=2.5;
0.5 mg/kg: -25.6 ± 1.7 %, P<0.0002, t(2,5)=2.5; 1.0 mg/kg: -41.9 ± 2 %, P<0.0001, t(2,5)=2.5; 2.0
mg/kg: -62.6 ± 3.1 %, P<0.00003, t(2,5)=2.5; mean 50% maximum saline ICPSDs = 136.9 ± 14.3;
n=6), compared to saline control. The IC50 for lidocaine on ICPSDs was approximately 1.2
mg/kg, based on linear interpolation from the dose-response curve (r²=0.91). Intravenous
administration of methamphetamine did not significantly alter VTA GABA neuron ICPSDs
(Figure 2C; 0.25 mg/kg: -10 ± 5 %, P>0.05, t(2,4)=2.7; 0.5 mg/kg: -5 ± 6.2 %, P>0.05, t(2,3)=3.1;
1.0 mg/kg: -8 ± 5.2 %, P>0.05, t(2,3)=3.1; mean 50% maximum saline ICPSDs = 74.9 ± 9.5;
n=15), compared to saline control.

**Dopamine Pharmacology of Cocaine Effects on VTA GABA Neuron Evoked Spikes in vivo**

In order to evaluate the role of DA in mediating cocaine inhibition of VTA GABA
ICPSDs we tested the effects of select DA receptor subtype antagonists on cocaine inhibition of
VTA GABA neuron ICPSDs at the 1.0 mg/kg dose level 1 min after injection, and compared to iv saline (Figure 2D). Intravenous administration of the D1/D5 DA receptor antagonist SCH23390 (1.0 mg/kg) had no effect on VTA GABA neuron ICPSDs (104.2 ± 7.9 %; P>0.05; n=5), and did not alter cocaine’s ability to reduce ICPSDs at the 1.0 mg/kg dose level (56.2 ± 4.0 %) when administered 10 min after injection of SCH23390. Similarly, iv administration of the D2/D3 antagonist eticlopride (1.0 mg/kg) had no effect on VTA GABA neuron ICPSDs (102.7 ± 6.6 %; P>0.05; n=5), and did not alter cocaine’s ability to reduce ICPSDs (51 ± 2.1 %) when administered 10 min after injection of eticlopride.

**Expression of Select Genes in Dopamine vs GABA Neurons in Mature Rats: Quantitative Single-cell RT-PCR**

In order to further evaluate the role of D2 receptors in DA activation of VTA GABA neurons, we evaluated the quantitative expression of D2 receptor gene products in these neurons. In addition, in order to validate the electrophysiological criteria used to distinguish VTA neurons, we compared the expression of TH in VTA GABA and DA neurons. Figure 3 summarizes the differences between VTA DA and GABA neurons for the expression of TH and D2 mRNA transcripts in adult male rats. Tyrosine hydroxylase expression in DA neurons was significantly greater than GABA neurons (P=0.0002, t(1,23)=4.5; mean DA neuron TH expression = 6.12 ± 0.77 and mean GABA neuron TH expression = 0.07 ± 0.01; n=33). The cycle threshold for 18s, the housekeeping gene that was used for the quantification of the relative expression of each of the gene products, was not significantly different between DA and GABA neurons (P=0.35, t(1,31)=0.95; mean DA cell 18s CT = 12.53 ± 0.18 and mean GABA cell 18s CT = 12.87 ± 0.34). The expression of D2 was significantly greater in DA neurons than GABA neurons (P=0.019; mean DA cell D2 expression = 3.39 ± 0.59 and mean GABA cell D2 expression =
The cycle threshold for 18s was not significantly different between groups (P=0.82, t(1,27)=0.23; mean DA cell 18s CT = 12.53 ± 0.18 and mean GABA cell 18s CT = 12.63 ± 0.35).

**Effects of Cocaine on Current-Evoked VTA GABA Neuron Spikes in vitro**

Based on *in vivo* studies, cocaine did not appear to be acting through DA to reduce VTA GABA neuron firing rate or ICPSDs. Thus, we tested the effect of cocaine on VTA GABA neuron current-evoked spiking *in vitro* to determine whether cocaine might reduce spiking similar to ICPSDs *in vivo*. VTA GABA neurons displayed an average spiking frequency of 22.8 ± 9.9 Hz at threshold (25 pA) and 180 ± 49.7 Hz at the maximum current step (400 pA). Not surprisingly, 50 µM cocaine markedly reduced spiking at current levels 100-400 pA (85.7 ± 13.8 Hz, P < 0.05, t(2,4)=2.8 at 400 pA; **Figure 4AB**). Since cocaine reduced current-evoked spikes *in vitro* similar to ICPSDs *in vivo* we postulated that cocaine was blocking ICPSDs, current-evoked spikes, and reducing firing rate via its VSSC blocking properties. To provide evidence to support this assumption, we next examined cocaine’s effects of VTA GABA neuron sodium currents.

**Effects of Cocaine on VTA GABA Neuron Sodium Currents in vitro**

Cocaine appeared to exert greater inhibition on the plateau rather than initial phase of ICPSDs *in vivo* (see **Figure 2A**) and current-evoked spiking *in vitro* (see **Figure 4A**). This use-dependent inhibition suggested that cocaine was acting via the well-established mechanism of preferentially binding the sodium channel in the open, inactivated conformational state (G. Strichartz, 1976; B. P. Bean et al., 1983; S. W. Postma and W. A. Catterall, 1984; M. E. O'Leary and M. Chahine, 2002). To confirm that cocaine was inhibiting ICPSDs and current-evoked spiking by blocking VSSCs in a use-dependent manner we tested the effect of cocaine on VTA
GABA neuron whole-currents elicited using a voltage waveform command protocol (R. Llinas et al., 1982; M. T. Do and B. P. Bean, 2003; A. Enomoto et al., 2006). The voltage waveform command protocol was created from a typical voltage trace obtained in current clamp mode (Figure 5Ai) of a VTA GABA neuron driven to fire at 172 Hz, a frequency consistent with what we have previously determined for ICPSDs in vivo (M. B. Lassen et al., 2007) at 50% maximum stimulus level, and at sub-max current levels for spikes driven in current-clamp mode. This current-clamp derived voltage waveform command protocol has the advantage of clamping both spike dynamics and frequency, compared to a sequence of square waves. All inward current events obtained using the voltage waveform command were abolished by TTX (0.5 µM), indicating that they were sodium currents. Figure 5Aii-iv illustrates the effects of cocaine (7 µM) and TTX (0.5 µM) on sodium currents evoked by the voltage waveform command in a typical VTA GABA neuron. We postulated that if cocaine was blocking VSSCs in a use-dependent manner, suggested by the effect observed on current-evoked spikes, cocaine would have its greatest effect during the plateau phase of the waveform trace. We therefore measured cocaine’s effect on the amplitude of the first inward current event, the mean of all or total inward current, and the mean inward current of the last 25 events- the plateau phase of the trace (Figure 5B,C). The superimposed traces of the first current event and last 5 current events in panel Figure 5B taken from the raw traces in Figure 5A illustrate this. Cocaine was tested at concentration levels 3, 7, 15 and 50 µM and significantly reduced VTA GABA neuron TTX-sensitive sodium currents in a concentration-dependent manner, exerting its greatest VSSC-blocking effect at each dose on the plateau phase of the trace (percent reduction of plateau phase inward current: 3 µM, 25.8 ± 2.9%, P < 0.001, t(2,4)=2.7; 7 µM, 36.6 ± 3.3%, P < 0.01, t(2,3)=3.1; 15 µM, 69.1 ± 4.6%, P < 0.001, t(2,3)=3.1; 50 µM, 76.9 ± 4.5%; P < 0.0001, t(2,5)=2.5; Figure
Cocaine’s IC<sub>50</sub> on sodium currents in VTA GABA neurons was calculated to be 13 µM, using a least squares fit line to the points generated from 3, 7, 15 µM, (r<sup>2</sup> = 0.90, y = -3.1x + 90.4).

**Effects of Cocaine on VTA DA Neuron Evoked and Spontaneous IPSCs in vitro**

Since cocaine inhibited putative VTA GABA neuron sodium currents as well as current-evoked spikes *in vitro*, and local circuit GABA neurons are presumed to inhibit VTA DA neurons, we evaluated the effects of cocaine (7, 13 and 50 µM) on DA neuron IPSCs evoked by local stimulation. The concentrations tested were based on the assumption that if cocaine blocked evoked IPSCs, the IC<sub>50</sub> for this effect would be similar to the IC<sub>50</sub> of cocaine’s blocking effects on putative VTA GABA neuron waveform-evoked sodium currents (13 µM). Indeed, cocaine significantly reduced evoked VTA DA neuron IPSCs within 8 to 10 minutes (*Figure 6A*) at all concentrations tested, with an IC<sub>50</sub> of 13 µM (7 µM, 18 ± 2.9%, P < 0.001, t(2,3)= 4.18; 13 µM, 48.3 ± 4.3%, P < 0.01, t(2,3)=3.18; 50 µM, 79.7 ± 2.9%; P < 0.001, t(2,3)= 3.18; *Figure 6B*). Since it has been shown that changes in transmitter release affect the paired-pulse ratio (R. Khazipov et al., 1995; S. Mennerick and C. F. Zorumski, 1995; P. A. Salin et al., 1996; S. C. Steffensen et al., 1999) we examined IPSC paired pulse ratio at 50 msec to determine whether cocaine’s reduction of evoked IPSCs was correlated to a change in the probability of transmitter release. Calculating the paired-pulse ratio using the test/conditioning IPSC peak amplitude ratio (i.e., IPSC2/IPSC1), 13 µM cocaine increased DA neuron IPSC paired-pulse ratio from 1.16 ± 0.11 to 1.94 ± 0.19 (P = 0.006, t(2,4)= 2.44; *Figure 6C*), suggesting cocaine was acting presynaptically and decreasing the probability of GABA release. To further investigate cocaine’s effects on GABAergic synapses on VTA DA neurons, and to confirm whether cocaine was acting presynaptically or postsynaptically, we examined cocaine’s effects on spontaneous
action potential-dependent GABAergic IPSCs (sIPSCs). Cocaine (13 µM) reduced sIPSC frequency 33.7 ± 7.2% (P= 0.018, t(2,3)= 3.18; **Figure 6D**), but did not significantly affect sIPSC amplitude (control, 37 ± 10.2 pA; n=4; cocaine, 40.4 ± 9.1 pA; n=4; p=0.31; **Figure 6D**).

Finally, in order to determine whether cocaine was reducing the action potential-dependent or independent components of sIPSC activity, or both, we sought to isolate this effect by examining the action potential-independent, TTX-insensitive or miniature IPSC (mIPSC) component alone. Cocaine did not significantly affect mIPSC frequency or amplitude (frequency: control, 2.78 ± 0.48 Hz; n=5; cocaine, 2.61 ± 0.46 Hz; n=5; p=0.3; amplitude: control, 33.8 ± 2.3 pA; n=5; cocaine, 33.4 ± 2.7 pA; n=5; p=0.88; **Figure 6E**), suggesting that cocaine was acting presynaptically as a VSSC blocker to reduce the action potential-dependent component of spontaneous IPSC activity.
DISCUSSION

We have previously demonstrated that DA enhances the firing rate of VTA GABA neurons (S. H. Stobbs et al., 2004). Here we ascribe this enhancement to be mediated by D2/D3 receptor activation. This activation may occur directly via D2 receptors located on GABA neurons, as has been observed in substantia nigra (A. Ruffieux and W. Schultz, 1981; B. L. Waszczak and J. R. Walters, 1986; S. R. Sesack et al., 1994), or indirectly, through some unknown mechanism involving D2/D3 autoreceptors on DA neurons (for review see (A. Adell and F. Artigas, 2004)). Accordingly, we evaluated the quantitative mRNA expression of D2 receptors in VTA neurons. Both DA and GABA neurons expressed D2 receptor mRNA, providing molecular support for our DA pharmacology studies that GABA neurons are excited by DA. Although D2 excitatory effects have been the subject of controversy (J. L. Waddington, 1997), D2 receptors have been demonstrated in GABA neurons in substantia nigra (A. Ruffieux and W. Schultz, 1981; B. L. Waszczak and J. R. Walters, 1986; S. R. Sesack et al., 1994), suggesting at least a role for D2 receptors in DA modulation of midbrain GABA neurons.

Cocaine enhanced VTA GABA neuron firing rate at low doses (0.25-0.5 mg/kg), but inhibited at higher doses (1.0-2.0 mg/kg), while the DAT inhibitor methamphetamine had only excitatory effects on VTA GABA neuron firing rate across all doses. The enhancement of firing rate by cocaine was not due to its peripheral effects, as neither systemic cocaine methiodide, which does not cross the blood brain barrier, but retains all of cocaine’s peripheral effects on blood pressure and temperature, nor systemic DA, which has similar effects on blood pressure, affected VTA GABA neuron firing rate. The VSSC blocker lidocaine had only inhibitory effects on VTA GABA neuron firing rate. While D2 receptor antagonists blocked low-dose cocaine activation of VTA GABA neurons, they had no effect on cocaine’s ability to inhibit firing rate at higher doses,
suggesting that cocaine enhances VTA GABA neuron firing rate at low doses via its central effects on DA release and D2 receptor activation by its DAT inhibiting properties, but at higher doses it inhibits firing rate by its central VSSC blocking properties.

Cocaine significantly reduced ICPSDs, a putative physiological index of electrical coupling (D. W. Allison et al., 2006) that is sensitive to NMDA agonists and antagonists (S. C. Steffensen et al., 1998; S. H. Stobbs et al., 2004), ethanol (S. H. Stobbs et al., 2004), and connexin-36 gap junction (GJ) blockers (D. W. Allison et al., 2006). The number of ICPSDs is a monotonic function of frequency, stimulus intensity, and pulse number (M. B. Lassen et al., 2007), rats will self-stimulate the IC, and the number of ICPSDs is directly proportional to responding for brain stimulation reward (M. B. Lassen et al., 2007). Neither local DA nor systemic methamphetamine had any significant effect on ICPSDs, evincing a lack of involvement of DA in cocaine reduction of ICPSDs, and distinguishing them mechanistically from spontaneous activity. Cocaine methiodide had no effect on ICPSDs, suggesting that cocaine’s peripheral actions were not responsible for its inhibitory effects. In support of the lack of DA involvement in cocaine’s effects on ICPSDs, systemic administration of D1/D5 or D2/D3 antagonists had no effect on cocaine reduction of ICPSDs. Lidocaine markedly reduced ICPSDs, nearly identical to cocaine. Thus, while ICPSDs depend on activation of NMDA receptors (which may induce oscillations by acting as current amplifiers due to their voltage dependence (A. S. Kuznetsov et al., 2006)), and appear to require electrical synaptic connectivity (S. H. Stobbs et al., 2004; D. W. Allison et al., 2006), cocaine appears to be reducing ICPSDs via block of VSSCs, and does not appear to affect them through DA neurotransmission.
While the characterization of VTA neurons *in vitro* is problematic, it seems VTA GABA and DA neurons each possess unique electrophysiological characteristics that may aid in distinguishing them (for overview see (E. B. Margolis et al., 2006)). In an effort to reconcile these electrophysiological characteristics to a post hoc test that might aid in distinguishing GABA from DA neurons, we examined the expression of TH mRNA in a separate set of adult male rats. Surprisingly, TH mRNA was detected in both putative DA (I_h positive, low input resistance, spike accommodating) and putative GABA (I_h negative, high input resistance, no spike accommodation) neurons in the VTA. However, TH was expressed 143X more in putative DA than putative GABA neurons, suggesting this small amount of TH mRNA detected in GABA neurons might simply be contamination-related background noise associated with the harvesting of cells from the slice. There is evidence, however, that TH protein is present in GABA neurons (R. Klink et al., 2001; V. G. Olson and E. J. Nestler, 2007). In spite of the possible disparity between detection of mRNA transcripts and the presence of encoded protein, and some persisting vagaries regarding how the VTA is defined anatomically (S. Ikemoto, 2007), this RT PCR data supports the idea that VTA neurons can be distinguished somewhat reliably based on certain electrophysiological characteristics in some areas of the VTA.

Based on cocaine’s reduction of ICPSDs and firing rate *in vivo*, and current evoked spikes *in vitro*, we performed voltage-clamp experiments in the midbrain slice preparation to evaluate cocaine’s effects on sodium current in VTA GABA neurons. Since VTA GABA neurons lack spike accommodation, very high-frequency (~200 Hz) sodium currents could be studied *in vitro* that might emulate the high-frequency spiking obtained in current clamp *in vitro* (i.e., current-evoked spiking) and *in vivo* (i.e., ICPSDs). A voltage waveform command (R. Llinas et al., 1982; M. T. Do and B. P. Bean, 2003; A. Enomoto et al., 2006) was created from a
typical recording of current-evoked spiking at 172 Hz, a frequency observed in both ICPSDs in vivo (Figure 2; (M. B. Lassen et al., 2007)) and current-evoked spikes in vitro (Figure 4). The inward currents obtained from this voltage waveform command were abolished by TTX, indicating they were sodium currents. Cocaine suppressed sodium currents with an IC$_{50}$ of 13 µM, a concentration consistent with its reinforcing properties (J. S. Fowler et al., 1998; E. A. Kiyatkin and P. Leon Brown, 2006). Of the three measurements of sodium currents (first current, total current, and last 25 currents), cocaine exerted its greatest effect on the amplitude of the last 25 currents or plateau phase of sodium currents, confirming that cocaine was acting in a use-dependent manner on VSSCs (G. Strichartz, 1976; B. P. Bean et al., 1983; S. W. Postma and W. A. Catterall, 1984; M. E. O'Leary and M. Chahine, 2002). While cocaine’s anesthetic effects are not specific to GABA neurons, the use-dependent nature of cocaine’s VSSC actions would exert a greater effect on relatively fast-firing, wide-bandwidth GABA neurons than DA neurons. VTA GABA neurons have baseline firing rates that are more than ten times higher than DA neurons, even in unanesthetized rats (R. S. Lee et al., 2001), which would render them more vulnerable to cocaine’s use-dependent VSSC blocking properties.

The prevailing view is that VTA GABA neurons may serve to inhibit VTA DA neurons locally. Given the sensitivity of VTA GABA neurons to cocaine’s VSSC blocking properties, we reasoned that cocaine may disinhibit VTA DA neurons, and sought to determine cocaine’s effects on GABA receptor-mediated evoked and spontaneous IPSCs in VTA DA neurons. Since somatodendritic release of DA elicits D2 receptor-mediated IPSCs in VTA DA neurons (M. J. Beckstead et al., 2004), we examined DA IPSCs in the presence of the D2/D3 receptor antagonist eticlopride. To our knowledge, we are the first to demonstrate that cocaine can reduce VTA DA evoked IPSCs at reinforcing dose levels (IC$_{50}$ = 13 µM). This same concentration
significantly increased the IPSC paired-pulse ratio, suggesting cocaine acts to decrease the probability of GABA release (R. Khazipov et al., 1995; S. Mennerick and C. F. Zorumski, 1995; P. A. Salin et al., 1996; S. C. Steffensen et al., 1999). Additionally, 13 µM cocaine reduced action potential-dependent spontaneous IPSC (sIPSC) frequency, but not amplitude, and did not reduce TTX-insensitive action potential-independent miniature IPSC (mIPSC) frequency or amplitude. Taken together, these observations support the idea that cocaine is acting presynaptically through its VSSC blocking properties on VTA GABA neurons to reduce activity-dependent GABA release in the VTA. This concept is supported by the fact that lidocaine (500 µM) is used to pharmacologically isolate mIPSCs (M. Melis et al., 2002). Given that acute intravenous injections of cocaine at 1.0 mg/kg or higher can lead to extracellular cocaine concentrations greater than 25 µM in the brain (H. T. Pan et al., 1991), it is somewhat surprising that lidocaine has not been used as a control in in vitro studies wherein cellular and molecular changes in VTA DA neurons have been attributed to high doses of cocaine (P. W. Kalivas and J. E. Alesdatter, 1993; M. A. Ungless et al., 2001; F. Sarti et al., 2007).

Conceivably, the effects of cocaine in the mesolimbic system are concentration-dependent: a low-dose cocaine effect that enhances DA levels via DAT inhibition at the terminals of DA neurons, while moderate to higher dose level effects of cocaine may be mediated through both its DAT-inhibiting and VSSC-inhibiting properties. These actions would affect the midbrain regions containing DA neuron cell bodies, and their projection areas such as the nucleus accumbens, with cocaine's VSSC-inhibiting properties preferentially affecting fast-firing GABA neurons. In support of the data of this study, and our hypothesis that DA neurons may not be as sensitive as GABA neurons to cocaine's VSSC-blocking effects, Shi et al. (2004) demonstrated that cocaine (1.0 mg/kg) enhancement of DA neuron firing rate, bursting activity,
and low-frequency oscillations in vivo were unmasked by blocking DA neuron D2 autoreceptor inhibition. In that study, cocaine may have acted to increase DA neuron firing rate and bursting activity by reducing local GABA neuron inhibition when DA neuron autoreceptor inhibition was blocked. Additionally, procaine, which has VSSC blocking actions and minimal DAT inhibiting properties (K. M. Wilcox et al., 1999), slightly increased DA neuron firing rate in the VTA (L. C. Einhorn et al., 1988). We have previously demonstrated that chloral hydrate anesthesia, which was used in the Einhorn et al study, abolishes GABA neuron activity (R. S. Lee et al., 2001), suggesting that DA firing rate might have been enhanced even more if this anesthetic agent hadn’t markedly reduced GABA inhibition. Finally, facilitators of GABA neurotransmission significantly attenuate cocaine-induced locomotor sensitization, stereotypy and reinforcement (E. L. Gardner et al., 2002), providing behavioral support for a role for GABA in cocaine reinforcement. Thus, cocaine’s inhibition of GABA neuron VSSCs might synergize with its DAT blocking actions to enhance DA neurotransmission over that produced by its DAT inhibitory properties alone. Cocaine’s net effect on DA neuron activity in the VTA might result from a combination of cocaine inhibition of DA neurons by D2 autoreceptor activation (L. C. Einhorn et al., 1988), and disinhibition of DA neurons via GABA neuron inhibition by virtue of its VSSC blocking actions.

These in vivo and in vitro findings establish the physiological relevancy and mechanism of action for cocaine effects on VTA GABA neurons and GABA inhibition of DA neurons. We have shown that cocaine inhibits VTA GABA neuron excitability in vivo as well as sodium currents and inhibitory synaptic potentials in DA neurons in vitro at dose levels known to be reinforcing, providing compelling mechanistic evidence that cocaine is acting through its VSSC blocking effects to disinhibit VTA DA neurons. By virtue of their proximity to midbrain DA
neurons, wide dynamic range, widespread axonal distribution, potential electrical coupling via GJs, dependency on corticotegmental glutamatergic NMDA receptor-mediated input, and sensitivity to DA, VTA GABA neurons are in a critical position to regulate mesocorticolimbic DA neurotransmission. Although highly speculative at this time, along with VSSCs, GJ-mediated communication between VTA GABA neurons may be sensitive to cocaine. Modulation of GJ neurotransmission by DA is well-established in the retina and has been implicated in other regions of the brain containing DA (for review see (R. Weiler et al., 2000)), and GJ connexin proteins have been shown to be altered in the brain following cocaine self-administration (S. A. Bennett et al., 1999). Thus, while the role of VTA GABA neurons in mediating alcohol and opiate reinforcement is gaining wider acceptance, this study suggests a potential role for these neurons in cocaine reinforcement as well.
Figure 1: Dopamine pharmacology of cocaine effects on VTA GABA neuron firing rate in vivo

(A) The ratemeter record shows the effects of saline and 0.5 mg/kg cocaine on a representative VTA GABA neuron with a baseline rate of approximately 32 Hz. At this dose level, cocaine enhanced the firing rate of this neuron. (B) Cocaine significantly enhanced VTA GABA neuron firing rate at dose levels at or below 0.5 mg/kg, and modestly, but significantly, reduced firing rate at dose levels 1-2 mg/kg. Cocaine methiodide, which elicits cocaine’s peripheral, but not central effects, did not significantly alter firing rate at any dose level. Lidocaine significantly reduced firing rates at all cocaine dose levels. Methamphetamine significantly enhanced firing rate at all dose levels. Asterisks *,** represent significance level P<0.05 and P<0.01, respectively. (C) Compared to saline microelectrophoresis, DA significantly increased firing rate 162 % and microelectrophoretic application of the D2 antagonist eticlopride blocked DA activation. Intravenous administration of 1.0 mg/kg eticlopride blocked low-dose cocaine activation of firing rate, but not higher dose cocaine inhibition of firing rate. Asterisks * correspond to significance levels P<0.001, compared to saline control.
Figure 2: Cocaine inhibition of VTA GABA neuron ICPSDs in vivo: Time course and comparison to dopamine agonists, VSSC blockers and DAT inhibitors

Figure 2: (A) The inset shows a 1.0 sec trace of a representative VTA GABA neuron spike in association with brief (50 msec), high frequency (200 Hz) stimulation of the internal capsule (IC). The horizontal bar above the stimulus artifacts indicates the time of the stimulus train. VTA GABA neurons evince multiple spike discharges for hundreds of msec following IC stimulation. These are termed IC-induced post-stimulus spike discharges, or ICPSDs. The peri-stimulus spike histogram (PSH) below the trace shows the average of 12 IC stimulation trials on ICPSDs obtained from this representative VTA GABA neuron following iv saline. The stimulus artifacts in the PSH are omitted to illustrate spikes only. The PSH bottom shows the effects of 1.0 mg/kg iv cocaine on ICPSDs in this same neuron 1.0 min after cocaine injection. Note that cocaine markedly decreased VTA GABA neuron ICPSDs at this dose level. (B) This graph summarizes the time course of cocaine inhibition of VTA GABA neuron ICPSDs at the 1.0 mg/kg dose level. Note the marked and pronounced inhibition of ICPSDs at 1 min with recovery in 30 min (n=5). (C) Cocaine significantly reduced ICPSDs at all dose levels tested, cocaine methiodide did not significantly alter them at any dose level, lidocaine, similar to cocaine, significantly reduced them at all dose levels tested, and methamphetamine did not significantly alter them at any dose level. Asterisks *,**, represent significance levels
P<0.005 and P<0.0005, respectively. (D) This graph summarizes the effects of DA receptor antagonists on iv cocaine inhibition of VTA GABA neuron ICPSDs at 1.0 min after injection. The ability of 1.0 mg/kg cocaine to reduce ICPSDs was not affected by a dose of saline administered 10 min prior to cocaine. Intravenous administration of the D1/D5 receptor antagonist SCH23390 had no effect on ICPSDs, nor on the ability of 1.0 mg/kg cocaine to reduce them when administered 10 min prior to cocaine (n=5). Similarly, iv administration of the D2/D3 receptor antagonist eticlopride had no effect on ICPSDs, or on the ability of 1.0 mg/kg cocaine to reduce them when administered 10 min prior to cocaine (n=5). Asterisks * represent significance levels P<0.005.

**Figure 3: Expression of TH and D2 receptors in dopamine versus GABA neurons**

![Graph showing expression of TH and D2 receptors in dopamine versus GABA neurons]

**Figure 3:** Single-cell quantitative RT-PCR was performed on neurons in the VTA of adult male rats. Both DA and GABA neurons expressed TH and D2 transcripts. However, the expression of TH and D2 receptors was much greater in DA neurons than GABA neurons. Asterisks **,** represent significance levels P<0.0002 and P<0.02, respectively.
**Figure 4: Cocaine reduces current-evoked spiking of VTA GABA neurons in vitro**

(A) These are representative current-clamp traces of current-evoked VTA GABA neuron spikes recorded in the horizontal midbrain slice preparation. Note the lack of spike accommodation characteristic of VTA GABA neurons (D. W. Allison et al., 2006). Superfusion of cocaine (50 µM) markedly reduced spiking at this level of depolarization (250 pA; darker line of the current steps that were performed). (B) This graph summarizes the effects of 50 µM cocaine on the frequency of current-evoked spiking of VTA GABA neurons at all current steps tested (25-400 pA steps shown in A). Superfusion of cocaine significantly reduced spiking at current steps 100-400 pA in all cells tested. Asterisk * indicates significance level P<0.03 at each point.
Figure 5: Concentration-dependent and use-dependent effects of cocaine on sodium currents in VTA GABA neurons in vitro

(Ai) This graph shows the “voltage waveform command” applied to VTA GABA neurons in voltage-clamp mode to elicit TTX-sensitive sodium currents (Aii,iii,iv). (Aii) shows the control current response, (Aiii) the effect of cocaine (7 µM) after 8 minutes, and (Aiv) the effect of 0.5 µM TTX showing abolishment of sodium currents. (B) Superimposed traces of the first current events (taken from points indicated by filled circles in Aii,iii) and last 5 current events (taken from point indicated by line on current traces Aii,iii) of the control and cocaine effect. (C) This graph summarizes the dose-dependent effects of cocaine on the first sodium current, the total of all currents, and the last 25 currents induced by the voltage waveform command. All concentrations of cocaine significantly reduced the total current and last 25 sodium current events. Only 15 and 50 µM significantly reduced the first current event. Asterisks **,***,**** indicate significance levels P<0.05, P<0.01 and P<0.001, respectively.
Figure 6: Cocaine reduces evoked and spontaneous GABA inhibitory synaptic transmission to VTA dopamine (DA) neurons in vitro

Figure 6: (A) This graph shows the time-course for the effects of cocaine (at the IC_{50} for inhibition of VTA GABA neuron sodium current) on VTA DA IPSCs. The inset shows representative superimposed traces of IPSCs (each trace is an average of 12 sweeps collected at 0.1 Hz) obtained in a DA neuron before (lighter trace) and 10 to 12 minutes after cocaine (darker trace). (B) Cocaine significantly decreased DA IPSCs at 7, 13 and 50 µM. (C) The inset shows superimposed representative traces of IPSCs (each is an average of 12 sweeps collected at 0.1 Hz) before (lighter trace) and 10 to 12 minutes after cocaine (darker trace). Cocaine increased the paired-pulse ratio of DA neuron IPSCs. (D) These insets show the effects of cocaine on spontaneous IPSCs (sIPSCs) recorded in a representative DA neuron. Cocaine (13 µM) significantly reduced the frequency, but not amplitude, of DA neuron
sIPSCs. (E) These insets show the effects of cocaine on miniature IPSCs (mIPSCs) recorded in a representative DA neuron after treatment with TTX. Cocaine (13 µM) had no effect on frequency or amplitude of TTX-insensitive miniature IPSCs. Asterisks *, **, *** indicate significance levels P<0.05, P<0.01 and P<0.001, respectively.
CHAPTER 3

Mefloquine Disrupts Ventral Tegmental Area Inhibitory and Excitatory Synaptic Activity via the Blockade of Connexin 36 Gap Junctions and Disruption of Intracellular Calcium Homeostasis

INTRODUCTION

The mesolimbic dopamine (DA) pathway is composed of DA neurons located in the ventral tegmental area (VTA) of the midbrain that project to the dorsal striatum, hippocampus, amygdala, and prefrontal cortex. This diffuse modulatory pathway is involved in attention to rewarding stimuli, learned behavior, and drug addiction (for review, (H. L. Fields et al., 2007; S. Ikemoto, 2007). It is believed that VTA DA neurons are inhibited by local GABA interneurons, and projection GABA neurons from other brain regions. There is evidence suggesting this DA pathway contains reciprocal excitatory, DAergic, and GABAergic communication with other brain regions (D. B. Carr and S. R. Sesack, 2000a, b; N. Omelchenko and S. R. Sesack, 2005, 2006; E. B. Margolis et al., 2008). Recent evidence suggests VTA GABA neurons express Connexin 36 (Cx36) gap junctions (GJs), dye couple (S. C. Steffensen et al., 1998; D. W. Allison et al., 2006), and form part of a larger syncytium of GABA neurons in the reticular formation that are linked by Cx36 electrical synapses (M. B. Lassen et al., 2007). Acute intoxicating doses of ethanol and GJ antagonists, including the Cx36-selective antagonist mefloquine, suppress electrical coupling between VTA GABA neurons in vivo (S. H. Stobbs et al., 2004).

Studying Cx36 GJ’s is made difficult by the lack of drugs that act specifically and exclusively on GJs. The most promising drugs are mefloquine (a Cx36 selective GJ blocker, MFQ) and carbenoxolone (CBX), both of which have non-GJ blocking affects (for review G. R.
Juszczak and A. H. Swiergiel, 2009). But despite their shortcomings, MFQ and CBX can be utilized to determine the neurophysiology of GJs if used in correctly designed and executed experiments that control for non-specific effects.

Mefloquine (MFQ) serves as a potent anti-malarial prophylaxis and treatment. It is well known that it may act as a neurotoxin due to a potential to cross the blood-brain barrier and accumulate to high concentrations (G. S. Dow et al., 2004). MFQ is linked to adverse side-effects ranging in severity from mild dysphoria to severe psychotic episodes or seizure (E. Wooltorton, 2002; T. M. Tran et al., 2006; P. Schlagenhauf et al., 2009). While MFQ’s adverse side-effects have not been attributed to any particular drug effect, its ability to affect synaptic activity suggests its neuropsychiatric side-effects derive from one, or a combination of its pharmacological actions. Owing to DA’s involvement in synaptic transmission, mood, and addiction, we hypothesized some of MFQ’s side-effects may result from MFQ’s blockade of Cx36 GJs in the VTA and point to the possible role for Cx36 GJs in regulation of DA. Specifically, we hypothesized that MFQ’s blockade of VTA Cx36 GJs might disrupt the network activity of electrically-coupled VTA GABA neurons, thereby altering inhibition of VTA DA neurons resulting in a change in DA neuron activity. To that end, we investigated the effects of MFQ and CBX on VTA DA and GABA neuron inhibition and excitation in both wildtype and Cx36 KO mice. We hypothesized that Cx36 GJs would play a significant role in regulating mesolimbic DA transmission.
METHODS

Preparation of Brain Slices

The care and use of animals and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University. Two different mouse models (age P21 – 35) were used in this study. A glutamic acid decarboxylase-67 (GAD67) linked to green fluorescent protein (GFP) knock-in mouse created on a CD1 background (N. Tamamaki et al., 2003), and a Cx36 KO mouse created on a C57BL6/129SvEv hybrid background, originally created by Dr. David Paul at Harvard Medical School (H. Arumugam et al., 2008) and donated to us by Dr. Marla Feller (University of California, San Diego, USA). The GAD67-GFP mice (also referred to as “WT”) afforded us the possibility of positively identifying and recording from GAD67 GABA neurons in the VTA. The Cx36 KO mice provided a comparison against mefloquine’s Cx36 GJ blocking effects on VTA dopamine (DA) neurons in GAD67-GFP mice. Midbrain slices were obtained as previously described (S. C. Steffensen et al., 2008). Briefly, mice were anesthetized with Ketamine (60 mg/kg), decapitated, and brains were quickly dissected and sectioned into 200 µm thick horizontal slices in ice-cold artificial cerebrospinal fluid (ACSF), bubbled with 95% O2 / 5% CO2. This cutting solution consisted of (in mM): 220 Sucrose, 3 KCl, 1.25 NaH2PO4, 25 NaH2CO3, 12 MgSO4, 10 Glucose, 0.2 CaCl2, and 0.4 Ketamine. These VTA-targeted horizontal slices were immediately placed into an incubation chamber containing normal ACSF at 34-35°, bubbled with 95% O2 / 5% CO2 at 36° consisting of (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 12 glucose, 1.5 MgSO4, 2 CaCl2, pH 7.3, and allowed to incubate for at least 45 minutes prior to being transferred to a recording chamber. Once transferred to a recording chamber with continuous normal ACSF flow (2.0 ml/min) maintained at 34-35° throughout the
experiment, the slices were then allowed to settle for an additional 15 minutes before recordings began. Cells were visualized with either a Nikon Eclipse FN1 or E600FN microscope in the transmitted de Sénarmont Differential Interference Contrast (DIC) / infrared (IR) configuration.

**Whole-cell Recordings in vitro**

Electrodes pulled from borosilicate glass capillary tubes were filled with one of two types of pipette solutions consisting of either (in mM): 128 KCl, 20 NaCl, 0.3 CaCl$_2$, 1.2 MgCl$_2$, 10 HEPES, 1 EGTA, 2 Mg-ATP, 0.25 Na-GTP and 4.5 QX314 (pH 7.3) for IPSCs, and 123 K-Gluconate, 8 NaCl, 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 2 Na3-GTP, and 4.5 QX314 (pH 7.3) for EPSCs and firing rate experiments. Pipettes had tip resistances of 2.5 - 4 MΩ, and series resistances typically ranging from 7 to 15 MΩ. Access resistance ($R_a$, typically 100 to 300 MΩ) and membrane resistance ($R_m$), were continuously monitored with a 5 mV voltage step delivered with each sweep (0.1 Hz) throughout the experiment. Only experiments that maintained stable $R_a$ and $R_m$ (less than 15% change) were included in this study. Voltage clamp recordings were filtered at 2 kHz while current-drive spikes were filtered at 6 kHz with an Axon Instruments Multiclamp 700B amplifier and digitized at 5 to 20 kHz respectively using an Axon 1440A digitizer. pClampv10, Mini Analysis (Synaptsoft: Decatur, GA), and Igor Pro (Wavemetrics: Oswego, OR) software packages software were utilized for data collection and analysis. Evoked and spontaneous IPSCs (sIPSCs) were recorded in the presence of D- 2-amino-5-phosphonopentanoic acid (D-APV, 50 μM), 6-cyano-23-dihydroxy-7-nitro-quinoxaline (CNQX, 30 μM), and eticlopride (100 nM) to block NMDA, AMPA, and DA D2-mediated synaptic currents respectively. Spontaneous and evoked EPSCs were recorded in the presence of bicuculline (10 μM), a concentration that eliminated all spontaneous inhibitory activity. Miniature IPSCs and EPSCs were isolated from all other spontaneous activity by addition of
lidocaine (500 µM). To evoke IPSCs and EPSCs, cells were stimulated at 0.1 Hz with a stainless steel-platinum/iridium concentric bipolar stimulating electrode placed ~100 µm rostral to the recording electrode. All evoked and spontaneous inhibitory and excitatory currents (except while examining AMPA to NMDA ratio) were inward at the holding potential of -70 mV and were completely blocked by bicuculline and APV, respectively.

Evoked current amplitudes were calculated by taking the difference between the 1.0 msec window around the peak and the 5.0 msec baseline window immediately preceding the stimulation artifact. The paired-pulse ratio was calculated by averaging traces from 2 minutes of data and then dividing the average secondary by the average primary. For spontaneous synaptic currents, the amplitude threshold was set to 5 times RMS, while area threshold was adjusted for excitatory and inhibitory currents respectively. Spontaneous and mini currents were then automatically detected and verified and corrected by hand. The AMPA to NMDA ratio was determined by first obtaining robust evoked EPSCs at -70 mV and then slowly stepping the holding potential to +30 mV. At +30 mV, a combined AMPA/NMDA current response was recorded for 2 min, then D-APV (50 µM) was applied. Once the NMDA current was completely diminished, the AMPA current response was recorded. Posthoc subtraction of the pure AMPA current from the combined AMPA/NMDA current yielded pure NMDA current. Each 2 minute recording period was averaged. The ratio was calculated as the peak average AMPA current amplitude of divided by the peak average NMDA current amplitude. Peak GABA(A) current (30 second bins) was acquired in the presence of the GABA(B) receptor antagonist CGP 35348 (100 µM) and calculated by comparing the average of 1 minute of control and test current at peak. Firing rate was calculated by comparing 5 minutes control and test data.
**Drug Preparation and Administration**

*In vivo:* MFQ (5mg MFQ, 800 µl distilled H2O, 200 µl Tween 80) was administered via an intra-peritoneal injection at 30mg/kg. *In vitro:* All drugs except D-APV, CNQX, CGP 35348, and bicuculline were prepared fresh. MFQ was prepared 1mg/mL water to obtain a 25 mM stock solution. In all experiments (except Figure 4) wherein brain slices were presoaked in MFQ, carbenoxolone (CBX), ondansetron (ODS), or mecamylamine hydrochloride (MEC), these drugs were added to the slice incubation chamber 10 minutes after slicing and allowed to presoak 60 to 90 minutes, then continuously perfused throughout the experiment. There are two advantages to this method of drug application over obtaining a viable recording and then applying drugs acutely and observing the effect progress over. First, any postsynaptic MFQ effects due to disruption of neuronal calcium levels are not washed out by pipette solution. Second, given that long quality recording periods can be problematical using the whole-cell patchclamp technique, presoaking enables long drug application times which more closely mimic the chronic exposure to MFQ in humans. For time course of MFQ effects (Fig 1) brain slices where allowed to settle after cutting for 45 minutes before recordings began.

**Characterization of Neuron Types**

*In vitro:* GAD67-GFP (wildtype) and Cx36 KO mice the combination of two electrophysiological characteristics, were used to identify putative DA neurons in this study. Specifically: neurons located in the VTA that exhibited a modest non-cation specific inward rectifying current ($I_h$) in combination with low input resistance were assumed to be DA neurons (S. W. Johnson and R. A. North, 1992; D. W. Allison et al., 2006; E. B. Margolis et al., 2006). In GAD GFP mice, GABA neurons were identified with the aid of fluorescence microscopy. Only neurons located in the VTA that exhibited robust GFP fluorescence were considered
GABAergic (N. Tamamaki et al., 2003). *In vivo:* all neurons classified as VTA GABA neurons were located in the VTA and met the criteria established in previous studies for spike waveform characteristics and response to IC stimulation (S. C. Steffensen et al., 1998; S. H. Stobbs et al., 2004; D. W. Allison et al., 2006)

**Statistical Analyses**

All results are presented as mean raw values and percent control ± SEM. Results between groups were compared using a two-tailed unpaired *t* test or ANOVA. Experiments relying on variance in time or current were analyzed using ANOVA with post hoc *t*-test at individual points. Statistical significance required ≥95% level of confidence (P≤0.05). Analysis software included Microsoft Excel, and Igor Pro (Wavemetrics, Oswego, OR) Stat Pak. Figures were constructed with Igor Pro software.
RESULTS

*Time Course of Mefloquine Effects VTA DA sIPSC Frequency*

In recent studies mefloquine (MFQ) was shown to quickly increase sIPSCs in substantia nigra pars compacta (SNc) dopamine (DA) neurons (EC$_{50}$ $\approx$ 3µM) (C. Zhou et al., 2006) and EPSPs in neocortical interneurons (IC$_{50}$ $\approx$ 15µM) (S. J. Cruikshank et al., 2004). However, the maximum effect times of MFQ differed in each study. We first determined the time required for 25 µM MFQ to reach maximum effect on VTA DA neuron sIPSC frequency, and whether there was a significant difference between WT and Cx36 mice in the time course of its effects. In Figure 1, MFQ (25 µM) was bath-applied in ACSF containing D-APV (50 µM) and CNQX (30 µM) and the frequency of VTA DA neuron sIPSCs was calculated at 15 minute intervals. MFQ increased sIPSC frequency incrementally and reached maximum effect at $\geq$60 minutes in wildtype. In wildtype mice, baseline sIPSC frequency increased from 7.3 ± 1.7 Hz (t=0) to: 8.4 ± 0.9 Hz (15 min); 18.9 ± 3.5 Hz (30 min); 19.2 ± 1.4 Hz (45 min); 59.8 ± 4.3 Hz (60 min); an increase of 772.3 ± 27.8% at the $\geq$60 minute time point. These sIPSCs were completely eliminated by bicuculline (10 µM, data not shown).

In Cx36 KO mice, MFQ also increased sIPSC frequency incrementally as in WT mice (Figure 1) and reached maximum effect at $\geq$60 minutes. However, in Cx36 KO mice the baseline frequency only increased from 7.3 ± 1.0 Hz (t=0) to: 7.5 ± 0.9 Hz (15 min); 13.7 ± 1.6 Hz (30 min); 21.9 ± 3.7 Hz (45 min); 32.2 ± 6.5 Hz (60 min); an increase of 441.4 ± 20.4% at the $\geq$60 minute time point.. The only point at which WT and Cx36 KO data differed significantly was at the $\geq$60 minute time point where MFQ increased WT mouse sIPSC frequency 331% in over Cx36 KO mice (ANOVA F=8.03, P=0.0001, DF=46).
Mefloquine Effects on VTA DA sIPSC Frequency, Amplitude, and Inter-event Interval in WT and Cx36 KO Mice

Having established the time course of the full-effect of MFQ on GABA(A) receptor-mediated DA neuron sIPSC frequency in WT and Cx36 KO mice, we next compared the effects of presoaked MFQ to other presoaked drugs on VTA DA neuron sIPSC frequency in wildtype and Cx36 KO mice. Because MFQ has effects other than being a selective Cx36 gap junction (GJ) blocker (such as the Ca^{2+} effect previously mentioned), we compared its effects to carbenoxolone (CBX, a less potent GJ blocker than MFQ) in WT and KO mice. We reasoned that if MFQ’s GJ blocking properties played a role in increasing sIPSC frequency, a less selective gap junction blocker such as CBX, which has different non-specific effects, would increase sIPSC frequency in similar to MFQ in wildtype mice, but perhaps not at all in Cx36 KO mice. As shown in Figure 2C, in WT mice MFQ (25 µM) increased VTA DA neuron sIPSC frequency from 7.7 ± 0.8 Hz to 40.9 ± 2.7 Hz (533 ± 35.4% of control, P=0.0001, MFQ n=28, control n=30). Carbenoxolone (100 µM) increased sIPSC frequency in WT mice from baseline to 16.2 ± 2.2 Hz (210.7 ± 28.3% of control, P= 0.0001, CBX n=9, Fig 2C). Only MFQ increased DA sIPSC average amplitude significantly (134 ± 13.8%, P<0.023; Figure 2C) suggesting a possible postsynaptic effect. MFQ and CBX both shifted the cumulative inter-event interval distribution plot to the left (Figure 2B), suggesting increased activity-dependent, and or non-activity dependent GABA release.

In Cx36 KO mice 25 µM MFQ increased DA neuron sIPSC frequency from 8.9 ± 1.7 Hz to 27.9 ± 3.7 Hz (311.4 ± 41.1% of control, P=0.0003, control n=10, MFQ n=8 Figure 2F), while CBX slightly reduced sIPSC frequency to 7.9 ± 0.6 Hz (88.5 ± 7.3% of control, P=0.64, CBX n=7, Figure 2F). As with WT mice, MFQ shifted the cumulative sIPSC inter-event
distribution plot markedly to the left in Cx36 KO mice (Figure 2E) indicating increased GABA release. This MFQ induced increase in Cx36 KO mouse VTA DA neuron sIPSC frequency was 58% of the total increase seen in WT mice (Figure 2C). This difference, in addition to the lack of CBX effect on sIPSC frequency in Cx36 KO mice (Figure 2E, F), reinforces the idea that the MFQ-induced increase in VTA DA neuron sIPSC frequency in WT mice was partly due to MFQ’s GJ blocking properties, and partly due to MFQ’s disruption of intracellular calcium (C. Zhou et al., 2006). CBX decreased DA sIPSC average amplitude significantly (decrease of 59.1 ± 3.6%, P<0.003; Figure 2H).

Although we doubted MFQ was acting as a GABA(A) receptor antagonist (A. J. Thompson et al., 2007), the possibility still existed it was increasing DA sIPSCs via 5HT3 receptor antagonism (A. J. Thompson and S. C. Lummis, 2008). To this end we also examined the specific 5HT3 receptor antagonist odansetron (ODS) in WT mice. Contrary to MFQ’s potentiation of sIPSCs, ODS markedly reduced VTA DA neuron sIPSC frequency from baseline to 2.0 Hz (decrease of 74 ± 7.1%, P=0.0006, ODS n=9, data only shown in bar graph, Fig 2C). A recent study by Zhou et al (2006) hypothesized that along with MFQ’s potent calcium-mediated effects it was also increasing sIPSC frequency by inhibition of cholinesterase (J. J. McArdle et al., 2006), thereby increasing GABA release via excess acetylcholine activation of presynaptic nAChRs. In that study, Zhou et al found pre-application of mecamylamine hydrochloride (MEC, a non- α7nAChR antagonist), which reduced baseline sIPSCs frequency by itself, also reduced the MFQ increase of DA sIPSC frequency ~70%. For this reason, we also examined the effect of presoaking slices with MFQ in the presence of MEC. Mefloquine + MEC presoaked together increased DA neuron sIPSC frequency similar to, and not significantly different from MFQ alone (488 ± 47.3% of control, MFQ + MEC n=6, P=0.58, Fig 2C), and
shifted the cumulative inter-event interval plot to the left similar to MFQ alone (Fig 2B). As neither ODS or MEC mimicked MFQ in WT mice, we did not examine these drugs in Cx36 KO mice.

It has been reported in rats that the co-activation of mGluR1 and NMDA glutamate receptors may increase sIPSC frequency, transforming dispersed GABAergic input on midbrain SNe DA neurons into a rhythmic pattern of sIPSCs frequency from 4 to 22 Hz (N. Berretta et al., 2001). Investigators in that study attributed this sIPSC frequency increase and onset of rhythmic sIPSC activity to increased electrical coupling of local GABA neurons connected via GJs. The present study demonstrates a marked increase in sIPSC frequency in part due to the blockade of Cx36 GJs in the presence of AMPA and NMDA receptor antagonists. As expected, we did not detect rhythmic sIPSC activity in either control or MFQ treated VTA DA neurons (data not shown), demonstrating increased sIPSC frequency and rhythmicity are not related.

**Mefloquine Effects on VTA DA mIPSC Frequency, Amplitude, and Inter-event Interval in WT and Cx36 KO Mice**

Spontaneous postsynaptic currents are a combination of activity-dependent (spike-dependent) and non-activity-dependent transmitter release. In order to determine what portion of MFQ and CBX-induced increase in sIPSC frequency was due to non-activity-dependent GABA release, we examined the effect of MFQ and CBX on WT and Cx36 KO mouse VTA DA neuron non-activity-dependent IPSCs or mini IPSC (mIPSC) frequency by adding lidocaine (500 uM) to the ACSF. In WT mice, MFQ (25 µM) increased VTA DA neuron mIPSC frequency from 7.6 ± 1.2 Hz to 21.9 ± 2.7 Hz (289.8 ± 35.5% of control, P<0.0001, control n=13, MFQ n=6, Figure 3C), had no significant affect on amplitude, but did significantly shift the mIPSC cumulative inter-event interval distribution plot to the left (Figure 3B), suggesting MFQ was acting
presynaptically to increase non-activity-dependent GABA release. Conversely, CBX (100 µM) had no significant affect on frequency, but significantly reduced mIPSC amplitude from 30.7 ± 6.5 pA to 13.8 ± 0.7 pA (-55.2 ± 2.3%, P<0.027, CBX n=11, Figure 3B, C).

In Cx36 KO mice, MFQ (25 µM) increased VTA DA neuron mIPSC frequency from 8.1 ± 1.8 to 29.0 ± 2.15 Hz (358.3 ± 43.0% of control, P=0.004, control n=4, MFQ n=5) and did not affect amplitude (Figure 3F). Similar to WT mice, Cx36 KO mouse mIPSC cumulative inter-event interval distribution plot was shifted to the left by MFQ (Figure 3E). We did not examine CBX effects on DA mIPSC frequency in Cx36 KO mice since it did not alter mIPSC frequency in WT mice.

The sIPSC and mIPSC data presented demonstrates MFQ and CBX were increasing activity-dependent and non-activity dependent GABA release in WT mice, but only non-activity dependent GABA release in Cx36 KO mice. This difference in effects between strains could possibly be explained by two separate mechanisms. We reasoned MFQ could be increasing the frequency of non-activity-dependent presynaptic GABA release in WT and Cx36 KO mice via MFQ’s presynaptic Ca\(^{2+}\) effects (G. S. Dow et al., 2003; C. Zhou et al., 2006; D. Caridha et al., 2008), and increasing activity-dependent presynaptic GABA release via both Ca\(^{2+}\) effects and selective blockade of Cx36 GJs (S. J. Cruikshank et al., 2004). The credibility of this scenario is bolstered by the results obtained from CBX, a drug which blocks GJs but does not increase intracellular Ca\(^{2+}\). Similar to MFQ, CBX increased DA neuron sIPSC frequency in WT. But unlike MFQ, it did not increase sIPSCs in Cx36 KO mice or increase mIPSCs in either mouse model.
Neither Presoaking nor Bath-application of Mefloquine Alters VTA DA Neuron Evoked IPSC Paired Pulse Ratio or IPSC Primary Current Amplitude

Because an increase in mIPSC frequency is generally associated with an increase in probability of transmitter release (R. C. Malenka and R. A. Nicoll, 1999), we next attempted to more firmly establish that MFQ was increasing VTA DA neuron sIPSC frequency, in part through a presynaptic mechanism, by examining MFQ’s effect on VTA DA neuron evoked IPSC (eIPSC) paired pulse ratio (PPR). A decrease in the PPR is commonly associated with an increase in probability of release at excitatory and inhibitory synapses in the hippocampus (R. Khazipov et al., 1995; S. Mennerick and C. F. Zorumski, 1995; D. Debanne et al., 1996; S. C. Steffensen et al., 1999), and the VTA (M. Melis et al., 2002). Contrary to what we expected, presoaking slices with MFQ (25 µM) did not significantly alter VTA DA neuron eIPSC PPR in WT mice (Control 0.65 ± 0.03, n=10; MFQ 0.71 ± 0.06, n=8, P=0.41, Figure 4A, C). To confirm that inhibitory synapses on VTA DA neurons obey the rule of exhibiting PPD during an increase in probability of release as observed by others (M. Melis et al., 2002; J. W. Theile et al., 2008), we conducted a separate set of experiments without MFQ in which extracellular Ca\(^{2+}\) was increased from 2 mM to 4 mM while monitoring primary IPSC amplitude, PPR, and sIPSC frequency. Indeed, as has been demonstrated ad nauseam (D. Debanne et al., 1996; L. E. Dobrunz and C. F. Stevens, 1997; M. O. Kravchenko et al., 2006), this increase of extracellular Ca\(^{2+}\) increased the primary IPSC amplitude 127.8 ± 7.2% of control (P=0.05), decreased the PPR 31 ± 2.1% (P=0.05), and increased sIPSC frequency from 7.0 ± 0.8 Hz to 15.1 ± 0.6 Hz (increase of 114%, P=0.04, data not shown). We reasoned MFQ’s robust ability to disrupt neuronal calcium stores (G. S. Dow et al., 2003; D. Caridha et al., 2008) might facilitate an increase in GABA release, but prevent the ability to measure this calcium-linked phenomenon via
established methods that rely on normal calcium-linked ready-releasable transmitter vesicle pool regulation (L. E. Dobrunz and C. F. Stevens, 1997).

Because MFQ’s ability to increase intracellular Ca\(^{2+}\) and block GJs may both contribute to increased sIPSC frequency, we reasoned that we might detect a change in the PPR ratio as MFQ begins to take effect at some time point from zero to 45 minutes. To explore this possibility we added mefloquine to ACSF containing D-APV and CNQX only after achieving a stable VTA DA neuron whole-cell recording, rather than presoaking the slice in MFQ (25 µM). Evoked IPSC primary current amplitude, PPR, and sIPSC frequency were measured continuously for up to 45 min. The average DA neuron evoked IPSC amplitude did not significantly change throughout these experiments (98.8 ± 6.5% of control, P=0.85, n=5, Figure 4B), and there was no significant change in the PPR (Control 0.7 ± 0.1, MFQ 0.7 ± 0.1, P=0.8, Figure 4B, C). However, the average VTA DA neuron sIPSC frequency following 45 minutes of MFQ perfusion was increased from 6.9 ± 1.4Hz to 14.8 ± 2.5Hz (219.7 ± 37.3% of control, P=0.02, Figure 4D, E). This MFQ-induced increase in sIPSC frequency obtained while continuously recording up to 45 minutes was not significantly different from the increase elicited by presoaked MFQ at the same time point diagramed in Figure 1.
Mefloquine Increases VTA DA Neuron GABA(A) Receptor Current

Given that MFQ increased the frequency and amplitude of activity-dependent VTA DA sIPSCs in WT mice (Figure 2C), we next examined the possibility of a postsynaptic component of MFQ effect. Bath application of GABA (100 µM) for 2.5 minutes in WT VTA DA neurons increased peak GABA current in MFQ presoaked slices from -51.7 ± 17.0pA to -211.4 ± 27.9pA (408.5 ± 54.0% of control, P=0.001, control n=6, MFQ n=10, Figure 5A, B), suggesting that in addition to an increase in GABA release presynaptically via Cx36 GJ blockade and possibly disruption of Ca$^{2+}$ homeostasis, MFQ was inducing a form of GABAergic postsynaptic LTP at VTA DA neuron inhibitory synapses. This GABAergic LTP could possibly involve the increase in number and or efficiency of postsynaptic GABA receptors on VTA DA neurons. Ca$^{2+}$-dependent long-term potentiation (LTP) at GABAergic synapses has been demonstrated in the hippocampus, (H. A. McLean et al., 1996; J. Kang et al., 1998; O. Caillard et al., 1999) as well as other brain regions including the cerebellum (M. Kano, 1994; S. Y. Kawaguchi and T. Hirano, 2007), neocortex (Y. Komatsu and Y. Yoshimura, 2000), and the VTA (F. S. Nugent et al., 2007). To our knowledge the present study is the first to demonstrate postsynaptic LTP at GABA(A) receptors on VTA DA neurons.

Mefloquine Does Not Affect VTA GABA Neuron sIPSC Frequency or eIPSC Paired-pulse Ratio

Mefloquine’s increase of VTA DA neuron sIPSC frequency is remarkable. We also examined MFQ’s effect on VTA GABA neuron sIPSC frequency, as well as eIPSC paired-pulse ratio in WT mice. Identification of VTA GABA neurons was aided by the use of GAD67-GFP mice (referred to as WT mice, N. Tamamaki et al., 2003). With the use of fluorescence microscopy, GAD67-GFP neurons could be easily identified. MFQ (25 µM) increased slightly,
but not significantly, sIPSC baseline frequency in VTA GABAergic neurons from 11.0 ± 1.4 Hz to 13.7 ± 1.9 Hz (124% of control n=17; MFQ n= 21, P=0.29, Figure 6 A, C). Mefloquine did not significantly alter sIPSC average amplitude or inter-event distribution plots compared to control (Figure 6 B, C). VTA GABA neuron control PPR was not changed in slices presoaked with 25 µM MFQ (control 1.0 ± 0.07, n=8; MFQ 0.84 ± 0.1, n=6, P=0.3, Figure 6 D, E). These finding demonstrate that mefloquine’s marked increase in VTA DA neuron sIPSC frequency was not a universal phenomenon common to all inhibitory synapses in the midbrain.

**Mefloquine Increases VTA DA Neuron sEPSC Frequency in Wildtype and Cx36 KO Mice**

When comparing experiments where slices were presoaked D-APV + CNQX + MFQ (Figure 1) verses MFQ alone (Figure 2C), we observed that presoaking slices with D-APV + CNQX + MFQ yielded a slight, but significant, increase in average sIPSC frequency over presoaking with MFQ alone in WT, but not Cx36 KO mice. This significant increase from 40.9 to 59.8 Hz (increase of 31.4 ± 2.3%, P<0.04) suggested a possible role for GJs in regulating excitatory synaptic activity in mouse VTA. Indeed, Cruikshank et al (2004) observed a MFQ (25 µM) increased sEPSPs in rat neocortex interneurons. In order to determine whether GJs play a role in regulating excitatory synaptic activity in mouse VTA, we examined MFQ effects on VTA DA neuron sEPSC frequency in wildtype and Cx36 KO mice, taking the same pharmacological approach as with VTA DA neuron sIPSCs; mainly examining the possibility that carbenoxolone (CBX) and odansetron (ODS) might mimic MFQ effects on sEPSC frequency. The reasoning for the use of these drugs was previously discussed. Briefly stated, CBX mimicking MFQ in wildtype but not Cx36 KO mice would portent the role for Cx36 GJs in regulating excitatory synaptic activity. In WT mice, VTA DA neuron sEPSC frequency was increased from 5.7 ± 0.6 Hz to 18.5 ± 3.0 Hz (324.5 ± 52.9% of control, control n=16, MFQ
n=15, P=0.0002, **Figure 7A, C** by MFQ (25 µM). CBX (3.9 ± 1.4 Hz, n=4, P=0.2, **Figure 7A, C**) did not mimic MFQ, suggesting that GJs are not involved in regulating GLUergic input onto VTA DA neurons. ODS did not mimic MFQ’s effects (4.9 ± 1.0 Hz, n=6, P=0.5, **Figure 7C**). We did not test the effects of CBX or ODS in KO mice, because they did not mimic MFQ in WT mice. In Cx36 KO mice, sEPSC frequency was increased from 6.8 ± 1.6Hz to 23.1 ± 4.2Hz (332.6 ± 59.9% of control, control n=6, MFQ n=10, P=0.01, **Figure 7D, F**). The difference in sEPSC frequency increase between WT and KO mice was not significant (P=0.3, **Figure 7C, F**). Mefloquine’s leftward shift of the sEPSC inter-event interval distribution plot was about equal in WT and KO mice as well (**Figure 7B, E**). Taken together, the similarity in MFQ effect on VTA DA neuron sEPSC frequency in WT and Cx36 KO mice suggests GJs do not play a role in regulating GLUergic input onto VTA DA neurons.

**Mefloquine Increases VTA DA Neuron mEPSC Frequency in WT Mice**

We next examined what portion of MFQ effects on VTA DA neuron sEPSC frequency were due to an increase in activity-dependent and non-activity-dependent GLU release and whether we should search for a postsynaptic component, we examined presoaked MFQ’s effects on mEPSC frequency. Since CBX did not increase sEPSC frequency, suggesting the lack of GJ involvement in MFQ’s effects on sEPSC, we conducted these experiments in WT mice only. Mini EPSCs were obtained by adding lidocaine (500 µM) to the ACSF. Mefloquine significantly increased mEPSC frequency from 6.7 ± 1.5 Hz to 16.9 ± 3.4 Hz (253.1 ± 51.4% of control, n=8; MFQ n=7, P=0.015, **Figure 8A, C**). This increase in mEPSC frequency was accompanied by a leftward shift in the inter-event-interval distribution plot (**Figure 8B**). The increase in mIPSCs was ~82% of total MFQ-induced sEPSC frequency increase. This difference
(while not significant) did hint at the remaining ~20% of MFQ increase could be due to an increase in activity-dependent GLU release alone and or a postsynaptic mechanism.

**Mefloquine Effects on VTA DA and GABA Neuron EPSC PPR and AMPAR to NMDAR Current Ratios**

As mentioned previously, an increase in mIPSC frequency is generally associated with an increase in probability of transmitter release (R. C. Malenka and R. A. Nicoll, 1999). We tested whether the MFQ-induced increase in mEPSC frequency was accompanied by EPSC paired-pulse depression in WT mice. Although excitatory synapses on DA neurons in the VTA have been shown to express PPD in association with an increase in probability of glutamate release (A. Bonci and R. C. Malenka, 1999), 25 µM MFQ did not increase the PPR in evoked EPSCs (control PPR 0.89 ± 0.04, n=10; MFQ PPR 0.88 ± 0.05, n=10, P=0.88; Figure 9A, B). Owing to observations in other studies that MFQ’s disruption of intracellular calcium may lead to alterations in synaptic activity (G. S. Dow et al., 2003; C. Zhou et al., 2006; D. Caridha et al., 2008), as well as the evidence in the this study suggesting a potent MFQ effect on intracellular calcium regulation, we reasoned that as with the IPSC PPR, MFQ’s disruption of intracellular calcium might increase non-activity-dependent transmitter release but somehow negate the ability to measure the increase in probability of transmitter release through the paired-pulse paradigm.

Given MFQ’s potent calcium affects, and the role calcium plays in affecting plasticity at excitatory synapses (for review R. C. Malenka and R. A. Nicoll, 1999) we examined the possibility of postsynaptic modification at VTA DA neurons. Presoaking slices with 25 µM MFQ increased the AMPA to NMDA current ratio of 0.40 ± 0.06 (n=5) to 0.67 ± 0.05 (n=5, P=0.01, Figure 9C, D).

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Because MFQ robustly enhanced sEPSC frequency at VTA DA neuron synapses through a non-GJ-blocking mechanism, we reasoned MFQ may have the same effect at excitatory VTA GABA neuron synapses. As with previous experiments examining VTA GABA neurons, these cells were identified by virtue of their ability to fluoresce. Using the same experimental protocol as in previous experiments; presoaking slices in 25 µM MFQ increased VTA GABA neuron sEPSC frequency from 7.4 ± 1.0 Hz to 22.4 ± 3.3 Hz (303.4 ± 42.6% of control, control n=14, MFQ n=13, P=0.0002, data not shown) and shifted the inter-event interval distribution plot to the left compared to control (data not shown). Consistent with VTA DA neuron IPSC and EPSC paired pulse ratio data presented previously, MFQ did not increase the VTA GABA neuron eEPSC PPR (Figure 9 E, F). Unlike VTA DA neurons, MFQ did not increase the VTA GABA neuron AMPA to NMDA current ratio (Control 0.84 ± 0.15, n=4; MFQ 0.65 ± 0.21, n=5, P=0.5, Figure 9 G, H) which is consistent with previous studies that demonstrate VTA GABA neurons to not express LTP (A. Bonci and R. C. Malenka, 1999). Mefloquine’s ability to increased sEPSC frequency presynaptically at VTA DA and GABA neuron excitatory synapses is suggestive of presynaptic homogeneity at excitatory synapses on both cell types.

**MFQ Reduces VTA DA Neuron Firing Rate and Current-evoked Spiking**

The data obtained thus far points to Cx36 GJs playing a role in regulating VTA DA neuron inhibition but little or no role in regulating excitation. But whether the increase in VTA DA neuron sIPSC frequency due to blockade of Cx36 GJs in the face of MFQ’s other non-specific effects (the increase in VTA DA and GABA neuron sEPSC frequency) would result in VTA DA dysregualtion remained unknown. To address this question we examined the net effect of MFQ on VTA DA neuron firing rate, current-evoked spiking, and resting membrane potential (RMP). VTA DA neuron firing rate was reduced by 25 µM MFQ from 1.7 ± 0.4 Hz to 0.6 ± 0.3
Hz (32.8 ± 17.0% of control, control n=11, MFQ=9, P=0.036, **Figure 10B**). Current-evoked spiking was also reduced significantly at all currents greater than 125pA (P=0.001, **Figure 10D**). Interestingly, there was no significant difference in VTA DA neuron RPM between control and MFQ treated slices (Control -47.1 ± 2.3mV, MFQ 47.25 ± 3.8mV, P=0.97).
DISCUSSION

The aim of this study was to determine the role Cx36 GJs play in regulating synaptic activity in the VTA in mice. We were able to identify VTA GABA neurons through the use of GAD67-GFP mice, while DA neurons in GAD67-GFP and Cx36 KO mice were identified by previously tested electrophysiological characteristics. The selective Cx36 GJ-blocker mefloquine (MFQ) and the less selective GJ-blocker carbenoxolone, in conjunction with the use of Cx36 KO mice, where utilized to determine the role Cx36 GJs play in VTA synaptic activity. Mefloquine’s presynaptic increase in VTA DA sIPSCs in WT mice can be divided into two components: an increase in non-activity-dependent GABA release, most-likely attributed to MFQ’s ability to increase intracellular calcium, and an increase in activity-dependent GABA release that we attribute to MFQ’s selective blockade of Cx36 GJs. This is evidenced by the fact that MFQ increased VTA DA neuron sIPSC frequency ~533% in WT mice, but only ~310% in Cx36 KO mice. Additionally, mIPSCs (a measure of non-activity-dependent GABA release) were increased ~300% in both WT and Cx36 KO mice, but carbenoxolone (CBX), a less specific GJ blocker then MFQ, only increased sIPSC frequency in wildtype mice and did not affect minis. In addition to this increase in presynaptic GABA release, MFQ also affected postsynaptic changes on VTA DA neurons by increasing the number and or function of GABA(A) receptors. Of great interest was our discovery that MFQ did not increase VTA GABA neuron sIPSCs, suggesting VTA GABA and DA neuron inhibitory synapses are not physiologically homogenous, and that Cx36 GJs are not involved in regulating VTA GABA neuron inhibition. The differential effect of MFQ on DA and GABA neuron sIPSCs establishes the possibility that the negative side-effects of MFQ seen in humans may be related to dysregulation of VTA DA neuron inhibition.
MFQ increased sEPSCs markedly in VTA DA and GABA neurons, a similar phenomenon to that observed by Cruikshank et al (2004) in rat neocortex. We could not attribute this increase to MFQ’s GJ blocking properties. Mefloquine’s actions at both VTA DA and GABA excitatory synapses were due to an increase in presynaptic GLU release. However, MFQ appeared to only have postsynaptic effects, in the form of an increase in AMPAR to NMDAR receptor mediated current ratio, in DA neurons. This increase in AMPAR to NMDAR ratio may involve the increase in number and or function of AMPARs or the decrease in number and or function of NMDARs, or some combination of the two. These findings validate observations by Bonci et al (1999), that only VTA DA neurons are capable of this excitatory postsynaptic plasticity.

We demonstrated the overall net physiologic effect of MFQ was to decrease in VTA DA neuron firing rate. This net effect of MFQ on VTA synaptic activity demonstrates the importance of GJs in regulating VTA synaptic activity and poses a scenario in which midbrain synaptic activity is disrupted sufficiently to provide the physiologic framework to explain many of MFQ’s adverse side effects in humans. Alterations at these important synapses may lead to downstream behavioral changes (J. L. Gaiarsa et al., 2002; F. S. Nugent and J. A. Kauer, 2008).

Typically, an increase in the probability of transmitter release would be accompanied by an increase in the PPR. This was not the case in this study with eIPSCs and eEPSCs. We attribute the absence of PPD in DA and GABA neurons to the possibility that MFQ’s potent disruption of intracellular calcium homeostasis prevented the ability to detect this calcium-linked phenomenon. It is believed that the entry of calcium through presynaptic voltage-sensitive
calcium channels leads to the release of transmitter via the fusion of synaptic vesicles from a readily-releasable pool of vesicles (D. Debanne et al., 1996; C. Rosenmund and C. F. Stevens, 1996; L. E. Dobrunz and C. F. Stevens, 1997). The synaptic machinery regulating the number and fate of vesicles in this readily-releasable pool are regulated in part by calcium levels within the presynaptic bouton. The paired-pulse test paradigm relies partly on the constant kinetics of the replenishment of this readily-releasable pool, and the role calcium plays in replenishing this pool. We theorize that MFQ’s potent ability to release calcium from internal stores and allow it to enter from the external environment (G. S. Dow et al., 2003; C. Zhou et al., 2006; D. Caridha et al., 2008) would alter all calcium-related processes in the presynaptic bouton, including the replenishment kinetics of the readily-releasable pool, decreasing the odds of a depleted pool in the face of increased probability of release. Additional evidence suggesting MFQ treated synapses violate traditionally accepted measures of synaptic activity is our observation that bath-applied MFQ did not increase the amplitude of the primary IPSC in conjunction with an increase in mIPSC frequency or increase in the AMPAR to NMDAR current ratio.

We are not alone in the observation that MFQ increases inhibition of midbrain DA neurons, or the idea that disruption of calcium regulation is involved. Zhou et al (2006) found that in acutely dissociated brain slices, 3 µM MFQ induced a small increase in sIPSC frequency in midbrain DA neurons. At 25 µM, we were unable to substantiate Zhour et al’s claims that MFQ’s increase in midbrain DA neuron sIPSC frequency was facilitated through cholinesterase inhibition, but we did provide support for the role calcium plays. A possible explanation for the difference in concentrations of MFQ in these studies may be that effectual concentrations of drug are lower in whole-brain tissue as a result of diffusion through brain slices. The difficulty of MFQ’s ability to penetrate brain slice tissue is made more difficult by its propensity to bind
tightly to phospholipid membranes. We tested the effects of odansetron as a control against MFQ’s reported 5HT3 receptor antagonism, to eliminate the possibility the increase in sIPSC and sEPSC frequency could be due to this MFQ effect. MFQ’s effects on sIPSCs and sEPSCs were not mimicked by odansetron, but odansetron suppressed both sIPSC and sEPSC frequency, presenting another possible non-specific drug effect of MFQ that could contribute to its side effects reported in humans.

Electrical synapses (composed of Cx36 GJs) connecting inhibitory interneurons are common in certain areas of the brain (for review B. W. Connors and M. A. Long, 2004), including the midbrain. We have previously shown VTA GABA neurons express Cx36-mediated electrical synapses (S. C. Steffensen et al., 1998), dye couple, can be blocked by MFQ (D. W. Allison et al., 2006), and appear to form part of a larger syncytium of GABA neurons in the reticular formation (M. B. Lassen et al., 2007). While there is evidence to suggest the current sharing and synchronizing ability of electrical synapses can be modulated (C. E. Landisman and B. W. Connors, 2005), to our knowledge we are the first to provide evidence of electrical synapses regulating interneuron inhibition of the primary neuron populations they target. A possible explanation for this data is that when MFQ blocks electrical synapses the electrically-coupled VTA GABA network becomes uncoupled, releasing each cell from the resistive network load. The net result being individual GABA neuron activity is no longer dampened, resulting in increased GABA release onto target neurons. Under this model, these electrical synapses may serve to synchronize spiking and propagate action potentials when the network is tightly coupled, or serve as resistive loads that decrease synchrony and dampen overall network activity when loosely coupled (R. W. Joyner et al., 1983; R. W. Joyner et al., 1984). The findings of this study
strengthen the argument that GJs serve as critical modulators of brain activity and targets for the clinical treatment of many disorders involving the mesolimbic DA system.
Figure 1: Time Course of Mefloquine Effects on VTA DA sIPSC Frequency

Figure 1: VTA DA neuron sIPSCs were recorded at 15 min intervals in wildtype (WT, open circle) and Cx36 KO (filled squares) mice with bath-applied MFQ (25 µM), D-APV (50 µM), and CNQX (30 µM). MFQ increased sIPSC frequency similar in both WT and Cx36 KO mice until the 60+ min time point when the percent increase difference between WT and KO mice was 331%, P=0.0001. Asterisks ** indicate significance level P<0.01.
Figure 2: Mefloquine Effects on VTA DA Neuron sIPSC Frequency, Amplitude, and Inter-event interval in WT and Cx36 KO Mice

Figure 2: This figure compares WT (A-C) and KO (D-F) VTA DA neuron IPSC data after being presoaked with either MFQ (25 µM), CBX (100 µM), ODS (25 µM), or MFQ (25 µM) + MEC (10 µM) for ≥60 minutes. In WT mice (C), sIPSCs were increased similarly by MFQ (533%) and MFQ+MEC (488%), CBX increased sIPSC frequency 211%, but ODS did not mimic MFQ. In WT mice MFQ and MFQ+MEC shifted the inter-event interval curve to the left (B), while CBX shifted the curve slightly to the left. In Cx36 KO mice, only MFQ increased sIPSC frequency (F) and shifted the inter-event interval curve to the left (E). Asterisks *, **, *** indicate significance levels P<0.05, P<0.01 and P<0.001, respectively.
Figure 3: Mefloquine Effects on VTA DA mIPSC Frequency, Amplitude, and Inter-event Interval in WT and Cx36 KO Mice

Figure 3: This figure compares WT (A-C) and KO (D-E) VTA DA neuron mIPSC data after being presoaked with MFQ (25 µM) or CBX (100µM) for ≥60 minutes. In WT mice (C), mIPSCs were increased (289%) by MFQ but not at all by CBX (100µM). In WT mice only MFQ shifted the inter-event interval curve to the left (B). In Cx36 KO mice, MFQ (25µM) increased sIPSC frequency 358% (F) and shifted the inter-event interval plot to the left (E). Asterisks *, **, *** indicate significance levels P<0.05, P<0.01 and P<0.001, respectively.
Figure 4: Neither Presoaking nor Bath-application of Mefloquine Alters VTA DA Neuron Evoked IPSC Paired-pulse Ratio or IPSC Primary Current Amplitude

Figure 4: This figure diagrams presoaked (A,C) MFQ (25µM) effects on VTA DA neuron eIPSC PPR, and bath-applied (B,C,D,E) MFQ (25µM) on VTA DA neuron eIPSC PPR and sIPSC frequency in WT mice. Panel A shows raw side-by-side VTA DA neuron evoked IPSCs paired-pulse traces (each trace is an average of 12 sweeps collected at 0.1 Hz) not presoaked (Control) and after being presoaked (Presoaked) ≥60 minutes with MFQ (25µM) in WT mice. (B) This panel shows similar traces to (A) except these traces represent the same neuron before and after bath-application of MFQ (25µM) for 45 minutes. Neither presoaking (A,C) nor bath-application of MFQ (25µM, B,C) changed the eIPSC PPR. Bath-applied MFQ, did not increase the amplitude of the primary eIPSC (B). Bath-applied MFQ did increase sIPSC frequency (220%). Asterisk * indicates significance level P<0.05.
Figure 5: Mefloquine Increases VTA DA Neuron GABA(A) Receptor Current

Figure 5: This figure illustrates MFQ (25µM) effects on WT VTA DA neuron GABA(A) receptor-mediated current in the presence of CGP 35348 (100 µM). Panel A contains 8.5 minutes of sample data where GABA (100µM) was bath-applied for 2.5 minutes in control and MFQ presoaked slices while recording baseline holding current in VTA DA neurons. The periodic outward events are capacitative current used to monitor Ra. Inward events are sIPSCs. (B) Bath-application of GABA (100µM) for 2.5 minutes evinced a 4 fold increase in GABAA-mediated current amplitude (30 second bins) in MFQ-presoaked slices over control. ** indicates significance level P<0.01.
Figure 6: Mefloquine Does Not Affect VTA GABA Neuron sIPSC Frequency or Paired-pulse Ratio

Figure 6: This figure diagrams presoaked MFQ (25µM) effects on WT VTA GABA neuron sIPSCs and eIPSC PPR. MFQ did not increase VTA GABA neurons sIPSC frequency (B) or shift the inter-event distribution plot (B). (D) Side-by-side VTA GABA neuron evoked IPSCs paired-pulse traces (each trace is an average of 12 sweeps collected at 0.1 Hz) before and after being presoaked ≥ 60 min with MFQ. (E) This plot summarizes the VTA GABA neuron paired-pulse ratio (PPR) data. MFQ yielded no significant change in the VTA GABA neuron IPSC PPR.
Figure 7: Mefloquine Increases VTA DA Neuron sEPSC Frequency in Wildtype and Cx36 KO Mice

Figure 7: This figure compares WT (A-C) and KO (D-E) VTA DA neuron sEPSC data after being presoaked with MFQ (25µM), CBX (100µM), or ODS (25µM) for ≥60 minutes. (C) In WT mice, sEPSCs were increased (325%) by MFQ but not by CBX. In WT mice, only MFQ shifted the sEPSC inter-event interval curve to the robustly to the left (B). In Cx36 KO mice, MFQ increased sEPSC frequency 333% (F) and shifted the inter-event interval curve to the left (E). Asterisks *, ** indicate significance levels P<0.05, and P<0.001, respectively.
Figure 8: Mefloquine Increases VTA DA mEPSC Frequency in WT Mice

Figure 8: This figure illustrates WT (A-C) VTA DA neuron mEPSC data after being presoaked with MFQ (25µM) for ≥60 minutes. (C), mIPSCs were increased (253%) by MFQ. MFQ shifted the inter-event interval curve to the left (B). Asterisk * indicates significance level P<0.05.
Figure 9: MFQ Effects on VTA DA and GABA Neuron EPSC PPR and AMPAR to NMDAR Current Ratio

This figure compares MFQ’s (25µM) effects on VTA DA (A-D) and GABA neuron (E-F) eEPSC PPR and AMPAR to NMDAR current ratios. Panel A and E show side-by-side sample VTA DA neuron and GABA neuron evoked EPSC paired-pulse traces respectively (Each trace is an average of 12 sweeps collected at 0.1 Hz). Neither the VTA DA nor GABA neuron PPRs were altered by MFQ (B and F). MFQ increased the AMPAR (thick line) to NMDAR (thin line) current ratio in VTA DA neurons from 0.40 ± 0.06 to 0.67 ± 0.05, P=0.01 (C and D), but did not significantly alter VTA GABA neuron AMPAR to NMDAR current ratio (G and H).
Figure 10: MFQ Reduces VTA DA Neuron Firing Rate and Current-evoked Spiking

Figure 10: This graph illustrates the effects of MFQ (25µM) on WT mouse VTA DA neuron spontaneous firing rate (A,B) and current-evoked spiking (C,D). (A) Raw traces of VTA DA neuron voltage spikes. (B) This panel shows the summary of all spontaneous firing rate experiments, control (lighter dots) MFQ (darker dots). Panel C shows raw traces of current-evoked spiking. MFQ reduced current-evoked spiking at all currents greater than 125pA (D).
CHAPTER 4

CONCLUSION

The rationale for the cocaine and mefloquine research presented here is predicated on the belief that advancement in the understanding of the brain mechanisms underlying the recreational use and abuse potential of cocaine, alcohol and other drugs of abuse will pave the way for more effective treatment strategies that would save lives and resources throughout the world. Specifically, a better understanding of the role Cx36 GJ connected VTA GABA neurons play in mediating cocaine addiction, and regulation of the mesolimbic dopamine (DA) pathway in the brain. To what end does the research presented here further the aim of that rationale, and what implications toward future work does it hold? Taking these two studies as one body of research, they provide compelling evidence for a non-DA-mediated pathway of cocaine addiction discussed previously. They also provide support for electrically coupled GABA neurons as key regulators of mesolimbic DA activity. In addition these studies present electrically coupled GABA neurons as possible candidates for a separate but parallel non-DA-dependent pathway of addiction for other drugs of abuse such as ethanol, heroin, and nicotine.

The in vivo and in vitro findings in Chapter 2 provide evidence for cocaine’s anesthetic actions to reinforce its DAT blocking properties, and support for the idea of a non-DA-dependent mechanism of cocaine reinforcement and reward. In vivo, cocaine and lidocaine both markedly reduced VTA GABA neuron firing rate and internal capsule driven postsynaptic discharges (ICPSDs), showing cocaine’s ability to block a putative measure of electrical coupling. In vitro, cocaine reduced current-driven spikes in VTA GABA neurons via its block of VSSCs leading to a marked decreased VTA GABA neuron inhibition of VTA DA neurons; demonstrating
cocaine’s addictive liability may be greatly increased if its DAT blocking and anesthetic actions can combine to simultaneously increase DA in the NAcc by blocking DAT and increase DA release by acting to disinhibit DA neurons in the VTA. This study underscores cocaine’s dangers as an illicit compound, provides direct evidence of VTA GABA neuron regulation of mesolimbic DA, and highlights the important role electrical connectivity of VTA GABA neurons plays in regulating VTA DA neurons.

It should be noted that any research examining the role that electrically-coupled VTA GABA neurons play in addiction and regulation of the mesolimbic DA system encounters the same stumbling blocks: the lack of specific GJ blockers and the inability to definitively indentify GABA neurons in vitro. The study presented in Chapter 3 attempts to overcome these stumbling blocks by combining the most selective, albeit non-specific, GJ blocking drugs, mefloquine (MFQ) and carbenoxolone (CBX), with the use of GAD67-GFP and Cx36 KO mouse models. Some studies have relied exclusively on the use of Cx36 KO mice to study electrical synapses. By utilizing wildtype and KO mice in conjunction with more than one GJ blocking drug we avoid many of the pitfalls associated with using only KO mice.

The integral role that electrically-connected VTA GABA neurons play in regulating VTA DA neurons was addressed in Chapter 3. This research demonstrated that inhibition of VTA DA neurons could be increased significantly by decreasing the electrical connectivity of VTA GABA neurons. To our knowledge we are the first to demonstrate GJs playing a critical role in regulating a well-established physiologically relevant activity in the brain such as excitatory and inhibitory synaptic activity. With this data, and other evidence suggesting electrical coupling may play a role in many phenomena such as: rhythmic activity (N. Berretta et al., 2001; M. A. Long et al., 2002; M. A. Long et al., 2005); propagation of slow calcium waves (L. R. Wolszon
et al., 1994); learning and memory; epilepsy (V. M. Nemani and D. K. Binder, 2005); etc (for review, B. W. Connors and M. A. Long, 2004), we are loath to envision GJs as anything less than critical brain machinery. This work supports the belief that by virtue of their dynamic range (high discharge rate and short refractory period), widespread distribution to structures implicated in drug reward, and their electrical network properties, VTA GABA neurons are a prime target and focal point for addiction research.
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