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I_h blockade reduces cocaine firing patterns of putative dopaminergic neurons of the ventral tegmental area in the anesthetized rat

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Abstract: The hyperpolarization-activated cation current (I_h) is a determinant of intrinsic excitability in various cells, including dopaminergic neurons (DA) of the ventral tegmental area (VTA). In contrast to other cellular conductances, I_h is activated by hyperpolarization negative to -55mV and activating I_h produces a time-dependent depolarizing current. Our laboratory demonstrated that cocaine sensitization, a chronic cocaine behavioral model, significantly reduces I_h amplitude in VTA DA neurons. Despite this reduction in I_h, the spontaneous firing of VTA DA cells after cocaine sensitization remained similar to control groups. Although the role of I_h in controlling VTA DA excitability is still poorly understood, our hypothesis is that I_h reduction could play a role of a homeostatic controller compensating for cocaine-induced change in excitability. Using in vivo single-unit extracellular electrophysiology in isoflurane anesthetized rats, we explored the contribution of I_h on spontaneous firing patterns of VTA DA neurons. A key feature of spontaneous excitability is bursting activity; bursting is defined as trains of two or more spikes occurring within a short interval and followed by a prolonged period of inactivity. Burst activity increases the reliability of information transfer. To elucidate the contribution of I_h to spontaneous firing patterns of VTA DA neurons, we locally infused an I_h blocker (ZD 7288, 8.3 μM) and evaluated its effect. I_h blockade significantly reduced firing rate, bursting frequency, and percent of spikes within a burst. In addition, I_h blockade significantly reduced acute cocaine-induced spontaneous firing rate, bursting frequency, and percent of spikes within a burst. Using whole-cell patch-clamp, we determine the progressive reduction of I_h after acute and chronic cocaine administration (15mg/kg intraperitoneally). Our data show a significant reduction (~25%) in I_h amplitude after 24 but not 2 hours of acute cocaine administration. These results suggest that a progressive reduction of I_h could serve as a homeostatic regulator of cocaine-induced spontaneous firing patterns related to VTA DA excitability.

Keywords: cocaine; ventral tegmental area; dopamine; electrophysiology; substance abuse disorder; rats; I_h current; neuronal excitability

1. Introduction

Hyperpolarization-activated cation current (I_h) is a significant modulator of intrinsic excitability on neurons of the mesocorticolimbic (MCL) system, including dopaminergic cells (DA) from the ventral tegmental area (VTA) [1–3]. Neuroadaptations in this network are hypothesized to trigger substance abuse disorder. In contrast to other ionic conductances, I_h is a slowly activating cation inward current activated by hyperpolarizing inputs negative to -55 mV. I_h activation depolarizes the membrane to a threshold level for the generation of action potentials [4]. Additionally, I_h is involved in various neuronal properties such as the control and modulation of the rhythmic activity of neural circuits [5,6], regulation of the resting membrane potential [7], firing frequency modulation [8,9], regulation of synaptic transmission [10,11] and dendritic integration of synaptic inputs [12–14]. On VTA DA neurons, I_h is classified as an excitatory driving force [15–18].

Neuromodulation of intrinsic properties is a decisive determinant of neuron excitability [19,20]. Intrinsic excitability is a measure of the neuron's inherent electrical properties that can be adjusted by numerous factors [21,22]. These adjustments arise through changes in morphological features and the expression level or biophysical properties of ion channels in the membrane [23]. The activity of a single neuron results from the summation of excitatory and inhibitory synaptic inputs, and the intrinsic membrane properties [24]. Neuroadaptations can be categorized as either homeostatic, that contribute to stabilizing neuronal excitability, or non-homeostatic, that produce an alteration from basal excitability [19,25,26]. Homeostatic changes in neuronal excitability can arise in response to long periods of altered activity [27–29]. For example, 3 hours after acute cocaine administration, activity of VTA DA neurons recorded in vivo, is significantly increased [30]. Our laboratory demonstrated that cocaine sensitization, a chronic cocaine behavioral model, significantly reduces I_h amplitude by ~40% in VTA DA neurons [31]. This reduction of current occurs concomitantly with the reduction of membrane surface HCN2 protein, the main HCN subunit in VTA DA neurons [32]. An important observation is that after the development of cocaine sensitization, the spontaneous firing of VTA DA cells remains similar to that of control groups [31]. These findings suggest that I_h reduction could reduce cocaine-induced excitability as a homeostatic adaptation to regulate neuronal excitability of VTA DA neurons.

I_h increases burst firing by enhancing rebound spiking after the disinhibition of VTA DA neurons [33]. Additionally, pharmacological blockade of this current on VTA DA neurons can significantly decrease (by 40%) the firing rate in the majority of these neurons; this reduction is suggested to be behaviorally significant [34]. While this study indicates that the modulation of I_h can have a role in controlling VTA DA neuronal excitability, the relationship is still poorly understood. Hence, the study of I_h modulation on spontaneous firing activity could lead us to elucidate its contribution to VTA DA neuronal excitability.

To determine if reduction of I_h can alter basal VTA DA neuronal excitability, we evaluated the effect of I_h blockade on spontaneous firing patterns using in vivo single-unit extracellular recordings. VTA DA neurons

can be divided by their firing patterns, classified by the average firing rate and the percentage of spike within a burst (%SWB) [35]. To investigate how I_h modulates cocaine-dependent excitability, we measured I_h using whole-cell patch-clamp electrophysiology, 2 and 24 hours after acute cocaine injection. Additionally, we determined the effect of I_h blockade on cocaine-induced spontaneous firing patterns using in vivo single-unit extracellular recordings. We postulate that a progressive I_h reduction serves as a homeostatic regulator to oppose cocaine-induced excitability in VTA DA neurons.

2. Results

2.1. Effect of blockade of I_h on in vivo anesthetized single-unit spontaneous VTA DA neuron firing.

To evaluate the effect of I_h blockade on in vivo anesthetized spontaneous firing, we used double barrel pipettes [36]. These pipettes were comprised of a recording electrode and a perfusion pipette that contained ZD 7288 (8.3 μ M). We recorded a minimum of 100 seconds of baseline extracellular firing activity and then locally perfused at the vicinity of the recorded cell, 60nL of ZD 7288 to see the effect of I_h blockade on neuronal firing patterns as seen on sample traces (**Figure 1A**). Local infusion of ZD 7288 significantly reduced the firing rate on VTA DA neurons (**Figure 1B**). The bursting frequency was also significantly reduced by I_h blockade (**Figure 1C**). This finding provides the first evidence that I_h modulates the bursting activity of VTA DA neurons. Moreover, the percent of spikes in bursts was also significantly decreased by I_h blockade (**Figure 1D**). The mean inter-spike intervals (ISI) from each recorded neuron were analyzed. As shown in (**Figure 1E**), the naïve group mean ISI was significantly increased after ZD 7288 local infusion (paired t test, $t = 3.075$, $df = 19$; $n = 8$ rats $c = 20$ cells). When comparing the total population of the naïve cells' with gaussian best fit for mean ISI, we found a significant increase from $0.1025 \pm 0.02122s$ to $0.1750 \pm 0.02497s$ after ZD 7288 infusion (**Figure 1F**). On (**Figure 1G**), a representative cell ISI histogram of the naïve group was plotted. Gaussian best fit values resulted in an increase baseline mean ISI from $0.0797 \pm 0.0003s$ to $0.2128 \pm 0.0046s$ after ZD 7288 infusion. These results suggest that I_h blockade can significantly reduce firing patterns in VTA DA neurons in part due to an increase in mean ISI. Additionally, through I_h modulation, we could control the firing activity of VTA DA neurons.

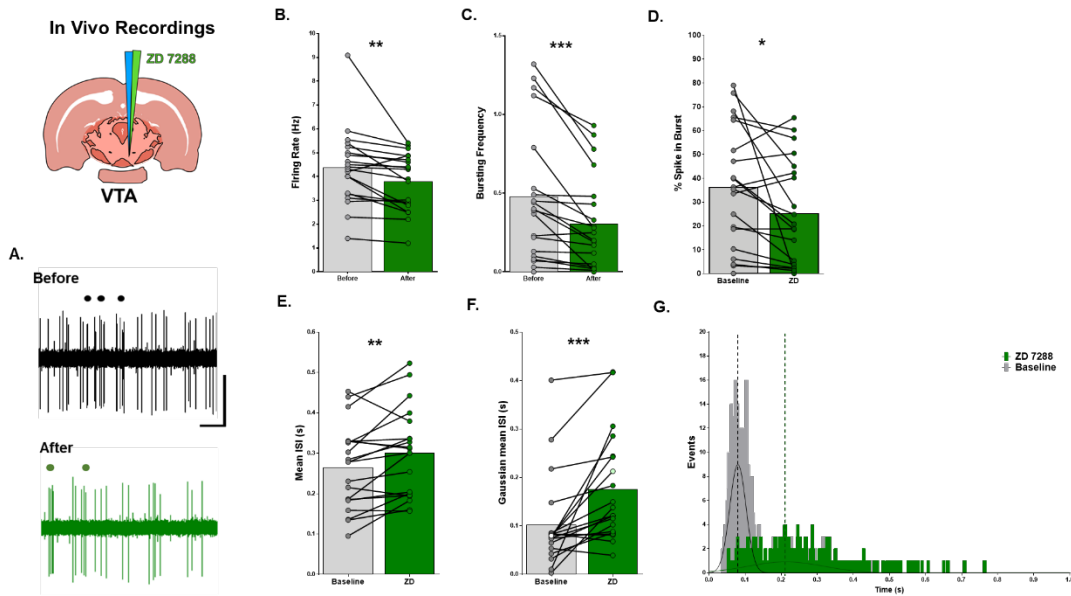


Figure 1. I_h blockade significantly reduced firing properties on in vivo extracellular recordings of VTA DA neurons. **1A.** Sample traces before and after I_h blocker ZD 7288 (8.3 μ m) perfusion. Each dot represents a burst event. Scale bars: horizontal, 1 s; vertical, 1 mV. **1B.** I_h blockade significantly reduced the firing rate (paired t test, $t=2.912$ $df=19$). **1C.** I_h blockade significantly reduced bursting frequency (Wilcoxon signed-rank test). **1D.** I_h blockade also significantly reduces the percent of spikes in bursts (paired t test, $t=2.428$ $df=19$, rats $n=8$, cells=20). **1E.** Mean Interspike interval was significantly increased after ZD 7288 infusion (paired t test, $t=3.075$ $df=19$, rats $n=8$, cells=20). **1F.** Gaussian fitting mean ISI significantly increased after ZD 7288 infusion. **1G.** A representative naïve cell ISI histogram. Gaussian best fit values resulted in an increase baseline mean ISI from 0.0797 ± 0.0003 s to 0.2128 ± 0.0046 s after ZD 7288 infusion. *Asterisk denotes significant differences ($p < 0.05$).

2.2. Effect of I_h blockade on VTA DA neuron firing modes.

Dopaminergic neurons of VTA can be categorized by the average firing rate and the percentage of spike within a burst (SWB; number of spikes within burst). These firing pattern classifications are based on characteristic interspike interval distribution. Low firing neurons (LF) fire at rates < 5 Hz, while High firing neurons (HF) fire at frequencies > 5 Hz. Low Bursting Activity is denoted as %SWB lower than 20%, while High Bursting Activity is classified as %SWB higher than 20% for neurons with firing rates < 5 Hz and %SWB higher than 40% for neurons with firing rates of > 5 Hz [35]. On (Figure 2A,2B), the naïve populational firing modes before and after ZD 7288 perfusion are plotted. Chi-squared analysis demonstrated significant difference in the HFHB and HFLB groups after ZD 7288 perfusion ($p < 0.05$; χ^2 test) (Figure 2C.). These results suggest that I_h blockade is sufficient to change basal firing mode categories.

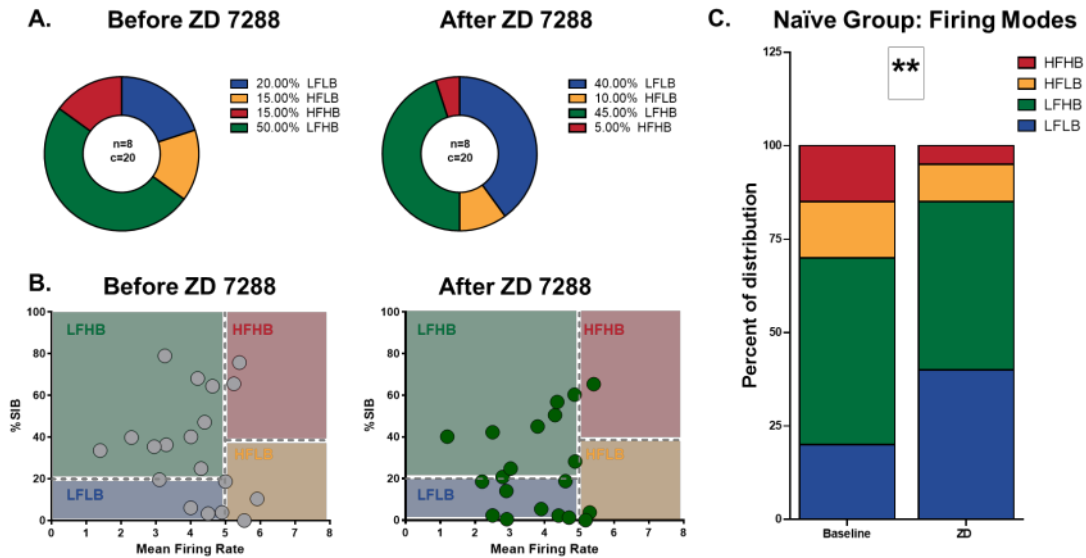


Figure 2. Effect of I_h blockade on VTA DA neuron firing modes. 2A. Pie chart of naïve group firing mode population before and after ZD 7288. 2B. Mean Firing rate vs percent of spikes in burst of each recording. Colors represent lighter versions of the ones depicted in the pie charts. 2C. Naïve group firing pattern percent of distribution demonstrates a significant difference in the HFHB and HFLB groups after ZD 7288 ($p < 0.05$; χ^2 test).

2.3. Effect of I_h blockade on in vivo extracellular firing patterns 24 hours after cocaine administration.

Using whole-cell patch-clamp voltage-clamp protocol we, evaluated I_h amplitude 2 and 24 hours after acute cocaine injection (15mg/kg) and after chronic (7 days) cocaine administration (cocaine sensitization) (**Figure 3A**). 2 hours after acute cocaine, there is no significant difference in I_h amplitude compared to control. However, 24 hours after acute cocaine, we found a significant reduction in I_h amplitude (~25%) in DA VTA neurons from cocaine sensitized rats. Note that after cocaine sensitization there was also a significant reduction in I_h amplitude (~40% (**Figure 3A**) and [31]). Mechanistically, the continuous I_h reduction after cocaine sensitization could serve as a homeostatic adaptation for neuronal excitability of VTA DA neurons.

Acute cocaine administration significantly increases firing activity of in vivo VTA DA neurons [30]. I_h blockade decreases firing rate through an increase in rebound delay and interspike interval [17,33,34,37]. Thus, I_h blockade may reduce cocaine-induced enhanced in vivo spontaneous activity. To evaluate the effect of I_h blockade on spontaneous firing of DA VTA neurons after acute cocaine administration in anesthetized rats, we used double barrel micropipettes. These pipettes are composed of a recording glass electrode (1-2 μ m, 10-12M Ω filled with 2.0% pontamine sky blue in 0.5M sodium acetate and a perfusion pipette that contained ZD 7288 (8.3 μ M in ACSF). We recorded a minimum of 100 seconds of baseline extracellular firing activity and perfused

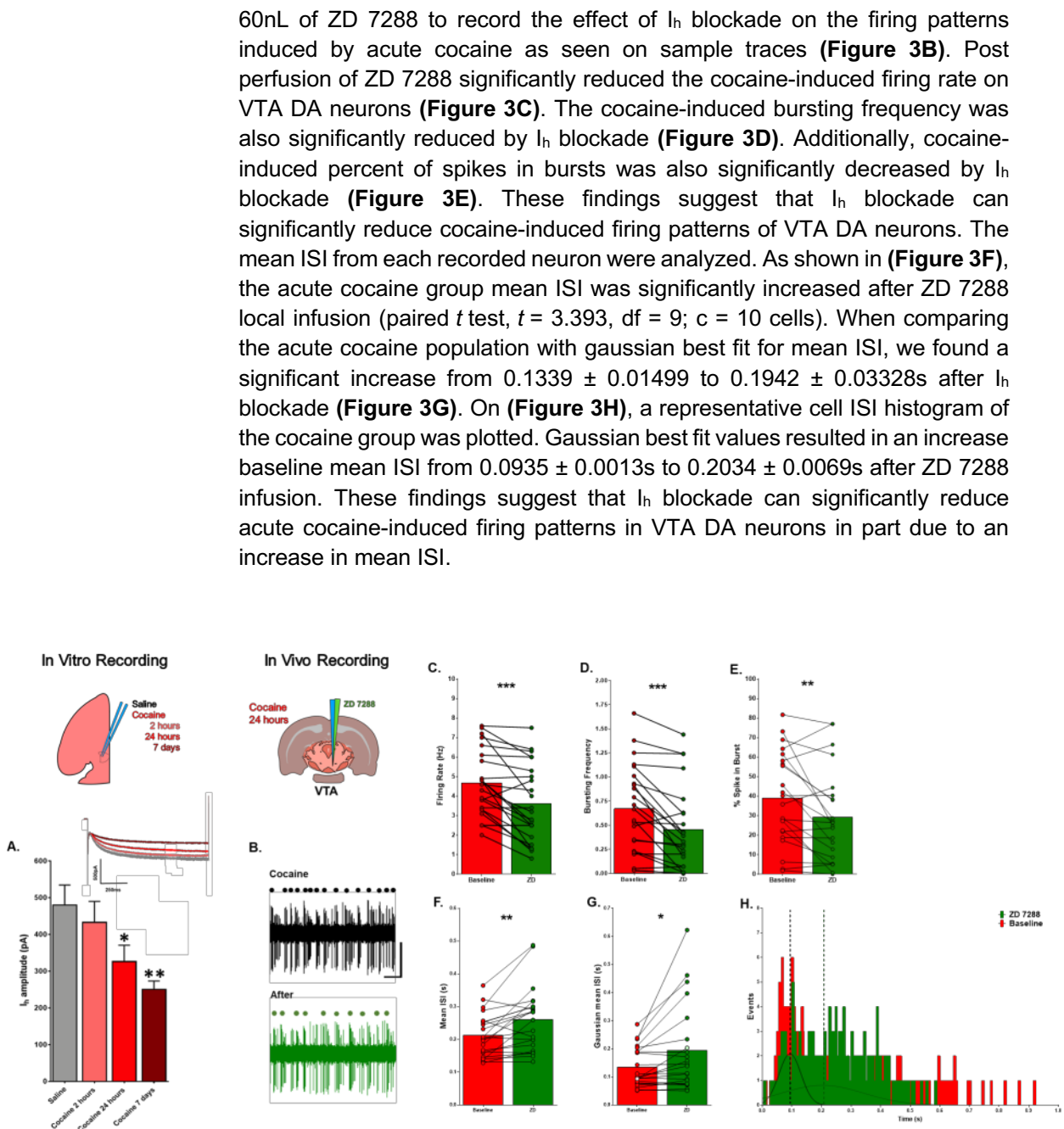


Figure 3. Post perfusion I_h blockade significantly reduced firing properties after acute cocaine on in vivo extracellular recordings of VTA DA neurons. **3A.** Whole cell patch-clamp voltage-clamp protocol I_h amplitude 2 and 24 hours after acute cocaine injection (15mg/kg) and after chronic (7 days) cocaine administration. A significant I_h reduction occurs 24 hours after acute cocaine administration. One-Way ANOVA, Newman-Keuls multiple comparison test $F(3,42) = 5.659$. **3B.** Sample traces of acute cocaine injection and after I_h blocker ZD 7288 (8.3 μ m) perfusion. Each dot represents a burst event. Scale bars: horizontal, 1 s; vertical, 1 mV. **3C.** I_h blockade significantly reduced the cocaine-induced firing rate (paired t test, $t = 4.633$ $df = 22$). **3D.** I_h blockade significantly reduced cocaine-induced bursting frequency (paired t test, $t = 4.383$ $df = 22$). **3E.** I_h blockade also significantly reduces the percent of spikes in bursts. (paired t test, $t = 2.428$ $df = 19$, rats $n = 5$, cells $n = 23$). **3F.** Mean Interspike interval was significantly increased after ZD 7288 infusion. **3G.** Gaussian fitting mean ISI significantly increased after ZD 7288 infusion. **3H.** A representative cell ISI

histogram. Gaussian best fit values resulted in an increase baseline mean ISI from $0.0935 \pm 0.0013s$ to $0.2034 \pm 0.0069s$ after ZD 7288 infusion. *Asterisk denotes significant differences ($p < 0.05$).

2.4. Effect of I_h blockade on acute cocaine-induced VTA DA firing modes.

Since VTA DA neurons can be categorized by the average firing rate and the percentage of spike within a burst, we tested if ZD 7288 infusion can alter acute cocaine treated animals firing modes. On (Figure 4A, B), the acute cocaine populational firing modes before and after ZD 7288 perfusion are plotted. Chi-squared analysis demonstrated no significant difference in any category after ZD 7288 infusion (Figure 4C.). Although I_h blockade can reduce acute cocaine-induced firing patterns, these results suggest that I_h inhibition is not sufficient to alter the acute cocaine firing mode categories.

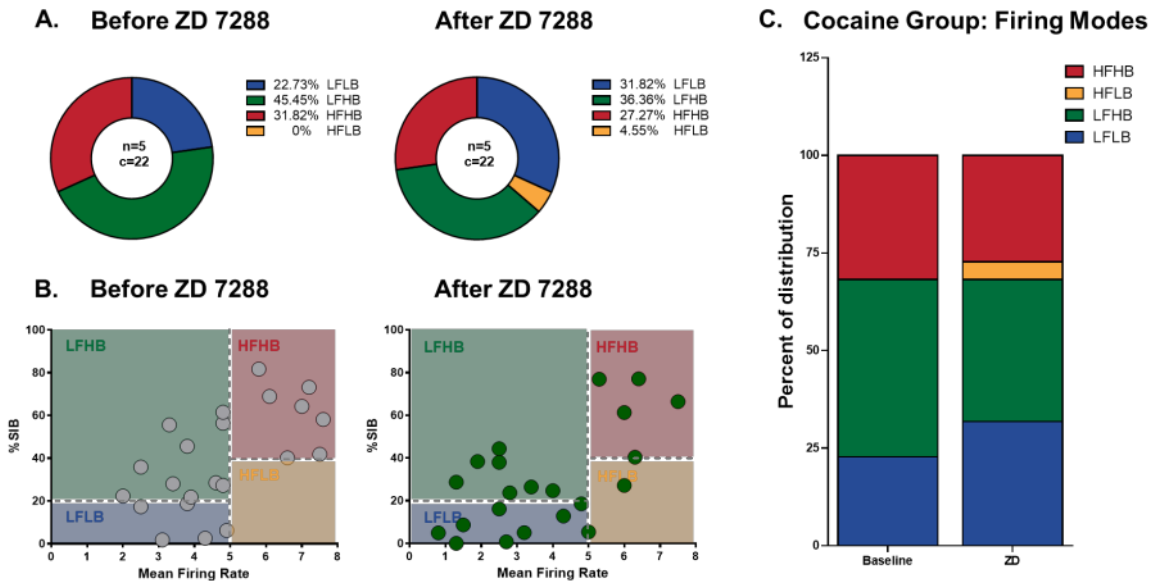


Figure 4. Effect of I_h blockade on acute cocaine induced VTA DA firing modes. 4A. Pie chart of cocaine group firing mode population. **4B.** Mean Firing rate vs percent of spikes in burst of each neuron recorded in the cocaine group. Colors represent lighter versions of the ones depicted in the pie charts. **4C.** Cocaine group firing pattern percent of distribution did not demonstrate significant differences in groups after ZD 7288 ($p < 0.05$; χ^2 test).

3. Discussion.

We have previously shown that cocaine sensitization reduces I_h in VTA DA neurons [31]. In the present study we report that I_h blockade reduces spontaneous firing parameters in VTA DA neurons of rats. We elucidated the contribution of I_h to spontaneous activity using in-vivo extracellular recording in anesthetized rats. Through local infusion of ZD 7288 from a micropipette in the vicinity of the recording electrode, we determined that I_h blockade significantly reduces VTA DA neurons firing rate and bursting activity. Moreover, this diminution in activity is due to an increase in the inter-spike interval induced by I_h blockade. In addition, we explored the contribution of I_h

to changes in firing patterns induced by acute cocaine. Infusion of an I_h blocker locally into the VTA of rats treated with acute cocaine significantly decreased cocaine-induced firing activity. Both spontaneous firing activity and percent of spikes within bursts was significantly reduced after I_h blockade. In addition, I_h blockade in this group also increased its inter-spike interval. Finally, we measured the I_h amplitude in VTA DA cells after an acute cocaine injection. We observed that a significant I_h reduction occurs 24 hours after drug administration. These results reinforce our earlier observation that I_h is reduced after cocaine sensitization, and suggest that a progressive reduction of I_h current after cocaine exposure is a possible homeostatic regulator of VTA DA neuronal excitability.

Neuromodulation of intrinsic properties is a significant determinant of neuronal excitability [20,21]. Intrinsic excitability can be modified by altering the neuron's inherent electrical properties [22,23]. These adjustments in excitability arise through changes in morphological features and the expression level or biophysical properties of ion channels in the membrane [24]. The final response of a single neuron emerges through the summation of the effects of excitatory and inhibitory synaptic inputs, and intrinsic membrane properties [25]. Homeostatic changes in neuronal excitability can arise in response to long periods of altered activity [27–29]. Such periods of altered activity may be a consequence of drug exposure.

The I_h current modulates electrophysiological properties of neurons in the Mesocorticolimbic (MCL) system, including DA neurons of the VTA, in a variety of ways. Neuroadaptations in this network are hypothesized to contribute to the neuropathology of substance abuse disorder [38,39]. Dopaminergic neurons of the VTA are important elements that are activated by rewarding experiences [40–42]. In addition, alteration of normal functionality in the VTA underlies aspects of drug abuse and other neuropsychiatric disorders [43–46]. One of the most explored reward-related circuits is the VTA DA projection to the nucleus accumbens [47–49]. The alteration of normal dopamine neurotransmission by persistent drug administration can trigger both molecular and cellular mechanisms in this circuit that can lead to substance abuse disorder [40,49,50].

Different studies have identified I_h as an excitatory driving force in VTA DA neurons [18,19]. Pharmacological I_h blockade suppresses DA neuronal firing in both VTA and substantia nigra zona compacta neurons in vivo [33,34]. After pharmacological blockade, an artificially mimicking I_h via current injection via dynamic clamp restored the normal intrinsic DA cell membrane potential phenotype [33]. I_h modulation has been demonstrated to compensate for alterations in CA1 pyramidal neuronal excitability. Chronic pharmacological enhancement or excitability deprivation triggers a bidirectional plasticity of I_h and leads to regulation of both input resistance and intrinsic excitability [51]. This evidence supports the possibility that I_h modulation can alter VTA DA excitability.

I_h current participates in burst formation in various neurons [52,53]. For example, rhythmic burst firing in thalamic relay neurons arises mainly from the interaction of two dominant currents, I_h and T type calcium channels (I_{Cat}) [7]. The I_h activation depolarizes the membrane potential towards threshold activating I_{Cat} and generating low-threshold Ca^{2+} spike. The generation of

Ca²⁺ spikes activate a burst of fast Na⁺ and K⁺ dependent action potentials. This provides a possible mechanism for how I_h could influence burst firing in VTA DA neurons. Our results show that spontaneous firing activity of VTA DA neurons is significantly reduced by in vivo delivery of an I_h blocker (**Figure 1**). Bursting activity and percent of spikes within the burst were also reduced. We can speculate that a similar mechanism of action as in the thalamus might be acting in the VTA for the generation of bursting activity. Further studies should address this hypothesis.

VTA DA neurons can be categorized in four main modes of firing pattern with respect to the average firing rate and the percentage of spikes within a burst [35]. Each group is also associated with a characteristic ISI histogram distribution. I_h blockade affected all VTA DA neurons firing patterns. I_h blockade increases the afterhyperpolarization potential in cell-attached recordings of VTA DA neurons, indicating a physiological mechanism for the increase in ISI produced by these blockers [18]. Here we provide evidence that after the I_h blockade there is an increase in the ISI while also reducing the quantity of spiking activity (**Figure 1 and 2**). These results lead us to postulate that I_h modulation in VTA DA neurons is important for the homeostatic regulation of excitability.

Drugs of abuse such as cocaine can induce acute and sustained changes in DA neuronal firing activity. A single exposure to cocaine increases firing rate and bursting activity of VTA DA neurons [30] concomitantly with a synaptic potentiation of these cells [54,55]. Furthermore, after the development of cocaine sensitization, a model of chronic cocaine exposure, the spontaneous firing rate of VTA DA cells remains similar to control groups. In these neurons, cocaine sensitization is associated with a reduction in I_h amplitude by ~40% [31]. The observed current reduction coincides with a HCN2 membrane surface protein diminution, the main HCN subunit in VTA DA neurons [32]. In addition, I_h current reduction persists after a 7-day cocaine withdrawal period [13]. Our evidence demonstrates a significant (~25%) reduction in I_h amplitude 24 but not 2 hours after acute cocaine administration (**Figure 3A**). It has been demonstrated that an increase in bursting activity appears beginning at 24 hours following an acute cocaine injection [30]. High frequency stimulation of VTA DA neurons can also trigger a significant reduction in I_h amplitude [56]. Thus, we hypothesize that cocaine-induced burst firing could trigger I_h modulation as a homeostatic regulator to oppose the alteration of excitability induced by cocaine.

In **Fig 2**, we evaluated the I_h blockade effect on naïve animals VTA DA recordings. The chi-squared analysis demonstrated a significant difference in the HFHB and HFLB groups after ZD perfusion ($p < 0.05$; χ^2 test). These results suggest that I_h blockade is sufficient to change the firing mode of VTA DA subpopulations within this category system. On the other hand, in **Fig 4**, we evaluated the effect of I_h blockade on acute cocaine-treated rats. This group did not have any significant change in subpopulation firing modes. We hypothesize this could be because VTA DA neurons in the acute treated group fire more frequently. Under these circumstances, an I_h blockade would not be sufficient to change the cells' firing mode category. Although, when comparing the population mean ISI on the acute treated group, we can detect a significant increase in the time between spikes.

Our experiments demonstrate that 24 hours after a single cocaine injection, I_h blockade significantly reduces cocaine-induced firing and bursting activity. Similar to our first set of experiments, pharmacological blockade of I_h reduces the acute cocaine-induced increase in spontaneous firing and bursting activity by increasing the mean ISI. This provides support for the idea that reduction of I_h conductance could serve as a regulator of cocaine-induced excitability. Given the fact that the mean ISI on both naïve and acute cocaine groups significantly increased after ZD 7288, we propose that the reduction in firing patterns on both groups could be in part mechanistically due to the increase in time between spikes.

Our data demonstrated statistically significant differences on firing rate, bursting frequency and percent of spike in bursts. (**Figure 1bcd and Figure 3cde**). Fundamental studies have established that DA neuronal baseline activity is irregular with an average firing rate of 4.5 Hz [57]. Our results demonstrate that after ZD 7288 local infusion there was a significant reduction in firing rate of approximately 1 Hz in both naïve and acute cocaine groups. We suggest that the reduction in firing rate of a normally low firing DA neuron could be enough to alter cellular functionality. Indeed, it has been demonstrated that optogenetically-induced phasic firing on VTA DA neurons is sufficient to establish behavioral conditioning [58]. While optogenetically-induced tonic firing on VTA DA neurons decreases sucrose intake [59]. It has been demonstrated that burst formation provides a new platform of neuronal coding used to enhance the gain of neural signaling by increasing transmitter release and improving synaptic potentiation [60]. Bursts also enhance the reliability of information transfer and provide various stimulus features in the same spike train [61]. These studies provide evidence that our significant reduction in bursting activity and firing patterns after I_h blockade could also lead to distinctive physiological and behavioral alterations.

Although previous evidence and our current results can define I_h modulation as a possible homeostatic regulator of intrinsic excitability, it is one component of different interrelated biophysical properties that results in the regulation of spontaneous firing activity. For example, small conductance K^+ (SK) current amplitude is another determinant of endogenous DA firing patterns. Computational models evaluating VTA DA neurons firing patterns denote that high SK conductance is characteristic in low firing neurons, while low SK conductance is present in high firing neurons [62]. Further studies will evaluate the influence of other conductances in our experimental timepoints and how the integration and interplay of these diverse currents can alter VTA DA neuronal excitability.

Currently, little is known of how homeostatic regulation of neuronal excitability is initiated in the MCL **system**. We suggest these modifications could be triggered by cocaine-induced enhancement of glutamatergic excitatory afferent towards VTA DA neurons [54, 63]. Repeated exposure to cocaine can also disinhibit VTA GABA neurons thus increasing neuronal firing of VTA DA neurons [64]. Our hypothesis postulates that specific cocaine-induced enhancement of bursting patterns could result in alteration of I_h , since 24 hrs after acute cocaine administration, bursting activity increases, and this is correlated with a significant reduction in I_h current. Our evidence supports the idea that I_h reduction could be a homeostatic regulator that reduces

spontaneous firing rate after cocaine-induced firing patterns are present. I_h diminution can increase the ISI, thus reducing the probability of generating an action potential.

Previous studies have demonstrated that ZD 7288 can additionally block Na^+ currents in HEK293 cells transfected with Nav 1.4 plasmids [65]. This effect is unlikely to influence our results because local ZD 7288 infusions did not alter the action potential amplitude in our recordings. Studies have shown that isoflurane inhibits I_h current in mice cortical pyramidal neurons [66]. Our experimental design evaluated both baseline and the effect of I_h blockade on firing patterns from the same neuron of the anesthetized rat. We found that prior isoflurane alteration of I_h amplitude does not influence our experimental results after ZD 7288 infusions since the effect was only present after I_h blockade. Moreover, our VTA DA neuronal baseline firing patterns were similar to other experiments using different types of anesthetics such as chloral hydrate and sodium pentobarbital as well as in awake behaving animals [57, 67-69].

In sum, we conclude that I_h blockade can decrease the spontaneous firing activity of VTA DA neurons. Furthermore, I_h blockade can also reduce the acute cocaine-induced increase in spontaneous firing activity in VTA DA cells. It was found that the reduction in firing activity can be correlated with an increase in the inter-spike interval. In addition, there was a significant reduction in I_h current 24 hrs after cocaine administration. We have previously discovered that after the development of cocaine sensitization I_h is significantly reduced whereas the spontaneous firing activity of VTA DA cells remains unaltered [31]. Thus, the progressive current reduction suggested here from acute to chronic cocaine administration could serve as a homeostatic mechanism to reduce cocaine enhanced firing patterns. The study of the regulatory role of this current can provide novel insights on how changes in intrinsic neuronal properties might establish cellular homeostasis in the VTA DA system.

4. Experimental Procedure:

4.1. Animals

Procedures involving experimental animals were performed according to the U.S. Public Health Service Publication Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Puerto Rico Medical Sciences Campus. Behavioral and electrophysiological experiments were performed with male Sprague-Dawley rats (35–51 days postnatal). Animals were housed two per cage and were maintained at constant temperature and humidity with a 12:12-h light-dark cycle. Water and food were provided ad libitum.

4.2. Whole-cell Patch-Clamp Electrophysiology.

Midbrain horizontal slices (220 μ m) containing the VTA were prepared from male Sprague Dawley rats (35–51 days postnatal) as previously described [70]. Whole-cell voltage- and current-clamp recordings were obtained from visually identified neurons in the VTA with an infrared microscope with differential interference contrast (DIC) (BX51WI Olympus).

Putative DA neurons were identified by the presence of the I_h and were located lateral to the fasciculus retroflexus and medial to the medial terminal nucleus of the accessory optic tract (MT) [71]. Margolis et al. [72,73] clearly demonstrated that every cell that expresses tyrosine hydroxylase (TH) also coexpresses I_h , and hence every VTA DA cell displays I_h . The converse argument, however, is not always true, i.e., if I_h is present in a VTA cell then it may not be dopaminergic [63]. Nonetheless, we recorded from medial VTA neurons lateral to the MT, where other authors have reported that I_h and TH are colocalized in ~75% of cells [74]. Therefore, the contribution of non-DA cells to the data shown here is likely to be minimal. Recording pipettes (borosilicate glass, OD 1.5 mm, ID 1.0 mm; WPI, Sarasota, FL) had a resistance of 3–5 M Ω when filled with (in mM) 115 KCH₃SO₄ (methyl potassium sulfate), 20 KCl, 1.5 MgCl₂, 5 HEPES, 1 EGTA, 2 ATP, 0.2 GTP, and 10 creatine phosphate (CP), pH 7.25, 290 mOsm. (Na)GTP, (Mg)ATP, and (Na)CP were added fresh daily. Artificial cerebrospinal fluid (ACSF) contained (in mM) 127 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, and 25 D(+)-glucose, and were equilibrated with 95% O₂ 5% CO₂ at 35°C. Data sets were collected through an Axopatch 200B amplifier (Axon Instruments; Molecular Devices, Sunnydale, CA), digitized at 5kHz, filtered at 1kHz, and stored in a computer using pCLAMP 9 (Axon Instruments; Molecular Devices). Series resistance was monitored during the entire recording, voltage-clamp data were discarded if changes >15% in series resistance occurred.

4.3. *In Vivo Anesthetized Single-Unit Neuron Recordings.*

Male Sprague Dawley Rats were anesthetized with 4% of isoflurane 2 l/min air and O₂ for induction and placed in the stereotaxic frame. During the surgical procedures and neuronal recordings, 1.5% isoflurane 2 l/min air and O₂ were delivered through a facial mask via spontaneous respiration. Body temperature was maintained between 36–38°C with an animal temperature controlled electric heating pad during the procedure (World Precision Instruments, Sarasota, FL). A glass micropipette (1–2 μ m, 10–12M Ω filled with 2.0% pontamine sky blue in 0.5M sodium acetate) were lowered into the VTA. A double barrel pipette was used, similar to the ones used by Georges and Aston-Jones [36]. The perfusion pipette on the double barrel pipette was filled with ZD 7288 (8.3 μ M) an I_h blocker dissolved in ACSF. Electrodes were lowered in the anterior and lateral part of the VTA according to stereotaxic coordinates derived from rat brain atlas and corrected empirically (antero-posterior: -5.0–6.0 mm from bregma; mediolateral: 0.7–1.0 mm from midline; dorso-ventral: -7.8–8.4 mm from bregma). To distinguish dopamine from non-dopamine neurons, the following parameters were used: (1) an action potential width greater than 1.1 ms (measured from the start of action potential to the negative trough); (2) slow spontaneous firing rate (<10 Hz); (3) single and burst spontaneous firing patterns (characterized by spike-amplitude decrement). The extracellular potential was recorded with an Axoclamp-2B amplifier and filter (300 Hz/0.5 Hz: Georges and Aston-Jones, 2002). Single-neuron spikes were collected online (CED 1401; SPIKE 2;

Cambridge Electronic Design). A baseline of 100 seconds was recorded and then 100 seconds after delivery of 60nL of the ZD 7288 solution.

4.4. Data analysis.

All data were presented as mean \pm SEM. The statistical significance of two group comparisons were assessed performing paired-sample Student t-test when data followed normal distribution or Wilcoxon matched-pairs test when data did not follow normal distribution. For multiple comparisons, data values were subjected to a one-way ANOVA followed by Newman-Keuls multiple comparison as a post hoc analysis. P values were reported throughout the text and the significance was set at $p < 0.05$.

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