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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/k.g 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

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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.

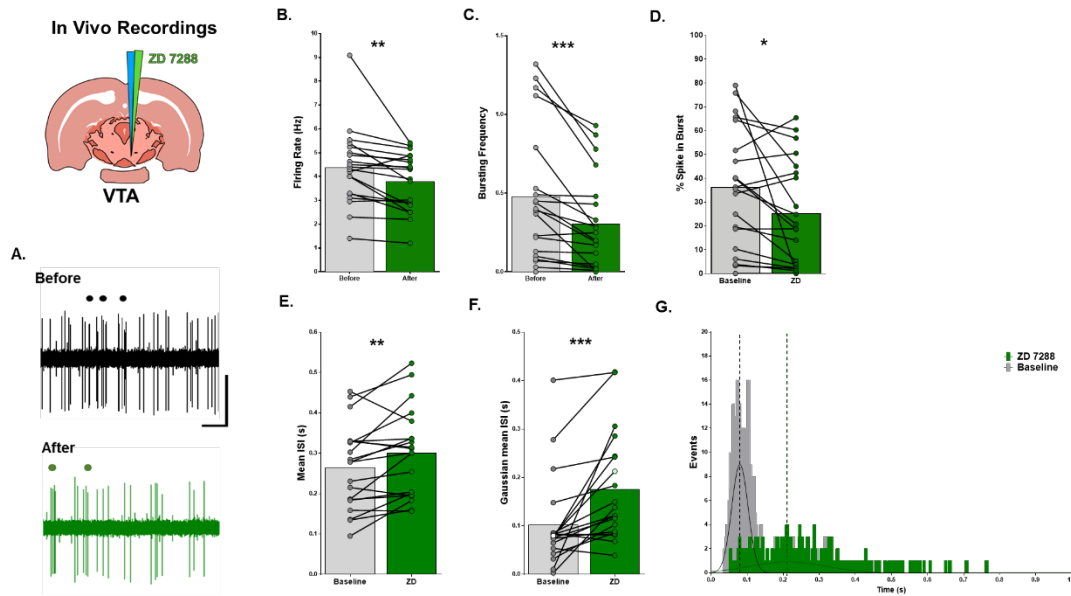
To determine if reduction of I_h can alter basal VTA DA neuronal excitability, we evaluated the effect of I_h blockade on spontaneous firing patterns using in vivo single-unit extracellular recordings. VTA DA neurons

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

102 *2.1. Effect of blockade of I_h on in vivo anesthetized single-unit spontaneous* 103 *VTA DA neuron firing.*

104 To evaluate the effect of I_h blockade on in vivo anesthetized spontaneous
105 firing, we used double barrel pipettes [36]. These pipettes were comprised of
106 a recording electrode and a perfusion pipette that contained ZD 7288 (8.3 μ M).
107 We recorded a minimum of 100 seconds of baseline extracellular firing activity
108 and then locally perfused at the vicinity of the recorded cell, 60nL of ZD 7288
109 to see the effect of I_h blockade on neuronal firing patterns as seen on sample
110 traces (**Figure 1A**). Local infusion of ZD 7288 significantly reduced the firing
111 rate on VTA DA neurons (**Figure 1B**). The bursting frequency was also
112 significantly reduced by I_h blockade (**Figure 1C**). This finding provides the first
113 evidence that I_h modulates the bursting activity of VTA DA neurons. Moreover,
114 the percent of spikes in bursts was also significantly decreased by I_h blockade
115 (**Figure 1D**). The mean inter-spike intervals (ISI) from each recorded neuron
116 were analyzed. As shown in (**Figure 1E**), the naïve group mean ISI was
117 significantly increased after ZD 7288 local infusion (paired t test, $t = 3.075$, df
118 $= 19$; $n = 8$ rats $c = 20$ cells). When comparing the total population of the naïve
119 cells' with gaussian best fit for mean ISI, we found a significant increase from
120 $0.1025 \pm 0.02122s$ to $0.1750 \pm 0.02497s$ after ZD 7288 infusion (**Figure 1F**).
121 On (**Figure 1G**), a representative cell ISI histogram of the naïve group was
122 plotted. Gaussian best fit values resulted in an increase baseline mean ISI
123 from $0.0797 \pm 0.0003s$ to $0.2128 \pm 0.0046s$ after ZD 7288 infusion. These
124 results suggest that I_h blockade can significantly reduce firing patterns in VTA
125 DA neurons in part due to an increase in mean ISI. Additionally, through I_h
126 modulation, we could control the firing activity of VTA DA neurons.



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128 **Figure 1. I_h blockade significantly reduced firing properties on in vivo extracellular recordings of VTA DA**
 129 **neurons. 1A.** Sample traces before and after I_h blocker ZD 7288 (8.3 μ m) perfusion. Each dot represents a burst event.
 130 Scale bars: horizontal, 1 s; vertical, 1 mV. **1B.** I_h blockade significantly reduced the firing rate (paired t test, $t=2.912$
 131 $df=19$). **1C.** I_h blockade significantly reduced bursting frequency (Wilcoxon signed-rank test). **1D.** I_h blockade also
 132 significantly reduces the percent of spikes in bursts (paired t test, $t=2.428$ $df=19$, rats $n=8$, cells=20). **1E.** Mean
 133 Interspike interval was significantly increased after ZD 7288 infusion (paired t test, $t=3.075$ $df=19$, rats $n=8$, cells=20).
 134 **1F.** Gaussian fitting mean ISI significantly increased after ZD 7288 infusion. **1G.** A representative naive cell ISI
 135 histogram. Gaussian best fit values resulted in an increase baseline mean ISI from $0.0797 \pm 0.0003s$ to $0.2128 \pm$
 136 $0.0046s$ after ZD 7288 infusion. *Asterisk denotes significant differences ($p<0.05$).

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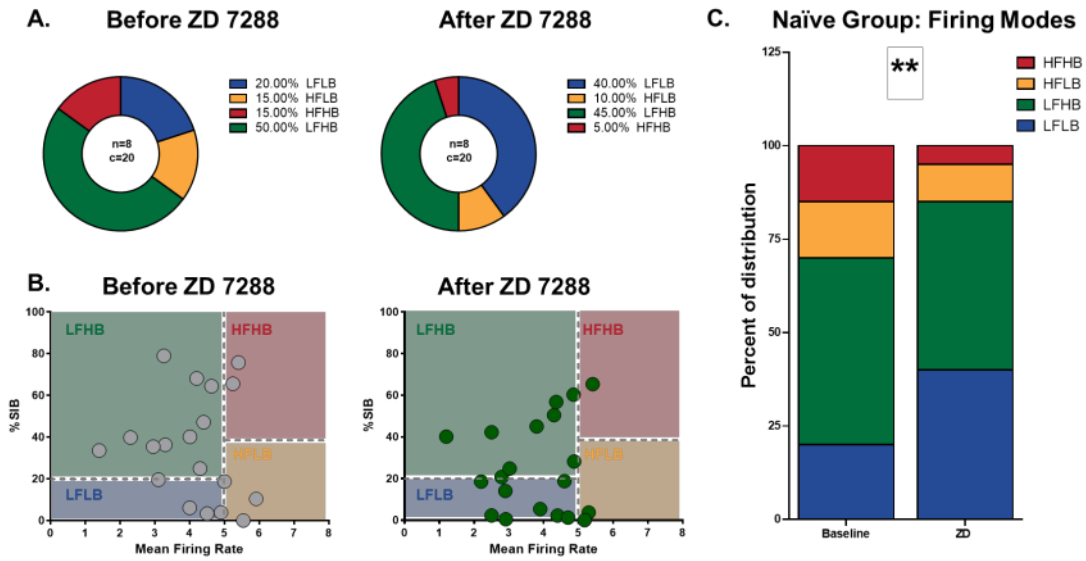
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2.2. Effect of I_h blockade on VTA DA neuron firing modes.

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

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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).

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

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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.

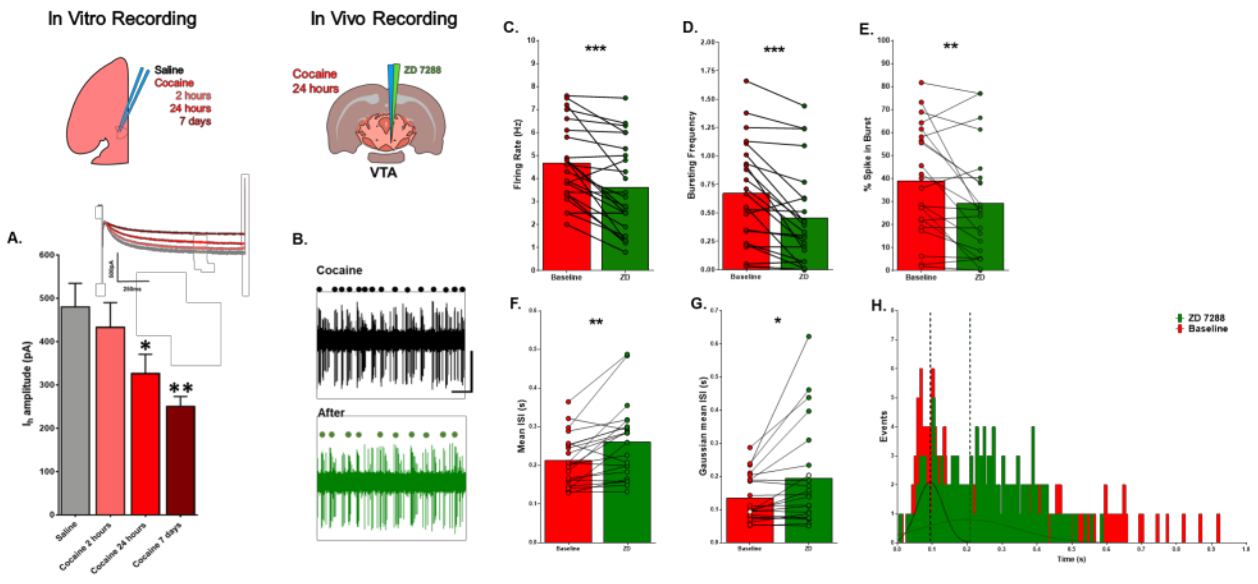
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Acute cocaine administration significantly increases firing activity of in vivo VTA DA neurons [30]. I_h blockade decreases firing rate through an increase in rebound delay and interspike interval [17,33,34,37]. Thus, I_h blockade may reduce cocaine-induced 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

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60nL of ZD 7288 to record the effect of I_h blockade on the firing patterns induced by acute cocaine as seen on sample traces (**Figure 3B**). Post perfusion of ZD 7288 significantly reduced the cocaine-induced firing rate on VTA DA neurons (**Figure 3C**). The cocaine-induced bursting frequency was also significantly reduced by I_h blockade (**Figure 3D**). Additionally, cocaine-induced percent of spikes in bursts was also significantly decreased by I_h blockade (**Figure 3E**). These findings suggest that I_h blockade can significantly reduce cocaine-induced firing patterns of VTA DA neurons. The mean ISI from each recorded neuron were analyzed. As shown in (**Figure 3F**), the acute cocaine group mean ISI was significantly increased after ZD 7288 local infusion (paired t test, $t = 3.393$, $df = 9$; $n = 10$ cells). When comparing the acute cocaine population with gaussian best fit for mean ISI, we found a significant increase from 0.1339 ± 0.01499 to 0.1942 ± 0.03328 s after I_h blockade (**Figure 3G**). On (**Figure 3H**), a representative cell ISI histogram of the cocaine group was plotted. Gaussian best fit values resulted in an increase baseline mean ISI from 0.0935 ± 0.0013 s to 0.2034 ± 0.0069 s after ZD 7288 infusion. These findings suggest that I_h blockade can significantly reduce acute cocaine-induced firing patterns in VTA DA neurons in part due to an increase in mean ISI.

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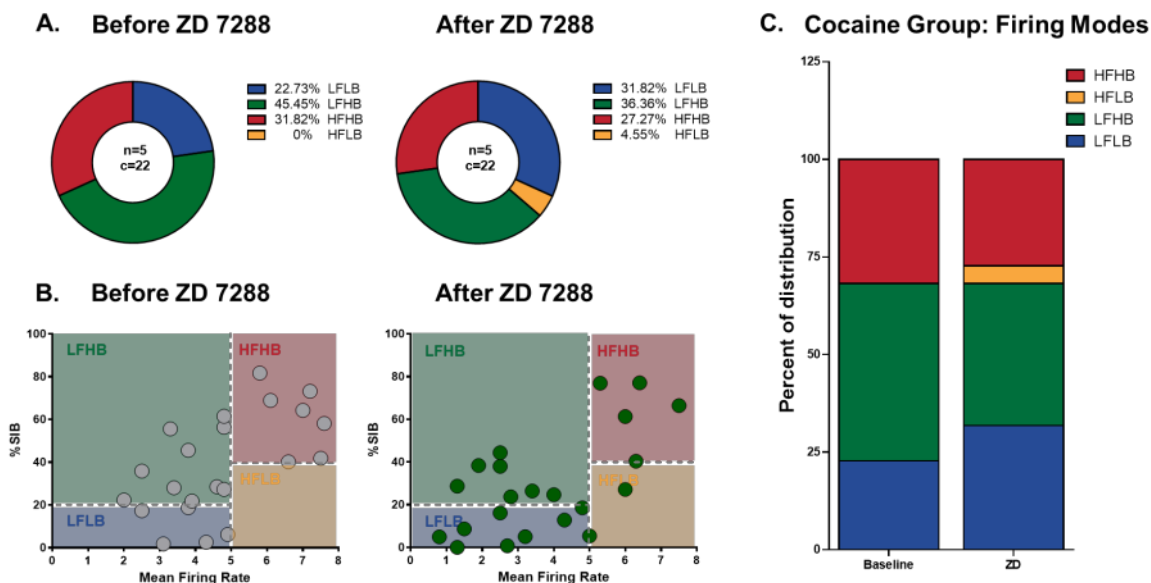
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204 **Figure 3. Post perfusion I_h blockade significantly reduced firing properties after acute cocaine on in vivo**
205 **extracellular recordings of VTA DA neurons. 3A.** Whole cell patch-clamp voltage-clamp protocol I_h amplitude 2 and
206 24 hours after acute cocaine injection (15mg/kg) and after chronic (7 days) cocaine administration. A significant I_h
207 reduction occurs 24 hours after acute cocaine administration. One-Way ANOVA, Newman-Keuls multiple comparison
208 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
209 dot represents a burst event. Scale bars: horizontal, 1 s; vertical, 1 mV. **3C.** I_h blockade significantly reduced the
210 cocaine-induced firing rate (paired t test, $t=4.633$ $df=22$). **3D.** I_h blockade significantly reduced cocaine-induced
211 bursting frequency (paired t test, $t=4.383$ $df=22$). **3E.** I_h blockade also significantly reduces the percent of spikes in bursts.
212 (paired t test, $t=2.428$ $df=19$, rats $n=5$, cells=23). **3F.** Mean Interspike interval was significantly increased after ZD 7288
213 infusion. **3G.** Gaussian fitting mean ISI significantly increased after ZD 7288 infusion. **3H.** A representative cell ISI

214 histogram. Gaussian best fit values resulted in an increase baseline mean ISI from $0.0935 \pm 0.0013s$ to $0.2034 \pm$
 215 $0.0069s$ after ZD 7288 infusion. *Asterisk denotes significant differences ($p < 0.05$).

216 *2.4. Effect of I_h blockade on acute cocaine-induced VTA DA firing modes.*
 217 Since VTA DA neurons can be categorized by the average firing rate and the
 218 percentage of spike within a burst, we tested if ZD 7288 infusion can alter
 219 acute cocaine treated animals firing modes. On (Figure 4A, B), the acute
 220 cocaine populational firing modes before and after ZD 7288 perfusion are
 221 plotted. Chi-squared analysis demonstrated no significant difference in any
 222 category after ZD 7288 infusion (Figure 4C.). Although I_h blockade can reduce
 223 acute cocaine-induced firing patterns, these results suggest that I_h inhibition
 224 is not sufficient to alter the acute cocaine firing mode categories.

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

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

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

253 Neuromodulation of intrinsic properties is a significant determinant of
254 neuronal excitability [20,21]. Intrinsic excitability can be modified by altering
255 the neuron's inherent electrical properties [22,23]. These adjustments in
256 excitability arise through changes in morphological features and the
257 expression level or biophysical properties of ion channels in the membrane
258 [24]. The final response of a single neuron emerges through the summation
259 of the of effects of excitatory and inhibitory synaptic inputs, and intrinsic
260 membrane properties [25]. Homeostatic changes in neuronal excitability can
261 arise in response to long periods of altered activity [27–29]. Such periods of
262 altered activity may be a consequence of drug exposure.

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

275 Different studies have identified I_h as an excitatory driving force in VTA
276 DA neurons [18,19]. Pharmacological I_h blockade suppresses DA neuronal
277 firing in both VTA and substantia nigra zona compacta neurons in vivo
278 [33,34]. After pharmacological blockade, an artificially mimicking I_h via current
279 injection via dynamic clamp restored the normal intrinsic DA cell membrane
280 potential phenotype [33]. I_h modulation has been demonstrated to
281 compensate for alterations in CA1 pyramidal neuronal excitability. Chronic
282 pharmacological enhancement or excitability deprivation triggers a
283 bidirectional plasticity of I_h and leads to regulation of both input resistance and
284 intrinsic excitability [51]. This evidence supports the possibility that I_h
285 modulation can alter VTA DA excitability.

286 I_h current participates in burst formation in various neurons [52,53]. For
287 example, rhythmic burst firing in thalamic relay neurons arises mainly from
288 the interaction of two dominant currents, I_h and T type calcium channels (I_{Cat})
289 [7]. The I_h activation depolarizes the membrane potential towards threshold
290 activating I_{Cat} and generating low-threshold Ca^{2+} spike. The generation of

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

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

310 Drugs of abuse such as cocaine can induce acute and sustained
311 changes in DA neuronal firing activity. A single exposure to cocaine increases
312 firing rate and bursting activity of VTA DA neurons [30] concomitantly with a
313 synaptic potentiation of these cells [54,55]. Furthermore, after the
314 development of cocaine sensitization, a model of chronic cocaine exposure,
315 the spontaneous firing rate of VTA DA cells remains similar to control groups.
316 In these neurons, cocaine sensitization is associated with a reduction in I_h
317 amplitude by ~40% [31]. The observed current reduction coincides with a
318 HCN2 membrane surface protein diminution, the main HCN subunit in VTA
319 DA neurons [32]. In addition, I_h current reduction persists after a 7-day
320 cocaine withdrawal period [13]. Our evidence demonstrates a significant
321 (~25%) reduction in I_h amplitude 24 but not 2 hours after acute cocaine
322 administration (**Figure 3A**). It has been demonstrated that an increase in
323 bursting activity appears beginning at 24 hours following an acute cocaine
324 injection [30]. High frequency stimulation of VTA DA neurons can also trigger
325 a significant reduction in I_h amplitude [56]. Thus, we hypothesize that cocaine-
326 induced burst firing could trigger I_h modulation as a homeostatic regulator to
327 oppose the alteration of excitability induced by cocaine.

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

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

350 Our data demonstrated statistically significant differences on firing rate,
351 bursting frequency and percent of spike in bursts. (**Figure 1bcd and Figure**
352 **3cde**). Fundamental studies have established that DA neuronal baseline
353 activity is irregular with an average firing rate of 4.5 Hz [57]. Our results
354 demonstrate that after ZD 7288 local infusion there was a significant reduction
355 in firing rate of approximately 1 Hz in both naïve and acute cocaine groups.
356 We suggest that the reduction in firing rate of a normally low firing DA neuron
357 could be enough to alter cellular functionality. Indeed, it has been
358 demonstrated that optogenetically-induced phasic firing on VTA DA neurons is
359 sufficient to establish behavioral conditioning [58]. While optogenetically-
360 induced tonic firing on VTA DA neurons decreases sucrose intake [59]. It has
361 been demonstrated that burst formation provides a new platform of neuronal
362 coding used to enhance the gain of neural signaling by increasing transmitter
363 release and improving synaptic potentiation [60]. Bursts also enhance the
364 reliability of information transfer and provide various stimulus features in the
365 same spike train [61]. These studies provide evidence that our significant
366 reduction in bursting activity and firing patterns after I_h blockade could also
367 lead to distinctive physiological and behavioral alterations.

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

379 Currently, little is known of how homeostatic regulation of neuronal
380 excitability is initiated in the MCL **system**. We suggest these modifications
381 could be triggered by cocaine-induced enhancement of glutamatergic
382 excitatory afferent towards VTA DA neurons [54, 63]. Repeated exposure to
383 cocaine can also disinhibit VTA GABA neurons thus increasing neuronal firing
384 of VTA DA neurons [64]. Our hypothesis postulates that specific cocaine-
385 induced enhancement of bursting patterns could result in alteration of I_h , since
386 24 hrs after acute cocaine administration, bursting activity increases, and this
387 is correlated with a significant reduction in I_h current. Our evidence supports
388 the idea that I_h reduction could be a homeostatic regulator that reduces

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

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

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

419 **4. Experimental Procedure:**

420 *4.1. Animals*

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

429 *4.2. Whole-cell Patch-Clamp Electrophysiology.*

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

435 Putative DA neurons were identified by the presence of the I_h and were
436 located lateral to the fasciculus retroflexus and medial to the medial terminal
437 nucleus of the accessory optic tract (MT) [71]. Margolis et al. [72,73] clearly
438 demonstrated that every cell that expresses tyrosine hydroxylase (TH) also
439 coexpresses I_h , and hence every VTA DA cell displays I_h . The converse
440 argument, however, is not always true, i.e., if I_h is present in a VTA cell then
441 it may not be dopaminergic [63]. Nonetheless, we recorded from medial VTA
442 neurons lateral to the MT, where other authors have reported that I_h and TH
443 are colocalized in ~75% of cells [74]. Therefore, the contribution of non-DA
444 cells to the data shown here is likely to be minimal. Recording pipettes
445 (borosilicate glass, OD 1.5 mm, ID 1.0 mm; WPI, Sarasota, FL) had a
446 resistance of 3–5 M Ω when filled with (in mM) 115 KCH₃SO₄ (methyl
447 potassium sulfate), 20 KCl, 1.5 MgCl₂, 5 HEPES, 1 EGTA, 2 ATP, 0.2 GTP,
448 and 10 creatine phosphate (CP), pH 7.25, 290 mOsm. (Na)GTP, (Mg)ATP,
449 and (Na)CP were added fresh daily. Artificial cerebrospinal fluid (ACSF)
450 contained (in mM) 127 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂,
451 1 MgCl₂, and 25 D(+)-glucose, and were equilibrated with 95% O₂ 5% CO₂
452 at 35°C. Data sets were collected through an Axopatch 200B amplifier (Axon
453 Instruments; Molecular Devices, Sunnydale, CA), digitized at 5kHz, filtered at
454 1kHz, and stored in a computer using pCLAMP 9 (Axon Instruments;
455 Molecular Devices). Series resistance was monitored during the entire
456 recording, voltage-clamp data were discarded if changes >15% in series
457 resistance occurred.

458 *4.3. In Vivo Anesthetized Single-Unit Neuron Recordings.*

459 Male Sprague Dawley Rats were anesthetized with 4% of isoflurane 2
460 l/min air and O₂ for induction and placed in the stereotaxic frame. During the
461 surgical procedures and neuronal recordings, 1.5% isoflurane 2 l/min air and
462 O₂ were delivered through a facial mask via spontaneous respiration. Body
463 temperature was maintained between 36–38°C with an animal temperature
464 controlled electric heating pad during the procedure (World Precision
465 Instruments, Sarasota, FL). A glass micropipette (1–2 μ m, 10–12M Ω filled with
466 2.0% pontamine sky blue in 0.5M sodium acetate) were lowered into the VTA.
467 A double barrel pipette was used, similar to the ones used by Georges and
468 Aston-Jones [36]. The perfusion pipette on the double barrel pipette was filled
469 with ZD 7288 (8.3 μ M) an I_h blocker dissolved in ACSF. Electrodes were
470 lowered in the anterior and lateral part of the VTA according to stereotaxic
471 coordinates derived from rat brain atlas and corrected empirically (antero-
472 posterior: -5.0–6.0 mm from bregma; mediolateral: 0.7–1.0 mm from midline;
473 dorso-ventral: -7.8–8.4 mm from bregma). To distinguish dopamine from non-
474 dopamine neurons, the following parameters were used: (1) an action
475 potential width greater than 1.1 ms (measured from the start of action
476 potential to the negative trough); (2) slow spontaneous firing rate (<10 Hz);
477 (3) single and burst spontaneous firing patterns (characterized by spike-
478 amplitude decrement). The extracellular potential was recorded with an
479 Axoclamp-2B amplifier and filter (300 Hz/0.5 Hz: Georges and Aston-Jones,
480 2002). Single-neuron spikes were collected online (CED 1401; SPIKE 2;

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

483
484 *4.4. Data analysis.*

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

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