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Decreased hyperpolarization-activated cation current (I_h): a response mechanism to reduce cocaine-induced excitability in VTA DA neurons.

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List of symbol and abbreviations

Abbreviation	Definition
ACSF	artificial cerebrospinal fluid
AMPA	(2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid)
BIC	bicuculline methiodide
DA	dopamine/ dopaminergic
GABA	gamma-aminobutyric acid
HCN	hyperpolarization-activated cation channel
i.p.	intra-peritoneal
I_h	hyperpolarization-activated cation current
MLS	mesolimbic system
nAcc	nucleus accumbens
NMDA	N-methyl-D-aspartic acid pyrimidinium chloride
SUD	substance use disorder
VTA	ventral tegmental area
ZD/ ZD7288	4-(N-Ethyl-N-phenylamino)-1,2 dimethyl-6-(methylamino)

Abstract:

The hyperpolarization-activated cation current (I_h) is a determinant of intrinsic excitability in dopaminergic neurons (DA) of the ventral tegmental area (VTA). I_h is a slowly activating cation inward current triggered by hyperpolarization. When elicited I_h depolarizes the membrane to threshold for the generation of action potentials. Our laboratory has previously demonstrated that cocaine sensitization, a chronic cocaine administration model, significantly reduces I_h amplitude in VTA DA neurons (Arencibia-Albite et al., 2012). Despite this current reduction, the spontaneous firing of VTA DA cells remained similar to control animals. The role of I_h in controlling VTA DA excitability is poorly understood. Our hypothesis was that I_h reduction could play a role as a homeostatic controller which compensates for cocaine-induced change in excitability. Our main goal was to understand how I_h contributes to VTA DA neuronal excitability. In our first aim, we tried to elucidate if I_h reduction can alter basal VTA DA neuronal excitability., We blocked I_h and evaluated firing properties, such as rebound spiking, using whole-cell patch-clamp electrophysiology from naïve rats. Rebound spiking is defined as the production of one or more action potentials in response to the cessation of a prolonged hyperpolarizing current step. We also determined the effect of I_h blockade on *in vitro* spontaneous excitability using cell-attached configuration from naïve rats. We hypothesized that I_h blockade will reduce rebound and spontaneous firing of VTA DA cells**.** It was found that I_h blockade can decrease evoked and spontaneous firing activity of VTA DA neurons. In addition, we evaluated the effect of I_h blockade

on spontaneous firing patterns using single-unit extracellular recordings from naïve rats. We found that I_h blockade can decrease evoked and spontaneous firing activity, while increasing the interspike interval of VTA DA cells.

To investigate how this current modulates cocaine-dependent excitability, we postulated that I_h reduction serves as a homeostatic regulator (or controller) to oppose cocaine-induced excitability. We measured I_h current using whole-cell patch-clamp electrophysiology, 2 and 24 hours after an acute cocaine injection. We found a progressive reduction of I_h starting 24 hours after acute cocaine administration. Thus, this progressive decline observed from acute to chronic cocaine administration could serve as a homeostatic mechanism to reduce cocaine enhanced excitability. In addition, we quantified the increase in rebound action potentials, 2 and 24 hours after an acute cocaine injection. We found that after 2, 24 hours or seven days of cocaine administration, there is a significant increase in rebound action potentials of VTA DA neurons. Interestingly, there is a higher number of rebound action potentials after 2 hours of cocaine in comparison to 24 hours. When ZD, an I_h blocker was perfused, there was a significant reduction in rebound action potentials. The progressive I_h reduction may be a key factor in the decrease of rebound action potentials found from 2 to 24 hours after cocaine injections. The effect of I_h blockade was also evaluated on *in vitro* spontaneous excitability using cell-attached configuration, 2 hours after an acute cocaine injection. When perfused with ZD, the spontaneous firing activity was also reduced,

while significantly increasing the interspike interval providing further evidence in favor of an I_h acting as a homeostatic regulator of VTA DA cell excitability.

The overall cocaine effect can be influenced by the environmental context in which the drug is experienced (Caprioli et al., 2007). One question we addressed is how exposure to cocaine in a novel context interferes with chronic alterations on synaptic potentiation and neuronal excitability in the mesolimbic system (MLS). To evaluate this question an acute cocaine injection was administered to subjects in both a novel context or their home-cage. We found a significant increase in the AMPA-to-NMDA receptor-mediated EPSC ratio of VTA DA neurons in both the novelty and the homecage groups. Cocaine experienced in a novel environment produced a higher degree of synaptic potentiation. In addition, we found that I_h blockade can also diminish the acute cocaine-induced increase in spontaneous firing activity in *in vivo* anesthetized rats. Again, the reduction in firing was correlated with an increase in the inter-spike interval.

Lastly, we evaluated if I_h can function as a homeostatic regulator of intrinsic excitability and can be modulated when an altered firing activity in VTA DA cells is present. We enhanced VTA DA excitability pharmacologically and recorded changes in evoked rebound action potentials and I_h. VTA brain slices from naive rats were incubated with bicuculline methiodide (BIC) as a mechanism to enhance excitability and promote rebound excitation through GABAergic disinhibition. Our hypothesis was that I_h will be decreased as a consequence of the pharmacological

enhancement of firing activity. We found that BIC-induced excitability facilitates a reduction in I_h conductance. Furthermore, I_h blockade can significantly reduce this enhanced BIC-induced excitability.

It has been reported that high frequency stimulation of the ventral subiculum (HFSvSUB) elicits *in vivo* persistent hyperactivity of VTA DA neurons (Glangetas et al. 2015). We elucidated the effect of I_h blockade on HFSvSUB induced potentiation on *in vivo* spontaneous excitability. We hypothesized that I_h blockade will significantly decrease HFSvSUB-induced firing patterns on VTA DA cells. We demonstrated that I_h blockade significantly reduces firing activity after HFSvSUB. Additionally, I_h blockade also significantly increased the interspike interval. These results suggest that I_h is an intrinsic mechanism that can modulate the excitability of VTA DA neurons after an hyperexcitable state is present.

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 and in conditions of altered excitability such as substance use disorder (SUD).

Chapter 1

Introduction

Substance Use Disorder

Substance use disorder (SUD), formally known as addiction, is a neurological disease that modifies the brain and behavior of a person, leading to an individual's inability to control their use of legal or illegal drugs. The Diagnostic and Statistical Manual of Mental Disorders, fifth version (DSM 5) classifies SUD as a chronic relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences. It is estimated that in the United States approximately $700 billion is spent for health care costs, loss of productivity, law-breaking offenses, and crime due to the abuse of drugs (National Drug Intelligence Centers, 2011). Furthermore, it is important to highlight that SUD has negative impacts on individuals, families, communities, and societies.

By 2020 in the United States, 21.4% (or an equivalent of 59.3 million people) among people aged twelve or older used some type of illicit drug (SAMHSA, 2021). Within that group, 3.7% (or 10.3 million people) misused central nervous system (CNS) stimulants, of which about a third used only cocaine (32.4 percent of CNS stimulant misusers or 3.3 million people). Also in 2020, 40.3 million people aged twelve or older (or 14.5%) were diagnosed with SUD, including 28.3 million who had an alcohol use disorder, 18.4 million who had an illicit drug use disorder, and 6.5 million people who had both an alcohol use disorder and an illicit drug use disorder. In the United States alone, the National Center for Health Statistics of the

Centers for Disease Control and Prevention reported 91,799 deaths from drug overdoses in 2020.

An epidemiological study conducted at Puerto Rico demonstrated that 11.5% of adults aged between 18 and 64 years met the diagnostic criteria for SUD (ASSMCA, 2020). Furthermore, 2.5% (57,301 individuals) of the adult population in Puerto Rico are estimated to need substance use services. Despite the high rate of diagnosed individuals, approximately seven out of ten adults (67.4%) who need substance services did not receive therapy or treatment.

Treatments are available for those suffering from substance abuse problems. Medication and behavioral therapies are accessible to some types of abused substances, which aim to detoxify the drug, followed by focusing on treatment and prevention of relapse. An important aim of SUD research is to understand the biological mechanisms underlying this neuropathology. The effect this disease has on individuals and our society defines the importance of fundamental research on SUD and the necessity of novel approaches for the treatment and recovery of this disorder.

Cocaine as a drug of abuse

Cocaine is an addictive stimulant drug purified from the leaves of the Erythroxylon coca plant (NIDA, 2021). Although it has been used by health care providers for

valid medical purposes, such as local anesthesia for particular surgeries, the recreational use of cocaine is illegal (NIDA, 2021). Cocaine in the brain blocks the dopamine transporter channel and other monoamine transporters, which inhibits the dopamine reuptake in the synapse after the neurotransmitter is released from the presynaptic neuron. By blocking this channel, DA will have more time in the synapse and can produce a longer interaction with postsynaptic dopamine receptors. Although the immediate pharmacological mechanisms of cocaine action are previously described, adaptive alterations caused by repeated cocaine use produces several types of health conditions and long-term modifications in the brain (Spronk et al, 2013; Potvin et al, 2014; Frazer et al., 2018; Wang, 2020). Some of these health risks are, but not limited to: headaches, convulsions and seizures, cardiac conditions, increased irritability, restlessness, panic attacks, paranoia, and even psychosis. The alteration of normal dopamine neurotransmission by persistent drug administration, such as cocaine, can trigger molecular and cellular mechanisms in the brain that can lead to develop SUD (Kauer, 2004; Everitt and Wolf, 2002; Kauer and Malenka, 2007).

In 2020, 1.9% (or 5.2 million individuals) of people aged twelve or older used cocaine, while 0.5% (or 1.3 million individuals) had a cocaine use disorder within the United States. Furthermore, it is estimated that 70% of these individuals tried cocaine for the first time between the ages 18 and 25 (SAMHSA, 2021). In the same year, overdose deaths that involved cocaine increased from 5,419 in 2014 to 19,447 in 2021. Although most of these cases have been attributed to opioid

overdose deaths, the combination of both types of drugs of abuse has been increasing steadily since 2014 and is the main driver of cocaine-involved overdose deaths (NIDA, 2020).

The Mesolimbic System (MLS)

Drugs of abuse, such as cocaine, have a prominent effect in the brain mesolimbic system (MLS), commonly known as the reward pathway. The MLS is a set of interconnect regions that regulate reward, pleasure, and motivation (Pierce and Kumeresan 2006). This pathway is composed of the ventral tegmental area (VTA) that projects toward the nucleus accumbens (nAcc), amygdala, hippocampus, medial prefrontal cortex (mPFC), and ventral pallidum. Dopaminergic neurons in the VTA innervate the nAcc and changes in dopaminergic transmission play a critical role in modulating the flow of information through the limbic circuit (Napier and Maslowski-Cobuzzi, 1994; Carr et al., 2000; Kalivas and Nakamura, 1999; Wise, 2002; Jay, 2003; Sesack et al., 2003). In addition, dopaminergic neurons of the VTA that innervate the orbital prefrontal cortex, the nAcc and the basolateral nucleus of the amygdala are involved in the reward actions produced by cocaine (Wise, 2004; Pierce and Kumaresan, 2006).

The use of cocaine can significantly alter the MLS. For example, a single exposure to cocaine has been reported to increase the firing rate and bursting activity of VTA DA neurons (Creed et al., 2016; Fois et al., 2022), concomitantly with alpha-amino-

3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor–mediated and N-methyl-D-aspartate (NMDA) receptor–mediated synaptic potentiation, which are glutamatergic receptors involved in long term potentiation, a cellular hallmark of memory formation (Ungless et al., 2001; Mameli et al., 2007). These changes by acute cocaine can alter both synaptic and intrinsic mechanisms that modulate the functionality of VTA DA neurons (Creed et al., 2016). It has also been reported that chronic exposure to cocaine enhances glutamatergic excitatory afferent towards VTA DA neurons (Ungless et al., 2001; Borgland et al., 2004). In addition, chronic cocaine also disinhibits VTA GABA neurons thus increasing the neuronal firing of VTA DA neurons (Bocklisch, 2013). Alterations in neuronal excitability of the VTA could underlie the formation of SUD. Thus, the study of this region is of main interest to elucidate how SUD is initiated and how we can develop treatments for this disease.

Behavioral Sensitization Model

To better understand the changes induced by drugs of abuse, animal models for SUD have been established that mimic these alterations and allow researchers to study important components of this disease, such as: escalation of drug use, withdrawal, seeking, craving, and relapse. Behavioral sensitization is a simple and environment-dependent model (Vezina et al. 1989; Anagnostaras & Robinson, 1996; Mattson et al. 2008; Vezina & Leyton, 2009) that replicates drug-induced plasticity in both dopamine and glutamate systems (Kalivas & Stewart, 1991;

Vanderschuren & Kalivas, 2000) in a long-lasting manner (Paulson et al. 1991). Sensitization is defined as an increased response to a stimulus after repeated exposure (Robinson & Becker, 1986; Kalivas & Stewart, 1991). This increased locomotion can persist for months after the last drug administration (Castner & Goldman-Rakic 1999; Robinson & Badiani 1998; Robinson & Berridge 1993; Paulson et al. 1991; Strakowski & Sax 1998). Behavioral sensitization as a model of drug addiction is based on the concept of sensitization and uses the experimenter-administered drug as a stimulus (non-contingent drug administration), and the augmented locomotion, resulting from a psychoactive drug like cocaine, as the measurable response.

The two main phases of the sensitization model are initiation and expression. Initiation corresponds to the initial changes produced by the drug in the MLS, and the VTA neurotransmission is hypothesized to be central to this phase (Kalivas and Weber, 1988; Kalivas & Stewart, 1991; Wolf, 1998; Vanderschuren & Kalivas, 2000; Wolf et al., 2004). Studies have correlated acute cocaine-induced locomotor activation with cocaine-induced synaptic potentiation, although after repeated administration of cocaine this correlation was no longer present (Borgland et al., 2004). These results suggest that modulation of VTA DA neurons is critical for the initiation of behavioral sensitization. The expression phase is maintained by the long-lasting changes that follow repetitive drug administration (Kalivas & Stewart, 1991) and is mediated by NAc (Shippenberg et al., 1996; Vanderschuren & Kalivas, 2000; Kalivas & Weber, 1988).

Homeostatic Regulation of Intrinsic Properties

Models of SUD have explored alterations on neuronal excitability in MLS as a key modulator of the development of this disease. Neuromodulation of intrinsic properties are a significant determinant of neuron excitability (Desai, 1999; Turrigiano, 2004). Intrinsic excitability is the modification of neurons' intrinsic electrical properties by neuronal or synaptic activity (Kemenes, 2006; O'Leary, 2011). These modifications are mediated by changes in morphological characteristics and the expression level or biophysical properties of ion channels in the membrane (O'Leary and Williams, 2013). The final response of a single neuron is defined by the combination of both excitatory and inhibitory synaptic inputs, and intrinsic membrane properties (Beck, 2008). These changes in excitability are classified as either those that contribute to the stabilization of neuronal firing (known as homeostatic regulation) or those that produce an alteration from basal neuronal activity (non-homeostatic regulation) (Desai, 1999; Howard, 2007, Wijesinghe, 2011). Homeostatic changes in neuronal excitability can occur in response to extended periods of altered activity (Turrigiano, 1999 & 2000; Keck, 2017). For example, cocaine abuse can alter DA transmission and firing properties of VTA DA neurons. The study of cocaine-induced alterations in the MLS can lead us to elucidate how modifications in excitability are initiated and how could we revert these changes through manipulation of homeostatic regulators.

I_h conductance and the hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN)

The hyperpolarization activated cation current (I_h) is a mixed cationic current produced by the hyperpolarization-activated cation channel, which is permeable to both Na+ and K+ ions (Doan and Kunze, 1999; Magee, 1999; Okamoto, 2006). In contrast to other ionic conductances, I_h is a slowly activating cation inward current that is activated upon hyperpolarization of the membrane potential and through binding of cyclic adenosine monophosphate (cAMP) conducting an inward excitatory current (Kusch, 2010; Wu, 2011). I_h activation depolarizes the membrane to a threshold level for the generation of action potentials. The HCN channels belong to the superfamily of voltage-gated pore loop channels encoded by the HCN1-4 gene family in mammals (Robinson and Siegelbaum, 2003). Interestingly, this channel does not exhibit voltage-dependent inactivation (Biel, 2009). The HCN channel is made up of four subunits (HCN1-4) creating a tetramer channel **(Figure 1)**. Formation of this HCN channel can be homomeric, of one subunit type or heteromeric of two different subunit types (Much et al., 2003). The specific subunit channel composition has been shown to alter biophysical properties if the channel, for example, the HCN1 subunit has the fastest activation kinetics while HCN4 has the slowest. The HCN2 and HCN3 subunits have intermediate activation kinetics. Furthermore, the HCN2 and HCN4 subunits show a high response to cyclic nucleotide binding compared to HCN1 and HCN3 (Ishii, Nakashima, Takatsuka, & Ohmori, 2007).

Each monomer consists of six transmembrane domains (S1–S6) with a positively charged voltage sensor region (S4), a pore region between (S5 and S6) carrying a GYG motif that forms the selective ion filter (Macri, 2012), following this domain a C-linker (80 residue) composed of six a-helices (A'–F') and the cyclic nucleotide binding domain (CNBD). This domain structure consists of three α-helices (A-C) and a β-roll between the A and B helices (Biel, 2009; Wicks, 2011). Together, the C-linker and the CBND are known as the 'cAMP-sensing domain' (CSD) because they are functionally significant for the positive cAMP-induced shift of voltage-dependent activation of HCN channels. Additionally, the CNBD favors a faster channel gating without protein phosphorylation (Santoro & Tibbs, 1999; Santoro, Wainger, & Siegelbaum, 2004; Wainger, DeGennaro, Santoro, Siegelbaum & Tibbs, 2001). It is suggested that cAMP shifts voltage-dependent activation of HCN2 and HCN4 to a positive direction (about 17 mV) increasing opening kinetics; while HCN1 and HCN3 have weak cAMP responsiveness (shifting approximately 2–4 mV) (Wang, 2001; Santoro, 1998; Wainger, 2001; Zagotta, 2003). Other molecules of cyclic nucleotides, such as cyclic guanosine monophosphate (cGMP) and cytidine 3',5'-cyclic monophosphate (cCMP), can modulate activation kinetics, although they have a lower binding affinity to the HCN channel activation gate (Wilson and Garthwaite, 2010; Zong, 2012). Both of these cyclic nucleotides induce a positive shift in the HCN2 and HCN4 activation curves of approximately 6–8 mV, increasing channel activation and slowing the deactivation kinetics of these channels. Furthermore, the HCN channel can also be altered through

phosphoinositide such as phosophatidylinositol-4, 5-bisphosphate (PIP2), which shifts the voltage-dependent activation about 20 mV and channel kinetics. The application of PIP2 significantly slows both the opening during hyperpolarizing steps and the closure after the membrane reaches the 40mV (Pian, 2006; Zolles, 2006). Other phosphoinositide, such as phosphatidic acid (PA) and arachidonic acid (AA), downstream products of diacylglycerol kinase and phospholipase A2, facilitate HCN gating by shifting voltage-dependent activation approximately 5-10 mV in the positive direction (Fogle et al., 2007).

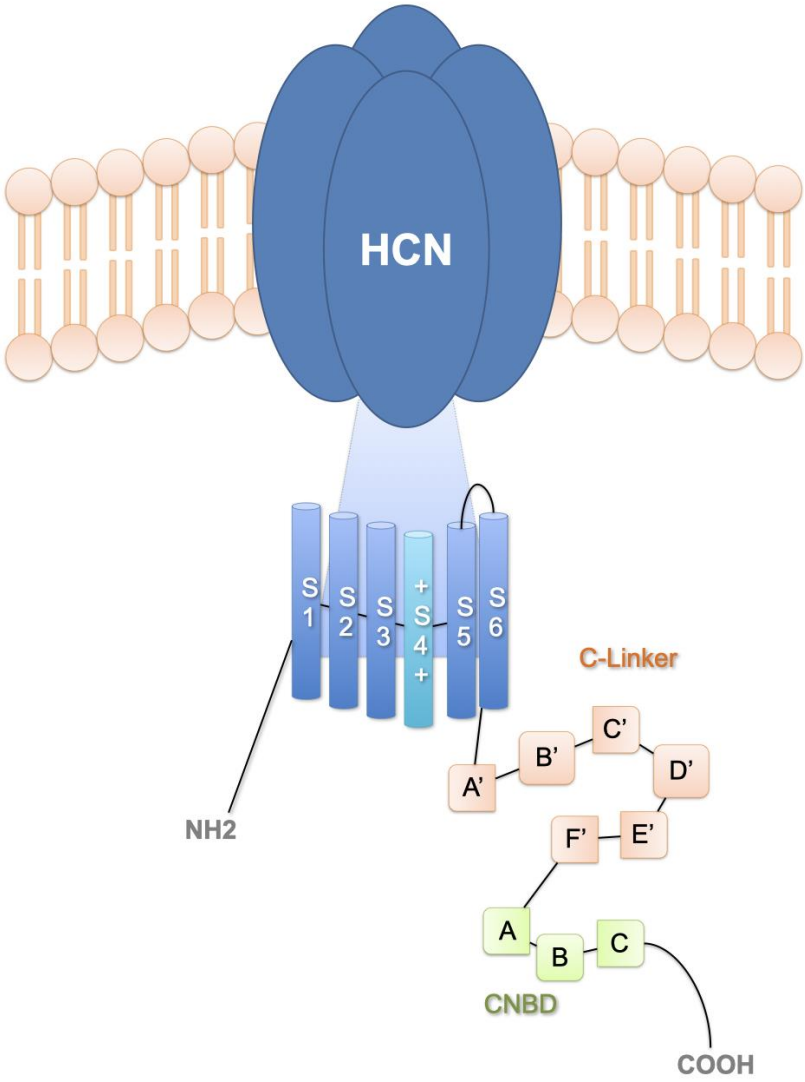

Figure 1. Structure of the HCN channel composed of four subunits (HCN1-4) creating a tetramer channel. The HCN monomer subunit is made up of six transmembrane segments (from S1 to S6). A positive charged voltage sensor (S4) and the pore region amongst S5 and S6. The C-terminal of this channel is composed of the C-linker and the cyclic nucleotide binding domain (CNBD). The C-linker consists of six a-helices: A' to F' The CNBD, or cAMP sensing domain, follows the C-linker domain and consists of α-helices A-C with a β-roll between the A and B helices.

Phosphorylation has also been documented to play a modulatory role in HCN channels. Studies conducted in hippocampal pyramidal neurons indicate that activation of protein kinase C (PKC) decreases I_h and HCN1, while inhibition of PKC increases I_h and HCN1, both mechanisms mediated by serine/threonine phosphorylation (Williams, 2015). In contrast, activation of PKC in dopaminergic neurons of the VTA causes a negative shift in the voltage dependence of I_h activation (Inyushin, 2010). Moreover, a small ubiquitin-like modifier (SUMO) peptide has also been demonstrated to participate in the regulation of protein-protein interactions of HCN channels. SUMOylation of HCN2 subunits increases current conductance and surface expression in HEK cells (Parker et al., 2017).

The HCN channel is also modulated by various scaffold auxiliary proteins that interact with the C-terminus. One example is Filamin A, which, upon binding to the HCN1 subunit, decreases I_h and reduces both the activation and the deactivation kinetics. This interaction occurs through the twenty-two amino acid regions located downstream of the CNBD, a region that is not conserved in HCN2, HCN3, or HCN4. (Gravante and Barbuti, 2004). Furthermore, rab proteins such as the tetratricopeptide repeat-containing Rab8b-interacting protein (TRIP8b), can influence the gating and kinetics of the HCN channel. Studies conducted with various TRIP8b splice variants neurons shifted the voltage gating of HCN channels in negative directions, reduced opening kinetics, and increased closing kinetics (Santoro, 2009; Zolles, 2009). These modifications are determined by interactions

with the C-linker/CNBD of the HCN1 subunits in the HCN channels (Han, 2011; Noam, 2010; Santoro, 2011). It is suggested that TRIP8b and cAMP are in the same HCN channel activation site on CNBD. Through increased cAMP levels, the interaction of TRIP8b with CNBD is disrupted (Han et al., 2011), moreover, TRIP8b expression alters cAMP-dependent activation in HCN2 and HCN4 channels but did not alter channel activation by phosphoinositide and basal hyperpolarizing activation (Zolles et al., 2009).

I_h functional complexity

I_h participates in many neuronal physiological activities. The specific location of the HCN channel within the neuron is associated with its physiological role in membrane excitability (He, 2014). Therefore, I_h could function as an excitatory or inhibitory current depending on its subcellular localization (Santoro, 2003; Wahl-Schott & Biel, 2009). Currently, the precise distribution of HCN channels in VTA DA neurons is still unknown. It has been postulated that the specific subunit composition of HCN channels could be involved in the possible output functionality; this could be caused by the different gating properties that each HCN subunit has on the channel function. Furthermore, it has also been reported that the location of specific excitatory and inhibitory inputs in CA1 pyramidal neurons could explain the opposing roles of the overall functionality of I_h **Figure 2.** (Santoro, 2003). For example, an increase in hyperpolarizing perisomatic inhibitory inputs to CA1 neurons will enhance I_h and firing rate, producing a hyperexcitable network in

seizure models (Chen, 2001). The presence of HCN channels in the distal dendrite, such as in pyramidal neurons of CA1, reduces and shortens the time course of excitatory postsynaptic potentials (EPSP), also known as the shuntting effect (Scharfman et al., 2003; Williams and Stuart, 2000; Lörincz, 2002; He, 2014; Sariati, 2017). This shunt effect decreases the amplitude of EPSP and dampens dendritic integration, thus repressing voltage-dependent activation of calcium channels (Pavlov, 2011**;** Tsay, 2007). Furthermore, the inhibitory effects of I_h have also been linked to its interaction with other ionic conductances, such as K + channels gated by voltage delay rectifiers (George, 2009). Therefore, by reducing I_h, dendritic integration improves through increased input resistance (Zhang, 2006; Fan, 2016). 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 (Gasselin et al., 2015). On the contrary, activation of I_h through an I_h potentiator (Lamotrigine) reduces the firing of action potentials (APs) when initiated by dendritic depolarization, but minimally alters APs initiated by somatic depolarization. I_h enhancement in CA1 neurons overall produces an inhibitory effect in these neurons. It also reduces input resistance, length of temporal summation, dendritic integration, and neuronal excitability (Poolos, 2002).

In contrast, I_h has also been classified as an excitatory driving force in DA neurons from VTA (Neuhoff, 2002; Wanat, 2008). For example, empirical data demonstrate

that ethanol significantly increases the firing frequency of VTA DA neurons and facilitates I_h voltage gating (Brodie, 1990; Okamoto, 2006). Furthermore, by repeated exposure to ethanol, there is a ~25% density downregulation of I_h. Also, pharmacological blockade of I_h attenuates DA neuron firing exposed to ethanol (Okamoto, 2006; McDaid, 2007). Ethanol increases AP firing through I_h activation. This firing rate enhancement is mediated by the increase in the activation kinetics of this channel (Okamoto, 2006). The change in activation kinetics is not present after chronic cocaine administration (Arencibia-Albite, 2012), which could be the basis for other types of neuroadaptations. Other publications have shown that pharmacological I_h blockade suppressed DA neurons firing in VTA and Substantia Nigra Compacta (SNc) neurons (Tateno, 2011; Seutin, 2001). After this pharmacological blockade, an artificial injection of I_h restored the normal intrinsic DA cell membrane potential phenotype (Tateno, 2011). Furthermore, in a social defeat stress model, susceptible depressed mice demonstrate hyperexcitability of the VTA DA neuron, which is caused by upregulation of I_h. Interestingly, resilient mice demonstrate higher I_h-conductance; concomitantly an increase in potassium channel currents (K +) was also present which regulated firing frequency (Friedman, 2014). These results indicate a pro-excitatory role for I_h in dopaminergic neurons in the midbrain. Taking into account these different grades of complexity of channel composition, membrane localization, activation kinetics, and overall response can lead us to discover important variations in the effect of I_h on neuronal excitability (**Figure 2)**. Dopaminergic neurons are electrotonically compact; different ionic densities are present in the soma compared to dendritic

compartments. This fact suggests that these neurons may have specialized electrical compartments with different functional roles (Gantz, 2017). The idea of specialized electrical compartments could explain the possibility of opposing functions of the I_h (dendritic and somatic); in addition, both excitatory and inhibitory responses could be possible.

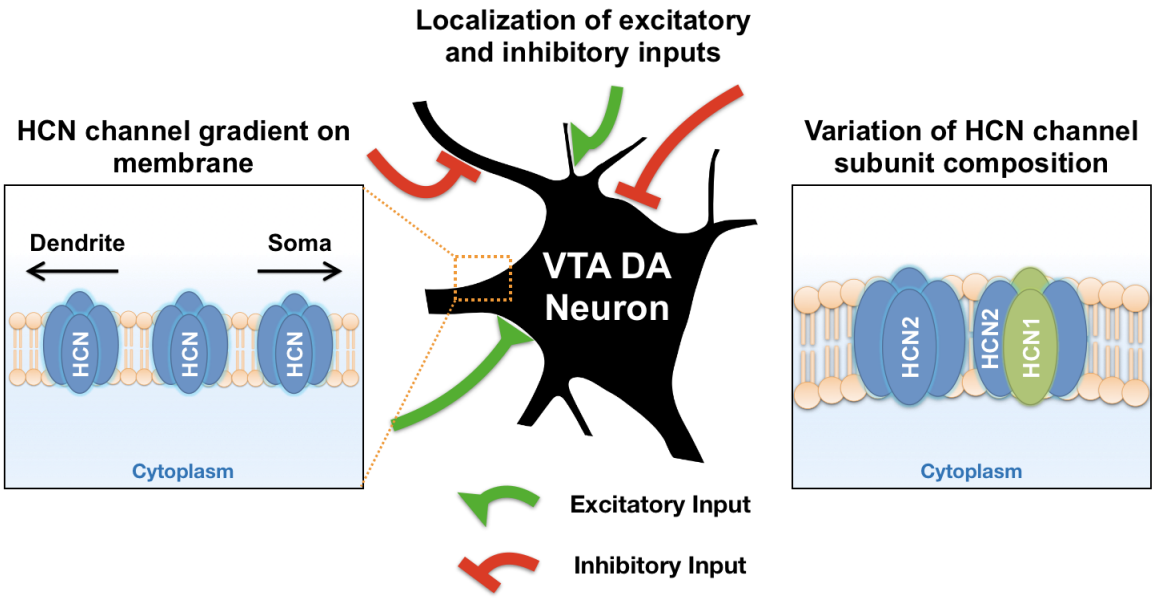

Figure 2. Functional complexity of the hyperpolarization-activated cyclic nucleotide-gated cation channels. The location of excitatory and inhibitory inputs in the soma or dendrite of dopaminergic neurons in the ventral tegmental areas can influence how the HCN channel contributes to increasing or decreasing neuronal excitability. The presence of an HCN channel in the soma and dendrite may create a specific gradient or a specialized electrical compartment, which could differentially regulate excitability. Additionally, variation in the subunit composition of the HCN channel can also influence channel activation and excitability.

I_h role in regulation of VTA DA excitability

The dual functional role of I_h leads us to question its participation in VTA DA neuronal excitability. For example, overexpression of HCN2 ionic channels in the VTA of rats increased voluntary alcohol consumption, locomotor activity, and dopamine release into the nAcc (Rivera-Meza, 2014). Reports have also indicated that optogenetic activation of VTA DA neurons can induce enhanced locomotor activity and phasic patterns of dopamine release in nAcc (Guo, 2014; Bass, 2013). Although without assuming a direct relationship, this could suggest that HCN2 overexpression can increase dopamine release into nAcc. Furthermore, the I_h potentiator (lamotrigine) significantly increased the firing rate of VTA DA neurons (Friedman, 2014). These studies shed light on the possibility that I_h up-regulation may facilitate AP firing by rapidly depolarizing the membrane near the resting membrane and the subthreshold potential. Furthermore, when the voltage dependence of HCN channels is shifted to the depolarizing direction by cAMP or Phosphatidylinositol 4,5-bisphosphate (PIP2), HCN channels can be activated at around the resting membrane potential and can act as an excitatory factor (Kase & Imoto, 2012). Up-regulation of the HCN channel is hypothesized to not alter the reversal potential; this is because a shift in the reversal potential of HCN channels has only been reported to be produced by an increase in outer potassium concentration (5 to 20 mM) in neurons of the postrema area (Funahashi, 2003). In addition, membrane input resistance can be reduced by HCN up-regulation; ethanol perfusion activates I_h and reduces input resistance in VTA DA neurons

(Okamoto, 2006). Up-regulation of HCN channels will not affect the activation curve. This is because cyclic nucleotide molecules such as cAMP directly modify the channel kinetics and it is not mediated by channel overexpression onto the membrane. Activation of the time constant, which is how rapidly current flow changes the membrane potential, is suspected to be reduced by I_h upregulation similar to the ethanol-induced time constant activation (Okamoto, 2006). I_h channels are also present in distal dendrites; their specific distribution and density can influence the processing of synaptic signals, preventing the transition from spontaneous firing to bursting activity of VTA DA neurons (Arencibia-Albite, 2007). Up-regulation of dendritic I_h could facilitate reduction of excitatory inputs to VTA DA neurons. Although this contradicts the pro-excitability role of I_h, discovering the specific location of the HCN channel gradient and localized channel activation could clarify I_h's effect on neuronal excitability. In contrast to the expressed above, down-regulation or blockade of I_h reduces the firing rate, increasing the interspike interval and causing a slower transition from the hyperpolarizing state to the resting membrane potential and the subthreshold potential in VTA DA neurons (Seutin, 2001; Tateno, 2011; Okamoto, 2006; McDaid, 2008; Zhong, 2017).

Hypothesis and Rationale:

I_h has been proven to be an essential modulator of various intrinsic mechanisms, membrane excitability, and synaptic properties such as dendritic integration. HCN channels present distinct levels of complexity, starting from the specific subunit

composition of the forming channel, where excitatory and inhibitory inputs project onto VTA DA neurons and the possibility of a specialized electrical compartment within the neuron. Although a dichotomy of the physiological role of I_h could still be argued, evidence suggests that it is a depolarizing current that influences the excitability of the VTA DA membrane.

Previous publications from our laboratory demonstrated that cocaine sensitization, significantly reduces the amplitude of I_h by ~40% in VTA DA neurons (Arencibia-Albite et al., 2012). This current reduction occurs concomitantly with the reduction of the membrane surface HCN2 protein, the main HCN subunit in DA neurons VTA (Santos-Vera, 2019). An important observation is that after the development of cocaine sensitization, the spontaneous firing of VTA DA cells remains similar to that of the control groups (Arencibia-Albite et al., 2012). These findings suggest that I_h reduction could decrease cocaine-induced excitability as a homeostatic adaptation to regulate the neuronal excitability of VTA DA neurons.

Our main goal is to understand how I_h contributes to VTA DA neuronal excitability. To elucidate if an I_h reduction can alter basal VTA DA neuronal excitability in our first aim, we will: 1A) Block I_h and evaluate firing properties such as rebound spiking using whole-cell patch-clamp electrophysiology from naïve rats. Rebound spiking is defined as the production of one or more action potentials in response to the cessation of a prolonged hyperpolarizing current step. 1B) Determine the effect of I_h blockade on *in vitro* spontaneous excitability using cell-attached

configuration from naïve rats. 1C) Evaluate the effect of I_h blockade on spontaneous firing patterns using single-unit extracellular recordings from naïve rats. **We hypothesize that I_h blockade will reduce rebound and spontaneous firing on VTA DA cells.**

To investigate how this current modulates cocaine-dependent excitability, we postulate that I_h reduction serves as a homeostatic regulation to oppose cocaine-induced excitability. For our second aim, we will: 2A) Measure I_h current using whole-cell patch-clamp electrophysiology, 2 and 24 hours after an acute cocaine injection. Determine the increase in rebound action potentials, 2 and 24 hours after an acute cocaine injection. Additionally, we will evaluate the effect of I_h blockade on rebound spiking 2 hours after an acute cocaine injection. 2B) Evaluate the effect of I_h blockade on *in vitro* spontaneous excitability using cell-attached configuration, 2 hours after an acute cocaine injection. 2C) We will measure changes in HCN2 subunit expression on the VTA using Western blot total protein quantification**.** Acute cocaine administration produces a significant increase in firing rate 3 hours after injection and lasts up to 5 days (Creed at al. 2016). The overall cocaine effect can be influenced by the environmental context in which the drug is experienced (Caprioli et al., 2007). One question we address is how exposure to cocaine in a novel context interferes with persistent alterations on neuronal excitability in the MLS. For this, 2D) an acute cocaine injection will be administered to subjects in both a novel context or their home-cage. 24 h after treatment, AMPA-to-NMDA receptor-mediated EPSC ratio will be quantified to

determine if cocaine experienced in a novel environment produces a higher magnitude of synaptic potentiation. Furthermore, we will evaluate the increase in firing rate 24 hours after an acute cocaine injection in a novel environment, to: Determine the effect of I_h blockade on cocaine-induced spontaneous firing patterns using single-unit extracellular recordings. **We hypothesize that acute cocaine will trigger a progressive reduction of I_h 24 hours after cocaine injection.** Additionally, it is hypothesized that I_h blockade will further decrease cocaine-induced rebound and spontaneous firing in VTA DA neurons. To our knowledge, our laboratory is the first one to address the role of the I_h current and HCN subunits in the VTA after cocaine administration.

For our third aim, we will pharmacologically enhance VTA DA excitability and record changes in evoked rebound action potentials and I_h. To address this naive rat VTA brain slices will be incubated with bicuculline methiodide (BIC) as a mechanism to enhance excitability and promote rebound excitation through GABAergic disinhibition. **Our hypothesis is that I_h will decrease as a consequence of the pharmacological enhancement in excitability, I_h blockade will further reduce this increase of excitability.** In addition, we will determine if I_h blockade can reduce VTA DA spontaneous enhanced excitability. We will induce synaptic potentiation through High-Frequency Stimulation of the Ventral Subiculum (HFSvSUB). It has been demonstrated that after 5 days HFSvSUB potentiation elicits *in vivo persistent* hyperactivity of VTA DA neurons (Glangetas, 2015). This stimulation has also been documented to elicit cocaine-

seeking behavior dependent on glutamate release in the VTA (Vorel et al. 2001). We will determine the effect of I_h blockade on HFSvSUB potentiated firing patterns using single-unit extracellular recordings. We postulate that I_h blockade will reduce HFSvSUB-induced excitability on VTA DA cells.

The study of the regulatory role of this channel can provide new insights on how the VTA DA system adjusts neuronal excitability and the way these modifications can affect efferent projections.

Chapter 2

To elucidate if an I_h reduction can alter basal VTA DA neuronal excitability.

Results obtained as part of this aim were published in the following article:

Bosque-Cordero, Karl Y., Rafael Vazquez-Torres, Cristhian Calo-Guadalupe, Daisy Consuegra-Garcia, Giulia R. Fois, François Georges and Carlos A. Jimenez-Rivera. "I_h blockade reduces cocaine-induced firing patterns of putative dopaminergic neurons of the ventral tegmental area in the anesthetized rat." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 112 (2022): 110431.

Specific Aim 1

Determine if I_h reduction can alter basal VTA DA neuronal excitability.

Hypothesis: I_h reduction will decrease basal VTA DA neuronal excitability.

Specific Aim 1A: Elucidate the effect of I_h blockade on Rebound Action Potentials of VTA DA neurons from naive rats.

Rationale: Rebound spiking is defined as the production of one or more action potentials in response to the cessation of a prolonged hyperpolarizing current step (Santos et al., 2021). Activation of HCN channels by hyperpolarization causes the membrane to rebound, increasing the probability of subsequent AP firing (Tsantoulas, 2016). If I_h contributes to VTA DA excitability, I_h blockade should reduce Rebound Action Potentials.

Specific Aim 1B. Determine if I_h blockade can alter *in vitro* spontaneous VTA DA neuronal firing.

Rationale: I_h blockade decreases firing rate through an increase in rebound delay and interspike interval (Seutin, et al,. 2001; Tateno, et al,. 2011; Migliomore, et al,. 2012 and Zhong et al,. 2018). Hence, I_h could play a prominent role in the spontaneous firing of VTA DA neurons. Consequently, I_h blockade will reduce the firing rate while increasing the interspike interval.

Specific Aim 1C. Effect of I_h blockade on *in vivo* anesthetized single-unit spontaneous VTA DA neuron firing.

Rationale: I_h increases burst formation by enhancing rebound spiking after the disinhibition of VTA DA neurons (Tateno, 2011). Although this has been suggested, the relationship between I_h and bursting has not been well established. We will explore the effect of I_h blockade on the firing patterns of VTA DA neurons (Firing Rate, Bursting Frequency, and Spikes in Burst). Our hypothesis is that I_h blockade will reduce firing rate of VTA DA neurons.

Experimental Procedures:

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.

Whole-cell and Cell-attached Patch Clamp Electrophysiology:

Midbrain horizontal slices (220 μm) containing the VTA were prepared from male Sprague Dawley rats (35–51 days postnatal) as previously described (Arencibia-Albite et al., 2007). Cell-attached and 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) (Paxinos and Watson, 2009). Margolis et

al. (Margolis et al., 2006a; Margolis et al., 2006b) 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 (Borgland et al., 2004). 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 (Hopf, 2007). 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 MΩ when filled with (in mM) 115 KCH^3SO^4 (methyl potassium sulfate), 20 KCl, 1.5 $MgCl^2$, 5 HEPES, 1 EGTA, 2 ATP, 0.2 GTP, and 10 creatine phosphate (CP), pH 7.25, 290 mOsm. Sodium (Na)GTP, (Mg)ATP, and (Na)CP was added fresh daily. Artificial cerebrospinal fluid (ACSF) contained (in mM) 127 NaCl, 2.5 KCl, 1.25 NaH^2PO^4, 25 $NaHCO^3$, 2 $CaCl^2$, 1 $MgCl^2$, and 25 D(+)-glucose, and was equilibrated with 95% O^2 5% CO^2 at 35 °C. Data sets were collected through an Axopatch 200B amplifier (Axon Instruments; Molecular Devices, Sunnydale, CA), digitized at 5 kHz, filtered at 1 kHz, 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.

***in vivo* 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 and 38 °C with an animal temperature controlled electric heating pad during the procedure (World Precision Instruments, Sarasota, FL). A glass micropipette (1-2 μm, 10-12 MΩ filled with 2.0% pontamine sky blue in 0.5 M sodium acetate) were lowered into the VTA. A double barrel pipette was used, similar to the ones used by Georges and Aston-Jones (Georges and Aston-Jones, 2002). The perfusion pipette on the double barrel pipette was filled with ZD 7288 (8.3 μM) an I_h blocker disolved 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 nondopamine neurons, the following parameters were used: (1) an action potential duration 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 s was recorded then 100 s after delivery of 60 nL of the ZD 7288 solution.

Data analysis:

All data were presented as mean ± 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 or two-way ANOVA followed by Newman-Keuls or Bonferroni multiple comparison as a post hoc analysis. P values were reported throughout the text and the significance was set at $p < 0.05$.

Results:

Specific Aim 1A. I_h blockade reduces rebound action potentials in VTA DA neurons

We evaluated the effect of I_h blocker on rebound spiking in VTA DA neurons of naïve animals. Rebound spiking was measured using whole-cell patch-clamp current-clamp configuration by first hyperpolarizing the neuron and then stimulating consecutive depolarizing current steps (increments of 50 pA, 1-s current pulse) **(Figure 3A)**. Post perfusion of ZD 7288(10uM) abolished I_h

amplitude **(Figure 3B)**. Additionally, rebound action potentials significantly reduced after ZD 7288 perfusion at current injection steps (50, 100, and 150 pA) **(Figure 3C)**. We conclude that I_h contributes to the formation of rebound action potentials. Moreover, I_h blockade is capable of reducing the probability of rebound action potentials formation.

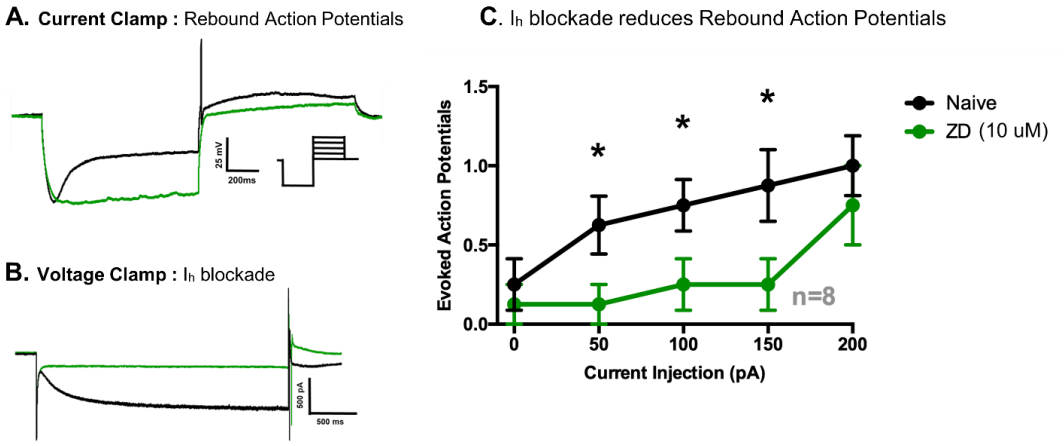

Figure 3. Post perfusion I_h blockade significantly reduced evoked rebound action potentials. *in vitro* whole-cell patch-clamp recording (current clamp) of VTA neurons demonstrating spike discharges in response to consecutive depolarizing current steps (increments of 50 pA, 1-s current pulse, see inset) after I_h activation. A. Sample trace of Current clamp Rebound protocol (Trace 3, 100pA) before and after ZD7288 (10μM) perfusion. B. Sample trace of Voltage Clamp I_h amplitude before and after ZD7288 (10μM). C. Rebound Action Potentials were significantly reduced by I_h blockade. Paired student t-test (n=8); *Asterisk denotes significant differences ($p<0.05$).

Specific Aim 1B. I_h blockade reduces *in vitro* spontaneous excitability and increases the interspike interval on VTA DA neurons

We evaluated the effect of I_h blockade on *in vitro* spontaneous excitability in VTA DA neurons of naïve animals using cell attached recordings. A minimum of 100 seconds of baseline spontaneous firing activity was recorded and then bath application of ZD 7288 (10μM) was perfused to see the effect of I_h blockade on spontaneous firing in the same neuron. To identify dopaminergic neurons Baclofen was perfused after ZD 7288 bath application **(Figure 4A)**. We found that I_h blockade significantly reduced firing rate (n=6, paired t-test, t = 2.633 df = 5) **(Figure 4B)**. In addition, we discovered a significant increase in the mean interspike interval of these recordings (paired t-test, t = 5.141 df = 5) **(Figure 4C)**. These results suggest that I_h blockade can significantly reduce spontaneous firing in VTA DA neurons in part due to an increase in the mean interspike interval. In addition, through I_h modulation we can regulate the spontaneous excitability of these cells.

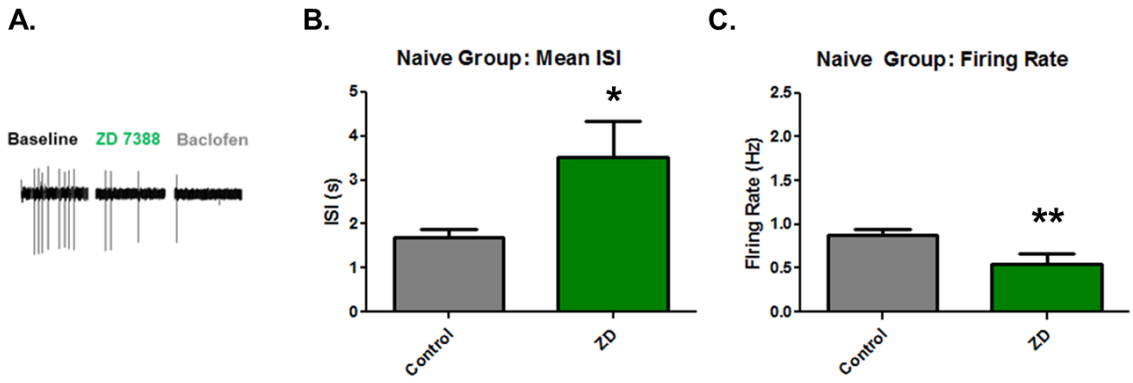

Figure 4. I_h blockade effect on *in vitro* cell-attached recordings of VTA DA neurons. A. Sample traces before and after I_h blocker ZD 7288 (10μM) bath application perfusion, baclofen (1μM) was perfused to identify dopaminergic neurons. B. The mean Interspike interval was significantly increased after ZD 7288 infusion (paired t-test, t = 5.141 df = 5. C. I_h blockade significantly reduced the firing rate (n=6, paired t-test, t = 2.633 df = 5).. *Asterisk denotes significant differences ($p < 0.05$).

Specific Aim 1C. Effect of I_h blockade 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 (Georges and Aston-Jones, 2002). These pipettes were comprised of a recording electrode and a perfusion pipette that contained ZD 7288 (8.3 μM). We recorded a minimum of 100 s of baseline extracellular firing activity and then locally perfused at the vicinity of the recorded cell, 60 nL of ZD 7288 to see the effect of I_h blockade on neuronal firing patterns as seen on sample traces **(Figure 5A)**. Local infusion of ZD 7288 significantly reduced the firing rate on VTA DA neurons **(Figure 5B)**. The bursting frequency was also significantly reduced by I_h blockade **(Figure 5C)**. 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 5D)**. The mean inter-spike intervals (ISI) from each recorded neuron were analyzed. As shown in **(Figure 5E)**, 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 ±0.02122 s to 0.1750 ± 0.02497 s after ZD 7288 infusion **(Figure 5F)**. On **(Figure 5G)**, 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 ±0.0003 s to 0.2128 ±0.0046 s 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.

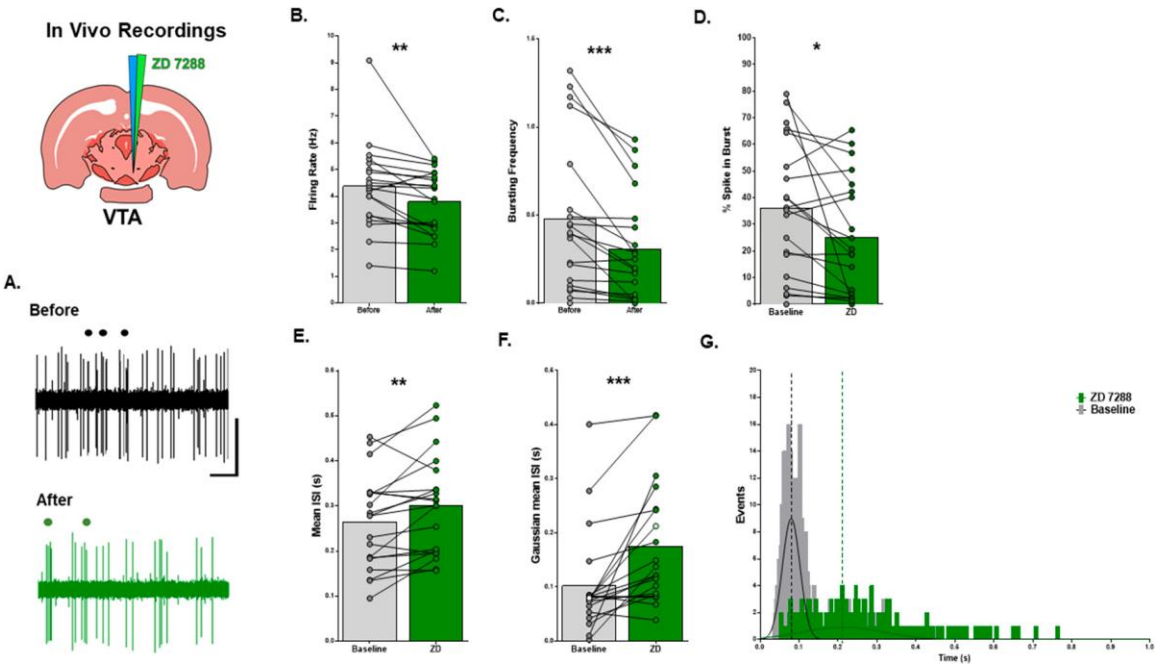

Figure 5. I_h blockade significantly reduced firing properties on *in vivo* extracellular recordings of VTA DA neurons. A. Sample traces before and after I_h blocker ZD 7288 (8.3μm) perfusion. Each dot represents a burst event. Scale bars: horizontal, 1 s; vertical, 1 mV. B. I_h blockade significantly reduced the firing rate (paired t-test, t = 2.912 df = 19). C. 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). E. Mean Interspike interval was significantly increased after ZD 7288 infusion (paired t-test, t = 3.075 df = 19, rats n = 8, cells = 20). F. Gaussian fitting mean ISI significantly increased after ZD 7288 infusion. G. A representative naive 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 ($p < 0.05$).

Specific Aim 1C. 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 inter-spike 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 (Mameli-Engvall et al., 2006). On **(Figure 6A, B)**, 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 ($p < 0.05$; $\chi 2$ test) **(Figure 6C)**. These results suggest that I_h blockade is sufficient to change basal firing mode categories.

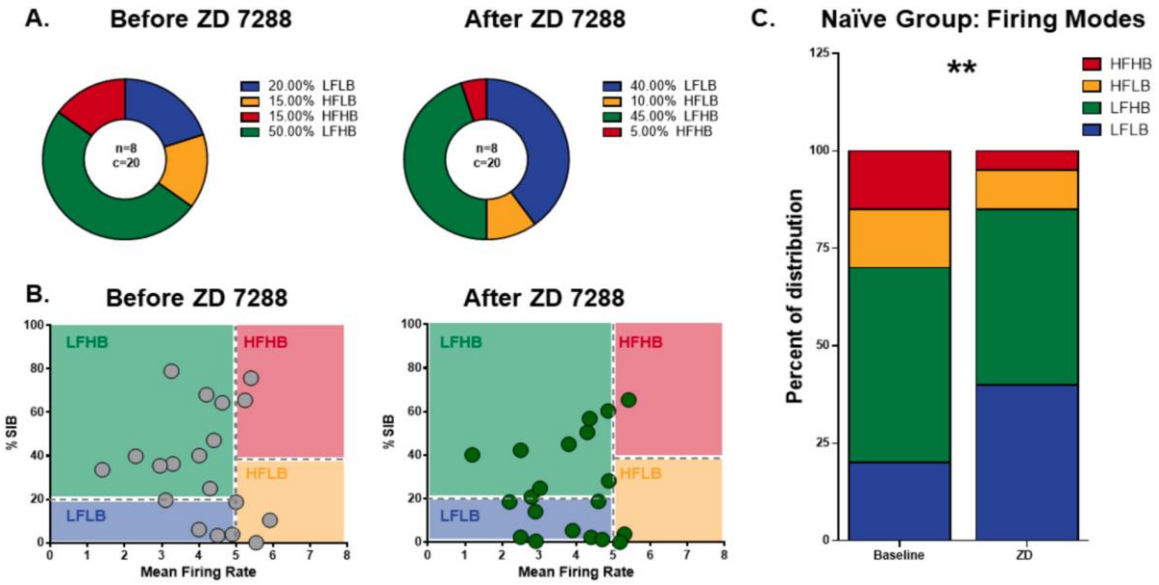

Figure 6. Effect of I_h blockade on VTA DA neuron firing modes. A. Pie chart of naïve group firing mode population before and after ZD 7288. B. Mean Firing rate vs percent of spikes in burst of each recording. Colors represent lighter versions of the ones depicted in the pie charts. C. Naïve group firing pattern percent of distribution demonstrates a significant difference in the HFHB and HFLB groups after ZD 7288 ($p < 0.05$; $\chi 2$ test).

Chapter 3

To demonstrate a progressive reduction of I_h by repeated cocaine administration. To determine if I_h reduction is a response mechanism that opposes cocaine-induced excitability.

Results obtained as part of this aim were published in the following articles:

Bosque-Cordero, Karl Y., Rafael Vazquez-Torres, Cristhian Calo-Guadalupe, Daisy Consuegra-Garcia, Giulia R. Fois, François Georges and Carlos A. Jimenez-Rivera. "I_h blockade reduces cocaine-induced firing patterns of putative dopaminergic neurons of the ventral tegmental area in the anesthetized rat." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 112 (2022): 110431.

Fois, Giulia R., **Karl Y. Bosque-Cordero**, Rafael Vazquez-Torres, Cristina Miliano, Xavier Nogues, Carlos A. Jimenez-Rivera, Stéphanie Caille, and François Georges. "Locus coeruleus activation during environmental novelty gates cocaine-induced long-term hyperactivity of dopamine neurons." *iScience* 25, no. 4 (2022): 104154.

Specific Aim 2

To demonstrate a progressive reduction of I_h by repeated cocaine administration. To determine if I_h reduction is a response mechanism that opposes cocaine-induced excitability.

Hypothesis: I_h will be progressively reduced by repeated cocaine administration as a response mechanism to oppose cocaine-induced excitability.

Specific Aim 2A. Elucidate the progressive reduction of I_h amplitude 2 or 24 hours after acute cocaine administration.

Rationale: Cocaine sensitization, a chronic cocaine behavioral model, significantly reduces I_h amplitude in VTA DA cells (Arencibia-Albite et al., 2012). Hence, we hypothesize that acute cocaine administration can trigger a progressive reduction of I_h after acute cocaine injection.

Specific Aim 2B. Determine if I_h blockade can reduce the increase on *in vitro* spontaneous firing after 2 hours of acute cocaine administration.

Rationale: Acute cocaine significantly increases firing activity after 3 hours of administration on *in vivo* VTA DA neurons (Creed, 2016). Here, we will measure spontaneous firing 2 hours after acute cocaine administration and explore the effects of I_h blocker on cocaine-induced spontaneous

excitability. We expect a significant increase in spontaneous firing activity 2 after acute cocaine administration.

Specific Aim 2C. Quantify HCN2 subunit protein expression on the VTA.

Rationale: Cocaine sensitization alters HCN channels expression in the VTA. We have demonstrated a significant increase in total protein of the HCN2 subunit on the VTA (Santos-Vera, 2014). Surface to intracellular membrane distribution of the HCN2 subunit demonstrated a significant reduction on the VTA (Santos-Vera, 2019). To elucidate if repeated cocaine progressively reduces the HCN2 subunit, we will quantify this protein 24 hours after acute cocaine injection and 24 hours after two consecutive days of cocaine injections.

Specific Aim 2D. Determine the effect of I_h blockade on *in vivo* extracellular firing patterns 24 hours after cocaine administration.

Rationale: Acute cocaine administration significantly increases firing activity on *in vivo* VTA DA neurons (Creed, 2016). I_h blockade decreases firing rate through an increase in rebound delay and interspike interval (Seutin, 2001; Tateno, 2011; Migliomore, 2012; Zhong, 2018). Thus, I_h blockade may reduce cocaine-induced enhanced in vivo spontaneous activity. For this, we will record single-unit extracellular recording and perfuse through a double-barrel pipette I_h blocker (ZD7288, 8.3um). We

hypothesize a significant reduction in the firing activity by I_h blockade in VTA DA neurons.

Experimental Procedures:

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.

Whole-cell and Cell-attached Patch Clamp Electrophysiology:

Midbrain horizontal slices (220 μm) containing the VTA were prepared from male Sprague Dawley rats (35–51 days postnatal) as previously described (Arencibia-Albite et al., 2007). Cell-attached and 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) (Paxinos and Watson, 2009). Margolis et al. (Margolis et al., 2006a; Margolis et al., 2006b) 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 (Borgland et al., 2004). Nonetheless, we recorded from lateral VTA neurons medial to the MT, where other authors have reported that I_h and TH are colocalized in ~75% of cells (Hopf, 2007). 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 MΩ when filled with (in mM) 115 KCH^3SO^4 (methyl potassium sulfate), 20 KCl, 1.5 $MgCl^2$, 5 HEPES, 1 EGTA, 2 ATP, 0.2 GTP, and 10 creatine phosphate (CP), pH 7.25, 290 mOsm. Sodium (Na)GTP, (Mg)ATP, and (Na)CP was added fresh daily. Artificial cerebrospinal fluid (ACSF) contained (in mM) 127 NaCl, 2.5 KCl, 1.25 NaH^2PO^4, 25 $NaHCO^3$, 2 $CaCl^2$, 1 $MgCl^2$, and 25 D(+)-glucose, and was equilibrated with 95% O^2 5% CO^2 at 35 °C. Data sets were collected through an Axopatch 200B amplifier (Axon Instruments; Molecular Devices, Sunnydale, CA), digitized at 5 kHz, filtered at 1 kHz, 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.

In vivo 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 and 38 °C with an animal temperature controlled electric heating pad during the procedure (World Precision Instruments, Sarasota, FL). A glass micropipette (1-2 μm, 10-12 MΩ filled with 2.0% pontamine sky blue in 0.5 M sodium acetate) were lowered into the VTA and a double barrel pipette was used, similar to the ones used by Georges and Aston-Jones (Georges and Aston-Jones, 2002). The perfusion pipette on the double barrel pipette was filled with ZD 7288 (8.3 μM), an I_h blocker disolved 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 nondopamine 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 s was recorded then 100 s after delivery of 60 nL of the ZD 7288 solution.

Tissue Extraction:

Using an intraperitoneal (i.p.) injection of chloral hydrate (400mg/kg), animals were deeply anesthetized and immediately decapitated, 24 hours after acute cocaine injection. Brains were quickly removed from the skull and placed in ice-cold PBS (0.01 M phosphate buffered solution, pH 7.4, Sigma-Aldrich, St. Louis, MO). Immediately coronal sections of 200-400 µm at the level of the VTA will be made following Paxinos and Watson Rat Brain Atlas (2012) as dissection guide, using a Leica® Vibratome VT1000S (Leica, Germany). Brain slices were then mounted onto a glass surface and immediately frozen with dry ice. Two micropunches of the VTA were removed using a 0.8 mm stainless steel hypodermic tube (Fine Science Tools), transferred to ice cold microtubes 1000µL with lysis buffer and stored at -80°C.

SDS-PAGE and Western Blot:

Dissected tissue were thawed and homogenized through sonication in ice cold microtubes 1000µL with lysis buffer containing protease and phosphatase inhibitors (20 mM Tris, 150 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, pH 8) containing 2 µg/mL antipain, 10 µg/mL aprotinin, 5 mM benzamidine, 1 mM DTT,

10 µg/mL leupeptin, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 µg/mL trypsin inhibitors in the presence of 1% NP-40 detergent. Following centrifugation (20,000 g, 30 min at 4℃) the protein concentration of the supernatant fraction was determined using Bio-Rad's DC Protein assay (Bio-Rad Laboratories, Hercules, CA). Extracted proteins (40µg) were resolved in a 7.5% or 4-15% precast SDS gel and electrophoresed for 2.5 hours at 100 V (300 mA and 10 W at room temperature). This amount was used because is withing the linear detection range, without reaching saturations. Proteins was transferred to a nitrocellulose membrane using Turbo Trans-Blot transfer system (Bio-Rad Laboratories, Hercules, CA) with transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH, pH 8.3) for 3 min at 100 V constant voltage. To validate protein transference, the nitrocellulose membrane was stained with 0.1% Ponceau S solution, made in 0.1% glacial acetic acid for 10 min, and then rinsed with PBS (3x, 10 min/each). The nitrocellulose membrane was incubated in blocking solution (15% skim milk, 20 mM Tris-HCl, 150 mM NaCl, 0.01% Tween-20, pH 7.5) for 2 hours at room temperature. The membrane was probed with anti-mouse HCN2 monoclonal antibodies (1:500; NeuroMabs Labs/ Antibodies Incorporated, UC Davis, CA) in blocking solution (overnight, 4ºC). Next day, the membrane was washed in PBS-T (PBS with 0.01% Tween 20; 3x, 10 min/each) and then incubated with HRP-conjugated anti-mouse IgG produced in rabbit (1:5,000; Sigma, St. Louis, MO) for 1 hour at room temperature. Then, membranes were washed with PBS-T (2X, 10 min/each) and Tris-NaCl (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20, pH 7.5; 2X, 10 min/each). HRP-signal of protein bands corresponding to 110kDa (HCN2

glycosylated protein), 97kDa (HCN2 non-glycosylated protein) were enhanced with Super Signal West Dura extended version (Pierce, Rockford, IL) for 1 min according to manufacturer's instruction before exposure and development. The levels of HCN2 proteins were normalized to GAPDH (37kDa) immunoreactivity (1:5,000; Sigma-Aldrich, St.Louis, MO). The membrane densitometry analysis was performed using ChemiDoc™ Imaging System and ImageLab 5.1 Software® (Bio-Rad Laboratories, Hercules, CA).

Data analysis:

All data were presented as mean ± 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 $p < 0.05$.

Results:

Specific Aim 2A. I_h progressively reduces after cocaine administration

Using whole-cell patch-clamp voltage-clamp protocol we, evaluated I_h amplitude 2 and 24 h after acute cocaine injection (15 mg/kg, i.p.) and after chronic (7 days) cocaine administration (cocaine sensitization) **(Figure 7A)**. Two hours after acute cocaine, there is no significant difference in I_h amplitude compared to control. However, 24 h after acute cocaine, we found a significant reduction in I_h amplitude 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 7B)** and (Arencibia-Albite et al., 2012). Using a rebound spiking protocol, we found a significant increase in rebound action potentials after 2 hours of cocaine in comparison to 24 hours **(Figure 7C)**. I_h blockade significantly reduced rebound spiking in the 2 hour group. These results suggest that the progressive I_h reduction after cocaine administration could serve as a homeostatic adaptation for neuronal excitability of VTA DA neurons.

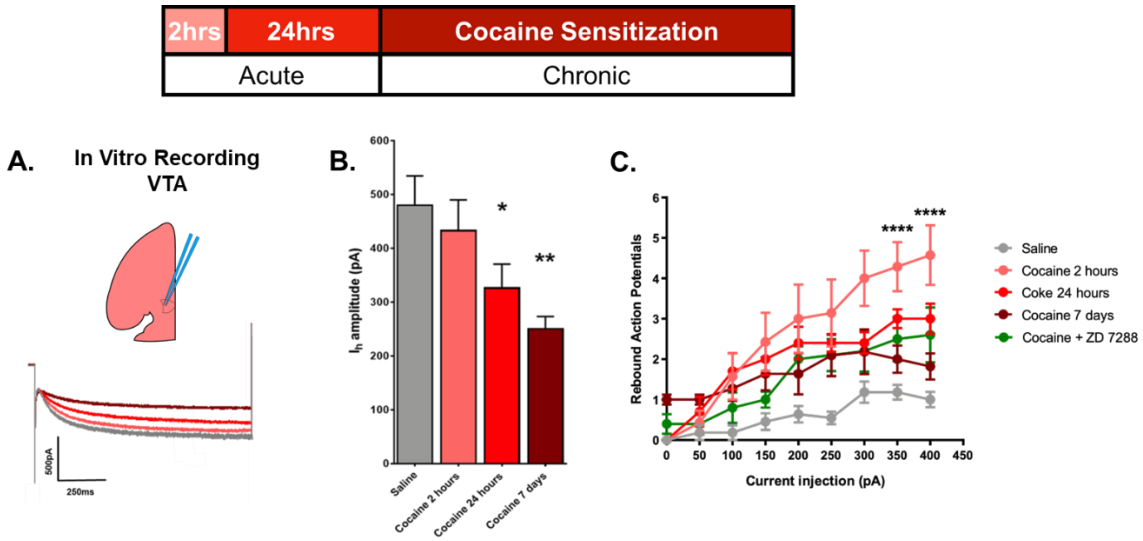

Figure 7. Progressive I_h reduction after acute cocaine administration. A. Whole cell patch-clamp voltage-clamp protocol I_h amplitude. B. Recordings 2 and 24 h after acute cocaine injection (15 mg/kg) and after chronic (7 days) cocaine administration. A significant I_h reduction occurs only 24 h after acute cocaine administration. One-Way ANOVA, Newman-Keuls multiple comparison test $F(3,42) = 5.659$. C. Current Clamp recordings of VTA DA neurons demonstrating spike discharges in response to consecutive depolarizing current steps (increments of 50 pA, 1-s current pulse) after I_h activation. Significant increase in rebound action potentials were obtained 2h, 24h and, 24h following 7-days cocaine administration. In addition, I_h blockade significantly reduces rebound action potentials. One-Way ANOVA, Newman-Keuls multiple comparison test. One-Way ANOVA, Newman-Keuls multiple comparison test. *Asterisk denotes significant differences ($p<0.05$).

Specific Aim 2B. I_h blockade reduces *in vitro* cocaine-induced spontaneous activity and increases the interspike interval in VTA DA neurons.

We evaluated the effect of I_h blockade on *in vitro* acute cocaine-induced spontaneous excitability in VTA DA neurons using cell attached recordings. A minimum of 100 seconds of baseline spontaneous firing activity was recorded and then bath application of ZD 7288 (10μM) was perfused to see the effect of I_h blockade on spontaneous firing in the same neuron. To identify dopaminergic neurons Baclofen was perfused after ZD 7288 bath application **(Figure 8A)**. We discovered a significant increase in the mean interspike interval of these recordings (paired t-test, t = 5.141 df = 5) **(Figure 8B).** In addition, we found that I_h blockade significantly reduced firing rate (n=6, paired t-test, t = 2.633 df = 5) **(Figure 8C)**. These results suggest that I_h blockade can significantly reduce spontaneous firing in VTA DA neurons in part due to an increase in the mean interspike interval. In addition, through I_h modulation we can regulate the spontaneous excitability of these cells.

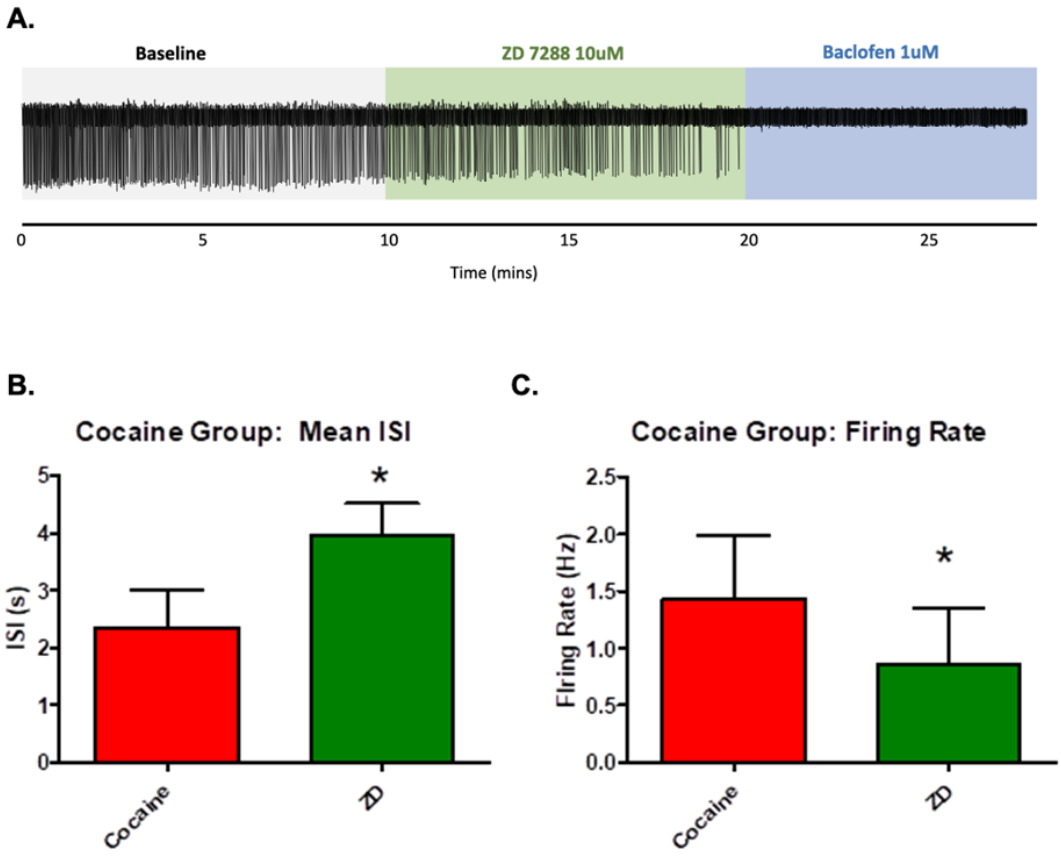

Figure 8. I_h blockade reduces *in vitro* acute cocaine-induced spontaneous excitability in VTA DA neurons. A. Sample traces before and after I_h blocker ZD 7288 (10μM) bath application perfusion, baclofen (1μM) was perfused to identify dopaminergic neurons. B. I_h blockade significantly reduced the firing rate (n=6, paired t-test, t = 3.332 df = 5). C. The mean Interspike interval was significantly increased after ZD 7288 infusion (paired t-test, t = 3.294 df = 5). *Asterisk denotes significant differences ($p < 0.05$).

Specific Aim 2C. Total HCN2 subunit protein expression in the VTA is significantly increased after two consecutive days of cocaine administration.

The total HCN2 protein expression in the VTA did not change after 24 hours of cocaine administration **(Figure 9A)** but increases significantly only after two consecutive days of cocaine administration **(Figure 9B)**. This finding aligns with the previous increase in total HCN2 subunit expression found after cocaine sensitization (Santos-Vera et al. 2013). However, a recent publication of our laboratory indicates that membrane HCN2 expression is significantly decreased after cocaine sensitization (Santos-Vera et al., 2019). These findings overall shows that there is an HCN channel membrane reduction after progressive cocaine administration.

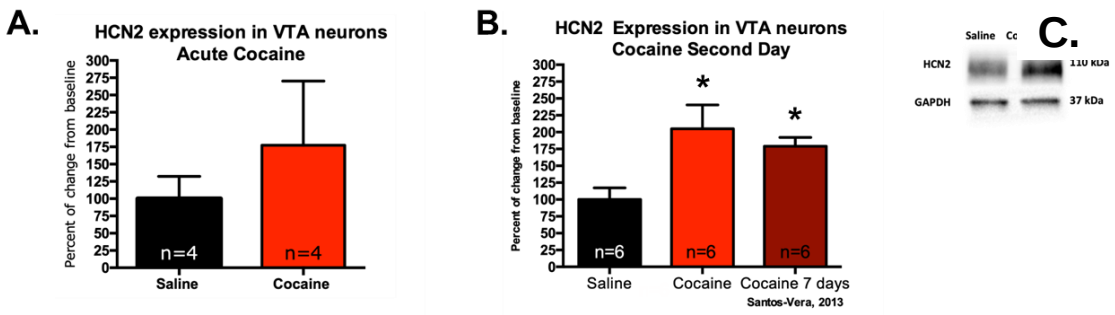

Figure 9. Western blots demonstrate the expression pattern of HCN2 subunit in the VTA. A. Two-hours after cocaine administration did not significantly change HCN2 protein expression. B. Densitometry analysis showed significant difference only 24 hours after second injection of cocaine ($t=2.671$, $df=10$). C. Representative western of proteins extracted after two consecutive cocaine administration. A single band of 110 kDA increased after cocaine exposure and the analysis performed relative to its GAPDH expression, which did not change with drug exposure. One-Way ANOVA, Newman-Keuls multiple comparison test. *Asterisk denotes significant differences ($p<0.05$).

Specific Aim 2D. Cocaine experienced in a novel context increases the AMPA/NMDA potentiation magnitude

To explore how cocaine experienced in novel context alters VTA DA excitability, an acute injection of cocaine was administered to subjects in both a novel context and their home cage. We found a significant increase in the AMPA-to-NMDA receptor-mediated EPSC ratio in both the novelty and the homecage groups **(Figure 10)**. The magnitude of this ratio is greater when rats are administered cocaine in a novel environment (Saline/homecage, n (neurons)=11; Cocaine/homecage, n=8, Saline/Novelty, n=7; Cocaine/Novelty, n=7) $p<0.05$. These results suggest that individuals may undergo higher synaptic potentiation that could explain the stronger motivation to seek and consume cocaine.

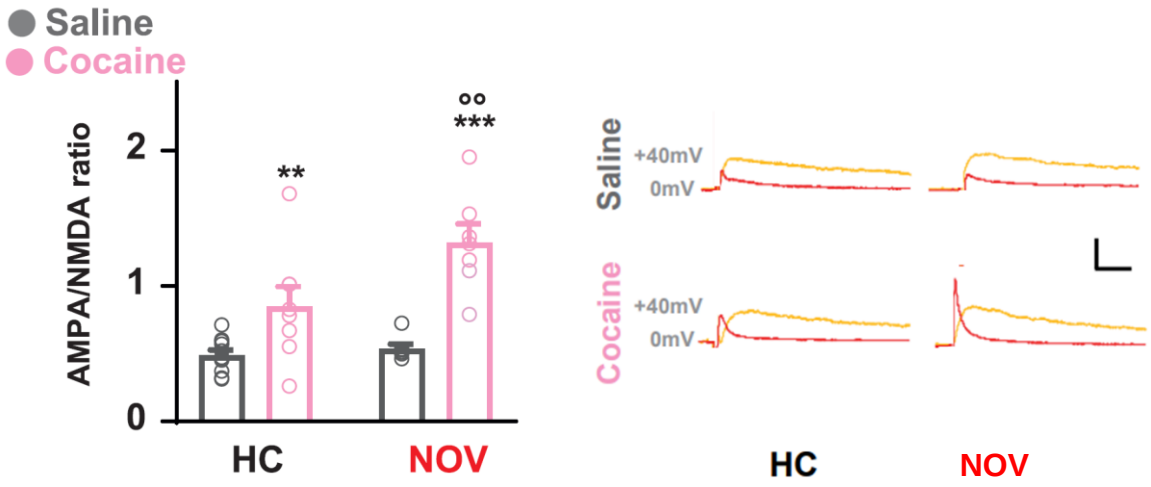

Figure 10. Quantification of the AMPAR/NMDAR ratio and representative glutamatergic synaptic currents in VTA dopamine neurons after cocaine administration in the home cage (HC) or a novel environment (NOV). *Significance compared to saline group; °significance compared to cocaine (Fois et al,. 2022).

Specific Aim 2D. Effect of I_h blockade on *in vivo* extracellular firing patterns 24 h after cocaine administration.

Acute cocaine administration significantly increases firing activity of *in vivo* VTA DA neurons (Creed et al., 2016). I_h blockade decreases firing rate through an increase in rebound delay and interspike interval (Wanat et al., 2008; Tateno and Robinson, 2011; Seutin et al., 2001; Migliore and Migliore, 2012). 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 μm, 10-12 MΩ filled with 2.0% pontamine sky blue in 0.5 M sodium acetate and a perfusion pipette that contained ZD 7288 (8.3 μM in ACSF). We recorded a minimum of 100 s of baseline extracellular firing activity and perfused 60 nL 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 11B)**. Perfusion of ZD 7288 significantly reduced the cocaine-induced firing rate of VTA DA neurons **(Figure 11C)**. The cocaine-induced bursting frequency was also significantly reduced by I_h blockade **(Figure 11D)**. Additionally, cocaine-induced percent of spikes in bursts was also significantly decreased by I_h blockade **(Figure 11E)**. 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 11F)**, the acute cocaine group mean ISI was significantly increased after ZD 7288 local

infusion (paired t-test, t = 3.393, df = 9; c = 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 11G)**. On **(Figure 11H)**, 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.

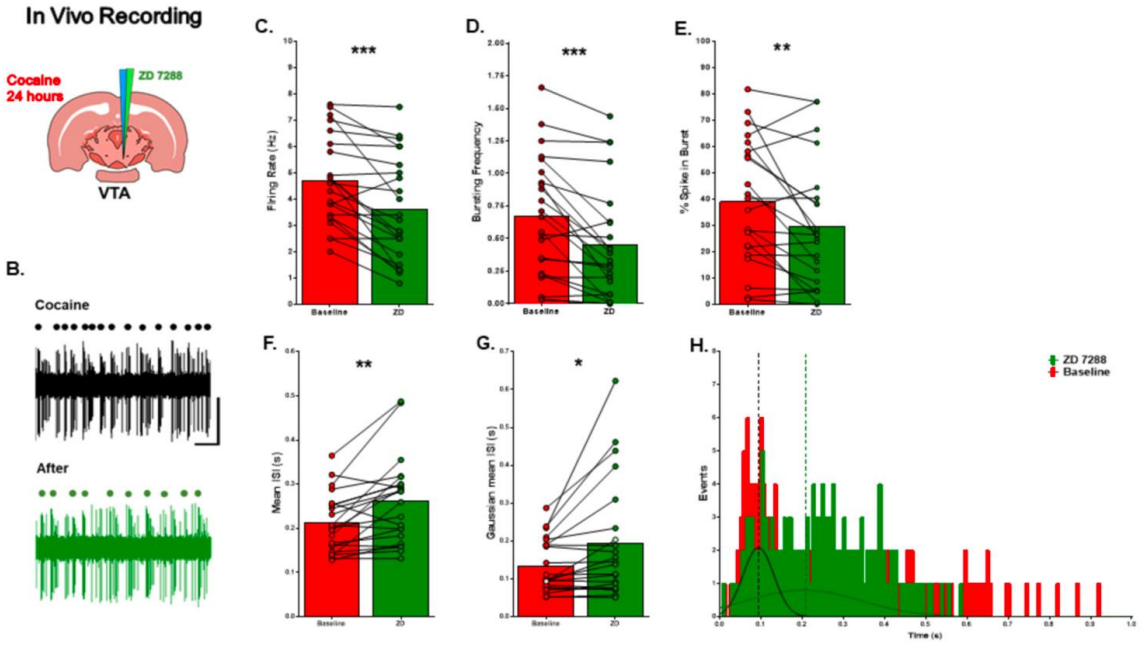

Figure 11. Post perfusion I_h blockade significantly reduced firing properties after acute cocaine on *in vivo* extracellular recordings of VTA DA neurons. A. VTA Double barrel pipette illustration. B. Sample traces of acute cocaine injection and after I_h blocker ZD 7288 (8.3μm) perfusion. Each dot 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 = 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 ± 0.0013 s to 0.2034 ± 0.0069 s after ZD 7288 infusion. *Asterisk denotes significant differences ($p < 0.05$).

Specific Aim 2D. 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. (12A, 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 12C)**. 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.

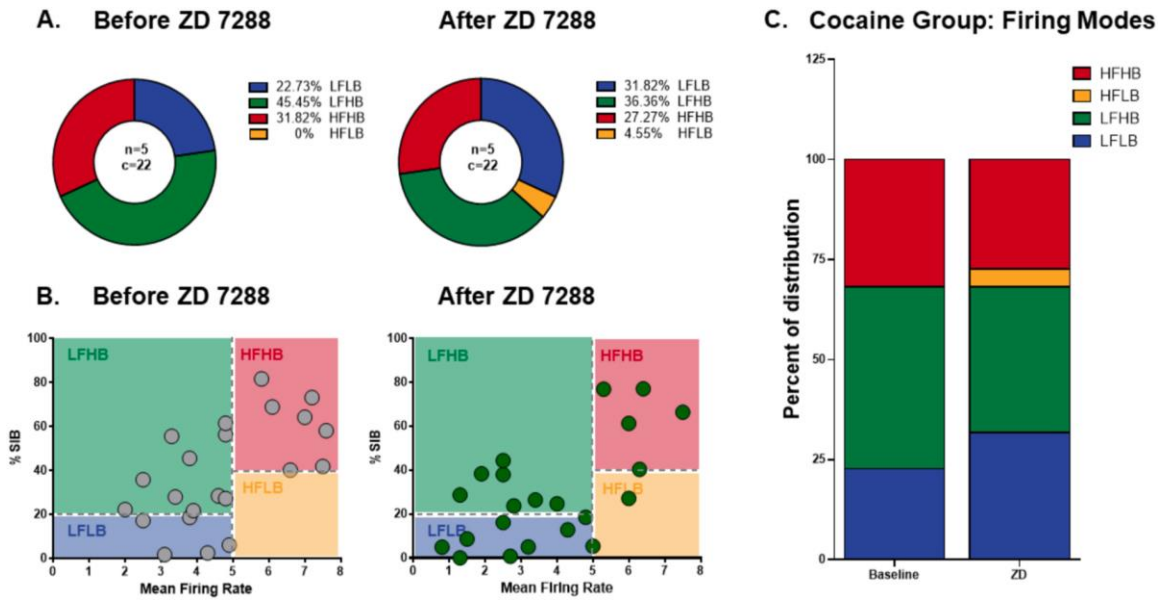

Figure 12. Effect of I_h blockade on acute cocaine induced VTA DA firing modes. A. Pie chart of cocaine group firing mode population. B. Mean Firing rate vs percent of spikes in burst of each neuron recorded in the cocaine group. Colors represent lighter versions of the ones depicted in the pie charts. C. Cocaine group firing pattern percent of distribution did not demonstrate significant differences in groups after ZD 7288 ($p > 0.05$; $\chi 2$ test).

Chapter 4

To elucidate if there is a reduction in I_h as a consequence of VTA DA enhanced excitability through pharmacological GABAergic disinhibition. To determine if I_h blockade can diminish VTA DA enhanced excitability by high frequency stimulation of the ventral subiculum.

Specific Aim 3

To elucidate if I_h reduces as consequence of VTA DA enhanced excitability through pharmacological GABAergic disinhibition. To determine if I_h blockade can reduce VTA DA enhanced excitability by high frequency stimulation of the ventral subiculum.

Hypothesis: I_h will be progressively reduced after an initial hyperexcitability state is present. I_h blockade will decrease the HFSvSUB-induced enhancement of VTA DA neuronal excitability.

Specific Aim 3A. Evaluate if bicuculline-induced increase in VTA DA excitability is sufficient to trigger I_h reduction.

Rationale: Bicuculline (BIC) administration has been used as a mechanism to enhance excitability and promote rebound excitation through GABAergic disinhibition (Paul et al,. 2003). We hypothesize that bicuculline-induced increase in excitability will trigger I_h reduction in VTA DA excitability. In addition, I_h blockade will further decrease bicuculline-induced excitability.

Specific Aim 3B. Elucidate the effect of I_h blockade on HFSvSUB induced potentiation on *in vivo* anesthetized single unit recordings.

Rationale: HFSvSUB elicits *in vivo* persistent hyperactivity of VTA dopamine neurons (Glangetas et al. 2015). This stimulation has been

documented to elicit cocaine-seeking behavior dependent on glutamate release in the VTA (Vorel et al. 2001). We hypothesize that the pharmacological I_h blockade will significantly decrease HFSvSUB-induced firing patterns on VTA DA cells.

Experimental Procedures:

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.

Drugs:

The pharmacological agents used in this study are bicuculline methiodide (50µm) and ZD7288 (10µm). All chemicals were purchased from Sigma and were made fresh on the day of experimentation.

Whole-cell and Cell-attached Patch Clamp Electrophysiology:

Midbrain horizontal slices (220 µm) containing the VTA were prepared from male Sprague Dawley rats (35–51 days postnatal) as previously described (Arencibia-Albite et al., 2007). Cell-attached and 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) (Paxinos and Watson, 2009). Margolis et al. (Margolis et al., 2006a; Margolis et al., 2006b) 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 (Borgland et al., 2004). 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 (Hopf, 2007). 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 MΩ when filled with (in mM) 115 KCH^3SO^4 (methyl potassium sulfate), 20 KCl, 1.5 $MgCl^2$, 5 HEPES, 1 EGTA, 2 ATP, 0.2 GTP, and 10 creatine phosphate (CP), pH 7.25, 290 mOsm. Sodium (Na)GTP, (Mg)ATP, and (Na)CP was added fresh daily. Artificial cerebrospinal fluid (ACSF) contained (in mM) 127 NaCl, 2.5 KCl, 1.25 NaH^2PO^4, 25 $NaHCO^3$, 2 $CaCl^2$, 1 $MgCl^2$, and 25 D(+)-glucose, and was equilibrated with 95% O^2 5% CO^2 at 35 °C. Data sets were collected through an Axopatch 200B amplifier (Axon Instruments; Molecular Devices, Sunnydale, CA), digitized at 5 kHz, filtered at 1 kHz, 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.

In Vivo 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 and 38 °C with an animal temperature controlled electric heating pad during the procedure (World Precision Instruments, Sarasota, FL). A glass micropipette (1-2 μm, 10-12 MΩ filled with 2.0% pontamine sky blue in 0.5 M sodium acetate) were lowered into the VTA. A double barrel pipette was used, similar to the ones used by Georges and Aston-Jones (Georges and Aston-Jones, 2002). The perfusion pipette on the double barrel pipette was filled with ZD 7288 (8.3 μM) an I_h blocker disolved 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 nondopamine 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 s was recorded then 100 s after delivery of 60 nL of the ZD 7288 solution.

High Frequency Electrical Stimulation of the Ventral Subiculum (HFSvSUB):

Bipolar electrical stimulation of the vSUB were performed with a concentric electrode (Phymep) and a stimulus isolator (500 ms duration; 0.2–1 mA; Digitimer). Baseline were recorded for 10 min (23100 pulses: 0.5 Hz). High-frequency stimulation were performed in the vSUB (HFSvSUB) (0.1–1 mA; same intensity use as that used for the baseline). HFSvSUB consisted of 50 trains (500 pulses at 400 Hz; 250 ms duration pulse) presented as bursts of five trains. The frequency of the five trains was 1 Hz. Each burst of five trains was presented five times at 1 min interval.

Data analysis:

All data were presented as mean ± 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 $p < 0.05$.

Results:

Specific Aim 3A. I_h blockade reduces bicuculline-induced evoked and rebound action potentials in VTA DA neurons.

Our experiments elucidated that after 30 minutes of BIC incubation there was a significant increase in the number of action potentials (AP) within the rebound excitation (No BIC, AP 1±0.22 vs. BIC 30 minutes, AP 18±11) **(Figure 13A).** There was a reduction in rebound excitation after 2 hours BIC incubation, but it was still enhanced compared to control (No BIC AP 1±0.22 vs. BIC 120 min AP 5±1.4). I_h blockade ZD bath perfusion (10uM) reduced the rebound excitation of 30 minutes BIC treated slices to baseline level (No BIC AP 1±0.22 compared to BIC 30 minutes +ZD AP 1±0.4). B. When using a depolarizing current clamp protocol 30 minutes of bicuculline incubation significantly increased rebound action potentials (n=8) in comparison to control groups (n=11). In addition, we also found a significant increase in evoked action potentials at higher depolarizing steps after 2 hours of bicuculline incubation (n=8) **(Figure 13B)**. I_h blockade reduced bicuculline-induced action potentials to baseline levels (n=4). These results

suggest that I_h reduction can decrease bicuculline-induced enhancement in both evoked and rebound spiking.

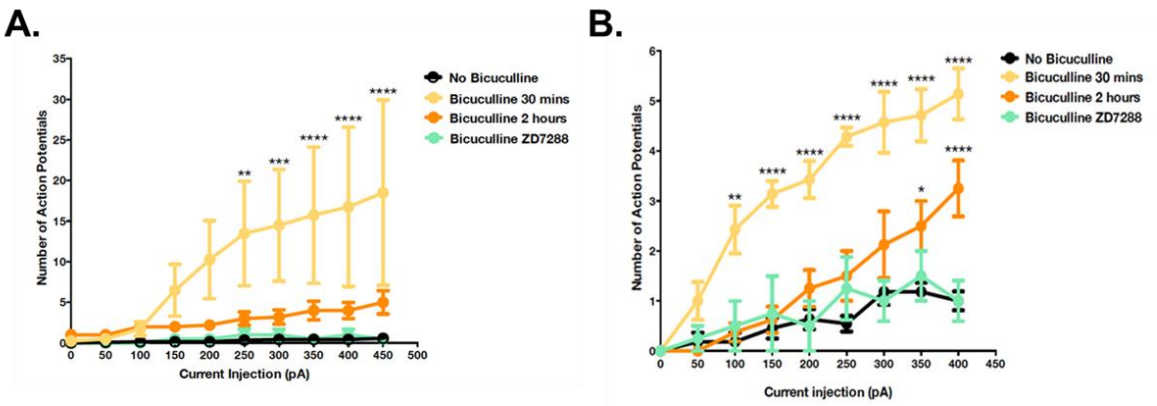

Figure 13. I_h blockade reduced bicuculline-induced increase in rebound and evoked action potentials on dopaminergic neurons in the VTA. A. *In vitro* whole-cell patch-clamp recording (current clamp) of VTA neurons demonstrating spike discharges in response to consecutive depolarizing current steps (increments of 50 pA, 1-s current pulse) after I_h activation. One-Way ANOVA, Newman-Keuls multiple comparison test demonstrates an increase in number of APs after 30 mins of bicuculline (yellow, n=4), but no significant increase after 2 hrs (orange, n=5) in comparison with control (black, n=14) perfusion. Additionally, I_h blockade reduces bicuculline-induced APs to baseline level (green, n=2). B. Current Clamp depolarizing protocol of VTA DA neurons demonstrating spike discharges in response to consecutive depolarizing current steps (increments of 50 pA, 1-s current pulse). One-Way ANOVA, Newman-Keuls multiple comparison test demonstrates increase in number of evoked APs after 30 mins (yellow, n=8) and 2 hrs bicuculline (orange, n=8) incubation in comparison with controls (black, n=11). I_h blockade reduces bicuculline-induced APs to baseline level (green, n=4). *Asterisk denotes significant differences ($p<0.05$).

Specific Aim 3A. Bicuculline incubation decreases I_h conductance in VTA DA neurons

The VTA slices were incubated with bicuculline for 30 or 120 minutes, after incubation we evaluated I_h amplitude at these time points. We registered a significant 69% I_h reduction after 30 minutes of bicuculline incubation (No BIC, -465pA vs. BIC 30 minutes, -144pA, ****p<0000.1) **(Figure 14B, C)**. A significant 25% I_h current reduction was also recorded after 120 minutes of BIC incubation (No BIC, -465pA vs. BIC 120 minutes, -351pA, *p>0.01) **(Figure 14B, C)**. We also measured the I_h activation curve after bicuculline incubation. We demonstrate a significant reduction in channel kinetics only after 30 minutes of bicuculline incubation **(Figure 14D).** These results suggest that I_h reduction occurs as a consequence of the bicuculline enhancement of VTA DA excitability, and the first 30 minutes of I_h reduction after bicuculline incubation are due to slower channel kinetics.

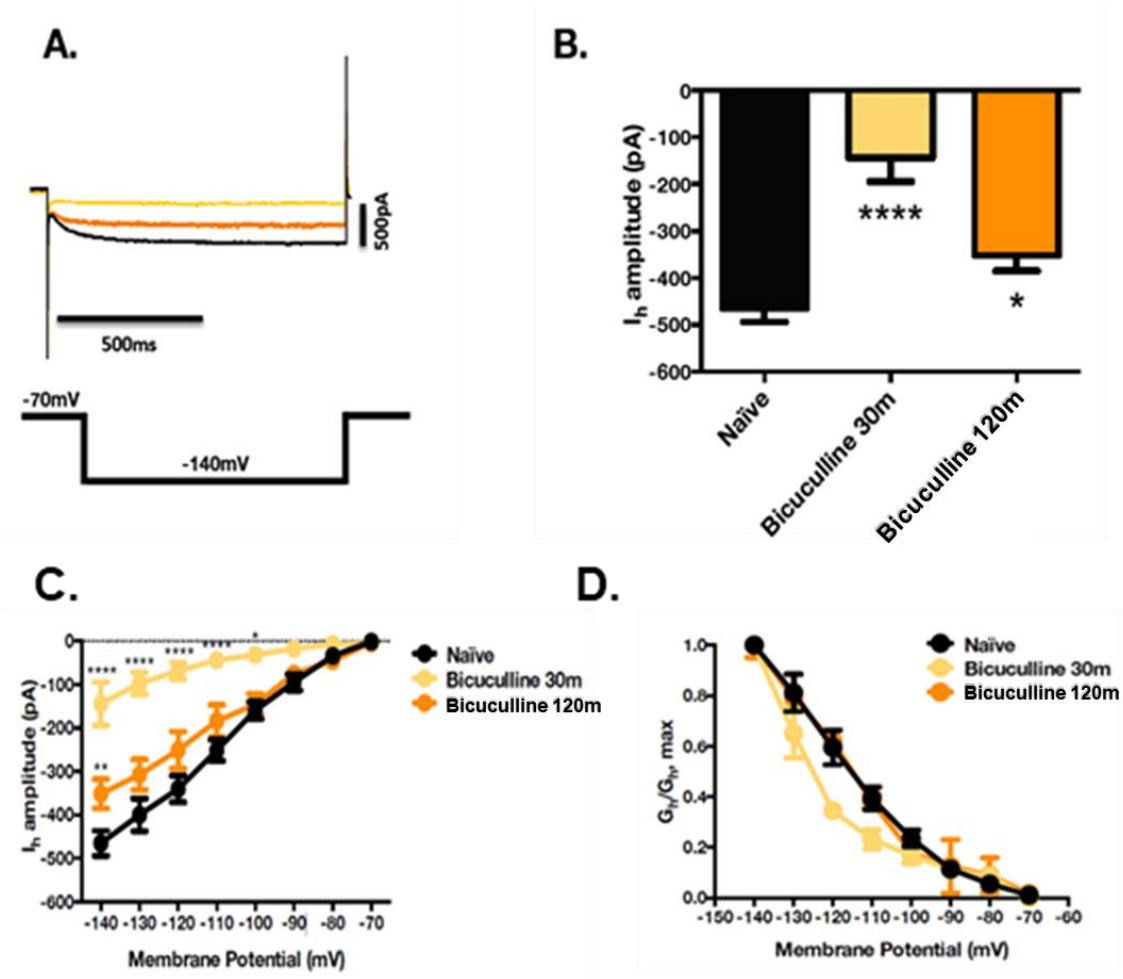

Figure 14. I_h amplitude after bicuculline incubation. A. Representative voltage-clamp traces demonstrating the effect of bicuculline (50μM) after incubations periods of 30 and 120 minutes on VTA DA neurons. B. Summary bar demonstrating the effects of bicuculline incubation on I_h conductance. One-way ANOVA, Newman-Keuls multiple comparison test demonstrates bicuculline incubation for 30 minutes (yellow, n=4) induced a 69% reduction in I_h conductance, whereas 2 hour incubation of bicuculline (orange, n=5) induced a 24% reduction on I_h current in comparison with naïve group (black, n=7). C. I-V curve of I_h after bicuculline incubation. IV relationship of the effects of bicuculline incubation after a period of 30 minutes (yellow, n=4), 2 hours incubation (orange, n=5) and naive group (black, n=7). One-Way ANOVA, Newman-Keuls multiple comparison test demonstrates significant reduction of I_h amplitude after 30mins and 2hrs bicuculline incubation. I_h activation curve after bicuculline incubation. Graph demonstrates slower channel kinetics after 30 minutes of bicuculline perfusion (yellow, n=4). No change in activation curve is seen after 2 hours of bicuculline perfusion (orange, n=5) in comparison with naïve group (black, n=7). *Asterisk denotes significant differences ($p<0.05$).

Specific Aim 3B. The effect of I_h blockade on HFSvSUB induced potentiation on in vivo anesthetized single unit recordings.

To evaluate the effect of I_h blockade on in vivo HFSvSUB induced hyperactivity of VTA DA neurons. We recorded using double barrel pipettes. These pipettes are composed of a recording electrode and a perfusion pipette that contained ZD 7288 (8.3uM). We recorded a minimum of 100 seconds of baseline extracellular firing activity and perfused 60nL of ZD 7288 to evaluate the effect of I_h blockade on HFSvSUB-induced firing patterns **(Figure 15A)**. Post perfusion of ZD 7288 significantly reduced the HFSvSUB-induced firing rate on VTA DA neurons **(Figure15B)**. The HFSvSUB-induced bursting frequency was also significantly reduced by I_h blockade **(Figure 15C)**. Additionally, the HFSvSUB-induced percent of spikes in bursts was also significantly decreased by I_h blockade **(Figure 15D)**. These findings suggest that I_h blockade can significantly reduce synaptic potentiated VTA DA firing patterns from Bed Nucleus Stria Terminalis (BNST) glutamatergic synapses. This finding provides further evidence that I_h reduction could serve as a homeostatic regulator of VTA DA neuronal excitability.

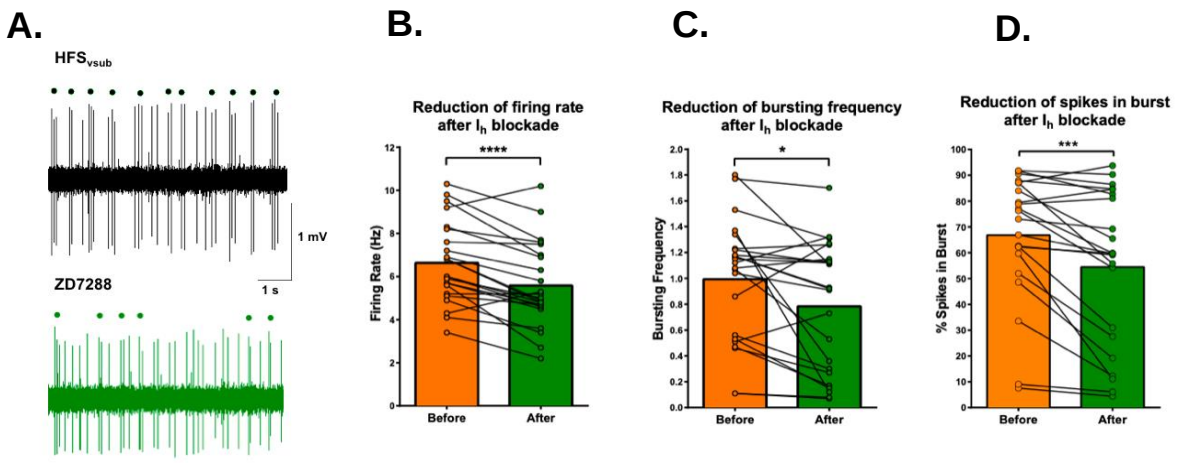

Figure 15. Post perfusion I_h blockade significantly reduced HFSvSUB-induced increase in firing properties of in vivo extracellular recordings of VTA DA neurons. A. Sample traces of HFSvSUB hyperactivity and after I_h blocker ZD7288 (8.3um) perfusion. B. I_h blockade significantly reduced the HFSvSUB-induced firing rate. C. The frequency of I_h blockade. D. I_h blockade also significantly reduces the percent of spikes in bursts. Paired student t-test (n=24); *Asterisk denotes significant differences ($p<0.05$).

Specific Aim 3B. The effect of I_h blockade on HFSvSUB induced potentiation in VTA DA firing modes

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 HFSvSUB induced treated animals firing modes. On **(Figure. (16A, B)**, the populational firing modes before and after ZD 7288 perfusion are plotted **(Figure 16C)**. I_h blockade significantly reduced firing rate in high firing rate, high bursting neurons. **(Figure 16D)** The percentage of spikes in bursts was also significantly reduced by I_h blockade. **(Figure 16E)** I_h blockade significantly increased the mean interspike interval. These results suggest that I_h blockade can significantly reduce the HFSvSUB-induced excitability and could serve as a modulator of VTA DA excitability.

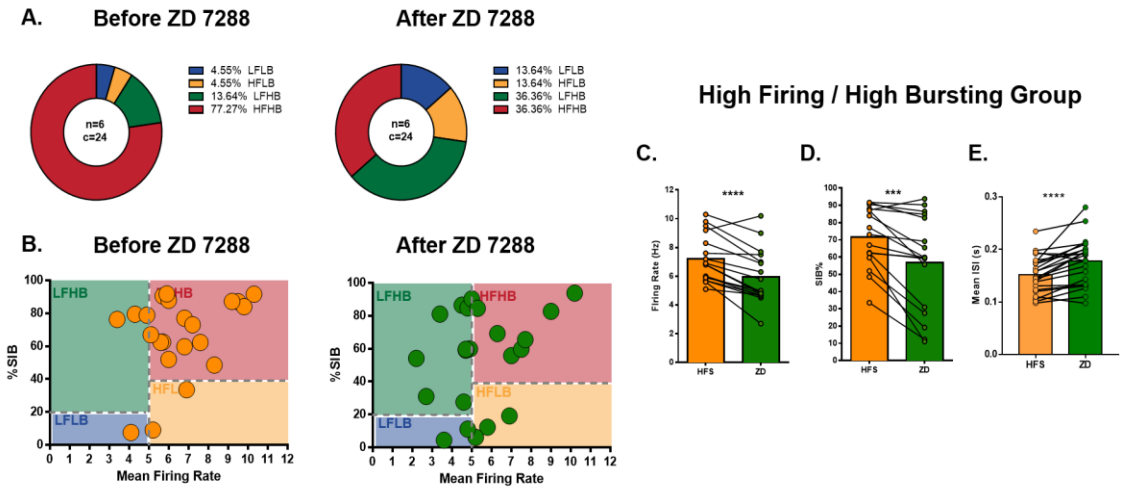

Figure 16. Effect of I_h blockade on HFSvSUB-induced VTA DA firing modes. A. Pie chart of HFSvSUB group firing mode population (n=6, cells=24). B. Mean Firing rate vs percent of spikes in burst of each neuron recorded. Colors represent lighter versions of the ones depicted in the pie charts. C. I_h blockade significantly reduced firing rate in high firing rate, high bursting neurons. D. The percentage of spikes in bursts was also significantly reduced by I_h blockade. E. I_h blockade significantly increased the mean interspike interval. *Asterisk denotes significant differences ($p<0.05$).

Chapter 5
Discussion & Conclusion

Discussion

The discovery of possible homeostatic regulators of biophysical properties allows us to identify important physiological components that can serve as modulators of neuronal excitability. Intrinsic excitability is a measure of the neuron's inherent electrical properties that can be adjusted through changes in morphological features and the expression level or properties of ion channels in the membrane (Beck and Yaari, 2008; Kemenes et al., 2006; O'Leary, 2010). These neuroadaptations can be categorized as either homeostatic, that contribute to stabilizing neuronal excitability, or non-homeostatic, that produce an alteration from basal excitability (Desai et al., 1999; Howard et al., 2007; Wijesinghe and Camp, 2011). Homeostatic changes in neuronal excitability can arise in response to extended periods of altered activity (Turrigiano Gina, 1999; Turrigiano and Nelson, 2000; Keck, 2017). Such periods of altered activity may be a consequence of exposure to drugs such as cocaine. I_h is a major determining factor in the control of intrinsic excitability (DiFrancesco, 1981; Adams and Halliwell, 1982; Kase and Imoto, 2012). I_h also modulates electrophysiological properties of neurons in the MLS, including DA neurons of the VTA. Neuroadaptations in this network are hypothesized to contribute to the neuropathology of SUD (Kauer, 2003; Chen et al., 2001). Cocaine sensitization significantly reduces I_h amplitude, concomitantly spontaneous firing in VTA DA neurons remains similar to control groups (Arencibia-Albite et al., 2012). Thus, I_h could be a possible candidate for a homeostatic regulator of VTA DA neurons. It has been demonstrated that I_h

modulation compensate for bidirectional alterations in CA1 pyramidal neuronal excitability. Chronic enhancement or excitability deprivation triggers bidirectional plasticity of I_h and leads to regulation of both input resistance and intrinsic excitability (Gasselin et al., 2015). This evidence suggests the possibility that I_h modulation can alter VTA DA excitability as a homeostatic regulator under hyperexcitable conditions.

In the present study we explore the functional role of I_h in VTA DA neuronal excitability. Given the fact that I_h serves as an excitatory current in VTA DA neurons (Tsantoulas et al., 2016), we hypothesized that I_h blockade can reduce basal VTA DA excitability. On Aim 1, we provided evidence that I_h blockade can reduce rebound action potentials using whole-cell patch clamp. Previous publications have elucidated that inhibition of I_h in VTA DA neurons increases rebound spike delay in current clamp protocols (Tateno et al. 2011), in concordance with our findings. But we were the first to show that in *in vitro* recordings I_h blockade can significantly reduce rebound action potentials at different depolarization steps. This finding gives us insight on how I_h modulates integration at different levels of excitation.

With the use of cell attached recordings of VTA DA neurons we found that I_h blockade significantly reduces firing rate and increases interspike interval. The cell attached configuration was employed as a method to increase the time of recordings of spontaneous firing activity and to reduce the probability of activity loss by a dialysis effect as can be seen on recorded neurons in whole-cell patch clamp configuration. The increased interspike interval by I_h inhibition has been

previously demonstrated in computational models (Migliore M and Migliore. 2012). These results are also supported by a previous study using *in vitro* extracellular recordings which found a decrease in the firing rate of DA neurons by ZD bath application. It has been suggested that this reduction in firing activity through I_h modulation is likely to be significant in altering behaviors (Seutin et al., 2001). I_h blockade has also been successful in reducing *in vitro* firing rate in both chronic mild stress rats and their control group (Zhong et al. 2018).

In another set of experiments, we elucidated the I_h contribution to spontaneous cell 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 found that I_h blockade significantly reduces VTA DA neurons' firing rate and bursting activity. Moreover, this diminution in activity is at least partly due to an increase in the interspike interval induced by I_h blockade. I_h current participates in burst formation in various neurons (Hughes et al., 1998; Ying et al., 2006). 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 (lCat) (Doan and Kunze, 1999). The I_h activation depolarizes the membrane potential towards threshold activating ICat and generating low-threshold Ca^{2+} spike. The generation of Ca^{2+} spikes activate a burst of fast Na^+ and K^+ dependent action potentials. This provides a possible mechanism for how an I_h decrement could influence burst firing in VTA DA neurons. We can speculate that a similar mechanism of action as in the thalamus might be taking place in the VTA for the generation of bursting activity.

Further studies should address this hypothesis. When using firing mode categorization, we found a significant difference between the HFHB and HFLB groups after I_h blockade. These results suggest that I_h blockade is sufficiently effective in reducing the basal firing-mode categories. Overall, this first aim demonstrated that I_h reduction in VTA DA neurons decreased neuronal excitability and could be a potential target of intervention for conditions related to hyperexcitability of VTA DA neurons.

Our laboratory has previously shown that cocaine sensitization reduces I_h in VTA DA neurons (Arencibia-Albite et al., 2012). For Aim 2, we measured the *in vitro* I_h amplitude in VTA DA cells after an acute cocaine injection. We observed that a significant I_h reduction starts to occur 24 hours after drug administration. On the other hand, total HCN2 protein expression in the VTA demonstrated a trend to increase 24 hours after an acute cocaine injection. This increase is significant after two consecutive days of cocaine administration. This is the first evidence that HCN2 protein expression is increased during early stages of cocaine sensitization. These latter results agree with the previous increase in total HCN2 subunit expression found after cocaine sensitization (Santos-Vera et al, 2013). However, a recent publication of our laboratory indicates that membrane HCN2 expression is significantly decreased after cocaine sensitization (Santos-Vera et al., 2019). We speculate that this increase in total HCN2 protein expression is a consequence of higher protein transcription and translation in VTA DA neurons. Future studies should address how these HCN2 subunits are modulated through various

trafficking proteins that may explain the membrane reduction of HCN channels in VTA DA neurons. Overall, these findings suggest that there is an HCN channel membrane reduction after progressive cocaine administration. Interestingly, using current clamp protocols we found that after 2 or 24 hours and also after seven days of cocaine administration, there is a significant increase in rebound action potentials of VTA DA neurons. There is a higher number of rebound action potentials after 2 hours in comparison to 24 hours of cocaine administration. When ZD was perfused, there was a significant reduction in rebound action potentials. Thus, the significant reduction in I_h after acute cocaine administration could explain the decrease in rebound action potentials found from 2 to 24 hours after cocaine injections. These results reinforce our earlier observation that I_h is reduced after cocaine sensitization and suggest that a progressive reduction of this current could be a possible homeostatic regulator of VTA DA neuronal excitability.

VTA DA cell spontaneous firing activity was recorded through cell attached experiments. When perfused with an I_h blocker, the spontaneous firing activity was reduced, while significantly increasing the interspike interval. This evidence further confirms that I_h blockade reduces firing activity by increasing the time between generation of action potentials.

When cocaine is administered in a novel environmental context, animal subjects express greater psychomotor sensitization (Caprioli et al, 2007). Novel stimuli exposure increases DA release in the nAcc. It was found that inhibition of the

ventral subiculum and the VTA can abolish this increase in DA release (Legault et al, 2001), which could lead us to hypothesize that novel stimuli could alter motivational behavior. Therefore, cocaine administered in a novel context could facilitate both cocaine-seeking and cocaine-motivation. In our experiment, an acute injection of cocaine was administered to subjects in both a novel context and their home cage. Twenty-four hours after treatment, we found a significant increase in the AMPA-to-NMDA receptor-mediated EPSC ratio in both the novelty and the homecage groups. The novelty group demonstrated a higher AMPA/NMDA ratio in potentiated neurons compared to the homecage. These results suggest that when cocaine is experienced in a novel context, individuals may undergo higher synaptic potentiation that could explain the stronger motivation to seek and consume cocaine. Given the fact that all our acute cocaine experiments were conducted in a novel context, we used the same model and sought to explore the contribution of I_h to changes in spontaneous *in vivo* firing patterns induced by acute cocaine administered in a novel context. 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, and percent of spikes within bursts are significantly reduced after I_h blockade. In addition, there was an increase in the inter-spike interval. When evaluating the VTA DA firing modes, our analysis did not show significant differences in any category after I_h blockade. Because in experimental animals cocaine-induced firing is significantly higher than in naive rats, it could be possible that the reduction of firing activity by I_h blockade in those conditions is less effective than in cells with low firing activity

(i.e. naïve rats) On the contrary, the naive group of Aim 1 did undergo significant changes in the firing modes category. A possible explanation for these differences could be that 24 hours after acute cocaine administration there is a significant reduction in I_h present. This progressive reduction could mask the effect of I_h blockade changing firing mode categories on acute cocaine groups. Other neuroadaptations induced by cocaine in which I_h is not involved might have a stronger influence on the firing category profile. In sum of Aim 2, these results demonstrate that I_h reduction serves as an intrinsic homeostatic modulator of VTA DA cocaine-induced firing activity **(Figure 17)**. Furthermore, I_h blockade can decrease VTA DA cocaine-induced spontaneous firing activity but is not sufficient to change firing modes, which leads us to hypothesize I_h reduction as an intrinsic modulator of excitability but given an increased state of firing by cocaine might not drastically diminish cocaine-induced excitability.

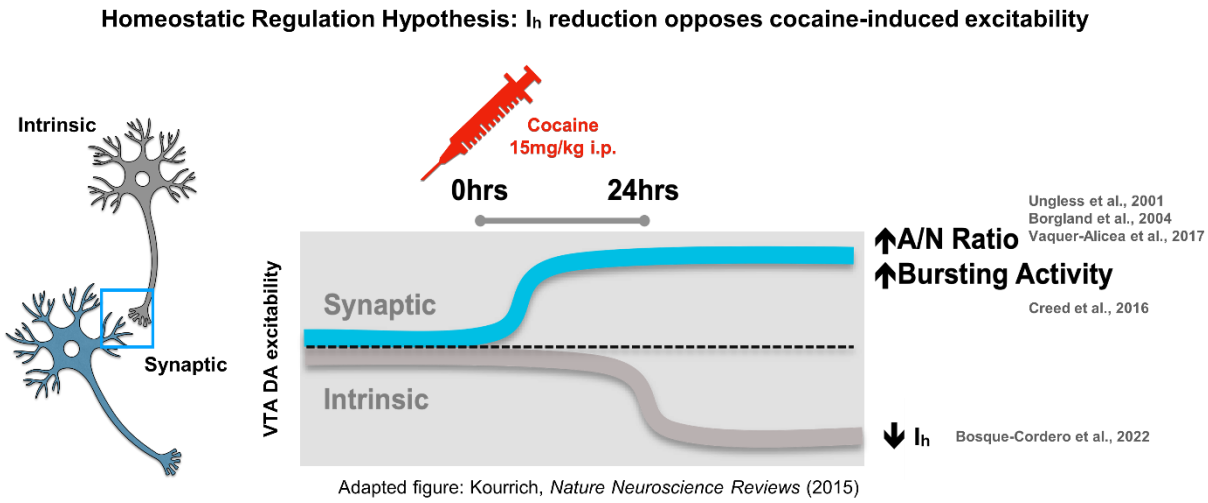

Figure 17. Progressive I_h reduction opposes cocaine-induced enhancement of VTA DA neuronal excitability. After an acute cocaine administrations VTA DA neurons significantly increase the AMPA-to-NMDA receptor-mediated EPSC ratio (Ungless et al., 2001 and Borgland et al.,2004). In addition, firing activity including bursting are also significantly increased (Creed et al., 2016). We propose that I_h progressive reduction is a homeostatic compensation triggered by the increased bursting activity after cocaine administration.

Fundamental studies have established that DA neuronal baseline activity is irregular with an average firing rate of 4.5 Hz in anesthetized recordings (Grace and Bunney, 1984). Our results demonstrate that after local infusion of ZD 7288 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 the firing rate of a normally low firing DA neuron could be enough to alter cellular functionality. Indeed, it has been demonstrated that optogentically-induced phasic firing on VTA DA neurons is sufficient to establish behavioral conditioning (Tsai, 2009). Optogenetically-induced tonic firing on VTA DA neurons decreases sucrose intake (Mikhailova et al., 2016). It has also 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 (Cooper, 2002). Bursts also improve the reliability of information transfer and provide various stimulus features in the same spike train (Zeldenrust et al., 2018). 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.

While 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 (Arencibia-Albite et al. 2021). For example, small conductance K+ (SK) current amplitude is another determinant of endogenous DA firing

patterns. Computational models evaluating VTA DA neuronal activity denote that high SK conductance is characteristic in low firing neurons, while low SK conductance is present in high firing cells (Oster et al., 2015). Further studies should evaluate the influence of other conductances on our experimental time points and how the integration and interplay of these diverse currents can alter the neuronal excitability of VTA DA neurons. Currently, little is known of how homeostatic regulation of neuronal excitability is initiated in the MLS. We suggest that these modifications could be triggered by cocaine-induced enhancement of glutamatergic excitatory afferent toward VTA DA neurons (Ungless et al., 2001; Borgland et al., 2004). Repeated exposure to cocaine can also disinhibit VTA GABA neurons thus increasing neuronal firing of VTA DA cells (Bocklisch, 2013). Our hypothesis postulates that specific cocaine-induced enhancement of bursting patterns could result in alteration of I_h, since 24 hours after acute cocaine administration, bursting activity increases, and this is correlated with a significant reduction in the I_h current. I_h diminution can increase the interspike interval, thus reducing the probability of generating action potentials to oppose for the increase brusting activity induced by cocaine.

Although our results support a homeostatic regulation mechanism after cocaine administration, individuals still sensitize and develop chronic cocaine behaviors (Arencibia-Albite et al., 2012). We postulate that changes in intrinsic properties like I_h conductance are sufficient and necessary to maintain a working neuron and/or a circuit. As an intrinsic modulator, we can speculate that I_h modulation can change

how information is received from presynaptic networks and does not necessarily compensate for altered presynaptic activity. Although this compensation by itself could not necessarily overturn addictive behavior, it can lead us to consider the study and regulation of both synaptic and intrinsic mechanisms to alleviate SUD symptoms.

On Aim 2 we demonstrated that progressive I_h reduction occurs 24 hours after acute cocaine administration. It has been demonstrated in hippocampal CA1 neurons that I_h current can be up or down regulated following chronic activity enhancement or activity deprivation, respectively (Gasselin et al, 2015). Chronic hypoactivity through kynurenate acid decreased I_h while it enhanced both input resistance and intrinsic excitability. Contrarily, I_h was upregulated by chronic hyperactivity through the $GABA_A$ receptor antagonist picrotoxin, subsequently reducing input resistance and intrinsic neuronal excitability. Somatic high frequency stimulation of VTA DA neurons can also trigger a significant reduction in I_h amplitude (Wei et al., 2013). These findings suggest that I_h can act as a homeostatic regulator of intrinsic excitability and can modulate it when altered firing activity is present. For our Aim 3, we enhanced VTA DA excitability through pharmacological and high frequency stimulation to determine if I_h homeostatic compensation is produced or if it is a regulation only present after cocaine administration. Bicuculline incubation has been used as a mechanism to enhance excitability of VTA DA neurons through GABAergic disinhibition (Paul et al. 2003). Our hypothesis establishes that there will be a reduction in I_h as a homeostatic

compensation to reduce bicuculline-induced increased VTA DA excitability. We again demonstrated the I_h-induced homeostatic compensation using bicuculline-mediated increase in VTA DA excitability. Our results indicate that bicuculline incubation induced a significant reduction in I_h conductance. The bicuculline incubation for 30 minutes induced a 69% reduction in I_h conductance, whereas 2 hour bicuculline incubation induced a 24% reduction. The I_h activation curve demonstrated slower kinetics after 30 minutes, but not after 2 hours of incubation. These results led us to conclude that the initial 30 min incubation produced a reduction in the activation kinetics of I_h. However, there was a reduction of I_h after 2 hour bicuculline incubation that could be explained by a reduction in channel expression at the membrane. There was an increase in the number of action potentials after 30 min of bicuculline incubation in comparison to 2 hours using the rebound excitation protocol. I_h blockade reduced the number of rebound APs to baseline levels in bicuculline incubated slices. In a current pulse protocol bicuculline also increased the number of evoked APs after 30 minutes and 2 hours incubations. I_h blockade also reduced bicuculline-induced evoked APs to baseline levels under a current pulse protocol, demonstrating a regulatory role of I_h in bicuculline-induced excitability. We suggest that I_h modulation is a mechanism to regulate bicuculline-induced excitability and could act as a homeostatic mechanism in an event in which VTA DA neurons are hyperexcited.

It has been reported that high frequency stimulation of the ventral subiculum (HFSvSUB) elicits *in vivo* persistent hyperactivity of VTA DA neurons (Glangetas

et al. 2015). This stimulation has been shown to induce cocaine-seeking behavior dependent on glutamate release in the VTA (Vorel et al. 2001). As part of this last aim, we investigated the effect of an I_h blockade on HFSvSUB induced potentiation on *in vivo* spontaneous excitability. We hypothesize that I_h blockade will significantly decrease HFSvSUB-induced firing patterns on VTA DA cells. We demonstrated that I_h blockade significantly reduces firing rate, bursting frequency and percent of spikes in burst after HFSvSUB. Additionally, the I_h blockade also increased the interspike interval of this group significantly. It is important to note that HFSvSUB increases VTA DA excitability by a higher magnitude than the acute cocaine groups. When evaluating the firing mode patterns, we demonstrated a significant shift from HFHB to HFLB, leading us to postulate that I_h blockade may have a greater influence on the reduction of bursting patterns, although our results also confirm that the firing rate is significantly reduced. These results suggest that I_h blockade is sufficient to significantly reduce the firing activity of HFSvSUB. It also makes us conclude that I_h blockade can reduce excitability after the stimulation of *in vivo* persistent hyperactivity of VTA DA neurons. These findings add to the idea that an I_h reduction serves as a homeostatic regulator of VTA DA hyperexcitability, by progressively reducing this conductance and increasing the interspike interval after an initial hyperexcitability state is present.

Previous studies have shown that ZD 7288 can additionally block Na + currents in HEK293 cells transfected with Nav 1.4 plasmids (Wu et al., 2012). This effect is unlikely to influence our results because local ZD 7288 infusions did not alter the

amplitude of the action potential in our recordings. Furthermore, it has been reported that ZD 7288 bath perfusion only affect spontaneous firing rate of TH-GFP+neuron in the VTA (Krashia et al., 2016). The development of subunit-specific HCN channel blockers would reduce any off-target effect that ZD 7288 could have. Furthermore, the use of specific HCN2 shRNAs targeted at VTA DA neurons could overcome these limitations in pharmacological experiments. Studies have shown that isoflurane inhibits the I_h current in mice cortical pyramidal neurons (Chen et al., 2009). Our experimental design evaluated both the baseline and the effect of I_h blockade on firing patterns from the same neuron of the anesthetized rat. We found that the prior isoflurane alteration of the I_h amplitude does not influence our experimental results after ZD 7288 infusions, since the effect was only present after I_h blockade. Furthermore, our baseline VTA DA neuronal firing patterns were similar to other experiments using diverse types of anesthetics, such as chloral hydrate and sodium pentobarbital, as well as in awake behaving animals (Grace and Bunney, 1984; Marinelli et al., 2006; Pan et al., 2005; Hyland et al., 2002).

Future experiments should evaluate the rheobase of rebound excitation spiking after I_h blockade using whole-cell patch-clamp recordings; this could potentially elucidate the contribution of HCN channels to subthreshold potentials and the facilitation of AP firing, since HCN channels have been shown to be tonically active at these potentials (He, 2014). Current evidence does not support the notion that down-regulation of I_h will change the reversal potential of the HCN channel.

Pharmacological blockade or reduction of I_h after cocaine sensitization does not alter input resistance in VTA DA neurons. (Okamoto, 2006; Arenbicia-Albite, 2012, & 2017). Furthermore, the activation time constant is suspected to increase with I_h reduction, since after cocaine sensitization the time constant increases (Arencibia-Albite, 2012). I_h blockers have been shown to increase temporal summation in axons with dendrites, but without a significant change in soma and non-axon dendrites (Engel & Seutin, 2015). This evidence leads us to believe that down-regulation of I_h will increase synaptic integration in the dendrite, although somatic down-regulation may not alter this integration. This could explain the dual functional role of I_h although further experiments would need to be addressed to prove this hypothesis.

Another interesting set of experiments could be to test if whether I_h homeostatic compensation is present in other models of SUD. For example, whether this homeostatic regulation is present in a non-contingent model and if I_h blockade could alter motivational behavior on animal subjects. Unpublished results from our laboratory concluded that intra VTA I_h blockade is able to significantly reduce acute cocaine locomotor behavior (Santos-Vera, unpublished). Long lasting reduction of I_h conductance and excitability in VTA DA neurons has been linked to pro resilience behaviors (D'Addario et al. 2021). In addition, it has been reported that an increase VTA DA neuronal excitability drives motivational behavior through the facilitation of action initiation (Boekhoudt et al., 2018). Furthermore, inhibition of these neurons reduces sucrose preference which is rescued through VTA DA phasic

activation (Tye et al., 2013). The optogenetic activation of VTA GABA neurons also suppresses the activity and excitability of DA neurons, suggesting that connections between VTA DA and GABA neurons can control the initiation and termination of reward-related behaviors (Van Zessen et al., 2012). This in turn could make I_h modulation a candidate for the treatment of addictive behaviors by reducing VTA DA neuronal activity.

In sum, the main contribution of this work suggests that I_h serves as a homeostatic regulator of VTA DA hyperexcitability that could arise from multifactorial conditions **(Figure 18)**.

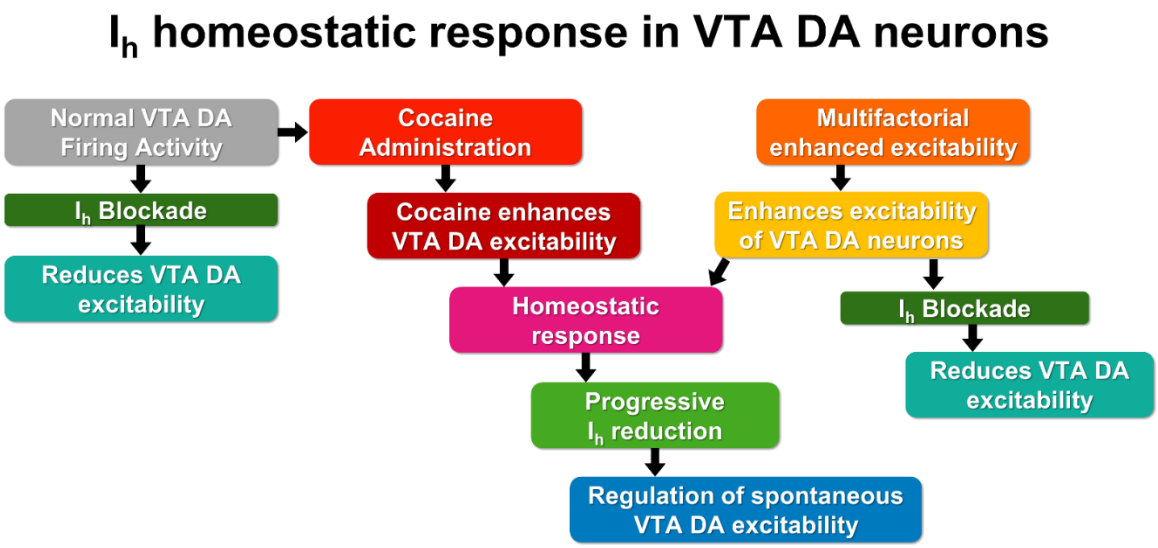

Figure 18. Overview of thesis findings. We demonstrated that I_h reduction in VTA DA neurons decreases neuronal excitability and could be a potential target of intervention for conditions related to hyperexcitability of VTA DA neurons. Progressive I_h reduction serves as an intrinsic homeostatic modulator of VTA DA cells by decreasing cocaine-induced firing activity. Finally, I_h could serve as a homeostatic regulator of VTA DA hyperexcitability that arises from diverse conditions, progressively reducing this conductance after initial hyperexcitability state is present.

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