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Protein and surface expression of HCN2 and HCN4 subunits in mesocorticolimbic areas after cocaine sensitization

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ABSTRACT

The I_h is a mixed depolarizing current present in neurons which, upon activation by hyperpolarization, modulates neuronal excitability in the mesocorticolimbic (MCL) system, an area which regulates emotions such as pleasure, reward, and motivation. Its biophysical properties are determined by HCN protein expression profiles, specifically HCN subunits 1–4. Previously, we reported that cocaine-induced behavioral sensitization increases HCN2 protein expression in all MCL areas with the Ventral Tegmental Area (VTA) showing the most significant increase. Recent evidence suggests that HCN4 also has an important expression in the MCL system. Although there is a significant expression of HCN channels in the MCL system their role in addictive processes is largely unknown. Thus, in this study we aim to compare HCN2 and HCN4 expression profiles and their cellular compartmental distribution in the MCL system, before and after cocaine sensitization. Surface/intracellular (S/I) ratio analysis indicates that VTA HCN2 subunits are mostly expressed in the cell surface in contrast to other areas tested. Our findings demonstrate that after cocaine sensitization, the HCN2 S/I ratio in the VTA was decreased whereas in the Prefrontal Cortex it was increased. In addition, HCN4 total expression in the VTA was decreased after cocaine sensitization, although the S/I ratio was not altered. Together, these results demonstrate differential cocaine effects on HCN2 and HCN4 protein expression profiles and therefore suggest a diverse I_h modulation of cellular activity during cocaine addictive processes.

1. Introduction

The repertoire of ionic channel composition in the plasma membrane critically contributes to the modulation of neuronal activity. One of these structures is the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel. HCN channels are activated by membrane hyperpolarization and their openings generate a mixed cation current known as I_h, a key modulator of neuronal excitability (Doan and Kunze, 1999; Magee, 1999; Okamoto et al., 2006).

There are four different subunits (HCN1-4) forming the HCN channel (Notomi and Shigemoto, 2004), all of which exhibit unique activation kinetics, voltage dependence and modulation by cyclic nucleotides (DiFrancesco and Tortora, 1991; Ludwig et al., 1998). Therefore, the biophysical properties of I_h are largely determined by the expression profiles of the HCN channel's subunit composition (Santoro et al., 2000; Ludwig et al., 1998).

HCN channels are widely expressed in the mesocorticolimbic system (MCL) (Chu and Zhen, 2010). The MCL is a set of interconnected brain regions that regulate pleasure, reward and motivation (Robinson and Berridge, 2003; Wolf, 2010). Amongst the regions comprising this system are the ventral tegmental area (VTA), the nucleus accumbens (NAc), the prefrontal cortex (PFC), and the hippocampus (HIP) (Hyman et al., 2006). Drugs that produce psychoactive and addictive effects act primarily on the MCL.

In recent years, research efforts have emerged to elucidate the role of I_h and HCN channels in addictive processes, specifically within the MCL. It has been shown that chronic ethanol exposure reduces I_h density in VTA dopaminergic (DA) neurons (Okamoto et al., 2006). Also, overexpression of HCN2 subunits in the VTA increases the rewarding effects of ethanol (Rivera-Meza et al., 2014). Moreover, in the

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HIP, an imbalance of HCN1 and HCN2 expression has been observed after chronic morphine exposure (Zhou et al., 2015) characterized by an increase in HCN2 together with a decrease in HCN1 subunits. Interestingly, methamphetamine exposure increases both, HCN1 and HCN2 mRNA's expression in the PFC (Gonzalez et al., 2016). It is noteworthy that VTA DA cells mainly express HCN2 whereas HCN4 subunits are exclusively detected in GABAergic neurons (Monteggia et al., 2000; Notomi and Shigemoto, 2004; Margolis et al., 2012). All these findings suggest an HCN channel dysfunction (i.e. channelopathy) as a common mechanism in several addiction models.

Here we used the behavioral sensitization model characterized by a progressive escalation in locomotor response after repeated exposure to psycho-stimulant drugs such as cocaine (Wolf, 2002). This paradigm is thought to reflect adaptations in the neural components of the MCL that determine the motivational value of external cues (Robinson and Berridge, 2003; Wolf, 2002). We have previously reported a decrease in I_h current density (~45%) and cell size in putative VTA DA cells after cocaine sensitization (Arencibia-Albite et al., 2012). In addition, we have also observed an increase in HCN2 subunit protein expression in all MCL structures (Santos-Vera et al., 2013). These results reveal an apparent contradiction between a decreased I_h current density and an increased HCN2 subunit expression that calls for clarification. To resolve this discrepancy, we decided to investigate the potential changes in HCN subunits compartmental distribution in animals exposed to cocaine sensitization. We also measured and analyzed HCN4 protein expression considering that VTA GABAergic cells possess I_h, mainly mediated by HCN4 subunits (Margolis et al., 2012). To estimate the HCN subunits distribution, the surface HCN2 and HCN4 subunits protein expression were evaluated using the protein cross-linking assay bis (sulfosuccinimidyl) suberate (BS3) (Boudreau et al., 2012). This technique distinguishes between cell surface (S) and intracellular (I) localization of protein pools by molecular weight. The trafficking of channel subunits from intracellular compartments to the plasma membrane is of utmost importance for the regulation of cell excitability (Noam et al., 2010; Santoro et al., 2004). We hypothesized that the compartmental distribution of HCN subunits in all areas of the MCL is altered with the development of cocaine sensitization. Our results represent an important finding in the understanding of molecular alterations of ion channels implicated in cocaine-induced neuroadaptations.

2. Materials and methods

2.1. Animals

The study was carried out with male Sprague-Dawley rats (6 weeks, 250–300 g) purchased from Taconic Farms, Germantown, N.Y., and used after a 7-day quarantine period for acclimatization to the facilities. Rats were housed in pairs in a temperature- and humidity-controlled colony room with a 12-h light/dark cycle where they had access to food and water *ad libitum*. Before the start of experiments, the subjects were randomly assigned to a group that received either cocaine (15 mg/kg in saline) or saline injections. All animal protocols employed in this study were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Puerto Rico Medical Sciences Campus Institutional Animal Care and Use Committee.

2.2. Behavioral sensitization to cocaine

Behavioral studies were performed as previously described (Arencibia-Albite et al., 2012; Santos-Vera et al., 2013). After group selection rats were transferred to a sound-attenuated chamber (Whisper Room Inc. Morristown, TN) where they were placed in clear plastic cages equipped with parallel infrared (IR) beams to detect locomotor activity (Accuscan Instruments, Columbus, OH). Interruptions of IR beams as the animals moved were used to categorize their behavior. The computer software used (Versamax®) to analyze the counting distinguished between two locomotion patterns: (1). Stereotypic movement when it detected repeated breaking of a single IR beam, and (2) ambulatory activity generated by the sequential interruption of two or more different beams. All rats were habituated for 1 h to the locomotion box for two consecutive days before the start of the behavioral measurement. On the experimental day, following a 15 min habituation period, animals were injected with either 15 mg/kg i.p., cocaine hydrochloride (Sigma, St. Louis, MO) or isovolumetric saline injections. Animal activity was immediately recorded for 1 h in the saline and cocaine-injected groups. The behavioral study was carried out for a total of 7 days.

2.3. Tissue extraction for western blot

Twenty-four hours after the last injection (on day 7), rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and euthanized by guillotine. Animals' brains were dissected out and placed in ice-cold PBS (0.01 M phosphate buffered solution, pH 7.4, Sigma-Aldrich, St. Louis, MO). A Leica® Vibratome VT1000S (Leica, Germany) was utilized to obtain coronal sections (200–400 μm) of the VTA, PFC, NAc and HIP as identified on the stereotaxic rat brain atlas of Paxinos and Watson (1996). Brain sections were rapidly frozen with dry ice and mounted onto glass microscope slides (Fisherbrand®). A 0.8 mm–1.5 mm stainless steel hypodermic tube (Fine Science Tools) was employed to prepare tissue micropunches of the sectioned brain areas. The collected tissue was transferred to ice-cold microtubes with artificial cerebrospinal fluid (aCSF) for further processing.

2.4. BS3 crosslinking assay

Tissue samples were incubated in ice-cold aCSF and BS3, bis (sulfosuccinimidyl) suberate (2 mM) (Pierce, Rockford, IL). The incubation was performed with mild agitation for 30 min (min) at 4 °C. Crosslinking was terminated by quenching the reaction with glycine (100 mM) for 10 min at 4 °C. The treated tissues were pelleted by brief centrifugation (12,000 rpm, 1 min) and the supernatant was discarded. Pellets were then re-suspended in ice-cold lysis buffer containing protease and phosphatase inhibitors. To view a detailed protocol, refer to Boudreau et al., (2012).

2.5. SDS-PAGE and western blot

Dissected tissue was homogenized by sonication in ice-cold 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) the protein concentration of the supernatant fraction was determined using Bio-Rad's DC Protein assay (Bio-Rad Laboratories, Hercules, CA). Extracted proteins (40 μg) were resolved in a 7.5% or 4–15% precast SDS gel and electrophoresed (2.5 h at 100 V, 300 mA and 10 W at room temperature). Proteins were 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 30 min at 100 V constant voltage. The nitrocellulose membrane was stained with 0.1% Ponceau S solution, made in 0.1% glacial acetic acid (10 min) and then rinsed with PBS (3x, 10 min) for validation of protein transfer. Blocking of the nitrocellulose membrane was done at room temperature with a 2 h exposure to the following solution (15% skim milk, 20 mM Tris-HCl, 150 mM NaCl, 0.01% Tween-20, pH 7.5). The membrane was incubated overnight (4 °C) with anti-mouse HCN2 or HCN4 monoclonal antibodies (1:500; NeuroMabs Labs/Antibodies Incorporated, UC Davis, CA) in blocking solution. Next day, the membrane was washed in PBS-T

(PBS with 0.01% Tween 20; 3x, 10 min) and incubated with HRP-conjugated anti-mouse IgG produced in rabbit (1:5000; Sigma, St. Louis, MO; 1 h at room temperature). Subsequently, membranes were washed with PBS-T (2X, 10 min) and Tris-NaCl (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20, pH 7.5; 2X, 10 min). HRP-signal of protein bands corresponding to 110 kDa (HCN2 glycosylated protein), 97 kDa (HCN2 non-glycosylated protein) or 130 kDa (HCN4 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. HCN2 or HCN4 protein levels were normalized to GAPDH (37 kDa) immunoreactivity (1:5000; Sigma-Aldrich, St. Louis, MO). The ChemiDoc™ Imaging System and ImageLab 5.1 Software® (Bio-Rad Laboratories, Hercules, CA) were employed to perform the membrane densitometricanalysis.

2.6. Statistical analysis

Statistical analysis was performed using Prism 5® (GraphPad Software Inc., San Diego, CA). Total locomotor activities were expressed as photocell counts and analyzed using One-way ANOVA followed by a post-hoc analysis employing the Bonferroni's method for multiple comparisons. We used the Wilcoxon matched-pairs signed rank test for all comparisons between two groups and against a theoretical mean of one. A Kruskal-Wallis procedure followed by Dunns multiple comparison test was also employed whenever there was multiple comparison analysis between experimental groups. Significance was set at $P < 0.05$.

3. Results

3.1. Cocaine behavioral sensitization

Cocaine sensitization produces several neurophysiological changes in the MCL. Previously, our lab reported an increase in HCN2 subunit expression in all MCL areas after cocaine sensitization (Santos-Vera et al., 2013). To determine if cocaine produce region-specific changes in HCN2 and HCN4 subunits compartmental distribution, first, animals were subjected to a behavioral sensitization protocol. We examined the progressive increment in locomotor responses to a daily cocaine dose. During seven consecutive days, animals were administered a single cocaine (15 mg/kg i.p.) or saline (isovolumetric dose) injection. Each day, total locomotor responses were recorded for 60 min, after injection. As expected, cocaine-treated animals showed a progressive increase in locomotor activity, measured as photocell counts (Fig. 1). Cocaine treated animals showed a significant increase in locomotor activity by day seven compared to day one (acute response) or saline-injected controls (One way ANOVA $F(15,245) = 47.05$; $p < 0.0001$, Day 7 Cocaine vs Day 1 Cocaine $p < 0.0001$; Day 7 Cocaine vs Day 7 Saline, $p < 0.0001$, Bonferroni's multiple comparison test).

3.2. The BS3 crosslinking assay as a surface expression analysis tool for HCN2 detection

The samples were incubated with BS3, a membrane-impermeable bifunctional cross-linker that only binds to membrane proteins and do not cross to the cell's intracellular compartment. This crosslinking assay increases the apparent molecular weight of surface proteins, while intracellular ones are not modified. Thus, surface and intracellular protein pools of HCN2 subunits can be separated and quantified using SDS-PAGE and immunoblotting with a monoclonal NeuroMabs anti-HCN2. Fig. 2 shows a western blot analysis from naïve samples with or without protein cross-linking. The results indicate that BS3 cross-linking assay was successful since the high molecular weight band is only present in the BS3 treated samples (X-linked).

3.3. HCN2 surface expression is altered in the VTA and PFC after cocaine sensitization

To determine the basal pattern of expression of HCN2 subunits in all MCL areas, we compared the S/I ratio between control animals (saline injected rats). The HCN2 S/I ratio in the VTA was found to be greater than one ($p < 0.05$, Wilcoxon signed rank test against a theoretical mean of 1), suggesting that normally, this subunit is mainly expressed at the cell surface (Fig. 3). In the PFC and NAc, the average S/I ratio was below 1 ($p < 0.05$) indicating that this subunit is expressed mainly intracellularly. In the HIP there was no significant difference ($p = 0.68$) suggesting an even compartmental distribution of HCN2 subunits under control conditions.

To explore whether cocaine sensitization can affect the HCN2 subunits compartmental distribution, animals were sacrificed 24 h after the last injection. Protein samples were incubated with BS3 and protein extract was probed against monoclonal anti-HCN2 for Western Blot analysis. In the VTA, the HCN2 S/I ratio of cocaine-sensitized animals compared to saline was significantly decreased (Fig. 4A; Wilcoxon matched-pairs signed rank test, $p = 0.05$, $n = 7$), indicating that there are fewer HCN2 subunits present in the membrane. There was no compartmental redistribution. Different results were obtained from the others MCL areas. In the PFC, HCN2 S/I ratio of sensitized animals compared to saline is significantly increased, indicating the presence of more membrane HCN2 subunits (Fig. 4B; Wilcoxon matched-pairs signed rank test, $p < 0.05$, $n = 6$). Since the S/I ratio was changed to a number greater than one, there is subunit redistribution from the intracellular to the membrane compartment, suggesting the presence of more subunits at the surface. The HCN2 S/I ratio in the NAc (Fig. 4C; Wilcoxon matched-pairs signed rank test, $p = 0.31$, $n = 8$) and HIP (Fig. 4C; Wilcoxon matched-pairs signed rank test, $p = 0.08$, $n = 7$) of cocaine-sensitized animals did not change, indicating an even compartmental distribution of HCN2 subunits.

3.4. HCN4 subunit expression in the MCL system

Immunohistochemical and mRNA expression shows HCN4 subunit expression among areas of the MCL system (Margolis et al., 2006; Notomi and Shigemoto, 2004). However, the evidence of the protein expression levels of this subunit is limited. The HCN4 subunit protein expression was analyzed in HIP, NAc, PFC and VTA, using Western Blot Analysis. Samples from naïve animals from these four areas were dissected using micropunches (Kim et al., 1993). We used rat's heart lysates as positive control tissue since they express HCN4 subunit (Shi et al., 1999; Ludwig et al., 1998). Spleen lysates were used as negative controls because they do not express HCN4 subunits (Arroyo et al., 2006). Total protein samples were resolved in 7.5% precast SDS gel and western blots were performed. Using an anti-HCN4 monoclonal antibody, a single immunoreactive band (130KD MW) was detected in a linear phase, from 10 µg to 60 µg total protein sample (data are not shown). Using 40 µg we were able to obtain the proper protein expression of HCN4 subunits in all four areas tested (Fig. 5A). The 130 kD single band suggests that in contrast to HCN2 subunits, HCN4 is in its native isoform and probably did not undergo glycosylation. The HCN4 relative expression among MCL areas is NAc > PFC > HIP > VTA.

3.5. Cocaine sensitization induces HCN4 subunit down-regulation in the VTA

To examine the HCN4 subunits total protein expression after cocaine sensitization animals were also sacrificed 24 h after the last injection. The HCN4 immunoreactive bands were normalized against GADPH (37 kDa) protein. It was found that the HCN4 protein expression in the VTA is decreased after cocaine sensitization. VTA samples from cocaine-treated animals showed a significant (41%) decrease in HCN4 expression compared to saline control (Fig. 5B; Wilcoxon

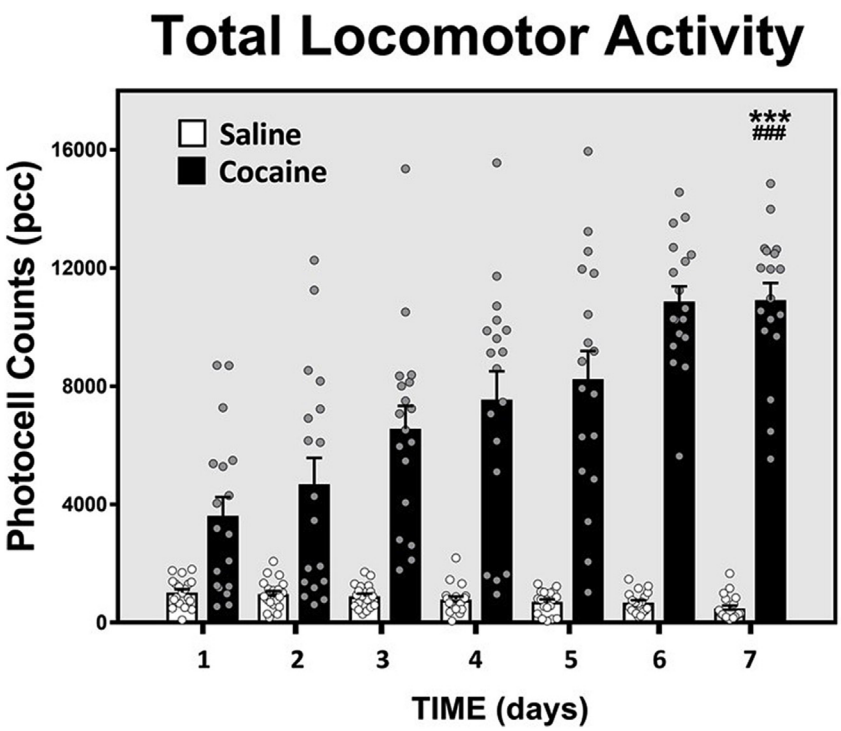

Fig. 1. Behavioral Sensitization to Cocaine. Mean total locomotor activity of all experimental subjects. Graph shows a progressive increase in locomotor activity of cocaine treated rats during seven days of cocaine administration. There is a statistically significant difference in locomotion between day 7 of cocaine treated rats vs. day 1, and between day 7 cocaine and day 7 of saline treated rats (One way ANOVA $F(15,245) = 47.05$; $p < 0.0001$, *Day 7 Cocaine vs Day 1 Cocaine $p < 0.0001$; #Day 7 Cocaine vs Day 7 Saline, $p < 0.0001$, $n = 15$, Bonferroni's multiple comparison test).

matched-pairs signed rank test, $p = 0.03$, $n = 6$). The HCN4 expression was also evaluated in the PFC (Fig. 5B; Wilcoxon matched-pairs signed rank test, $p = 0.95$, $n = 8$), NAc (Wilcoxon matched-pairs signed rank test, $p < 0.99$, $n = 6$) and HIP (Wilcoxon matched-pairs signed rank test, $p = 0.44$, $n = 6$). There were no significant densitometric changes observed in HCN4 subunit expression for these three areas after cocaine sensitization.

3.6. HCN4 surface expression in the MCL system does not change after cocaine sensitization

The HCN4 S/I ratio in all areas of the MCL system of cocaine-sensitized animals did not change, indicating even compartmental distribution of HCN4 subunits in VTA (Fig. 6; Wilcoxon matched-pairs signed rank test, $p = 0.16$, $n = 6$), PFC (Wilcoxon matched-pairs signed rank test, $p = 0.74$, $n = 8$), NAc (Wilcoxon matched-pairs signed rank test, $p < 0.99$, $n = 6$) and HIP (Wilcoxon matched-pairs signed rank test, $p = 0.16$, $n = 6$). Interestingly, HCN4 subunits were mainly present at intracellular compartments since all S/I ratios of saline animals were less than one.

4. Discussion

To understand the cellular mechanisms that underlie addictive behaviors, it is important to elucidate how diverse ion channel populations modify intrinsic and synaptic neuronal activity. The structure of the HCN channels is crucial for the functionality and biophysical activity of the I_h current (Herrmann et al., 2015; He et al., 2014). The I_h provides a significant fine tune to the modulation of cell excitability (Chen et al., 2001). Several studies have shown differential roles of I_h. For example, at the soma, during hyperpolarizing events, the HCN channels open allowing the I_h to act as an inward depolarizing current. In this manner, the current helps the cell to reach its resting membrane potential more rapidly, facilitating excitability (Biel et al., 2009; Chen et al., 2001). On the other hand, it has been shown that I_h plays a role in the dendritic integration of synaptic inputs. The presence of HCN channels in dendrites lowers membrane resistance, attenuating the amplitude of excitatory postsynaptic potentials. This event has been referred to as "the shunting effect" (Noam et al., 2011). Thus, whether the I_h can exert an excitatory or inhibitory role, will depend on the somatodendritic expression of HCN channels and cell type (Biel et al., 2009; Chen et al., 2001).

HCN channels' subunit composition determines I_h kinetics, voltage dependency and cyclic nucleotide sensitivity (Wainger et al., 2001; Berrera et al., 2006; Kusch et al., 2012). Each channel is composed of four subunits forming a tetramer that can be either homomeric (same subunit) or heteromeric (different subunits). The HCN1 and HCN2 are more likely to form heteromers than any other subunit (Much et al., 2003; Hegle et al., 2010). To date, there is no evidence that HCN2 and HCN4 can form these heteromers. Therefore, we can speculate that an increase in the expression levels of these two subunits can result in the formation of homomeric channels. Furthermore, the voltage-dependent activation of each HCN subunit differs: HCN1 activates at – 70 mV, HCN2 at – 95 mV, HCN3 at – 77 mV and HCN4 at – 95 to – 100 mV (Wahl-Schott and Biel, 2009). This voltage dependency is positively regulated by cyclic nucleotides. The kinetics of activation also differs between the HCN channel's subunits. HCN1 has the fastest channel kinetics while HCN4 has the slowest. HCN2 and HCN3 activate with kinetics in between HCN1 and HCN4 (Wahl-Schott and Biel, 2009; Wainger et al., 2001). HCN2 and HCN4 subunits have higher sensitivity to second messenger cascades involving cAMP (Wainger et al., 2001). Therefore, any variation in subunit composition might result in a significant change in the I_h biophysical properties and modulation of cell excitability.

Even though the HCN channel subunits are expressed on the entire MCL system, their role in modulating neural activity in these areas is poorly understood. Here we showed that cocaine sensitization alters the expression of HCN2 and HCN4 subunits in different areas of the MCL system. Using the BS3 crosslinking assay combined with western blot techniques, we were able to measure the surface expression of these ionic channels. This assay has been used before to detect changes in glutamate receptor distribution in dissociated cells, tissue slices and more recently, in tissue obtained after in-vivo treatments (Hall and Soderling, 1997; Grosshans et al., 2002a, b; Gerges et al., 2004; Boudreau and Wolf, 2005). We modified the test to identify the surface expression pattern of HCN channels in saline or cocaine-treated rats.

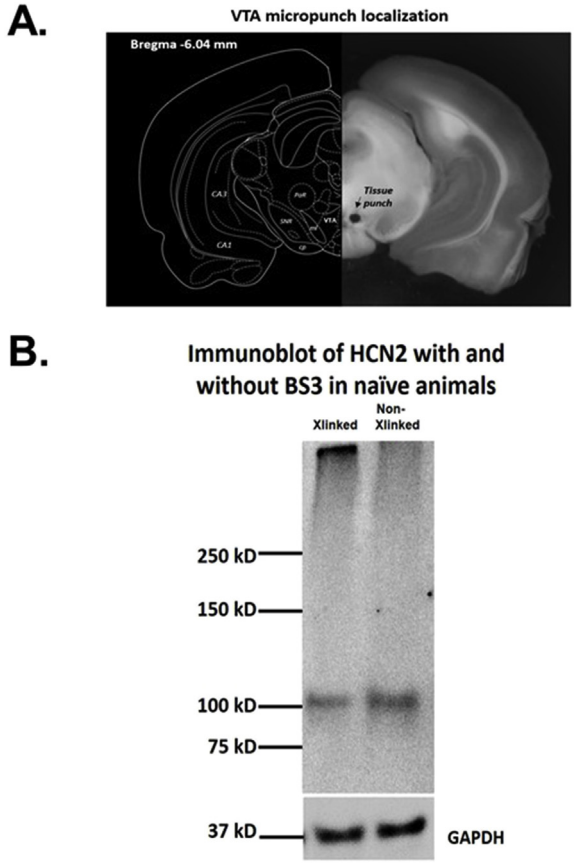

Fig. 2. Micropunched region and HCN2 BS3 Crosslinking from naïve rats. **A.** Representative image of a coronal brain section showing the size and localization of a VTA micropunch. PaR = pararubral nucleus, cp = cereberal peduncle, ml = medial lemniscus, SNR = substantia nigra par reticulata, VTA = ventral tegmental area, CA1 = field CA1 of hippocampus, CA3 = field CA3 of hippocampus. **B.** Immunoblot demonstrating a successful BS3 crosslinking assay since a high molecular weight band is only present in the BS3 treated samples (X-linked). The intracellular compartment of the HCN2 protein band is present at its native (97kD) and glycosylated (110kd) isoforms.

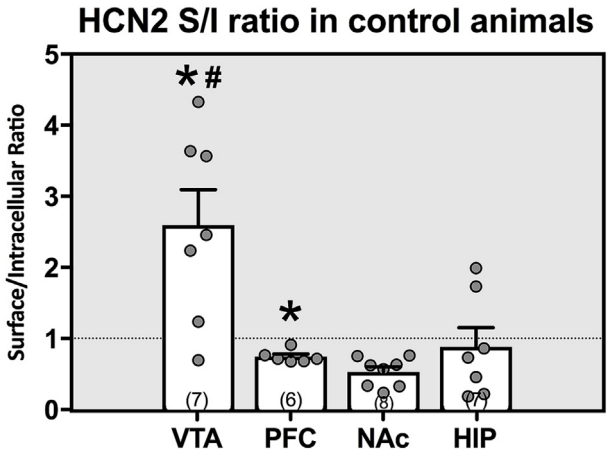

Fig. 3. HCN2 Subunit Compartmental Distribution in Saline Control Animals. Graph compares the HCN2 subunit compartmental distribution in control animals. The HCN2 S/I ratio in the VTA was found to be greater than one (*p < 0.05, Wilcoxon signed rank test against a theoretical mean of 1), suggesting that normally, this subunit is mainly expressed at the cell surface). In the PFC and NAc, the average S/I ratio was below 1 (*p < 0.05) indicating that this subunit is expressed mainly intracellularly. There is a significant difference (Kruskal-Wallis followed by Dunns test, #p < 0.05) between VTA HCN2 distribution and NAc.

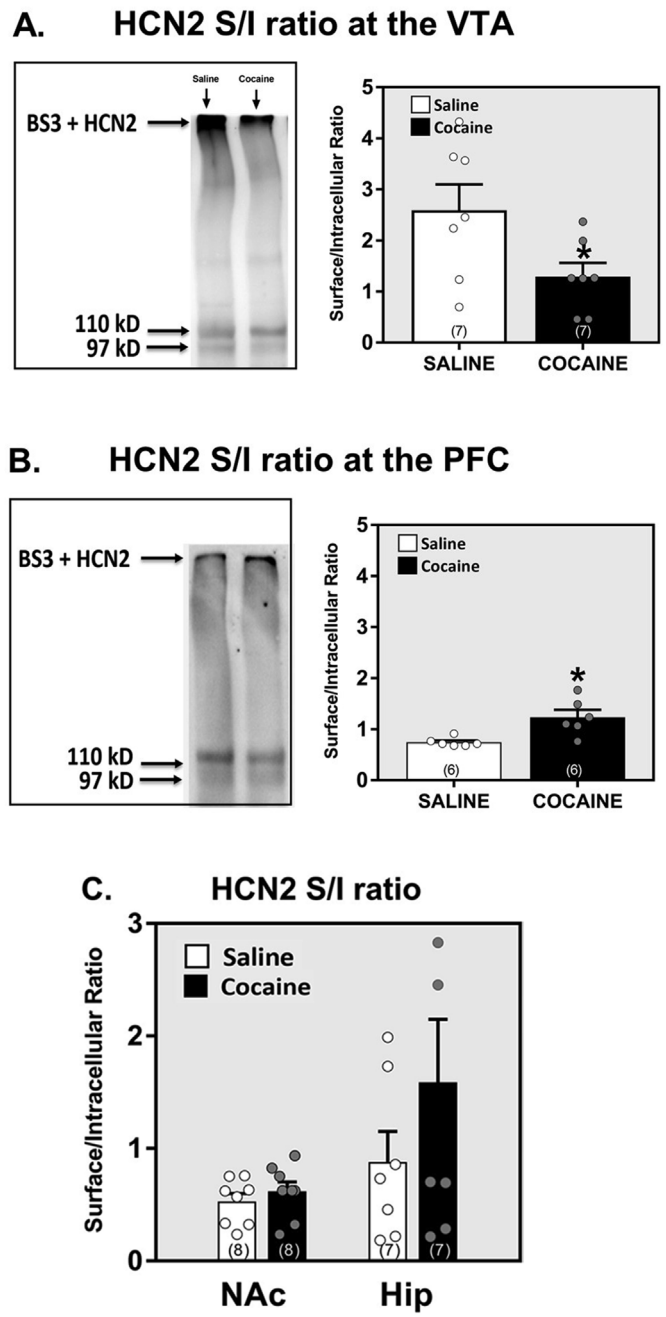

Fig. 4. A. HCN2 subunit S/I Ratio is decreased in the VTA after Cocaine Sensitization. Graphs show the HCN2 S/I ratio in the VTA of saline and cocaine treated animals. Figure represents the densitometry analysis of bands obtained by Western Blot using monoclonal antibody against HCN2 from NeuroMabs Labs. Statistical analysis using Wilcoxon matched-pairs signed rank test, *p < 0.05, n = 7, shows a significant decrease in S/I HCN2 subunit ratio in VTA compared with saline treated animals. **B. Cocaine sensitization increases the HCN2 S/I Ratio in the prefrontal cortex**. Graphs show the HCN2 S/I ratio in the PFC of saline and cocaine treated animals. Figure represents the densitometry analysis of bands obtained by Western Blot against HCN2. Statistical analysis using Wilcoxon matched-pairs signed rank test, p < 0.05, n = 6, shows a significant increase in S/I HCN2 subunit ratio in PFC compared with saline treated animals**. C. Cocaine sensitization does not affect the HCN2 subunit S/I ratio in NAc or HIP.** Graph shows the HCN2 S/I ratio in the NAc of saline and cocaine treated animals. Statistical analysis shows no significant changes in S/I HCN2 subunit ratio in NAc (Wilcoxon matched-pairs signed rank test, p = 0.31, n = 8) or HIP (Wilcoxon matched-pairs signed rank test, p = 0.08, n = 7), indicating an even compartmental distribution of HCN2 subunits.

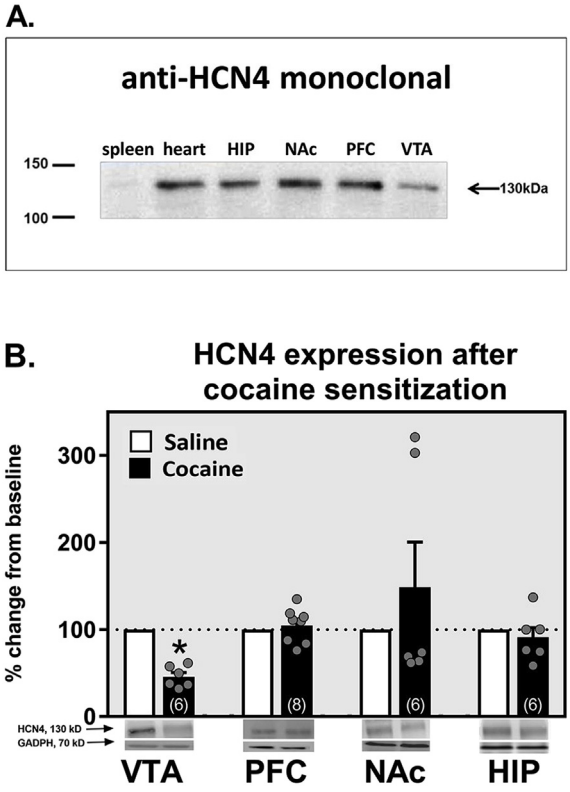

Fig. 5. A. HCN4 subunit expression in areas of the MCL system from naïve rats. Western Blots show the expression pattern of the HCN4 subunit (130 kDa) in all MCL areas, HIP, VTA, NAc, PFC, in naïve animals (n = 3). Bands were normalized using GAPDH expression (37kD). The 130 kDa band is in the expected position. Heart and spleen lysates are presented as positive and negative controls, respectively. **B. HCN4 subunit expression is decreased in the VTA after Cocaine Sensitization**. Graphs show the HCN4 Protein Expression expressed as Band Intensity in the VTA, PFC, NAc and HIP of saline and cocaine treated animals. Figure represents the densitometry analysis of bands obtained by Western Blot using monoclonal antibody HCN4 from NeuroMabs Labs. Statistical analysis for VTA shows a significant (41%) decrease in HCN4 expression compared to saline control (Wilcoxon matched-pairs signed rank test, $p = 0.03$, $n = 6$) whereas there were no significant densitometric changes observed in HCN4 subunit expression in the PFC (Wilcoxon matched-pairs signed rank test, $p = 0.95$, $n = 8$), NAc (Wilcoxon matched-pairs signed rank test, $p < 0.99$, $n = 6$) or HIP (Wilcoxon matched-pairs signed rank test, $p = 0.44$, $n = 6$).

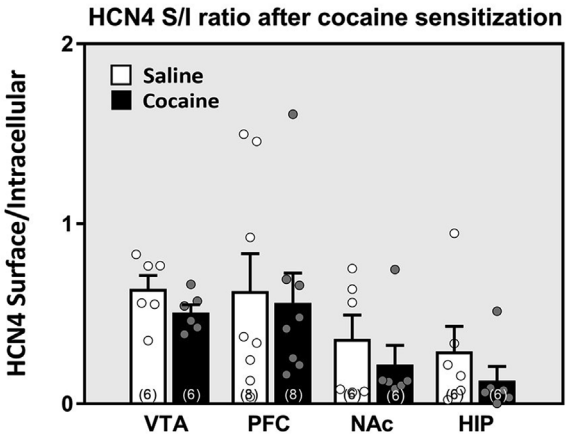

Fig. 6. HCN4 subunit S/I Ratio is not affected in the VTA, NAc, PFC or HIP after Cocaine Sensitization. Graph shows the HCN4 S/I ratio in the VTA, PFC, NAc and HIP of saline and cocaine treated animals. Figure represents the densitometry analysis of bands obtained by Western Blot using monoclonal antibody against HCN4 from NeuroMabs Labs. Statistical analysis using sample T-test shows no significant changes in S/I HCN4 subunit ratio in VTA (Wilcoxon matched-pairs signed rank test, $p = 0.16$, $n = 6$), PFC (Wilcoxon matched-pairs signed rank test, $p = 0.74$, $n = 8$), NAc (Wilcoxon matched-pairs signed rank test, $p < 0.99$, $n = 6$) or HIP (Wilcoxon matched-pairs signed rank test, $p = 0.16$, $n = 6$). Interestingly, HCN4 subunits were mainly present at intracellular compartments since all S/I ratios of saline animals were less than one.

The results confirmed that the surface/intracellular (S/I) ratio of these subunits is differentially altered after chronic cocaine exposure.

Previous studies have established that the HCN2 subunit is the most expressed in the MCL system, especially in VTA DA cells (Notomi and Shigemoto, 2004; Monteggia et al., 2000; Margolis et al., 2012). Here we found that in the VTA region, HCN2 subunits are mostly localized in the cell surface according to S/I ratios measured in control samples. There is no compartmental redistribution of HCN2 subunits in the VTA, since the measured ratios remained above a theoretical mean of one, even after cocaine treatment. Moreover, it was demonstrated that in the VTA the HCN2 S/I ratio is decreased. This reduction in S/I ratio suggests that there are less functional subunits expressed at the surface. These changes observed at the surface are in accordance with our previous investigations demonstrating that even though there is an increase in HCN2 total protein expression, there is a ~45% decrease I_h current density in VTA DA cells after cocaine sensitization (Santos-Vera et al., 2013; Arencibia-Albite et al., 2012). It was also shown that cocaine sensitization preferentially increased the glycosylated HCN2 isoform. The N-glycosylation of HCN2 subunits promotes adequate subunit trafficking and surface expression (Much et al., 2003; Hegle et al., 2010). It has been shown that HCN2 interacts with scaffold proteins tamalin, S-SCAM and Mint2, through different binding domains (Kimura et al., 2004; Wahl-Schott and Biel, 2009). The HCN2 surface expression is increased when co-expressed with Mint2, suggesting this co-expression as an indispensable mechanism for HCN2 surface trafficking (Kimura et al., 2004). In view of these findings we suggest, that in cocaine-sensitized animals, there is a possible disruption of the HCN2 trafficking to the VTA cell membrane, leading to a lower I_h current in this area. The mechanism by which cocaine can affect the glycosylated state of HCN2 subunits is unknown. It also remains to be elucidated if indeed cocaine disrupts the trafficking of these subunits in the VTA or if this disruption is a compensatory mechanism (homeostatic compensation) against a possible heighten excitability induced by the insertion of these HCN2 subunits at the membrane. Several studies propose that, when perturbed, neurons have a remarkable ability to maintain their specific functional properties (Liss and Roeper, 2008; O'Leary et al., 2014; Gasselin et al., 2015).

In the PFC of cocaine-sensitized animals, the HCN2 S/I ratio was found to be increased. This finding suggests the presence of more HCN2 subunits in the membrane since the total protein expression in the PFC is increased after cocaine sensitization. Since the S/I ratio in cocaine-treated animals changed to a number greater than a theoretical mean of one, we postulate that there is a subunit redistribution from the intracellular to the surface membrane compartment, suggesting the presence of more functional subunits at the surface site. It is likely that these added functional subunits will enhance I_h density, however, it needs to be further verified by electrophysiological measurements. Despite the increase in the HCN2 total subunit protein expression in the NAc and Hip of cocaine-sensitized animals, the S/I ratio did not change, indicating an even compartmental distribution of HCN2 subunits in these areas.

The HCN4 subunit is expressed among all areas of the MCL system (Notomi and Shigemoto, 2004; Santoro et al., 2000). GABAergic neurons in the VTA possess an I_h current which is mainly mediated by HCN4 subunits (Margolis et al., 2012). Therefore, we analyzed the HCN4 total subunit expression in all MCL regions. To our knowledge, this study is the first one showing the presence of HCN4 subunits at the

protein level in the VTA, PFC and NAc. In addition, in contrast to HCN2, HCN4 subunits in MCL areas do not undergo post-transcriptional modifications as suggested by the observation of a single immunoreactive band following the BS3 procedure. Based on the determined S/I ratios from control groups, HCN4 subunits are mostly localized at the intracellular compartment in all MCL areas (data not shown). The densitometric analysis demonstrates that HCN4 total protein expression in MCL system is not affected by cocaine sensitization, except in the VTA, where HCN4 expression is decreased. However, for this HCN subunit, the S/I ratio in all MCL regions tested did not change upon cocaine sensitization. These results demonstrate that cocaine treatment preferentially alters the HCN4 expression profile in VTA cells, possibly decreasing I_h density in GABAergic cells. The specific changes observed in the VTA could be related to the principal role this structure has in the initiation/development of cocaine sensitization which is the protocol used in this study. There is a consensus that the VTA is the most important area for the initiation of sensitization whereas NAc appears to be the fundamental area involved in the expression of sensitization after a withdrawal period (Cador et al., 1995; Kalivas and Stewart, 1991; Vanderschuren and Kalivas, 2000).

Our previous work suggested possible behavioral outcomes that can be derived from a decreased I_h function. We proposed that I_h channels are mainly located at dendrites of VTA DA cells and, thus, their inhibition may facilitate the transition from single-spike activity to burst firing (Arencibia-Albite et al., 2007) with the concomitant enhanced DA release. Thus, it is possible that the I_h inhibition seen under the development of locomotor sensitization could contribute to cocaine addiction by increasing bursting probability in VTA DA cells (Arencibia-Albite et al., 2012). Furthermore, this I_h diminution persisted after 7 days of drug withdrawal demonstrating a long-lasting effect (Arencibia-Albite et al., 2017). Recently, Zhong et al. (2018) have reported that HCN2 knockdown in the VTA induced an anxiety like behavior in response to chronic mild unpredictable stress (CMS) whereas VTA HCN2 overexpression largely prevented the CMS-induced behavioral deficits. The results implicate a critical role for the I_h current in VTA DA neuronal firing observed during this behavioral disorder.

5. Conclusion

It was demonstrated that cocaine sensitization differentially alters the protein expression of HCN2 and HCN4 subunits. We speculate that the I_h current reduction in VTA DA neurons previously observed might be due to selective changes in HCN2 S/I ratio present after chronic cocaine administration. However, the somatodendritic pattern of HCN channels in the VTA needs to be elucidated in order to attribute it a specific excitatory or inhibitory role in cell excitability. Our findings represent a potential molecular mechanism underlying neurobiological changes of cocaine addiction.

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Appendix A. Supplementary data

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