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Modulation of Hyperpolarization-Activated Cation Currents (I_h) by Ethanol in Rat Hippocampal CA3 Pyramidal Neurons

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ABSTRACT

It is well established that ethanol (EtOH), through the interaction with several membrane proteins, as well as intracellular pathways, is capable to modulate many neuronal function. Recent reports show that EtOH increases the firing rate of hippocampal GABAergic interneurons through the positive modulation of the hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels. This effect might be consistent with the increase of GABA release from presynaptic terminals observed in both CA1 and CA3 inhibitory synapses that leads the enhancement of the GABAergic system induced by EtOH. The activation of HCN produced an inward currents that are commonly called I_h. I_h play an important role for generating specific neuronal activities in different brain regions, including specific sub-regions of the hippocampal formation, such as CA1 and CA3 pyramidal neurons and hippocampal GABAergic interneurons. The main physiologic effect mediated by HCN-induced I_h is directed to the control of the neuronal resting membrane potential and action potential (AP) discharge as well as dampen synaptic integration. Since robust I_h are also present in CA3 glutamatergic neurons, I here investigated whether the action of EtOH in the control of CA3 excitability can be correlated with its possible direct interaction with these cation channels. For this purpose, patch-clamp experiments were performed in CA3 pyramidal neurons from hippocampal coronal slices obtained from male Sprague-Dawley rats. The data obtained demonstrated that EtOH is able to modulate I_h in biphasic manner depending on the concentrations used. Low EtOH concentrations enhanced I_h amplitude, while high reversibly reduced them. This biphasic action induced by EtOH reflects on firing rate and synaptic integration. In addition, in this reports it has been shown that EtOH modulates the function of HCN channels through interfering with the cAMP/AC/PKA intracellular pathways, an effect that is mimicked also by other endogenous compounds such as dopamine through D1 receptors activation. These data suggest that the HCN-mediated I_h currents in CA3 pyramidal neurons are sensitive to EtOH action, which at low or relevant concentrations is able to increase or reduce their function respectively. Altogether these data suggest a potential new mechanism of EtOH actions on hippocampal formation and may help to better understand the depressant central activity showed by this drug of abuse

ABBREVIATIONS

AMPA-R: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor

ButOH: buthanol

CA: cornu ammonis

cAMP: 3'-5'-cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CNG: cyclic nucleotide-gated

CNS: central nervous system

EPSP: excitatory post-synaptic potential

EtOH: ethanol

GABA: γ-aminobutyric acid

GHB: γ-hydroxybutyrate acid

HCN: hyperpolarization-activated and cyclic nucleotide

MAPK: Mitogen-activated protein kinase

NMDA-R: N-methyl-D-aspartate receptor

PKA: Protein Kinase A

PKC: Protein Kinase C

RMP: resting membrane potential

VTA: ventral tegmental area

τ: time costant

I_h: hyperpolarization-activated currents

INTRODUCTION

1. Ethanol

Ethyl alcohol, or ethanol (EtOH), is regarded as one of the most widespread drugs of abuse known to man, capable of exerting many effects on various physiological systems, in particular the central nervous system (CNS), where it acts as a depressant agent. Moreover, this substance is extensively studied because of its unique and complex pharmacology and toxicology. Elucidation of its molecular mechanism of action is regarded a crucial in order to find therapies that are effective in the treatment of alcohol dependence and relapse. EtOH's consumption is widely diffused in the Western world and its abuse represents a serious societal problem. The use of alcoholic beverages dates back to 10.000 years BC (Hanson, 1995) and furthermore, Greeks, Romans and inhabitants of Babylon consumed alcohol during religious festivals and for medicinal practices; however, the use of these beverages has been reported also during daily life to facilitate socialization and was considered as a source of nutrition. The role of EtOH in society has been documented in most cultures; for example, in pre-Columbian America, in 200 AD, and in the Islamic world, in the 700-800s, distillation was discovered; in fact, the word alcohol is derived from the Arabic "something subtle". The adverse and toxic effects of heavy consumption of EtOH were discovered early in India, Greece and Rome, but its use greatly increased in 1800s during the phase of industrialization. In our modern society, EtOH shows a widespread use, and statistical studies reported an average age of first intoxication of 11-15 years in most western countries, with a higher prevalence in men than women. Chronic use of EtOH may lead to a condition called "alcoholism" or "alcohol dependence",which is characterized by compulsive use and dangerous social and medical consequences. Moreover, its condition contributes to brain damage and to severe deficits in cognitive functioning. Indeed, in the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), alcohol dependence is defined as a cluster of cognitive, behavioral, and physiological symptoms that leads the individual to continue the consumption of alcoholic beverages despite significant alcohol-related problem. In Italy, abuse of EtOH is associated with

about 50.000 new alcoholics every year and 40.000 alcohol-related deaths. In the U.S.A., there are about 14 million people that normally abuse EtOH. Consequently, EtOH abuse is a one of the major health problems, with annual costs associated with this problem that have been estimated in the range of $ 185 billion each year in U.S.A. In addition, alcohol causes about 100.000 deaths annually, including 20.000 fatal car accidents related to EtOH consumption (Harwood *et al*., 1999; Rehm *et al*., 2007). EtOH is considered a substance that contributes to more morbidity, mortality, and public health costs than all the other illicit drugs. For this purpose, researchers of various fields studied extensively the complex actions of EtOH, in order to characterize the mechanism of action which is not yet sufficiently understood.

1.1. *Ethanol and Consumption*

With regard to the consumption of EtOH, it has been reported that this drug is consumed in gram quantities compared to most other drugs, which are assumed in milligram or microgram doses. EtOH content of alcoholic beverages typically ranges from 4% to 6% (volume/volume) for one glass of beer, 10% to 15% for wine, and from 40% to higher percentages for distilled spirits. Thus, a beer, a glass of wine or distilled spirits contains about 14 gr of alcohol or about 0.3 moles of EtOH. One method to measure the blood EtOH concentration (BEC) in human is represented by the measurement of alcohol levels in expired air; blood air partition is about 2000:1. Because the excessive EtOH consumption is a leading cause of more vehicular accidents, as stated above, there are laws that limit the use of vehicles when the driver has consumed alcohol. The allowed BEC for driving is set at or below 80 mg% (0.08% weight/volume; 80 mg ethanol per 100 ml blood), which corresponds to about 17 mM ethanol in blood.

It is important to remark that BEC depends on numerous factors, such as the rate of drinking, gender, body weight and water percentage, and the rates of metabolism and stomach emptying. It is well known that women, compared to men, have a different water percentage in the body, which results in a different distribution of EtOH. Moreover, alcohol-dehydrogenase (ADH) in the

females results to be less active; thus, women are expected to reach higher BEC and are more inclined to liver damages. In addition, age represents a factor also involved in the metabolism of EtOH, as this seems slower in advanced age. Body weight is responsible for the distribution of EtOH in the body. For example, in man with a strong build, the alcohol ingested is distributed at slower rate and with reduced toxic effects. Moreover, the effects of EtOH appears to be less harmful when the EtOH is consumed during the meals, because its gastric absorption is slowed down. The DSM IV distinguishes the consumption of EtOH in alcohol abuse, called also prolonged use, and chronic abuse.

Abuse of EtOH is defined as “chronic” when it is accompanied by tolerance, dependence, and craving for the drug. The pharmacological definition of tolerance is “the reduction in response to the drug after repeated administrations”, thus, in the presence of this phenomena a higher dose of EtOH is required to induce the same effects previously obtained with a lower dose. It is possible distinguish two different form of EtOH tolerance: pharmacokinetic and pharmacodynamic tolerance. Pharmacokinetic tolerance is characterized by changes in the distribution and/or metabolism of the EtOH after repeated administrations. Pharmacodynamic tolerance involves adaptive changes in the CNS, such as receptors and/or ion channels, affected by the substance, so that response to a given concentration of EtOH is reduced. Wilhelmsen *et al*. (2003) reported that the innate tolerance to EtOH is different among individuals and depends on family history of alcoholism. Moreover, the consumption of EtOH can produce greater tolerance and the BEC may range from 300 to 400 mg/dl. Heavy drinkers acquire physical dependence, which leads to drinking in the morning to restore the blood EtOH levels decreased during the night. Generally, the definition of physical dependence is “a state that develops as a result of the adaptation (tolerance) produced by a resetting of homeostatic mechanism in response to repeated drug use”. Moreover, physical dependence is correlated with the alcohol-withdrawal syndrome, which depends on the daily dose consumed and the length of consumption, and generally it is suppressed through resumption of alcohol ingestion. EtOH

withdrawal syndrome appears when administration of the drug is abruptly terminated and represents the only actual evidence of physical dependence. The symptoms that characterize the EtOH withdrawal syndrome involve tremor, irritability, nausea, sleep disturbance, tachycardia, hypertension, sweating, seizures, visual hallucinations, dilated pupils, confusion and severe agitation. Some individuals might experience *delirium tremens*, characterized by hallucinations, delirium, fever and tachycardia, and in some cases this phenomena may be fatal. Finally, other feature of the chronic abuse is craving and drug-seeking behaviour, also called *psychological dependence*.

1.2. Ethanol and Central Nervous System (CNS)

Generally, the public views considers EtOH as a stimulant drug; while this may be true to a certain point, this substance in fact exerts depressant activity in CNS. When the consumption is moderate, EtOH produces an anti-anxiety action and is responsible for the disinhibition; at higher doses it is possible to observe emotional outbursts and uncontrolled mood swings. Furthermore, in case of sever intoxication, CNS functions are markedly impaired and it is possible to observe a condition that may resemble general anesthesia. In this case, it is present a little margin between the anesthetic and lethal effects. Consumption of EtOH disrupts the delicate balance between inhibitory and excitatory neurotransmitters , resulting in anxiolysis, ataxia, and sedation (Valenzuela, 1997). Short-term alcohol exposure alters the balance toward inhibition by both enhancing the activity of inhibitory neurotransmitters and neuromodulators (γ-aminobutyric acid GABA, glycine and adenosine) and diminishing that of excitatory neurotransmission (glutamate and aspartate).

During EtOH withdrawal, the balance shifts toward a state of hyper-excitation characterized by seizures, delirium and anxiety. Experimental research documented that after long-term alcohol exposure, CNS restores the physiological equilibrium, with a decreased inhibitory neurotransmission and enhanced excitatory neurotransmission (Valenzuela, 1997). EtOH shows a complex array of neurochemical action which result from either direct and indirect interactions with distinct protein targets, although an unspecific

interaction with membrane lipids has not been ruled out. Indeed, early studies on acute alcohol effects lead to the Meyer-Overton rule, which defined the direct relationship between the hydrophobicity of an alcohol and its potency for producing intoxication (Meyer, 1901; Overton, 1896). Subsequently, experimental research identified the membrane proteins such as possible targets for EtOH, and this hypothesis was later confirmed by numerous evidences (Covarrubias *et al*., 1995; Peoples and Weight, 1995; Franks and Lieb, 1994; Slater, 1993). At pharmacological relevant concentrations, ranging from low to 100 mM, EtOH interferes with the function of several ion channels and membrane receptors (Vengeliene *et al*., 2008; Lovinger, 1997), and this is regarded as one of the most important action of EtOH that contributes to its overall CNS depressant effect.

1.3. Ethanol actions on synaptic transmission

As mentioned above, it is well established that the EtOH exerts its action on a large number of membrane proteins of the CNS (Li *et al*., 1994, Peoples and Weight 1995; Slater *et al*., 1993; Harris 1999), that are involved in synaptic transmission. The idea of the synapse as a substrate for the actions of EtOH emerged mostly from electrophysiological evidences that showed EtOH effects on the neuromuscular transmission and on synaptic transmission in CNS (Gage, 1965; Berry and Pentreath, 1980). In subsequent years, various experimental studies have confirmed this idea in a variety of CNS model (Siggings and Bloom, 1981; Siggings *et al*., 1987a, 1987b, 1999; Deitrich *et al*., 1989; Shefner, 1990; Weight, 1992; Criswell *et al*., 1993). In addition, many studies reported the postsynaptic consequences of EtOH interactions with transmitter receptors; Lovinger *et al*. (1989) demonstrated that EtOH was able to reduce N-methyl-D-aspartate (NMDA) function in a concentration dependent manner. On the other hand, GABA and glycine receptors activity is increased by EtOH (Mihic *et al*., 1997); the first evidences were shown by Allan and Harris (1987) and Sudzak *et al*. (1988), who observed GABA-induced chloride fluxes were enhanced by EtOH in brain preparations. Furthermore, EtOH actions on GABAergic synaptic inhibition derive from both pre and

postsynaptic actions, as many experimental studies demonstrated that the frequency of GABAA receptors-mediated spontaneous and miniature inhibitory postsynaptic currents (IPSCs) in hippocampal CA1 and CA3 pyramidal neurons were enhanced by EtOH (Ariwodola and Weiner 2004; Li *et al*., 2003, 2006; Sanna *et al*., 2004; Galindo *et al*., 2005). In addition, the frequency of synaptic events is altered in presence of EtOH as well as in the amygdala, cerebellum and ventral tegmental area (VTA) (Zhu and Lovinger, 2006; Kelm *et al*., 2007; Theile *et al*., 2008). As for the postsynaptic actions of EtOH, it was shown that amplitude and duration of GABAA and glycine receptors-mediated inhibitory postsynaptic currents were increased by EtOH (Sebe *et al*., 2003; Ziskind-Conhaim *et al*., 2003). Furthermore, 5-hydroxytryptamine type 3 (5-HT3) and neuronal nicotinic Ach receptors represent a further target for EtOH actions, since its acute exposure potentiates the function of these receptors (Lovinger 1999; Narahashi *et al*., 1999). As for the voltage-gated Ca^{2+}-channels, it was described that EtOH direct inhibition of these channels elicits a suppression of the release of several neurotransmitters (Wang *et al*., 1994). EtOH can also enhance the activity of G-protein-activated inwardly rectifying potassium channels (GIRKs) (Aryal *et al*., 2009; Kobayashi *et al*., 1999; Lewohl *et al*., 1999). To briefly summarize a large body of data collected in several decades of research, EtOH represents an unspecific pharmacological agent that has various primary targets, such as ion channels and membrane receptors.

More recently, another potential molecular target of EtOH has been characterized, namely the hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels, which mediate an inward cation current, termed I_h. Brodie and Appel (1998) in fact reported that EtOH enhances the amplitude of I_h in dopaminergic neurons VTA. Moreover, the blocker of I_h, ZD-7288, was reported to be able to reduce the increase of I_h amplitude induced by the EtOH in dopaminergic VTA neurons (Okamoto *et al*:, 2006). In addition, in hippocampal GABAergic interneurons, EtOH increases the firing rate in a concentration-dependent manner through the positive modulation of I_h, (Yan *et al*., 2009).

2. The hyperpolarization-activated current I_h

The hyperpolarization-activated currents, defined as I_h, were first discovered in the sino-atrial node tissue in 1976 (Noma and Irisawa, 1976), where they were named “funny currents” (I_f); thereafter, a similar I_h was determined in rod photoreceptors and hippocampal pyramidal neurons (Bader *et al*., 1979; Halliwell and Adams, 1982). The first characterization of this current was made by DiFrancesco and colleagues (DiFrancesco, 1981; DiFrancesco *et al*., 1986; Brown & DiFrancesco 1980; Brown *et al*., 1979a; 1979b). With regards to the biophysical properties, it is reported that I_h is a mixed cation current mediated by a channel that permeates both K^+ and Na^+ ions under physiological conditions (Biel *et al*., 2009; Wahl-Schott and Biel 2009). Generally, this cation current is activated in response to membrane hyperpolarization. In addition, one of the most important key feature of I_h is its modulation by cyclic nucleotides, such as cAMP (DiFrancesco and Tortora, 1991). For the pharmacological profile, it is well established that I_h are completely blocked by low concentrations of external caesium (CsCl) (Ludwing *et al*., 1998; DiFrancesco, 1982, Fain *et al*., 1978). The ion channels that mediate I_h were identified in the late 1990s and were termed hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels due to their complex activation kinetics (Ludwing *et al*., 1998,1999; Santoro *et al*., 1998; Seifert *et al*., 1999). Many physiological roles and neuronal functions have been attributed to I_h, for example this current is involved in the determination of the resting membrane potential (RPM) (Doan *et al.*, 1999; Lupica *et al*., 2001; Nolan *et al*., 2007; Pape *et al*., 1996). I_h plays also an important role in the other several neuronal process, such as dendritic integration (Magee, 1998, 1999, 2000) and synaptic transmission (Beaumont *et al*., 2002; Beaumont and Zucker, 2000).

2.1 The HCN Channel Family

Despite their important physiological roles, the identification of the genes that encode HCN channels has been achieved only recently. HCN channels together with cyclic nucleotide-gated (CNG) channels and the Eag-like K channels belong to the superfamily of voltage–gated pore loop channels (Craven and Zagotta, 2006; Yu *et al*., 2005; Kaupp and Seifert, 2002). HCN channels have been cloned from both Vertebrates and Invertebrates, but have not been found in *C. elegans* and yeast nor in a prokaryotic genome. Gauss *et al*. (1998) identified and cloned a single channel gene from testis of the sea urchin, *Strongylocentrotus purpuratus*, while in mice, four different genes have been characterized (Ludwing *et al*., 1999; Ranjan *at al*., 1998). Initially, these genes were called in different ways, but Clapham (1998) proposed the acronym HCN, which stands for hyperpolarization-activated and cyclic nucleotide-gated channels. In mammals, four isoforms of HCN channel (HCN1-4) encoded by the HCN 1-4 gene family have been identified (Robinson and Siegelbaum, 2003). These isoforms differ by patterns of gene expression and tissue distribution (Ludwing *et al*., 1998; Santoro *et al*., 1998). HCN1 isoform is expressed in brain, in particular in layer 5 pyramidal neurons of the neocortex, in hippocampal CA1 and CA3 pyramidal neurons, although with a stronger expression in hippocampal CA1 neurons compared to CA3 neurons. Furthermore, the expression of HCN1 in scattered neurons in the stratum oriens and lucidum of the hippocampus has been also reported. HCN1 is found in inhibitory basket cells and Purkinje neurons of the cerebellum (Santoro *et al*., 2000; Monteggia *et al.*, 2000; Moosmang *et al.*, 1999). In addition, retinal photoreceptors express high levels of HCN1 (Moosmang *et al*., 2001). In the heart, HCN1 is principally expressed in the sino-atrial (SA) node together with HCN2 (Shi *et al*., 1999). HCN2 expression have been identified in CA1 and CA3 pyramidal neurons, but higher levels in CA3 region are reported. HCN2 is also expressed in scattered neurons of stratum oriens and lucidum of the hippocampus. Vice versa, in the inhibitory thalamic reticular neurons, HCN2 seems to be the only isoform expressed (Santoro *et al*., 2000; Monteggia *et al*., 2000; Moosmang *et al*., 1999). Of the four HCN isoforms, HCN3 has the

weakest expression in the mammalian brain, but it is absent in heart (Ludwig *et al*., 1998, Moosmang *et al*., 2001). HCN4 shows high levels of expression in the thalamus, like HCN2, mainly in the excitatory thalamo-cortical relay neurons. In hippocampus and neocortex, the expression of HCN4 is low (Santoro *et al*., 2000; Monteggia *et al*., 2000; Moosmang *et al*., 1999). With regard to the expression in the heart, HCN4 is the predominant isoform (Shi *et al*., 2000, 1999); in particular, HCN4 transcript is found in cardiac Purkinje fibers and in the SA node. Structurally, HCN channels are complexes consisting of four subunits that are arranged around the centrally-located pore; these subunits form four different homotetramers with different and distinct biophysical properties (Ishii *et al*., 1999; Ludwing *et al*., 1999, 1998; Seifert *et al*., 1999; Santoro *et al.*, 1998). Each HCN channel subunit consists of the transmembrane core and the cyclic nucleotide binding domains (CNBD), which are highly conserved in all HCN isoforms, on the contrary, the amino (NH_2) and carboxy- (COOH) terminal cytoplasmic regions are variable (Kaupp and Seifert, 2001; Santoro and Tibbs, 1999). In the core transmembrane domain are located the gating machinery and the ion-conducting pore. Furthermore, the COOH-terminal domain is composed of the C-linker and the CNBD which mediates the response to cAMP (Craven and Zagotta, 2006). Both transmembrane core and COOH-terminal domain allosterically cooperate with each other during channel activation (Kaupp and Seifert, 2001).**(Figure 1)**.

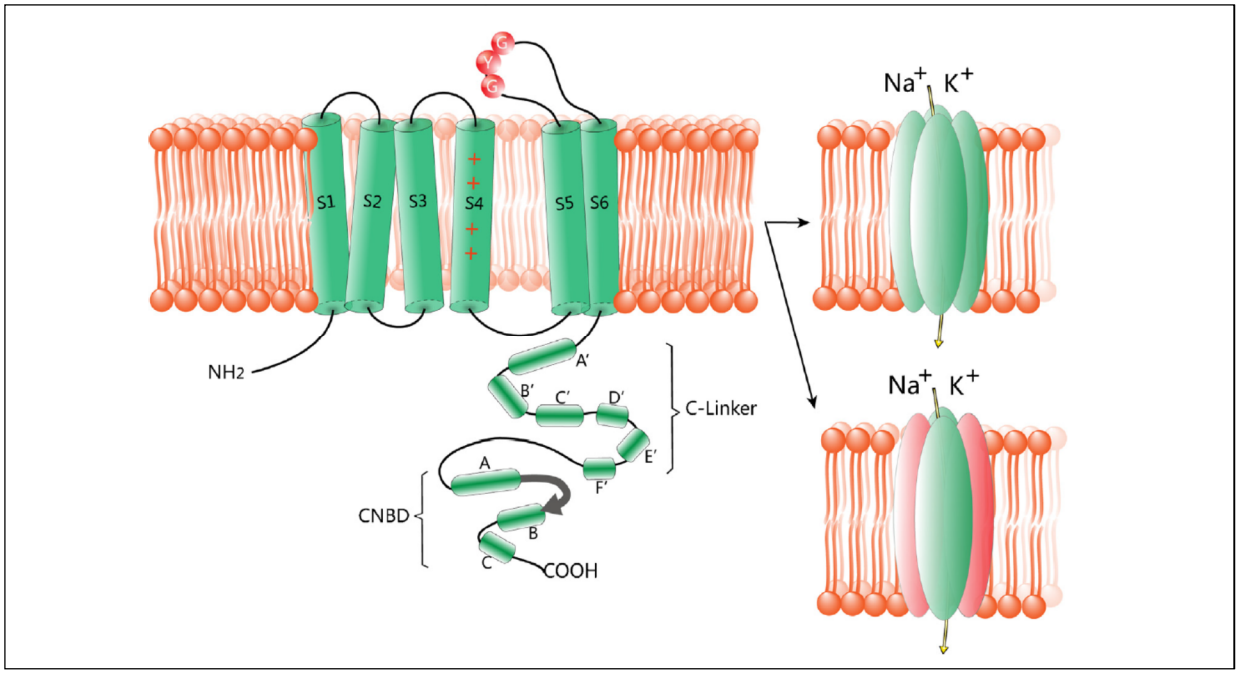

Figure 1. Molecular structure (From He *et al*., 2014).

2.2 Transmembrane Segments and Voltage Sensor

The transmembrane core of HCN channels is composed by six transmembrane helices (S1-S6), which include the voltage sensor (S4) and the ion-conducting pore loop between S5 and S6. In addition, this domain displays a highly conserved asparagine residue in the extracellular loop between S5, and the pore loop is glycosylated (N380 in murine HCN2). It was demonstrated that this post-translational channel modification is important and necessary for normal cell surface expression (Much *et al*., 2003). The S4 consists of four and eight basic residues (ariginines or lysines), regularly separated by two hydrophobic residues (Bezanilla, 2000; Vaca *et al*., 2000). The inward movement of S4 depends on the membrane potential that induces channel opening or closing (Bezanilla and Perozo, 2002; Männikkö *et al*., 2002). Furthermore, it was demonstrated that the loop connecting the S4 with the S5 segment is involved in the differential response to voltage change (Prole and Yellen, 2006; Decher *et al*., 2004). Experimental evidences led to hypothesize interactions between the S4-S5 linker and post-S6 region, precisely in the C-linker, which is linked with the CNBD, region sensitive to cyclic nucleotides that

modulate the activity of these channels (Prole and Yellen, 2006). Recently, in the HCN channel cloned from sea urchin sperm (spHCN) and in the HCN1 channel, it was described that the voltage-dependent activation can shift between two modes depending on the previous activity (Bruening-Wright and Larsson, 2007a, 2007b; Elinder *et al*., 2006; Männikkö *et al*., 2005). In mode I, gating charge movement and channel opening happen at very negative potentials, whereas in mode II, these processes occur at more positive potentials (Elinder *et al*., 2006). The transition from mode I to mode II is determined by the open state, while the shift from mode II to mode I generally is favored by the closed state. Thus, the kinetics of the channel activation and deactivation depends on these transition and this particular gating behavior is defined as *"voltage hysteresis"* (Elinder *et al*., 2006; Männikkö *et al*., 2005). Presumably, this complex mechanism is due to a slow conformational change of S4 (Bruening-Wright et al., 2007; Bruening-Wright and Larsson, 2007; Elinder *et al*., 2006; Männikkö *et al*., 2005).

2.3 Pore Loop and Selectivity Filter

It is well established that I_h is a mixed cation current of mainly K^+ and Na^+ ions. The permeability ratio of these two ions is comprised in a range from 3:1 to 5:1. Then, these channels have an high conductance for K^+ ions, but also allow an inward Na^+ current under physiological conditions (Pape, 1996). Moreover, this conductance is sensitive to external concentration of K^+. This mixed conductance is controlled by the pore loop sequence, which is highly similar to the selective K^+ channels (Yu and Catterall, 2004; Satoh and Yamada, 2002) and contains the glycine-tyrosine-glycine (GYG) motif that forms the selectivity filter for K^+ (Doyle *et al*., 1998; Zhou *et al*., 2001). Several experimental approaches have attempted to identify residues of the pore loop that confer this unique permeability of the HCN channels, but these attempts were unsuccessful (Much *et al*., 2003; Xue *et al*., 2002; Hu *et al*., 2002). Furthermore, HCN channels display a modest permeability for Ca^{2+}ions (Yu et al., 2007; 2004).

2.4 C-linker and Cyclic Nucleotide-Binding Domain

The activity of HCN channels is regulated by the binding of cyclic nucleotides, such as cAMP and cGMP, at the proximal portion of the cytosolic COOH-terminal (Zagotta *et al*., 2003). The COOH-terminal includes the CNBD and C-linker region that, together, can be named as "cAMP-sensing domain" (CSD). The CNBD is composed by 120 amino acids, while in the C-linker region were identified 80 amino acids. The C-linker, composed by six α-helices (A'-F'), connects the CNBD with the S6 segment of the channel core. The CNBD displays a highly conserved fold, including an initial α-helix, followed by an eight-stranded antiparallel β-roll (β1-β2), a short B-helix and a long C-helix (Wicks *et al*., 2011; Biel *et al*., 2009; Wahl-Schott and Biel, 2009; Zagotta *et al*., 2003). The binding pocket for cAMP is formed by a number of residues at the interface between the β-roll and C-helix (Flynn *et al*., 2007; Zhou and Siegelbaum, 2007; Zagotta *et al*., 2003). The residues of amino acids that interact with the ligand were characterized in the binding pocket of HCN2 channel: three amino acids are located in the β-roll (R591, T592, E582) and four in the C-helix (R632, R636, I636, K638), but only one of the C-helix controls the activity of cyclic nucleotide in channel opening (Zhou and Siegelbaum, 2007). It is not clear how these amino acids regulate cAMP selectivity, but it was proposed that this selectivity is due to the greater hydration energy of cAMP compared to cGMP (Zhou and Siegelbaum, 2008). With regards to allosteric changes of CNBD and C-linker during channel opening, a recent model showed that the C-linker in the absence of cAMP is in a "compact" conformation. This conformation leads to an inhibitory effect on channel opening, but the binding of cAMP to CNBD produces a conformational change in the C-helix that removes the inhibition of COOH-terminal (Craven and Zagotta,2006). Thus, the C-linker-CNBD is an auto-inhibitory domain since the absence of cAMP reduces open channel probability (Wang *et al*., 2007; Wainger *et al*., 2001).

The NH_2-terminal cytoplasmic region is different in all HCN isoforms (Santoro and Tibbs, 1999). However, Tran *et al*. (2002) found that a group of amino acids conserved in the different isoforms of HCN channels. These

residues are located near to S1 segment and seem to be involved in the subunit assembly of the channel.

2.5 Basic biophysical properties of I_h current

Extensive studies in the literature have described how the I_h currents mediated by HCN channels display important and distinct biophysical properties. Several properties appear to differ among the four HCN isoforms, such as channel gating, modulation by cAMP, ionic selectivity and pharmacological profile.

2.6 Channel Activation by Membrane Hyperpolarization

The first hallmark of HCN property is the channel activation by membrane hyperpolarization. I_h is evoked by hyperpolarizing steps to potentials more negative than -50 to -60 mV (Yanigihara and Irisawa, 1980). Furthermore, the voltage-dependent property varies among the various HCN channel isoforms. The half-maximal activation (midpoint) potentials ($V_{0.5}$) are -70 mV for HCN1, -95 mV for HCN2, -77 mV to -95 mV for HCN3, and -100 mV for HCN4 (Baruscotti *et al*., 2005; Stieber *et al*.,2005; Altomare *et al*., 2003). In addition, I_h are not characterized by voltage-dependent inactivation and two kinetic components are identified, a minor instantaneous current (I_{INS}) and a major slowly developing component (I_{SS}) (Macri and Accili, 2004; Macri *et al*., 2002; Proenza *et al*., 2002). The I_{INS} is fully activated within few ms, while the I_{SS} reaches its steady-state within a range of tens of ms to several s under fully activating conditions. Also, it is reported that I_{INS} is generated by cations that pass the pore loop, while the ionic nature of I_{SS} is not clear (Macri and Accili, 2004; Macri *et al*., 2002; Proenza *et al*., 2002). With regards to activation kinetic, in the literature it is been reported that most HCN channels activate quite slowly with time constants (τ_{act}) ranging between hundreds of ms to s (Edman *et al*., 1987), but also currents with faster activation are identified, such as in hippocampal CA1 pyramidal neurons (Maccaferri *et al*., 1993; Halliwell and Adams, 1982). In the various HCN isoforms, HCN1 results the fastest channel (τ_{act} : 25-300 ms) while HCN4 is the slowest channels (τ_{act} :

few hundred ms) (Ishii *et al*., 1999; Ludwing *et al*., 1999; Seifert *et al*., 1999, Santoro *et al*.,1998). HCN2 shows a τ_{act} ranging from 150 ms to 1 s (Ludwing *et al*., 1999, Stieber *et al*., 2005). The diversity of τ_{act} derives from several factors, including the diverse intrinsic activation properties of different HCN channel isoforms, cell type and experimental conditions (pH, temperature, patch configuration, ionic composition of solutions). Similarly to τ_{act}, also the half-maximal activation (midpoint) potentials ($V_{0.5}$) can vary due to a combination of intrinsic and extrinsic factors. The values are calculated with the Boltzmann functions (McCormick and Pape, 1990; Edman *et al*.,1987).

2.7 Modulation by Cyclic Nucleotides

Another peculiarity of HCN channels is their regulation by cyclic nucleotides (Di Francesco and Tortora, 1991). The mechanism by which cyclic nucleotides modulate HCN channel gating was demonstrated using current recordings in excised patches of sinoatrial node cells. Unlike other channels that are regulated by cAMP through protein kinase (PKA)-mediated serine or threonine phosphorylation (Park *et al*., 2008; Schulz *et al*., 2008; Yao *et al*., 2005), HCN channels are modulated also by cAMP-independent phosphorylation (DiFrancesco and Tortora, 1991), through the cAMP binding to CNBD on the COOH-terminal (Zagotta *et al*., 2003). Furthermore, there also are some reports of modulatory action due to PKA-mediated phosphorylation of HCN channels (Vargas and Lucero, 2002; Chang *et al*., 1991).Many experimental data confirmed that elevated cAMP intracellular levels facilitate activation of HCN channels through a shift of $V_{0.5}$ values to more positive values and increase and accelerate the channel gating kinetics (Chen *et al*., 2001; Santoro *et al*., 1998; Garratt *et al*., 1993; McCormick and Williamson, 1991; Tokimasa and Akasu, 1990; McCormick and Pape, 1990; Pape and McCormick, 1989). Wainger *et al*., (2001) demonstrated that the acceleration of the channel gating kinetics induced by cAMP can be attributed to the shift in voltage dependence of activation. The HCN2 and HCN4 voltage-dependent activation is shifted by cAMP to a positive direction, of about 17 mV, while for HCN1 it is reported a value about 2-4 mV. For the HCN3 isoform, it is reported

that, like HCN1, the cAMP responsiveness is weak (Chen *et al*., 2001; Santoro *et al*., 1998). To explain this important difference, a model of auto-inhibition was proposed. As mentioned above, the unliganded CSD limits the movement of the S6 segment leading to an inhibition of the HCN channels intrinsic activity. The binding of cAMP to CNBD abates this auto-inhibition and conduces the channels at the opening state; in this manner, it is possible the shift of the voltage activation. Because, the unlinganded CSD of HCN1 and HCN3 tetramer shows already several conformational changes, the binding of cAMP has a low effect on activation of these isoforms (Chow *et al*., 2012; Wicks *et al*., 2011; Craven and Zagotta , 2006; Wainger *et al*., 2001). Thus, HCN channel opening is faster and complete in presence of high cAMP concentrations, while low concentrations lead to an inhibition of channel activation through a shift of the activation curve to more hyperpolarized voltages (Jiang *et al*., 2008; Frere and Luthi, 2004; DiFrancesco and Mangoni, 1989; DiFrancesco and Tromba, 1988a; 1988b). Several studies have reported that the voltage activation of HCN channels also controls the cAMP binding affinity; thus, it seems that the ligand binding and voltage activation on HCN channels are mutually interdependent (Wu *et al*., 2011; Kush *et al*., 2010). In order to explain such interconnection between voltage and cyclic nucleotide-dependent gating, a kinetic model has been proposed (Altomare *et al*., 2001; Di Francesco, 1999). The classic Monod-Wyman-Changeux (MWC) model advanced for haemoglobin (Monod *et al*.,1965) was adapted to HCN channel behavior. The MWC model proposes that each subunit of the tetrameric channel is independently gated by voltage. When the voltage sensor shift from the inactivated state to the activated state, the probability of gating increases. The opening/closing reactions leads to allosteric changes and transitions in all subunits. This transition takes place if the channel is unliganded, partially liganded or fully liganded and is stabilized by cAMP binding. In addition, cAMP shows a higher affinity to open than closed channels (Altomare *et al.,* 2001; Wang *et al*., 2002).Like cAMP, cGMP and cCMP also modulate HCN channel activity. Recent evidences reported that cGMP and cCMP shift the voltage dependence of HCN2 and HCN4 channels activation to more positive values, in order to accelerate

activation and reduce speed of deactivation kinetics (Zong *et al*., 2012; Wilson and Garthwaite, 2010). Mechanistically, this regulation occurs through direct binding of cGMP and cCMP to CNBD, but these show a low binding affinity compared to cAMP (Zong *et al*., 2012; Wilson and Garthwaite, 2010).

2.8 Ion Selectivity

As reported in the section of Pore Loop and Selectivity filter, the current mediated by HCN channels is described such as a mixed cation current that is carried by both K^+ and Na^+ under normal physiological conditions (Ludwing *et al*., 1998; Santoro *et al*., 1998; Frace *et al*., 1992b); DiFrancesco, 1986; 1981). It is been reported that the ratio of the K^+ and Na^+ permeability of these channels goes from 3:1 to 5:1, while the reversal potential ranges from -25 mV and -40 mV (Ludwing *et al*., 1998; Ho *et al*., 1994; Maccaferri and McBain, 1996; Pape, 1994; Van Ginneken and Giles, 1991; McCormick and Pape, 1990; Mayer and Westbrook, 1983). The current amplitude and the ratio P_k:P_{Na} of I_h currents are controlled by the extracellular K^+ concentration (Ludwing *et al.*, 1998). In addition, extracellular concentration of K^+ induces an increase of current amplitude and decrease of the selectivity for K^+ over Na^+ (Frace *et al*., 1992a; Wollmuth and Hille, 1992). On the other hand, the reduction of extracellular Na^+ concentration alters the driving force (Pape, 1996; Di Francesco, 1981). The other feature of ion selectivity of I_h is that HCN channels do not conduct anions; however, they seem to be sensitive to external Cl^- (Frace *et al*., 1992b). Furthermore, recently it has been demonstrated a small but significant Ca^{2+} permeability in the HCN channels (Yu *et al*., 2007; 2004). With regard to the size of the single-channel conductance of I_h there is an ongoing controversy. Initially, the first experimental evidences reported a single-channel conductance in the range of 0-98 pS (DiFrancesco, 1986). Kole *et al*., (2006) confirmed this data, but different single-channel conductance have been reported for cloned HCN channels as well as for native cardiac and neuronal HCN (Michels *et al*., 2005; Simeone *et al*., 2005). This difference is not explained, but may derive from different experimental patch configurations.

2.9 Pharmacological Profile

One of the most interesting features of HCN channels is their pharmacological properties. I_h have been distinguished from other K^+ currents through its sensitivity to low concentrations of external Cs^+ ions (Ludwing *et al*., 1998; DiFrancesco, 1982; Fain *et al*., 1978). Millimolar concentrations of Cs^+ ions are able to block HCN channels. Furthermore, I_h results to be insensitive to millimolar concentrations of external Ba^{2+} and tetraethylammonium (TEA) (Ludwing *et al*., 1998). These currents are also insensitive to 4-aminopyridine, a blocker of the voltage-gated K^+ channels. A group of organic compounds that block the activity of HCN channel specifically, such as ZD-7288 (Shin *et al*., 2001; Gasparini and DiFrancesco, 1997), UL-FS49 (zatebradine) (DiFrancesco, 1994; Goethals *et al*., 1993), and S-16257 (ivabradine) (Bois *et al*., 1996) have been identified and characterized. Recently, it is been reported that loperamide, a μ-opioid-receptor agonist, is able to block I_h of dorsal root ganglion at concentrations between 5 and 10 μM (Vasilyev *et al*., 2007). Additionally, it is been demonstrated that the capsazepine, an inhibitor of the vanilloid-receptor (TRPV1), blocks HCN1 in a concentration-dependent manner (Gill *et al*., 2004).The I_h blockers are potentially useful in the therapy of epilepsies since Kitagawa *et al*., (2006) showed that the ZD-7288 reduced the generation of hippocampal epileptiform discharges in rabbit. Furthermore, several local and general anesthetics such as lidocaine, halothane and isoflurane inhibit at clinically relevant concentrations the activity of HCN channels, (Zhou *et al*., 2012; Budde *et al*., 2008; Chen *et al*., 2005). Finally, it is been shown that also the intravenous general anestetic propofol modulates I_h activity of thalamo-cortical neurons (Ying *et al*., 2006) and inhibits the fast-activating I_h in cortical neurons at a clinically relevant concentrations (Chen *et al*., 2005).

3. Regulation of HCN channel function

It is been reported that the physiological activity of HCN channels is regulated by the dynamic neurochemical environment. Thus, these channels

are targets of numerous cellular signals to regulate neuronal responses to different stimuli (Wang *et al*., 2011, 2007). Accordingly, HCN channels interact with many intracellular molecules, such as small molecules (protons, cAMP), protein kinases (p38-MAPK, PKC) and interacting proteins, and such interactions are able to regulate and modulate HCN channel gating, kinetics and surface expression (Lewis *et al*., 2010; Wahl-Schott and Biel, 2009).

3.1 Regulation by Acidic Lipids

Recent, experimental evidences reported that phosphatidylinositol 4,5-bisphosphate (PIP_2) can cause a hyperpolarizing shift of the HCN activation curve to positive a direction of about 20 mV (Pian *et al*., 2006; Zolles *et al*., 2006). Furthermore, the modulation by PIP_2 is independent of the presence of cyclic nucleotides and adjusts the voltage-dependent gating of HCN channels to a range relevant for the physiological function of I_h. PIP_2 is able to control also many ion transporters and ion channels, such as K^+ channels, TRP channels and Ca^{2+} channels (Suh and Hille, 2008; Gamper and Shapiro, 2007). The PIP_2 binding domain is located to the transmembrane region of channel subunits containing pore and voltage sensing domains (Flynn and Zagotta, 2011; Zolles *et al*., 2006). Moreover, a recent evidence described that other acidic lipids, such as phosphatidic acid (PA) and arachidonic acid (AA), which are products of diacylglycerol kinase and phospholipase A2 may also modulate HCN channels. Both PA and AA directly facilitate HCN channel gating through a shifting the $V_{0.5}$ to more positive values (Fogle *et al*., 2007). The modulation of HCN channel voltage-dependent gating by membrane acidic lipids depends on the stabilization of the open state of the pore loop or the activated state of the voltage sensor (Flynn and Zagotta, 2011). This regulation leads to a physiological effects to control neuronal excitability (Ying *et al*., 2011). It has been described that PIP_2 increased I_h in mouse thalamic intergeniculate leaflet (IGL) neurons, and this positive modulation involved an increase in low-threshold burst firing and spontaneous oscillations (Ying *et al*., 2011). Moreover, the reduction of PIP_2 down-regulated the excitability of OGL neurons. Because IGL neurons project to the supra-chiasmatic nucleus, the

modulation of HCN channel by PIP_2 seems to be involve in the control of sleep and circadian rhythms (Ying *et al*., 2011).

3.2 Regulation by Protons

Another mechanism that regulate the activity of HCN channels is represented by intracellular and extracellular concentrations of protons. Acidic intracellular (pH_i) is able to shift the voltage-dependent activation of HCN channels in the negative direction. Moreover, it leads to a reduction in the rate of activation and to the down-regulation of I_h. On the contrary, alkaline pH_i (pH_i 9.0) shifts the voltage-dependent activation in the positive direction and enhances I_h (Zong *et al.,* 2001; Munsch and Pape, 1999).The intracellular pH_i sensitivity depends on a histidine residue (His-321) located at the boundary between the voltage-sensing S4 helix and the cytoplasmic S4-S5 linker (Zong *et al*., 2001).Thus, the concentration of intracellular protons regulate the I_h in the brain, and this regulation represents a mechanism for the anti-epileptic action of acetazolamide, which is a carbonic anhydrase inhibitor. Inhibition of the carbonic anhydrase, in thalamo-cortical neurons, induces a reduction of intracellular concentrations of protons; consequently a shift of the voltage-dependent activation in the positive direction of HCN channels and an enhancement of I_h occur (Munsch and Pape, 1999). In addition, it is reported that acidic extracellular pH (pH_e < 5.0) modulates HCN channels expressed in rat taste cell (Stevens *et al*., 2001).

3.3 Regulation by Chloride ions

Even though HCN channels mediate a cationic current, it has been demonstrated that its conductance is regulated by the concentration of Cl^- (Frace *et al*., 1992b; Wahl-Schott *et al*., 2005). The effect of Cl^- on I_h is more pronounced in HCN2 and HCN4, since in these isoforms it is present an arginine residue in the pore region that is a molecular determinant of extracellular Cl^-sensitivity (Wahl-Schott *et al*., 2005). It was observed that a reduction of the amplitude of sino-atrial I_h mediated by HCN2 and HCN4 is involved in the generation of arrhythmias associated to hypochloremia.

Moreover, Cl^- is able to modulate HCN channels also from the intracellular site. It was reported that an increase of intracellular Cl^- abolished I_{INS} of I_h (Mistrik *et al*., 2006). In spite of these experimental evidence, the physiological relevance of this regulation is still not known.

3.4 Regulation by Protein Kinases

Several experimental evidences showed that different kinases are able to modulate HCN channel function and surface expression. For example, genistein, a tyrosine kinase inhibitor, reduces the amplitude of I_h and leads to a negative shift of the voltage-dependent activation (Yu *et al*., 2004; Wu and Cohen, 1997). The experiments performed on tyrosine kinases (Src) demonstrated that the Src SH3 domain interacts with a sequence of HCN channels, from the C'-helix of the C-linker to the COOH terminal of the CNBD. Such interaction might modulate HCN channels through the phosphorylation of channel proteins (Zong *et al*., 2005). The block of Src activity induces a shift in a negative direction of HCN2 and HCN4 activation and a reduction of the activation kinetics (Li et al., 2008; Arinsburg *et al.,* 2006). The effects of Src on HCN channel kinetics seem to be regulated through a highly conserved tyrosine residue (Tyr476 in HCN2, Tyr554 in HCN4) localized in the B'-helix of C-linker in all isoforms of these channels. When this tyrosine residue is replaced by a phenylalanine in either HCN2 and HCN4, the channels are not regulated by Src, but the Src inhibition on voltage-dependent activation is unmodified (Li *et al*., 2008; Zong *et al*., 2005). On the other hand, in the HCN4 channels the actions of Src on the gating depends on the phosphorylation of Tyr531 since the replacement of Tyr531 with phenylalanine abolished the effects of the Src inhibitor on HCN channel gating (Li *et al*., 2008). In addition, serine/threonine kinases, such as p38-mitogen-activated protein kinase (p38-MAPK), modulate HCN channels. Poolos and colleagues (2006) described that blockade of p38-MAPK in hippocampal pyramidal neurons caused a hyperpolarizing shift in HCN voltage-dependent activation, hyperpolarized the RPM and increased neuronal input resistance and temporal summation. Vice versa, the pharmacological activation of p38-MAPK significantly shifted the

voltage-dependent activation towards more positive potentials, depolarized the RPM and reduced input resistance and temporal summation. It is not clear if the regulation of HCN channels mediated by p38-MAPK depends on direct phosphorylation of the channels or by phosphorylation of another protein interacting with HCN channels. However, the blockade of other MAPKs had no effect on modulation of I_h (Poolos *et al*., 2006). The regulation of HCN channels by p38-MAPK is involved in pathological conditions. It is reported that the activity of p38-MAPK was reduced in vivo in the CA1 hippocampal region of epileptic animals, but when the activity of p38-MAPK was restored, the seizures induced in vitro hyperpolarized shift in HCN channel gating (Jung *et al*., 2010). Activation of protein kinase C (PKC) causes the down-regulation of HCN channel activity. Pharmacological activation with phorbol 12,13-diacetate (PDA), a specific PKC activator, reduced the amplitude of I_h and shifted the voltage-dependence activation towards negative potentials in dopaminergic neurons of the ventral tegmental area (VTA) (Inyushin *et al*., 2010; Liu *et al*., 2003). It is evident from several experimental evidences that PKC is an important downstream target of neurotransmitters to inhibit HCN channels because, for example, neurotensin modulates in negative manner the amplitude of I_h in the substantia nigra trough the activation of PKC (Cathala and Paupardin-Trisch, 1997). Additionally, serotonin down-regulates HCN channel activity in dopaminergic VTA neurons since the interactions with serotonin 5-HT_2 receptors activate PKC (Liu *et al*., 2003). Norepinephrine abolishes I_h in the pyramidal neurons of the prefrontal cortex and dopaminergic neurons of the VTA because the activation of α_2-adreniceptors stimulates PKC (Inyushin *et al*., 2010; Carr *et al*., 2007). In addition, orexin-A is able to inhibit I_h currents in pyramidal neurons of mouse pre-limbic cortex through the activation the PLC-PKC intracellular pathway of (Li *et al*., 2009). In CA1 pyramidal neurons, a down-regulation of HCN channels activity consequent to the activation of group 1 metabotropic glutamate receptors, which stimulate PKC has been reported (Brager and Johnston, 2007).As mentioned above for p38-MAPK, also for PKC the mechanism underlying the regulation of HCN channels remains elusive. Additional experimental studies

are required to characterize this mechanism of modulation, in order to unravel a direct or indirect interactions with HCN channel (Fogle *et al*., 2007). Recently, one study has described that cGMP-dependent protein kinase II (cGKII) has an inhibitory effect on the voltage-dependent gating of the HCN2 channel (Hammelmann *et al*., 2011). The cGKII phosphorylates HCN2-S641 residue and causes a hyperpolarizing shift of the voltage-dependent activation (Hammelmann *et al*., 2011). Data published suggest that calmodulin-dependent protein kinase II (CaMKII) might also control activity-dependent HCN channel trafficking and surface expression. In cultured hippocampus neurons, experiments of time-lapse demonstrated that GFP-fused HCN channels were localized in vesicle-like dendritic structures. The activation of either NMDA or AMPA-type ionotropic glutamate receptors induces Ca^{2+} influx and consequently activates CAMKII. This effect, in turn, induces an increase in HCN1 channel surface expression and an increase of I_h (Noam *et al*., 2010). Presently, also the mechanism underlying the modulation of HCN channel expression by Ca^{2+}/CaMKII has not been clarified yet.

3.5 Regulation by Interacting Proteins

Several proteins interacting with HCN channels have been identified, such as scaffold proteins, that interact with the COOH-terminal of these channels in the nervous system and are able to regulate channel voltage gating and kinetics. It was discovered that HCN1, but not HCN2 or HCN4, interacts with filamin A through a 22-amino acid sequence located downstream from CNBD (Gravante *et al*., 2004). Filamin A is a cytoplasmic scaffold protein that binds to actin and links transmembrane proteins, among which the K^+ channels Kv4.2 and Kir2.1. (Sampson *et al*., 2003; Petrecca *et al*., 2000). This interaction between HCN1 and filamin A significantly hyperpolarized the activation of HCN channels and modulated in a negative manner activation and deactivation kinetics (Gravante *et al*., 2004). Other scaffold proteins are able to interact with HCN channels; for example, tamalin binds to HCN2 through PDZ binding motif and the internal C-terminal tail of HCN2 subunits. Furthermore, the PDZ domain of S-Scam binds the CNBD and the CNBD-

downstream sequence of the COOH-terminal of HCN2 channels. However, also the Mint2 forms with HCN2 a protein macro-complex via the Mint2 munc18-interacting domain with the CNBD-downstream sequence of HCN2 (Kimura *et al*., 2004). Recently, several studies on TPR-containing Rab8b interacting protein (TRIP8b) demonstrated that this protein is able to influence HCN channel surface expression, localization, and function in the nervous system (Bankston *et al*., 2012; Zolles *et al*., 2009; Li *et al*., 2008). With regards to the binding domain, it is reported that TRIP8b interacts with a conserved tripeptide sequence in the CCOH-terminal of HCN channels (Santoro *et al*., 2004). In fact, TRIP8b co-localized with HCN1 channels in the distal dendrites of neocortical and hippocampal pyramidal neurons; in this last area it was demonstrated that the amplitude of I_h was diminished in TRIP8b knockout mice (Lewis *et al*., 2011) likely due to the reduction of HCN channels surface expression.

Additionally, it was shown that TRIP8b splice variants modulated the voltage gating of HCN channels in the negative direction (Santoro *et al*., 2009; Zolles *et al*., 2009). This effects depends on the binding between TRIP8b and the C-linker of CNBD of HCN1 channels (Han *et al*., 2011; Noam et al., 2010). Given that the CNBD represents the binding site for cAMP, an increase of intracellular concentration of cAMP blocks the binding between TRIP8b and the CNBD (Han *et al*., 2011). Thus, TRIP8b and cAMP are competitive ligands for CNBD domain of HCN channels and are able to modulate HCN channel gating, kinetics and trafficking.

3.6 Regulation by extracellular neurotransmitters

As mentioned above, HCN channels are regulated by several intracellular molecules, but also changes in the extracellular signals modulate the activity and function of these channels. Among the various extracellular signals, the inhibitory effect of acetylcholine (ACh) on currents mediated by HCN-channels was reported. The group of Heys (2010) described that the activation of muscarinic Ach receptors (mAChRs) induced a reduction of the voltage sag amplitude and slowed the time constant of the I_h in stellate neurons from layer

II of medial enthorinal cortex (EC). The changes of these parameters caused an increase of membrane resistance, associated with a reduction of the resonance frequency and strength (Heys *et al*., 2010). Various types of mAChRs, have been described and the m_2AChRs are coupled to G_i protein, which is able to reduce intracellular cAMP concentrations (Wilson *et al*., 2004). Then, it is possible to predict that the negative modulation of I_h via AChRs depends on m_2AChRs, since these receptors inhibit intracellular cAMP signalling. On the contrary, it is reported that Ach can also up-regulate currents mediated by HCN channels (Pian *et al*., 2007). This data was demonstrated in HCN1 and HCN2 channels expressed in Xenopus oocytes. In this experimental model, Ach activate m_1AChRs which are coupled to $G_{q/11}$ and PLC and induced a positive shift in activation, and blocked the deactivation kinetics of channels. This modulation is cAMP-independent because PLC increases local concentrations of PIP_2 which modulates the HCN channels as mentioned previously. HCN channels represents important targets of monoaminergic neurotransmitters, including norepinephrine (NE), serotonin and dopamine. NE inhibits HCN channels and increases prefrontal cortical networks functional connectivity, and subsequently influences working memory (WM) (Barth *et al*., 2008; Carr *et al*., 2007; Wang et al., 2007). NE is able to modulate in negative manner these channels, because, through α_{2A}-ARs, wich are coupled to G_i, it inhibits adenylyl cyclase (AC), which leads to a decrease of cAMP levels, and in turn suppress the channel activation (Wang *et al*., 2007). Indeed, Carr et colleagues (2007) confirmed that α_{2A}-AR activation suppressed the function of HCN channels, but independently from the reduction of cAMP in PFC pyramidal neurons of rat. It was described that this modulation was mediated through the PLC-PKC signalling pathway. Moreover, suppression of HCN channels function leads to an enhancement in dendritic temporal integration (Carr *et al*., 2007). It was also demonstrated that the α_{2A}-ARs modify Ca^{2+} transient through HCN channel inhibition in PFC slice preparations (Barth *et al*., 2008). The regulation of HCN channels via α_{2A}-ARs activation was found also in dopaminergic neurons of the rat VTA (Inyushin et al., 2010). Indeed, the application of α_{2A}-ARs agonists reduces the I_h amplitude and slows

the I_h activation rate. The actions of serotonin on I_h have been extensively studied in different populations of neurons. For example, in a guinea pig and cat medial and lateral geniculate thalamus relay neurons, a positive modulation of HCN channel activity was reported (Garratt *et al*., 1993; McCormick and Pape, 1990a; Bobker and Williams, 1989). This positive modulation is dependent on the stimulation of AC, leading to an increase of cAMP levels through the activation of 5-HT_1Rs. On the other hand, 5-HT_2Rs are coupled to G_q, which activates the p38-MAPK signalling pathway (Ramanjaneya *et al*., 2008; Yamauchi *et al*., 1997). p38-MAPK increases the activity of HCN channel in motoneurons of the facial motor nucleus. In addition, the inhibitory action of serotonin on HCN channel has also been described in cerebellar PCs and dopaminergic neurons of VTA (Li *et al*., 1993). Experimental data indicated that serotonin reduced the slope of the conductance-hyperpolarized potential curve via 5-$HT_{2/IC}$Rs activation.

With regards to the role of serotonin on HCN channel in rat brain VTA slices, Liu *et al*. (2003) described that serotonin is able to reduce the amplitude of the currents mediated by HCN in a concentration dependent manner. Moreover, serotonin shifted the voltage dependence of I_h activation to negative direction and reduced the maximal I_h conductance, suggesting that serotonin can modulate also HCN channels expression. The modulatory mechanism involves the PLC-PKC signalling pathway, and was observed the DA inhibition of the firing rate of dopaminergic VTA neurons subsequently HCN inhibition (Liu *et al*., 2003).Similarly to NE and serotonin, dopamine (DA) decreases the excitability of layer V EC pyramidal neurons via HCN channel (Rosenkranz and Johnston, 2006). This effect is mediated by the activation of D_1Rs, that consequently activate AC and increase cAMP concentrations. In EC pyramidal neurons, most HCN1 channels are located on dendrites and the effect of DA on HCN channels modulates dendritic synaptic integration. This modulatory effect is reported also in PFC pyramidal neurons, where D_1R stimulation leads to an increase of spatial turning through the suppression of responses to non-preferred directions (Vijayraghavan *et al*., 2007; Gibbs and Esposito, 2005). With regards to the molecular mechanism, it is possible, but

further studies are necessary to confirm, that DA up-regulates the intracellular cAMP levels via D_1Rs activation, and consequently increases the activity of HCN channels and reduces the membrane resistance. Recently, it was found that the glutamate can regulate the dentritic membrane expression of HCN1 channels in the hippocampus (Wollmuth and Sobolevsky, 2004). Physiological glutamatergic transmission is involved in the control of the subcellular distribution pattern of HCN channels (Shin and Chetkovich, 2007). Moreover, the increase of glutamate release might upregulate the surface expression of HCN channels in dendrites. It has been described that LTP (long-term potentiation) mediated by NMDA receptors (induced with a theta-burst stimulation protocol) increased HCN channel surface expression in CA1 pyramidal neurons (Fan *et al*., 2005). This data was later confirmed by the study of Noam et al. (2010), where it was reported that the activation of NMDA or AMPA-type ionotropic glutamate receptors was accompanied by the increased surface expression of HCN1 channels. This mechanism depends on the Ca^{2+} influx through ionotropic channels and activation of Ca^{2+}/CAMKII (Noam *et al*., 2010; Shin and Chetkovich, 2007). In addition, it was found that also long-term depression (LTD) is implicated in the surface expression of HCN channels. After LTD at the Schaffer collateral-CA1 pyramidal neuron synapses, a down-regulation of dendritic HCN channels was observed, because the voltage sag and sub-threshold resonance frequency were decreased. In this case, the effects of glutamate seem to be mediate by group 1 mGluRs, which activate PLC-PKC signalling pathway (Brager and Johnston, 2007; Honnuraiah and Narayanan, 2013). Recently, several research groups have demonstrated that ATP and adenosine modulate the activity of HCN channels in the nervous system and control neuronal excitability. Indeed, adenosine regulated excitability in rat EC stellate neurons and mouse pyramidal neurons of PFC through inhibition of HCN channels (Li *et al*., 2011; Yan Jie *et al*., 2008). This regulatory action is mediated by activation of adenosine A_1 receptors, which via G_i binding which in turn inhibit AC and reduce the cAMP levels. ATP is reported to increase the activation of HCN channels via $P2Y_1$ receptors in mesencephalic trigeminal neurons (Huang *et al*., 2010). HCN channels are the

targets of nitric oxide (NO) in deep cerebellar neurons, trigeminal motoneurons, thalamo-cortical neurons and hypoglossal motoneurons (Wenker *et al*., 2012; Wilson and Garthwaite, 2010; Abudara *et al*., 2002). NO is able to increase in a reversible manner the amplitude of I_h because it depolarizes the voltage dependent activation (Wenker *et al*., 2012; Wilson and Garthwaite, 2010). NO modulates the HCN channel voltage gating via intracellular cGMP formation, and, as mentioned above (in the section Modulation by cyclic nucleotides), cGMP directly binds to HCN channels and facilitates channel opening. Finally, in the group of extracellular neurotransmitters that regulated the HCN channels activity it should also be mentioned the neuropeptides, including orexins, opioid peptides, neuropeptide Y (NPY), neurotensin (NT) and substance P (SP). Neuropeptides regulate in a negative manner the I_h through the inhibition of AC or activation of the PLC-PKC signalling pathway.

A role of orexin-A has been demonstrated in prelimbic cortex, where it abolished I_h, decreased voltage sag and shifted to more negative values the HCN channel voltage-dependence (Li *et al*., 2009). The effects of the endogenous opioid peptides on HCN channels has been studied in the hippocampus network, where the reduction of excitability of interneurons depends on the inhibition of I_h (Svoboda and Lupica, 1998). Giesbrecht *et al*. (2010) reported the NYP-induced inhibition of I_h through the Y_1 receptor in basolateral amygdala pyramidal cells. The reduction of I_h amplitude in the rat substantia nigra pars compacta seems to be mediated by NT, which activates PKC pathways (Cathala and Paurpadin-Tritsch, 1997). Finally, SP arrests the activation of HCN channels by shifiting the I_h activation curve in vagal sensory neurons (Jafri and Weinreich, 1998).

4. Physiological roles of HCN channels in neurons

HCN channels are implicated in many physiological functions that range from cellular to behavioral levels and are dependent on channel subtypes, subcellular distribution (soma and proximal dendrites, distal dendrites, axonal terminals), and intracellular/extracellular environment. In the nervous system, I_h are involved in the regulation of resting membrane potential and membrane

input resistance, normalization of synaptic inputs and selective filtering for coincident inputs. Moreover, these functions are classified into three groups: excitatory, inhibitory and modulatory functions.

4.1 HCN channels in controlling resting membrane potential

Generally, it is reported that HCN channels are activated at membrane potentials more negative than to -50 mV, but a small fraction of these channels is tonically activate at RPM. This tonic activation contributes to the stabilization of RPM through lowering the membrane resistance (R_m); R_m is defined as the relationship between voltage change and the current injected (Ludwing *et al*., 2003; Nolan *et al*., 2007, 2004, 2003; Maccaferri *et al*., 1993). Futhermore, it has been described that tonic opening of HCN channels seems to behave as a slow "voltage clamp mechanism" since these currents try to stabilize the membrane potential through depolarizing or hyperpolarizing inputs (Hu *et al*., 2002). Indeed, when the membrane is hyperpolarized and more HCN channels are activated, a depolarizing inward current is generated that takes back the membrane potential to the initial potential. This regulatory effect is called "depolarizing voltage sag" (Robinson *et al*., 2003; Pape, 1996). On the contrary, during depolarization of membrane, HCN channels are deactivated and consequently reduce the inward current and restore the membrane potential; this partial hyperpolarization is called "hyperpolarizing voltage sag" (Robinson *et al*., 2003; Pape, 1996). This important modulatory property depends on the relationship between the activation curve and the reversal potential of I_h, because the values of the reversal potential declines to the values of its activation curve (Nolan *et al*., 2007; Lupica *et al*., 2001). HCN channels expressed in dendrites, when are activated tonically, modulate the passive propagation of excitatory postsynaptic potentials (EPSPs) (Magee, 1998, 1999, 2000). This modulatory function will be discussed in the next section " HCN channels and dendritic integration".

4.2 HCN channels in controlling resonance and neuronal oscillation

A neuron is able to respond to inputs at a preferred frequency, and this property is called “resonance” (Hutcheon and Yarom, 2000). Experimentally, the resonance can be evoked through application of a current stimulus with linear increasing frequency. This protocol is known as “ZAP current”. The resonance depends on properties of a low-pass filter and of a high-pass filter (Buzsaki and Draguhn, 2004; Hutcheon and Yarom, 2000; Hutcheon *et al*., 1996), and the HCN channels seem to possess features that allow them to control these properties. Indeed, at low frequency HCN channels have a sufficient amount of time to activate and control changes in membrane potential (high-pass filter), while during high frequencies the channels have not time to open; however, in this case the neuron is responsive for fast trains of spikes. The involvement of I_h in the generation of membrane resonance has been described for several neurons such as neocortical pyramidal neurons (Ulrich, 2002), subicular pyramidal neurons (Wang *et al*., 2006), and in neurons from the sensorimotor cortex of juvenile rats (Hutcheon *et al*., 1996). The currents mediated by HCN channels are described also such as “pacemaker currents” because the activation of these channels in the soma induces an inward current which depolarizes RMP; consequently, HCN channels initiate spontaneous neuronal firing and modulate subthreshold membrane potential oscillations (Jahnsen and Llinas, 1984; Llinas and Jahnsen, 1982; McCormick and Pape, 1990b). The oscillations derive from synchronized activity of neurons and seem to be involved in information processing by neuronal networks. The role of HCN channels in oscillations has been well demonstrated in stellate neurons of medial EC, where there is an interplay between I_h and low-threshold persistent Na^+ current (I_{NaP}). During an oscillation, HCN channels are activated and the membrane is depolarized; I_{NaP} is activated and, in turn, it increases the rate of membrane depolarization to induce the HCN channel deactivation. The decrease of HCN channel activity reduces the depolarization progress and induces the repolarizing of the oscillation, and subsequently the inactivation of I_{NaP}. Inactivation of I_{NaP} causes the hyperpolarization of the membrane, which leads to activation of HCN

channels and a new oscillatory cycle (Fransen *et al*., 2004; Dickson *et al*., 2000; Alonso and Llinas, 1989).

4.3 HCN channels activity in presynaptic terminals

HCN channels expressed in presynaptic fibers play different roles on synaptic transmission. Experimental studies reported that presynaptic HCN channels in mouse EC and globus pallidus are able to inhibit glutamatergic and GABAergic transmission, because the application of ZD-7288, selective blocker of HCN, increases the frequency of miniature postsynaptic currents (PSCs) (Huang *et al*., 2011; Boyes *et al*., 2007). The mechanisms through which the HCN channels control neurotransmitter release depends on depolarization of the membrane and inactivation of voltage-dependent Ca^{2+} channels. Conversely, it has been shown that presynaptic I_h enhances synaptic transmission in the hippocampus, cerebellar cortex, basolateral amygdala and spinal dorsal horn (Park *et al*., 2011, Aponte *et al*., 2006; Lupica *et al*., 2001; Southan *et al*., 2000). These data suggest that the role of presynaptic HCN channels is determined by the neuron type and its location. In hippocampal CA1 region, cerebellum and basolateral amygdala, ZD-7288 reduces the frequency of spontaneous action potential-dependent IPSCs, but not action potential-independent miniature IPSCs (Park *et al*., 2011; Lupica *et al*., 2001; Southan *et al*., 2000). In spinal dorsal horn and dentate gyrus, it has been observed a reduction miniature IPSCs frequency (Takasu *et al*., 2010; Aponte *et al*., 2006). These data confirm that the effects of HCN channels depend on subcellular location, because in the first case HCN channels are located in remote regions from the release machinery and the blockade of channels hyperpolarizes the axonal membrane increasing the threshold for action potential generation (Park *et al*., 2011). In the dentate gyrus and spinal dorsal horn, HCN channels seem to be located in proximity to the release machinery and their activation depolarize the membrane and activate P/Q-type Ca^{2+} channels, which in turn facilitate neurotransmitter release (Aponte *et al*., 2006; Awatramani *et al*., 2005).It has also been described that presynaptic HCN channels influenced the short and long-term synaptic plasticity. Dietrich

et al. (1997) observed that short-term depression evoked by field EPSPs in medial perforant path-granule cell synapses. Long term potentiation (LTP) in CA3 pyramidal neurons seems to be mediated by presynaptic HCN channels (*Mellor* et al., 2002) since repetitive stimulation induced a Ca^{2+}- and HCN-dependent long-lasting depolarization, which in turn enhances transmitter release. On the contrary, Chevaleyre and Castillo (2002) demonstrated that mossy fiber LTP is independent of HCN channels.

4.4 HCN channels and dendritic integration

Many studies reported that the I_h currents mediated by HCN channels dampens dendritic temporal integration during a burst of EPSPs. Dendritic integration is an important and crucial process for signal processing in most neurons, and has been characterised in CA1 hippocampal and neocortical pyramidal neurons (Magee, 2000). This process is a crucial one because single EPSPs are too small in order to bridge the gap between the RPM and the action potential threshold in the initial segment. Thus, the soma receives multiple synaptic inputs which must to be integrated to produce action potential firing. Since this process is fundamental for information processing, dendritic integration must be highly controlled both in space and time. Many experimental evidences have been reported that HCN channels play an important role in regulating dendritic integration (Magee, 1998, 1999, 2000; Williams and Stuart, 2000; Tsay *et al*., 2007). The EPSP summation in time depends on kinetic filtering by passive cable properties of the dendrites. Indeed, dendritic filtering works to slow down the time course of EPSPs resulting in somatic EPSPs that rise and decay more slowly if they are generated in distal dendrites with respect to proximal dendrites. As a consequence of this filtering, one would observe that repetitive EPSPs arising from more distal synapses should summate at the soma in greater manner and over a longer time course than EPSPs generated in proximal dendrites. However, this hypothetic consequence was not observed, for example, in CA1 pyramidal neurons and neocortical layer 5 pyramidal cells (Magee, 1998, 1999, 2000; Williams and Stuart, 2000). This discordance is due to a different

gradient of HCN channel density, which increases by more than six-fold with distance from the soma (Magee, 1998, 1999, 2000; Williams and Stuart, 2000; Lorincz *et al*., 2002). This gradient of HCN channels counterbalances dendritic filtering by lowering R_m Furthermore, these channels are deactivated during the rising phase of an EPSP and hyperpolarize the plasma membrane accelerating the decay of each EPSP. The distal EPSPs are shorter and decay faster. This mechanism leads to a temporal summation that is more attenuated for distal than for proximal inputs; thus, the temporal summation of all inputs reach the soma at the same time. Consequently, HCN channels behave as spatial filters that preferentially dampen distal input. These results indicated that HCN channels are involved in dendritic integration and their role depends on an increasing somato-dendritic HCN gradient.

5. The Hippocampal Formation

From the first brain investigations, the hippocampal formation has fascinated members of the Alexandrian School of Medicine because this structure showed an elegant, curved morphology, which when seen with its contralateral half, resembles strongly the coil of a ram. For this reason, this brain structure was called *"cornu ammonis"*(CA), Latin for horn of the ram. In 1564, the Italian anatomist Giulio Cesare Aranzi coined the name *" hippocampus"* because it resembled the tropical fish. The hippocampal formation differs from other cortical areas and is part of a system that controls many body functions, the limbic system, located in medial temporal lobe of brain. This structure plays important roles in several neurophysiologic processes, in particular it is involved in information encoding, learning and memory. The hippocampal formation is composed of six regions: the hippocampus proper, dentate gyrus, subiculum, presubiculum, parasubiculum, and enthorhinal cortex. Sometime, the entorhinal cortex, presubiculum, and parasubiculum are called "parahippocampal region". Generally, the term hippocampus is referred to the hippocampus proper and the dentate gyrus collectively. The hippocampus proper is characterized by three subdivisions: CA3, CA2 and CA1, where CA is the acronym of *"cornu ammonis"* (Lorente de

Nó, 1934). The cornu ammonis is constituted by glutamatergic pyramidal cells which occupy a single packed layer, while the dentate gyrus contains granule cells that, in the human brain, are about 18 million. Moreover, there is a variety of interneurons in the different structures of the hippocampal formation.The hippocampal formation communicates with other brain structures through the entorhinal cortex and fornix. The entorhinal cortex is described such as the neocortical gate-keeper, because it sends projections into the structure, and it receives its output and communicates with other neocortical structures. The fornix connects the hippocampal formation to various subcortical structures and sends outputs to the prefrontal cortex. Through this and other pathways, the hippocampal formation receives modulatory inputs from dopaminergic, norepinephrine, serotonin and acetylcholine systems.

The connectivity of the hippocampal formation is characterized by four important features:

1) information that arrives at the hippocampal formation has been highly processed by various neocortical pathways;
2) the connections between its regions are unidirectional;
3) the projections that depart from the dentate gyrus and go to CA3 are dense;
4) CA3 pyramidal neurons are highly self-connected.

The information follows four fiber pathways: two emerge from the entorhinal cortex and the first is represented by the perforant path; such input arises from its layer II and projects to the dentate and CA2/CA3. The second is called temporo-ammonic pathway, from layer III to CA1. In the mossy fiber pathway input arises from dentate gyrus and projects exclusively to CA3 pyramidal neurons. These synapses are defined such as “ detonator synapses” because of their strength in driving CA3 pyramidal neuron activity. The CA3 neurons project to CA1 neurons through the Schaffer collaterals pathway, and to many other CA3 neurons via recurrent collaterals. CA1 pyramidal neurons reach the

subiculum and the deep layer neurons of the entorhinal cortex and through the fornix to other structures. In addition, the subiculum, presubiculum, and parasubiculum show connections with the entorhinal cortex and with other structures.

5.1 CA3 pyramidal neurons

Neurons of the hippocampal formation have been studied extensively, and among the CA3 pyramidal neurons have received particular attention because of the unique functional specializations constituted by the mossy fiber inputs and the extensive collaterals between CA3 neurons, which formed an interconnected and excitable network, such as described above. With regards to the morphological structure, pyramidal neurons of CA3 are similar to CA1 pyramidal neurons: they consist of pyramid-shaped soma that gives rise to apical and basal dendritic trees, but they differ in the apical tree that bifurcates closer to the soma. Amaral *et al*. (1990) distinguished the CA3 pyramidal neurons in smallest and largest cells; the first group is located in the limbs of the dentate gyrus, while the second is found in the distal portion of the CA3 subfield. The dendritic arborizations of CA3 pyramidal neurons shows four main features:

1) basal arbor extends throughout the stratum oriens;
2) a short apical trunk in the stratum lucidum branches into two or more secondary trunks;
3) oblique apical dendrites in the stratum radiatum;
4) an apical tuft extends into the stratum lacunosum-moleculare.

The total length of the apical and basal dendritic arbors is ranging 9.3-15.8 mm in rat hippocampus (Henze *et al*., 1996; Ishizuka *et al*., 1995). Other hallmark feature of CA3 pyramidal neurons is bursting, that results to be more prominent with respect to CA1 region (Wong and Prince, 1978; Kandel and Spencer, 1961; Spencer and Kandel, 1961). Each burst is an “ all or nothing” event lasting 30 to 50 ms with the frequency of action potentials of 100-300 Hz (Traub and Miles, 1991; Wong and Prince, 1981). However, not all CA3 cells

respond to supra-threshold stimuli with intrinsic bursts. It had been documented that intrinsic bursting involved cells located in the distal portion of the CA3 subfield. Also, CA3 cells located close to the stratum pyramidal/oriens border show burst generation with respect to cells located closer to the stratum radiatum (Bilkey and Schwartzkroin, 1990). This difference is due to morphological distinction between these two cell groups in the length of the initial portion of their apical dendrite. Spruston and Johnston (1992) characterized the input resistance of CA3 pyramidal neurons at resting membrane potentials (-60/-70 mV) and with HCN channels blocked. The value of input resistance is about 160 MΩ and this results to be higher than the values found in CA1 pyramidal neurons. This high value for CA3 cells would be explained by the hypothesis that their dendrites may be electrically compact for the steady-state condition. Another hallmark of these neurons is the expression of HCN channels, as described in the first section of Introduction. In CA3 pyramidal neurons, HCN channels mainly HCN2 are distributed in dendrites (Santoro *et al.*, 2000), and are involved in synaptic integration (Makara and Magee, 2013), and, in thin dendrites of these cells, they integrate synchronous synaptic input in a highly supralinear fashion.

SPECIFIC AIMS AND EXPERIMENTAL DESIGN

Gaining of a deeper knowledge of the CNS effects induced by EtOH and the mechanism of its action in different brain areas has received, in the last decades, a great attention in many branches of the neuroscience research. EtOH exerts its action on a large variety of membrane proteins of the CNS that are involved in synaptic transmission (Lovinger and Roberto, 2013; Siggins *et al*., 2005; Harris 1999; Peoples and Weight 1995; Li *et al*.,1994; Slater *et al*., 1993). Different targets protein that are sensitive to EtOH at pharmacologically relevant concentrations have been identified. Several EtOH effects are directed at the modulation of ligand-gated as well as voltage-gated ion channels, but also protein involved in various intracellular signalling pathways (Kelm *et al*., 2008). One of the main overall effect induced by EtOH in the CNS is the alteration of the strict balance between inhibitory and excitatory systems (Valenzuela, 1997).

One of the first evidences, dating back to 1980s, suggests that the acute behavioural and cognitive effects of EtOH were mediated by an increase of GABAergic inhibition (Nestoros, 1980; Davidoff, 1973). Subsequent behavioral, neurochemical and electrophysiological studies provided evidence in support for this theory. Substantial data define the $GABA_A$ receptor as an important target for the EtOH actions. EtOH could directly and indirectly increase the function of $GABA_A$ receptors (Sanna *et al*., 2004; Aguayo *et al*., 2002; Allan *et al*., 1987; Suzdak *et al*., 1988). Acute EtOH exposure is able to increase the amplitude of $GABA_A$- and GlyR-mediated IPSCs (Sebe *et al*., 2003; Ziskind-Conhaim *et al*., 2003). Recent studies performed in brain slices and isolated neurons reported that spontaneous and miniature GABAergic IPSC frequency is enhanced at relevant EtOH concentrations suggesting a possible effect also at presynaptic terminals (Ariwodola and Weiner 2004; Zhu and Lovinger 2004; Theile *et al*., 2008; Roberto *et al*., 2003; Sanna *et al*., 2004; Kelm *et al*., 2007).Even though there is a wide body of literature reporting the effects of EtOH at GABAergic synapses, the mechanisms underlying EtOH presynaptic effect is still not completely clearified. Experiments performed in the VTA and cerebellum report that EtOH can increase calcium concentrations in the presynaptic terminal (Theile *et al*., 2009; Kelm *et al*., 2007). Furthermore, it

has been examined the intracellular pathways involved in this potentiating effects of EtOH. For example, increasing in GABA release in cerebellar Purkinje neurons is abolished when protein kinase A (PKA) (Kelm *et al*., 2008), and phospholipase C as well (PKC) are inhibited (Kelm *et al*., 2010).

Thus, EtOH potentiation of GABAergic synapses depends on both pre and postsynaptic actions. With regards to ionotropic glutamate receptors (iGLURs), it has been reported that EtOH is able to reduce the synaptic responses mediated by glutamate (Wang et al., 2007; Roberto et al., 2004b; Morrisett and Swartzwelder 1993; Lovinger *et al*., 1989). In addition, low EtOH concentrations exert inhibitory effects on the function of AMPARs (Akinshola *et al*., 2003; Wirkner *et al*., 2000; Dildy-Mayfield and Harris 1992), as well as KARs (Lack *et al*., 2008; Costa *et al*., 2000). EtOH inhibition of iGLURs results in dampening neuronal excitability by reducing excitatory synaptic drive. Okamoto *et al*. (2006) reported a potential new molecule target involved in the central effect of EtOH, the HCN channels, which mediate an inward cation current, defined as I_h. This study demonstrated that acute EtOH exposure induced a down regulation of HCN channels expression and function in dopaminergic neurons of the mesolimbic system. Furthermore, another study performed in hippocampal interneurons of male rats demonstrated that low EtOH concentrations are able to enhance the action potential firing rate via modulation of HCN channels (Yan *et al*., 2009). The involvement of HCN channels in the effects of EtOH was confirmed by Tateno and Robinson (2011) who showed that EtOH modulates I_h leading to a modified capacity of postsynaptic integration of GABAergic inhibitory potentials in DA neurons Since HCN channels are expressed in hippocampal neuron at great level, in this study my main objective has been to investigate and characterize the action of EtOH on HCN channels in CA3 pyramidal neurons of male rats. First, I have characterized the electrophysiological properties of I_h in CA3 pyramidal neurons present in rat hippocampal slices by using different electrophysiological protocols, including voltage- and current-clamp analysis.

Pharmacologically concentrations (10 to 80 mM) of EtOH have been used in order to study its effects on HCN channel activity, and I have tried to

elucidate the possible direct interaction with these membrane proteins and characterise some intracellular molecular mechanism trough which EtOH acts on HCN channels. Beacause these channels are modulated by different endogenous as well as synthetic compounds through different mechanism of actions, I have compared the observed EtOH effect with those of γ-hydroxybutirric acid (GHB), butanol, and dopamine (DA), all perfused in the hippocampal slices. Activation of D_1R_s by DA, increasing cAMP intracellular levels, modulates the HCN channel opening. Moreover, this indirect modulation of HCN channels by DA regulates physiological neuronal functions, such as excitability and synaptic integration *(*Rosenkranz and Johnston*,* 2006*;* Vijayraghavan *et al*., 2007; Gibbs and Esposito, 2005). In a different set of experiments I have evaluated in greater details the possible involvement of adenylyl cyclase/cAMP/ PKA intracellular pathway in the modulation of HCN channels by acute EtOH perfusion, in order to examine their contribution in the effects of EtOH. Since it has been reported that HCN channels are expressed in higher amount in distal dendrites, as opposed to proximal dendrites and soma, and are thus capable of modulating synaptic integration, I have tried also to characterize EtOH actions on this postsynaptic mechansim. Moreover, I have examined the possible effect of EtOH on neuronal excitability in relation with its activity of HCN channels, by using carbachol exposure to stimulate action potential firing (Jochems and Yoshida, 2013).

Furthermore, because it was shown that the subcellular distribution of HCN channels is subjected to developmental regulation (Brewster *et al*., 2007), I have evaluated HCN channels activity in animals exposed to early-life stress (post-weaning social isolation).

Finally, I have also evaluated the function of HCN channels in ratsthat were exposed chronically to EtOH for 21 days and rats undergoing withdrawal. The results that I have obtained reveal that EtOH modulates I_h currents mediated by HCN channels in a biphasic manner, with low concentrations (20 mM) increasing I_h amplitude, and high concentrations (60-80 mM) markedly inhibiting these currents. Although a direct interaction of EtOH with HCN cannot be ruled out, I found that the biphasic action involves adenylyl

cyclase/cAMP/PKA intracellular pathway, since inhibitors of adenylyl cyclase (DDA) and PKA (H89) prevents the effects of EtOH.

The data obtained from my work in CA3 pyramidal cells, together with those of others showing modulatory effects of EtOH on HCN expressed in other neurons populations (Okamoto et al., 2006; Yan et al., 2009)., indicate that these channels represent sensitive molecular targets of EtOH, and thus they may contribute in mediating some of the CNS actions of this drug.

MATERIALS AND METHODS

The present study was carried out in accordance with the current Italian legislation [D.L. 116, 1992], which allows experimentation on laboratory animals only after submission and approval of a research project to the Independent Committee of Bioethics of the University of Animal Testing (Cagliari, Italy) and to the Ministry of Health (Rome, Italy), and in strict accordance with the European Council directives on the matter [n.2007/526/CE]. All possible efforts were made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

1. Animals

Male Sprague Dawley CD young-adult (P30-60)rats (Charles River, Como, Italy) were bred in our animal facility and maintained under an artificial 12 h light, 12 h dark cycle (light on from 08:00 to 20:00 hours), at constant temperature of 22° ± 2° C, and a relative humidity of 65%. They had free access to water and standard laboratory food at all times.

2. Preparation of rat hippocampal slices

Animals were subjected to deep anesthesia by inhalation of chloroform vapour, and decapitated. The brain was rapidly removed from the skull and transferred into an ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 220 sucrose, 2 KCl, 1.3 NaH_2PO_4, 12 $MgSO_4$, 0.2 $CaCl_2$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, 10 D-glucose; pH 7.4 equilibrated with bubbling of 95% O_2 and 5% CO_2. Coronal brain slices (thickness of 250 μm) containing the hippocampus were cut with the use of a Leica VT1200s vibratome (Leica Microsystems, Milan, Italy). Slices were then transferred immediately to a nylon net submerged in normal ACSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 10 glucose, and 26 $NaHCO_3$, pH 7.4, equilibrated with 95% O_2 and 5% CO_2, first for 40 min at 34°C and then for at least 30 min at room temperature before beginning of the experiments. For all recordings, the temperature of the bath was maintained at 34 °C.

3. Whole-cell patch clamp recordings from CA3 hippocampal pyramidal neurons

After incubation, a hemi-slice was transferred to the recording chamber, which was constantly perfused with ACSF at a flow rate of ~ 2 ml/min. For all recordings, the temperature of the bath was maintained at 33 °C. Neurons were visualized with an infrared-differential interference contrast microscope. Recording pipettes were prepared from borosilicate glass (outer diameter, 1.5 µm) with the use of a Flaming Brown micropipette puller (Molecular Devices, Novato, CA, USA). Resistance of the pipettes ranged from 2.5 to 4.5 MΩ when they were filled with an internal solution containing (in mM): 140 potassium gluconate, 4 KCl, 0.1 EGTA, 10 Hepes, 2 MgATP, pH adjusted to 7.3 with KOH. In order to elicit the hyperpolarization-activated currents (I_h), voltage-clamp experiments were performed with incremental hyperpolarizing steps (10 mV) of the membrane from -65 to -115 mV. Membrane currents were recorded with an Axopatch 200-B amplifier (Axon Instruments, Foster City, CA, USA), filtered at 2 kHz, and digitized at 5 kHz, and pClamp 10.2 software (Molecular Devices, Union City, CA, USA) was used for acquisition, which allowed us to measure various characteristics of the neuronal membrane. Only recordings with access resistance of < 25-30 MΩ were used for analysis. Series resitance was not compensated, and cells were excluded from further analysis if access resistance changed by >20% during the course of the recording. Off-line analysis of I_h was performed with Clampfit 10.2 software (Molecular Devices, Union City, CA, USA). The amplitude of I_h was measured as the difference between the maximum sag reached by membrane current during every voltage step compared with the steady-state level at the end of every step.

In a different set of experiments, in order to evaluate synaptic integration, a bipolar concentric stimulation electrode (FHC, Bowdoin, ME, USA) was placed on the stratus radiatum of hippocampal CA3 subregion for distal dendrites glutamatergic afference and whole-cell recordings were made from the cell soma in response to a train of four stimuli at 20 Hz. Membrane potential was maintained at a value ranging from -75 to -90 mV in order to prevent action potentials. These experiments were performed in the presence of low[Ca^{2+}] to

suppress facilitation from repeated stimulation, 3 $MgSO_4$ to increase the NMDA occlusion, 20 μM bicuculline and 10 μM SCH50911 to block GABAergic transmission. In other set of experiments, in order to characterize the HCN channels activity, current-clamp experiments were performed with injected current steps of 400-ms duration and ranging in intensity from -80 to 0 pA in intervals of 20 pA in order to hyperpolarize the membrane potential.

4. Ethanol Dependence Induction

Ethanol dependence was induced by feeding rats with a liquid diet (Uzbay and Kayaalp, 1995). Male Sprague-Dawley CD adults rats (Charles River, Como, Italy) (n=18), body weigh 150-200 g at the beginning of treatment, were housed individually in Plexiglas cages and fed with a liquid diet, continuously available, prepared as follow: fresh whole cow milk: 1000 ml (Coapla, Italy), vitamin A (5000 IU/l), and 17 g of sucrose. This diet supplies 1000.7 kcal/l and it was freshly prepared daily and presented at the same time of the day (10:00 a.m.). No extra chow or water was supplied.

After animals had undergone an adaptation period to the liquid diet only with milk and sucrose, the diet was gradually enriched with 2.4% (days 1-4), 4.8% (days 5-8) and 7.2% (days 9-20) of ethanol and administered for at least 21 days. Body weight, as well as liquid intake, was monitored daily. Ethanol intake was measured and expressed as g/Kg. Control rats (CTRL) were pair fed with a liquid diet (without EtOH) enriched with sugar in order to equilibrate the calories of EtOH.

Chronic ethanol rats (EtOH-CHR) were sacrificed in the morning of the experiment immediately after termination of ethanol exposure, while for the group of withdrawal rats (EtOH-WDL) the ethanol treatment was suspended 12 hours before sacrifice for electrophysiological experiments. Withdrawal symptoms such as body tremors, tail rigidity, irritability to touch and ventromedial limb retraction were observed as previously reported (Spiga et al., 2014).

5. SI stress paradigm

Experimental animal model used for this set of experiments involved the use of male C57BL/6J mice (Charles River, Como, Italy). New-born pups were left undisturbed with their mothers until weaning (PND 21). After weaning, male C57BL6J mice were randomly assigned to be housed six per cage (group-housed, GH) or one per cage (SI) for 4-6 weeks.

6. *Statistical analysis*

Data are expressed as means ± SEM. Results were compared by columns statistics test and one-way analysis of variance (ANOVA) with the use of Prism software (version 6, GraphPad). A *P value* < 0.05 was considered statistically significant.

7. Drugs

All drugs were bath-applied unless otherwise indicated were purchased from Sigma (Sigma-Aldrich, Milan, Italy).

DRUGS	STOCK	CONCENTRATION
AP5	0.01 M	50 µM
Ascorbic Acid	1 M	1 mM
Bicuculline	0.1 M	20 µM
Caesium Chloride (CsCl)	1 M	5 mM
DDA	0.01 M	10 µM
Dopamine	0.01 M	10 µM
FORSKOLIN	0.01 M	0.1-1-10-30 µM
GHB	0.01 M	10-30 mM
H89	0.01 M	10 µM
SCH23390	0.01 M	5 µM
SCH50911	0.01 M	10 µM
ZD-7288	0.01 M	20 µM
Allopregnanolone	0.01 M	1 µM

Most drugs available in the laboratory were hydrochloride salts and were dissolved in ACSF to make stock solutions. Bicuculline was dissolved in methyl sulfoxide (DMSO) >99.9%, as stock solution, and after dilution, DMSO concentration was less than 0.1%.

The stock solutions were stored frozen in aliquots, and before each experiments they were diluted in ACSF to their final concentrations.

Ethanol used was at 96 % purity and was stored frozen. For the acute application it was dissolved in ACSF and used in a range at concentrations from 10 to 80 mM; the EtOH diluition were prepared fresh before each recordings.

Butanol was at 99.4 % purity and was dissolved in ACSF, and used at concentrations of 20 and 80 mM. Drugs were perfused into the recording chamber using a standard perfusion system (peristaltic pumps). EtOH and ButOH were added to the bath solution and perfused for 10-15 min followed by a washout period.

RESULTS

1. Characterization of I_h in CA3 pyramidal neurons

Initially, I have characterized the electrophysiological profile of I_h currents in rat CA3 pyramidal neurons. Under voltage-clamp conditions, hyperpolarizing voltage steps (from -65 mV to -115 mV) were applied in order to evoke HCN-mediated I_h **(Fig. 2A left panel)**. These had an averaged amplitude of -158.3 pA ± 8.96 (n= 87) when measured at -115 mV **(Fig. 2D)**, and a rise time constants τ_1=393.3 ms ± 55.72, τ_2 =82.97 ms ± 10.88 **(Fig. 2E)**. In **the Fig. 2A (right panel)**, a representative trace of voltage-clamp protocol (from -40 mV to -120 mV) is shown**.** I used similar I/V curves to calculate in reversal potential of the open channel, as displayed in the graph of the **Fig. 2C;** the analysis gave a value of -45 mV. The (I/V) relationship of these channels, calculated with hyperpolarizing voltage step (from -65 mV to -115 mV), is -65 mV (n=15) as reported in the **Fig. 2B**. Moreover, the channel conductance (G) is 75 $pSpF^{-1}$. As expected, I_h were abolished by the bath perfusion of 5 mM CsCl, a non-selective HCN blocker, and 20 μM ZD-7288, a selective blocker of HCN channels **(Fig. 3A)**.

2. Effects of EtOH on I_h in CA3 pyramidal neurons

It has been demonstrated that EtOH is able to modulate I_h recorded in rat hippocampal interneurons, with an increase in I_h amplitude and maximal conductance (Yan *et al*., 2009).

To evaluate if HCN-mediated currents were altered by EtOH also in CA3 pyramidal neurons, I initially tested a low (20 mM) and high (60 and 80 mM) concentrations of EtOH which were bath applied for 10-15 min under voltage-clamp conditions.

During the perfusion of 20 mM EtOH, I observed a significant increase in the amplitude of I_h (36.6 ± 5.89 %, n = 21, $P < 0.0001$) with respect to control **(Fig. 4B)**. Vice versa, 60 and 80 mM EtOH produced an opposite effect and reduced significantly the amplitude of I_h **(Fig. 4B)**. For 60 mM EtOH, the reduction was 17.36 ± 3.99 % vs control, (n = 48, $P < 0.0001$), whereas for 80 mM EtOH, I found a decrease of 53.11 ± 9.54 % vs control, n = 13,($P < 0.0001$) **(Fig. 4B)**. **Fig. 4A** shows representative traces of I_h recorded with the

hyperpolarizing step of -115 mV, during bath application of 20 and 80 mM EtOH.

To better characterize the effect of EtOH on I_h, I decided to test other concentrations of the drug. As shown in **Fig. 4C** EtOH modulates the activity of HCN channels with a biphasic manner. This modulation resulted significant. (One-Way ANOVA revealed $F_{(8,191)} = 7.340$, $P < 0.0001$).This modulatory effect of EtOH is evident after 10-15 minutes of continuous bath application, and it appears to be reversible upon a prolonged washout **(Fig. 5A)**.

Moreover, I analyzed the possible alterations in the I_h kinetic of activation during bath application of low and high concentrations of EtOH. The perfusion of 20 mM EtOH didn't alter this kinetic parameter **(Fig. 4E)**. Vice versa, the high 80 mM concentration of EtOH induced a significant increase 11± 4.56 %, n=14, $P < 0.05$ of the rise time constant compared to control values **(Fig. 4E)**. We also calculated the I/V relationship of I_h during acute application of 20 and 60 mM EtOH and we observed that this parameter is not altered with respect to controls **(Fig. 4F)**.

3. Effects of low and high concentration of EtOH on firing and synaptic integration in CA3 pyramidal neurons

HCN channels have been shown to be involved in action potential firing (Okamoto et al., 2006). In order to examine the effects of EtOH on neuronal firing, I bath applied the muscarinic receptor agonist carbachol to hippocampl slices. As shown by the recording illustrated in **Fig. 6**., CA3 pyramidal neurons display almost no spontaneous firing. Addition of 5 μM carbachol produces an immediate activity , characterized by a regular action potential firing **(Fig.6A-D)**. Carbachol induced firing is completely suppressed by co-application of CsCl (5 mM) or ZD-7288 (20 μM) (**Fig. 6A-B)**. Co-application of 20 mM EtOH enhanced the frequency of action potentials (**Fig. 6C).** Vice versa, 80 mM EtOH produced an opposite effect with a reduction of action potential firing **(Fig. 6D).**

It is well established that HCN channels play an important role in the control of temporal summation of post-synaptic potentials (PSPs) in dendrites and soma.

I then decided to measure and characterize the temporal summation in the presence of low and high concentrations of EtOH. The experimental protocol involved the delivery of 4 electrical stimuli of the same intensity and with a frequency of 20 Hz, to the distal dendrites of CA3 pyramidal cells. Such recording was done in the presence of $GABA_A$ and $GABA_B$ receptors antagonists, and in presence of high concentration of Mg^{2+}, so that the EPSPs recorded mediated by AMPA receptors. In addition, I lowered the Ca^{2+} concentrations to prevent the facilitation from repeated stimulation. Bath application of 20 mM EtOH led to a modest decrease of temporal summation compared to the controls, but this alterations was not statistically significant **(Fig. 7A-B upper panel)**. On the contrary, the paired t test values of EPSP4/EPSP1 ratio in the presence of 60 mM EtOH revealed a significant increase of temporal EPSP summation (n = 6, $P < 0.001$) **(Fig. 7A-B middle panel)**. Also, when I applied the selective I_h blocker ZD-7288 (20 μM), and as expected, I observed an increase of summation, measured as the EPSP4/EPSP1 ratio **(Fig. 7A-B bottom panel)**. Thus, the biphasic effect of EtOH on the temporal summation, together with the effect of ZD-7288, suggests that these effects may be mediated through the modulation of I_h.

4. Modulation of I_h by the dopamine/cAMP/PKA intracellular pathway

Experimental evidences have demonstrated that I_h are modulated by several extracellular and/or intracellular molecules. In particular, the physiological role of cyclic adenosine monophosphate (cAMP) on modulation of HCN channels has been well established. cAMP regulates the voltage-dependent gating of HCN channels, so that in this process are involved molecules and intracellular pathways associated with the production of cAMP. One of such examples involves dopamine that activates D_1 receptors (D_1Rs). D_1Rs activate the heterotrimeric G_s-protein, which is coupled positively with

adenylyl cyclase, thus elevating the production of cAMP and the activation of cAMP-dependent protein kinase A (PKA).

The role of dopamine in the activity of HCN channels in different brain areas it is well described (Rosenkranz and Johnston ,2006). To evaluate in my experimental conditions the modulatory effect of dopamine on I_h, I measured the amplitude of I_h in CA3 pyramidal neurons before and after 10 min of bath application of DA (10 μM). I observed a significant increase of I_h amplitude (48.8 ± 13.25 % vs control value, n = 4, $P < 0.0001$), and this effect was evident after 10 min after dopamine perfusion and it remained consistent for at least 60 min **(Fig. 8B)**.

This modulatory effect of dopamine on I_h was completely antagonized by SCH23390 and DDA **(Fig. 8C)**. In fact, pre-application of the selective antagonist of D_1Rs, SCH23390 (5 μM) for 15 min, abolished the effect of dopamine on the increase of I_h. A similar result was obtained with the pre-incubation of slices with 10 μM DDA, inhibitor of adenylyl cyclase, that precede that of 10 μM dopamine **(Fig. 8C)**.

5. Modulation of I_h by forskolin in CA3 pyramidal neurons

It is known that forskolin, an activator of adenylyl cyclase, increases the production of cAMP and consequently facilitates HCN channel voltage-dependent activation. To evaluate the modulatory effect of forskolin in CA3 pyramidal neurons. I tested different concentrations of forskolin, ranging from 0.1 to 30 μM **(Fig. 9B)**. I found that while 0.1 μM forskolin increased by 36.3 ± 11.25 % (n=5, P<0.05) the amplitude of I_h,the concentration of 30 μM produced an opposite effect, reducing the same parameter by 27.8 ± 7.37% (n=4, P<0.05).

Moreover, I wanted to evaluate the modulatory activity of forskolin in presence of selective inhibitor of PKA, H89 (10 μM). I observed that pre-application of H89 abolished the modulatory effect of forskolin at low and high concentrations. Forskolin 0.1 μM plus H89 modulated I_h amplitude by 17.47 ± 5.68 % (n=5, P<0.05), while forskolin 30 μM plus H89 modulated I_h amplitude by 20.19 ± 1.42 % (n=5, P<0.05) **(Fig. 9D)**.

6. Involvement of adenylyl cyclase/PKA intracellular pathway in the effects of EtOH on I_h

As shown in **Fig.8C ,** the modulatory effect of dopamine on I_h in CA3 pyramidal neurons was blocked by the prior application of 10 μM DDA. Based on this result and considering that cAMP is known to play an important role in the modulation of I_h (DiFrancesco and Tortora, 1991), I decided to evaluate the effect of EtOH in the presence of DDA and H89, selective inhibitors of adenylyl cyclase and PKA, respectively. Before the co-application of these inhibitors and EtOH, I decided to evaluate the possible effects of DDA and H89 alone on I_h. **Fig. 10A** reports representative traces of I_h before and after 20 min of drug application. In **Fig. 10B**, the graph shows that 10 μM DDA and 10 μM H89 did not change significantly the amplitude of I_h. On the other hand, perfusion of DDA and H89 blocked the modulatory effects of EtOH **(Fig. 10C)**. In fact, as shown in the **Fig. 10D,** the effect of 20 mM EtOH (28.7 ± 7.83 % vs control values, n = 4, $P < 0.05$), and of 80 mM EtOH (-43.7 ± 11.85 vs control values, n = 6, $P < 0.001$) were completely blocked by both DDA and H89.

7. Effects of butanol, GHB, and allopregnanolone on I_h in CA3 pyramidal neurons

It has been long known that there is a high correlation between the efficacy of different alcohols and lenght of their carbon chain, that is, their increasing hydrophobicity; this is known as the Meyer-Overton rule (Meyer, 1901). Thus, I reasoned that by using an alcohol such as butanol (ButOH), with a 4-C carbon chain, I might be able to detect stronger modulatory effects on I_h as compared to EtOH. As showing in **Fig. 11A-C**, ButOH at the concentrations of 20 and 80 mM produced an increase (13,4 ± 4.53% vs control values, n=5) and a decrease (58,93 ± 15.83 % vs control values, n= 5, P<0.05), respectively, effects that were very similar to those produced by EtOH.

γ-hydroxybutyric acid (GHB) is a compound that shares with EtOH a similar spectrum of pharmacological actions, including motor incordination, sedation and sleep (Schep *et al.*, 2012). Thus, two concentrations (10 and 30 mM) of

GHB were tested in my experimental condition. As illustrated in **Fig. 11B-D**, GHB at both concentrations produced a significant reduction in the amplitude of I_h in CA3 pyramidal neurons (for GHB 10 mM: 66.87 ± 4,19 % vs control values, n=5, P<0.05; for GHB 30 mM: 61.47 . ± 15.12 % vs control values, n=5, P<0.05)

Several studies performed in my (Sanna *et al*., 2004) and other laboratories (Morrow *et al*., 1999) have shown that EtOH can stimulate a rapid increase in the synthesis and release of neurosteroids such as allopregnanolone; these steroid hormones are very active at modulating the function of different synaptic proteins, in particular the $GABA_A$ receptors. Thus,

I wanted to test whether the effects of EtOH that I have found on I_h could involve the activity of these neurosteroids. I then examined whether the bath application of the neurosteroid allopregnanolone could alter the function of HCN channels in CA3 pyramidal neurons. This experiment, was performed in the presence of bicuculline in order to prevent indirect effect of allopregnanolone through the $GABA_A$ receptors. The results, shown in **Fig. 11E** demonstrated that allopregnanolone is completely devoid of effects on I_h.

8. Effects of chronic EtOH treatment and withdrawal on the function of HCN channels in rat CA3 pyramidal neurons

In a separate set of experiments, I have examined whether the function of HCN channels in the CA3 field could be altered following chronic EtOH treatment and during EtOH withdrawal. Rats were exposed for 21 days to a protocol of treatment whereby EtOH was administered through a liquid diet (cow milk with supplements). After completion of such regimen, rats were tested while still intoxicated (CHR-EtOH) or after 12 h of withdrawal. The results shown in **Fig. 12B** indicate that EtOH treatment and withdrawal only modestly reduced the amplitude of I_h, compared to controls, but this effect did not reach statistical significance.

9. HCN channels activity following social isolation stress

In my laboratory, studies on the effects of early-life stress are performed in adolescent C57BL/6J mice that are exposed to social isolation for 4-6 weeks starting from weaning (PND 21). Chronic stress has been shown to produce several effects on the hippocampal function, and among other, it induces a significant atrophy of dendritic arborisation of the CA3 field (Conrad *et al*., 1999). Thus, I recorded I_h in CA3 pyramidal neurons from mice exposed to social isolation and found that the amplitude of tehse currents was significantly reduced compared to the values measured in control animals (**Fig.13 A-B)**.

DISCUSSION

In the present work, I have attempted to characterize the modulatory activity of EtOH on the function of HCN channels in rat CA3 pyramidal neurons, and elucidated the molecular mechanism underlying such action.

Similarly to previous reports, also in my experimental model, HCN channels were activated upon hyperpolarization of the membrane potential. I_h currents generated by hyperpolarizing steps had an average amplitude of -158.3 ± 8.96 pA, a rise time constants (τ):τ_1= 393.28 ms and τ_2= 82.96 ms, and an apparent equilibrium potential of -45 mV. HCN can distinguished from other ion channels by their sensitivity to Cs^+, that is able to inhibit them at concentration as low as 2 mM (Frace *et al*., 1992b). Furthermore, the HCN-mediated I_h currents were suppressed pharmacologically with the perfusion of the selective blocker ZD-7288 (20 μM) (Gasparini and DiFrancesco, 1997).
I thus assessed the effects of acute EtOH perfusion in acute hippocampal slices on HCN-mediated I_h. Previous studies have reported that HCN channels are potential targets for EtOH actions in mouse midbrain dopamine neurons as well as in rat hippocampal interneurons (Okamoto et al., 2006; Yan et al., 2009).

My results show that a low EtOH concentration (20 mM) significantly increases I_h amplitude and, in parallel it reduces the rise time constant. On the contrary, higher EtOH concentrations (60-80 mM) modulate negatively I_h amplitude and increased the rise time constant. This biphasic modulatory activity of EtOH on I_h that I observed in CA3 pyramidal neurons is in part different with the results obtained in previous studies. In fact, in midbrain dopaminergic neurons and in hippocampal interneurons, perfusion of EtOH (100 mM) reversibly increased (26.2 ± 4.2%) HCN-mediated I_h amplitude. However, the positive modulation induced by the low EtOH concentration (20 mM) is similar to the effect reported by Okamoto *et al*. (2006) and Yan *et al*. (2009). These authors showed that EtOH, at 30 and 50 mM is able to modulate positively I_h amplitude. These data suggest that HCN channels expressed in CA3 pyramidal neurons display a different sensibility for the high EtOH concentrations of EtOH and that the mechanism involved in the action of EtOH on HCN channels appears to be much more complex and likely may depend on the brain area and neuronal subpopulation studied.

The biphasic effect of EtOH observed in my study may be in part associated with the differential expression of HCN channel subunit isoforms in different neuron types. In this regard, in the hippocampal formation, expression of different subunit of HCN channels was reported to be selective in specific brain sub-regions (Robinson and Siegelbaum, 2003). In fact, the HCN1 subunit is mainly expressed in CA1 pyramidal neurons, but less in CA3 pyramidal neurons. In contrast, the HCN2 subunit appears mainly expressed in the CA3 field, but much less in CA1 pyramidal neurons. On the contrary, scattered neurons, such as GABAergic interneurons in the stratum oriens and radiatum of the hippocampus express both HCN1 and HCN2 isoforms (Robinson and Siegelbaum, 2002). Thus, it would be very important to test the effects of EtOH on ricombinant HCN isoforms in order to verify whether each of such channels has indeed a differential sensitivity to EtOH, which could in part explain the difference between my results and those of Okamoto (2006) and Yan (2009).

Because the activity of HCN is considered a contributing factor in the fine-tuning of neuronal excitability (Beck and Yaari, 2008; Brager and Jhonston, 2007; Chen K. *et al*., 2001; Magee, 1998;1999), I studied the effect of EtOH on action potential firing in hippocampal CA3 pyramidal neurons. Firing in CA3 pyramidal cells is regulated by an intrinsic cellular mechanism. Recently, it was demonstrated that the majority of pyramidal cells in hippocampal CA3 region support persistent firing only in the presence of the cholinergic agonist carbachol (Jochems and Yoshida, 2013). Thus, because CA3 pyramidal neurons do not show a spontaneous firing, I performed my experiments in presence of carbachol. Perfusion of EtOH (20 mM) increased the carbachol-induced firing frequency of CA3 neurons, while higher concentrations (80 mM) reduced this parameter. The modulatory activity of EtOH on neuronal firing is well established by previously experimental evidences. Accordingly, EtOH reduces firing rate of pyramidal neurons with regular activity in somatosensory cortex (Sessler *et al*., 1998), and it inhibits neuronal activity in prefrontal cortical pyramidal cells (Tu *et al*., 2007). Other reports demonstrated that acute EtOH perfusion enhanced neuronal firing in dopaminergic cells in VTA (Appel *et al*.,

2003; Brodie and Appel, 1998; Koyama *et al*., 2007; Okamoto *et al*., 2006). The idea that HCN channels are strongly involved in the regulation of neuronal firing rate was confirmed by my data showing that perfusion of the selective HCN blocker, ZD-7288 (20 μM), as well as the non-selective blocker CsCl (5 mM) strongly decreased action potential firing rate recorded from CA3 neurons.

My data on the biphasic effect of EtOH are in agreement with previews reports by Tateno and Robinson (2011) who reported that EtOH (23 mM) increased, by acting on HCN channels, the firing frequency in VTA dopaminergic neurons. On the other hand Okamoto et al. (2006) demonstrated that EtOH interacting with HCN increased dopaminergic firing but only at high concentrations. A possible explanation for this discrepancy could be related to the different brain area studied as well as the different cells tested, but, nonetheless, it confirms the complex interaction between EtOH and HCN channels. HCN are also strongly involved in the control of synaptic integration and EPSP summation of both inhibitory and excitatory signals (Magee, 1999). In particular, EPSP summation is a process that results to be controlled and regulated by the remarkable distribution of HCN channels along the dendritic arborisation. This important role of HCN has been demonstrated in several neuron types such as the hippocampal pyramidal CA1 neurons (Magee, 1999).

It is accepted that a decrease in HCN expression and function results in an enhancement of EPSP summation (Magee, 1999). Based on such information, I performed a set of experiments that were directed at evaluating whether EtOH, through its action on HCN, could affect EPSP temporal summation. In CA3 pyramidal neurons, summation of 4 electrically-evoked AMPAR-mediated EPSPs was markedly enhanced by the perfusion of 60-80 mM EtOH. Because the recordings of EPSPs summation were performed in the presence of antagonists of $GABA_A$ and $GABA_B$ receptors, as well as in the presence of high Mg^{2+} concentrations to block NMDA-receptors, such effect appears consistent with the inhibitory action of the high concentration of EtOH on I_h.

In support to this hypothesis, perfusion of the HCN channel selective blockers ZD-7288 (20 μM) show a similar effect to that observed in the

presence of 60 mM EtOH, suggesting that pharmacological inhibition/antagonism of HCN results in a marked increase of dendritic EPSP summation. This data support the idea that both 60 mM EtOH and ZD-7288 act on the same target to induce their positive effect on EPSP summation at CA3 pyramidal neurons. This data are consistent with recent evidence which showing that pharmacological or molecular I_h ablation leads to the facilitation of EPSP summation of EPSPs in the soma (Kim *et al*., 2012).

These functional findings are also in agreement with other studies performed in nigral dopamine neurons of mice. Masi and coworkers demonstrated that 1-Methyl-4-phenylpyridinium (MPP^+), which markedly reduce HCN function, enhances the summation of EPSP in substantia nigra pars compacta dopamine neurons (Masi *et al*., 2013). Because of the opposite effect of EtOH on HCN channels, I also evaluated the effect of low EtOH concentrations on EPSP summation. Consistent with the increase of I_h amplitude, 20 mM EtOH produced a decrease in EPSP summation.

My data on the effect of EtOH on EPSP summation and regulation of action potentials firing rate appear in part inconsistent with some reports where a direct correlation with increase of firing rate and increase in EPSP summation has been suggested (Kim *et al*., 2012). A possible explanation of this discrepancies could be found not only in the different hippocampal sub-region studied (CA1 vs to CA3 pyramidal neurons), where CA3 pyramidal neurons may display peculiar features that contributed to different response, but also by the specific protocol of neuronal firing that I was used. In fact, in my experimental model, the persistent CA3 neuronal firing was evoked with the muscarinic agonist carbachol.

The function of HCN channels is also modulated by several exogenous synthetic as well as endogenous compound including intracellular messengers, protein kinases, and neurotransmitters such as DA. In order to characterize the possible molecular mechanism underlying the modulatory effect of EtOH on channels activity, I examined in great details the effects of DA on HCN channels in CA3 pyramidal neurons. In fact, DA is able to modulate I_h current by an intracellular cascade triggered by DA type 1 receptor (DA_1R) activation.

Bath perfusion of DA increases the amplitude of I_h in CA3 pyramidal neurons, and this effects is blocked by the selective DA_1R antagonist SCH23390, suggesting that the action of DA on HCN channels is in fact mediated by the interaction with D_1Rs. My data are in accordance with previous reports demonstrating that DA up-regulates I_h in pFC pyramidal neurons through D_1R stimulation (Vijayraghavan *et al*., 2007; Gibbs and D'Esposito, 2005). The activation of D_1Rs leads throught an activation of adenylyl cyclase, to an increase in intracellular cAMP levels,. In fact, I could prevent the effect of DA on I_h with the adenylyl cyclase inhibitor DDA. It is very interesting to note that bath perfusion of DDA alone modestly reduced the amplitude of I_h (**Fig**.**10A**); although this effect did not reach statistical significance, it suggests that in CA3 pyramidal cells, the basal activity of adenylyl cyclase maintains intracellular levels of cAMP that are sufficient to tonically activate HCN channels.

Thus, in CA3 pyramidal neurons DA displays the same action as that reported in layer V EC pyramidal neurons (Rosenkranz and Johnston, 2006). Because in my experimental conditions I_h currents mediated by HCN channels are modulated by DA through DA_1R_s and the consequent activation of the AC/cAMP pathway, I wanted to explore the role of this intracellular pathway by using the adenylyl cyclase activator forskolin. The data that I have obtained show, in fact, that forskolin mimics at least at lower concentrations (0.1μM), the potentiating effects of DA. However, when the concentration of forskolin was increased, up to 30 μM, on opposite inhibitory action was measured. Such biphasic profile of effects induced by forskolin strongly resembles effects of low and high concentrations of EtOH.

In addition, perfusion of the selective inhibitor of cAMP-dependent protein kinases A, H89, completely suppressed the modulatory activity on HCN channels evoked by forskolin. Vargas and Lucero (2002) reported that HCN channels can be modulated by PKA in cultured rat olfactory receptors neurons. Moreover, it is well reported that PKA phosphorylation-dependent sites are present in HCN channels (Santoro et al., 1998), and that the native channels can exist in a phosphorylated and more active state (Gauss *et al*., 1998).

Given that the AC/cAMP/ PKA intracellular pathway is involved in the regulation of HCN channel activity in CA3 pyramidal neurons, I evaluated the modulatory activity of EtOH on HCN channels in presence of the selective blocker of adenylyl cyclase, DDA, as well as in the presence of the selective inhibitor of PKA, H89.

Both DDA and H89 antagonized the modulatory effects of EtOH at lower and higher concentrations, suggesting that EtOH modulates I_h through its regulation of the cAMP/AC/PKA intracellular pathway.

As described in the Introduction chapter, the enhancement of cAMP intracellular levels conduces the channels at the opening state, increases the HCN-conductance and I_h amplitude. Indeed, the results discussed above demonstrated that EtOH modulates HCN channels in a biphasic manner. Moreover, lower and higher EtOH concentrations seem to exert their action through a similar intracellular mechanism. It well known that adenylyl cyclase is an important target of EtOH's action in the cAMP-generating system (Tabakoff et al., 2001; Kou and Yoshimura, 2007). Recently, it has been reported that EtOH showed two opposing effects on the activity of adenylyl cyclase; it can exert an enhancement as well as on inhibitory effects (Gupta et al., 2013). As reported in the present work it is possible that AC expressed in CA3 pyramidal neurons display a differential sensitivity to EtOH concentrations. Low EtOH concentrations may enhance cAMP levels, through activation of AC. As described in the Modulation by Cyclic Nucleotides section, enhanced cAMP will reduce the voltage required for HCN activation through the tetramerization of the intracellular C-linker,and relieves auto-inhibition of the inner pore (Akimoto et al., 2014).

On the other hand, high EtOH concentrations might exert an inhibitory action on AC; this will reduce the physiological concentrations of cAMP that are no longer sufficient to maintain HCN channel activity.

Altogether, the results of my work demonstrate that EtOH modulates the function of HCN channels in the rat CA3 pyramidal neurons. Such modulatory effect produced by EtOH appear to be relevant in many neurophysiological aspects that involve the activity of HCN channels, such as neuronal firing and

temporal summation od postsynaptic potentials in dendrites and cell body. Finally, although a direct interaction of EtOH with the HCN channels can not be ruled out, EtOH appears to regulate I_h through a molecular mechanism that involves the AC/cAMP/PKA intracellular pathway, that in CA3 pyramidal neurons in coupled to DA_1Rs.

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FIGURES

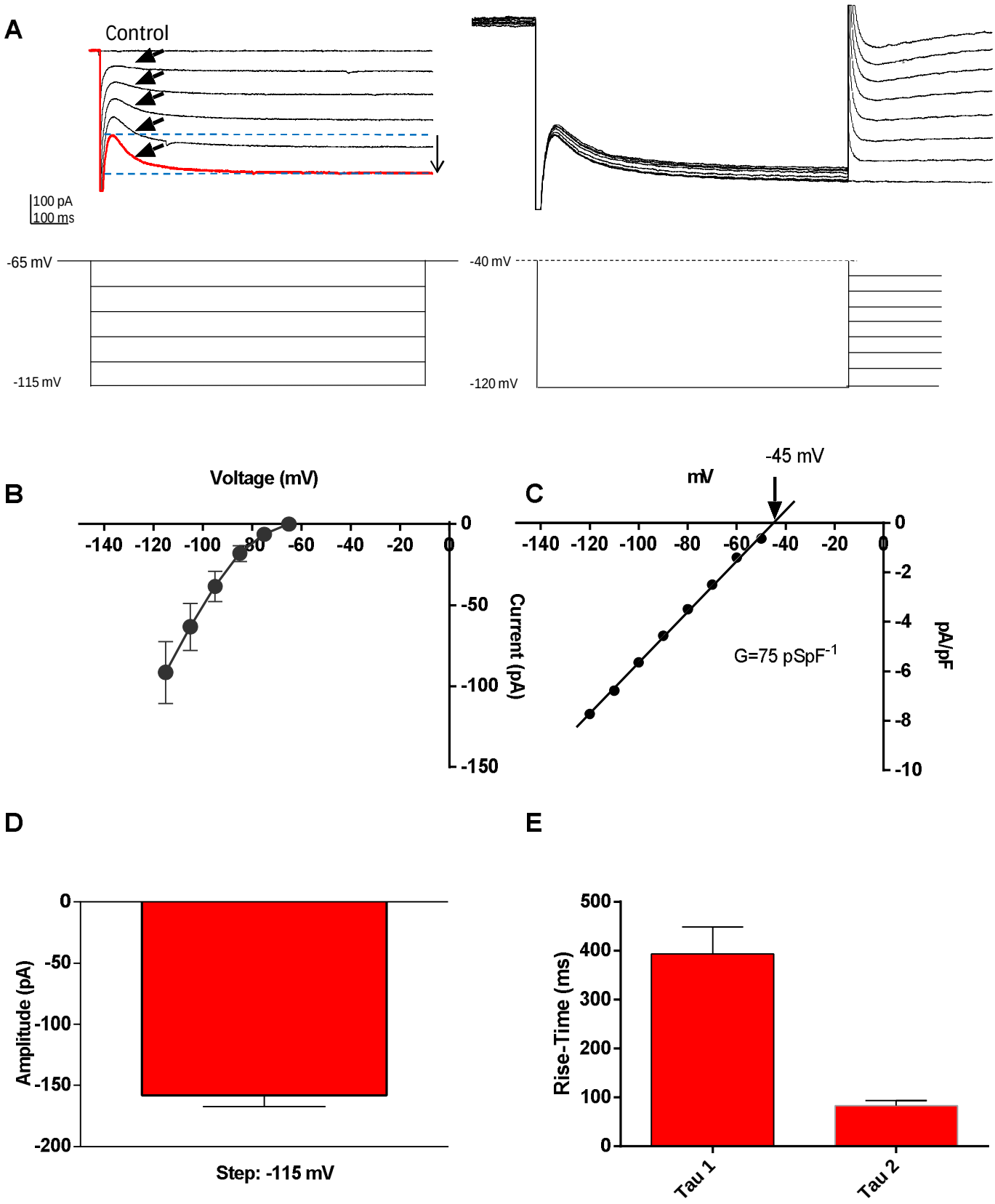

A
Control
100 pA
100 ms
-65 mV
-115 mV
-40 mV
-120 mV
B
Voltage (mV)
-140 -120 -100 -80 -60 -40 -20 0
0 -50 -100 -150
Current (pA)
C
-45 mV
mV
-140 -120 -100 -80 -60 -40 -20 0
0 -2 -4 -6 -8 -10
pA/pF
G=75 pSpF-1
D
Amplitude (pA)
0 -50 -100 -150 -200
Step: -115 mV
E
Rise-Time (ms)
500 400 300 200 100 0
Tau 1
Tau 2

Figure 2. Electrophysiological characterization of I_h recorded in CA3 pyramidal neurons of male rats.

A. Representative traces of I_h recorded from a single CA3 pyramidal neuron under voltage clamp conditions. On the left are showed I_h evoked by 1s hyperpolarizing steps (- 10 mV each) from -65 to -115 mV (bottom) in which the classical current sag is highlighted by black arrows. On the right is showed a sample trace of a fully activates I_h consisting (bottom) in a test pulse of -120 mV starting from – 40 mV followed by steps to different test potentials (- 20 mV each; from – 120 mV to - 40 mV). **B.** I-V plot obtained with voltage clamp protocol: hyperpolarizing steps from -65 to -115 mV. **C.** I-V plot constructed with a test pulse to -120 mV followed by steps to different test potentials. **D.** Bar graph reported average amplitude of currents mediated by HCN-channels in CA3 when an hyperpolarizing step of -115 mV was applied. Data are expressed as the mean of absolute values ± SEM. n=83. **E.** Bar graph reports values of fast and slow rise time constants of current sag elicited by the – 115 mV hyperpolarizing step. Values are expressed as the mean of absolute values ±SEM. n=10

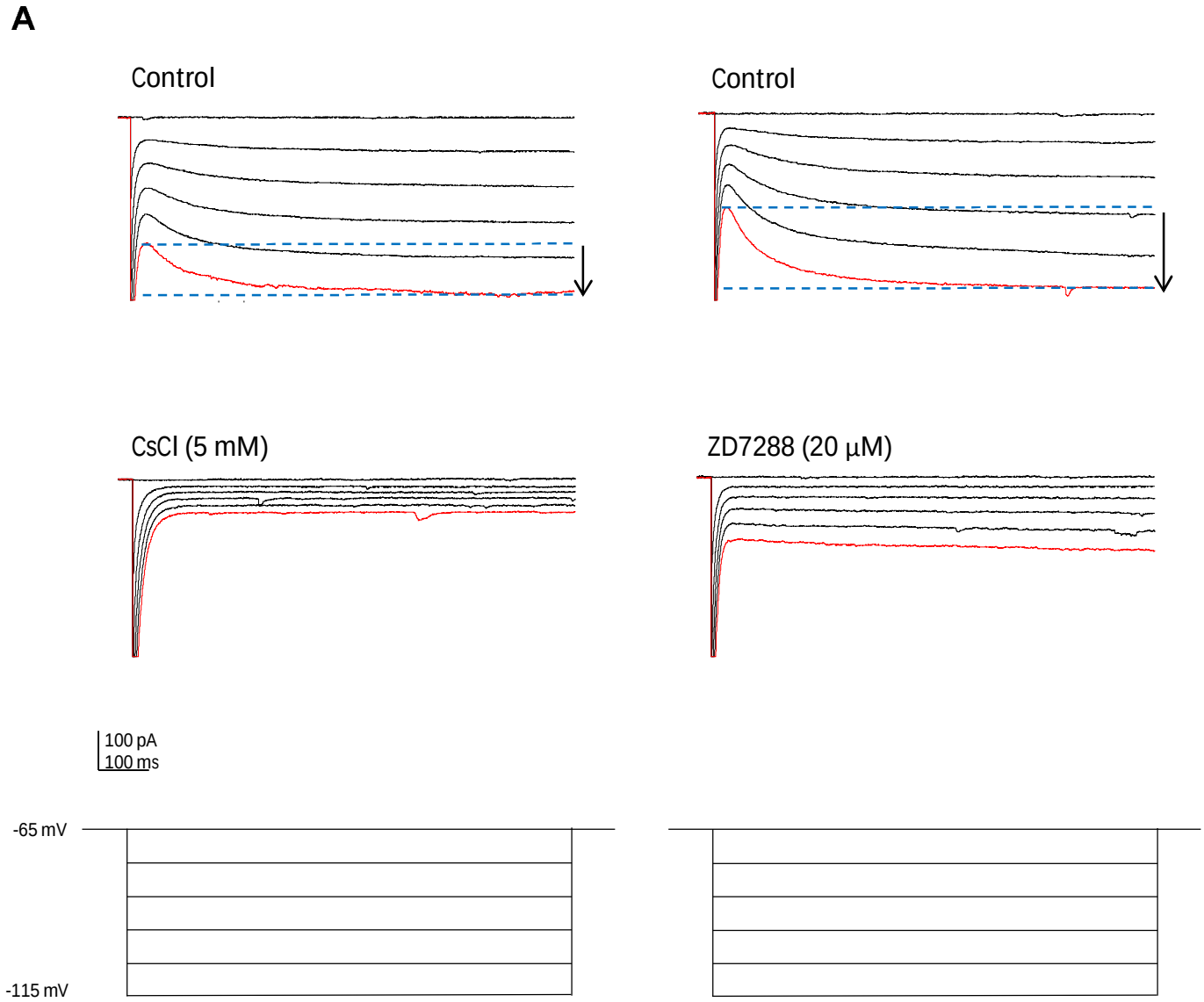


Figure 3. Pharmacological characterization of I_h recorded in rat CA3 pyramidal neurons.

A. Representative traces of I_h recorded from single CA3 pyramidal neuron under physiological conditions (ACSF) and after bath perfusion of non-selective blocker, CsCl (5 mM) (left), and a selective blocker ZD-7288 (20 µM) (right).

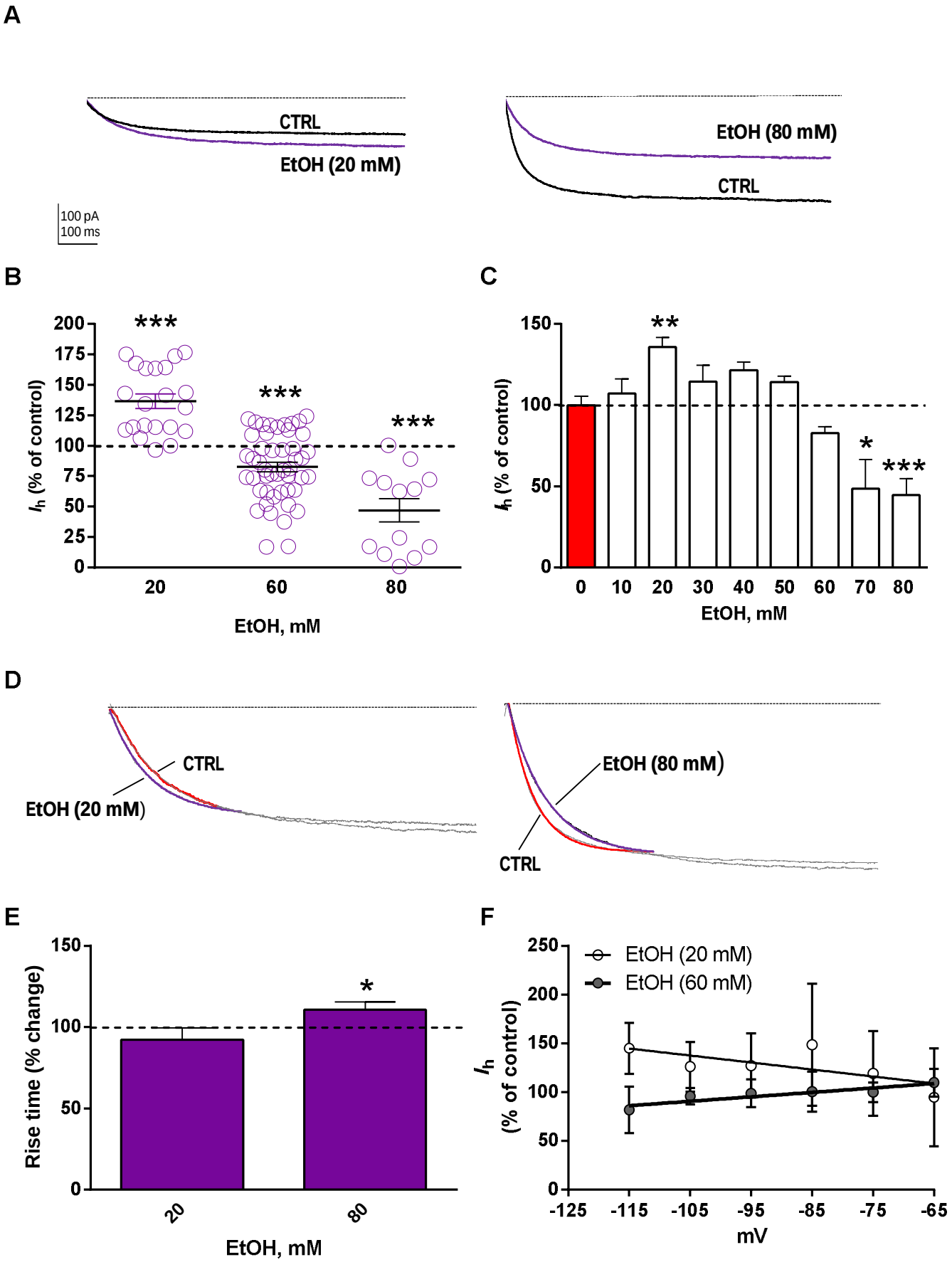

A
CTRL
EtOH (20 mM)
100 pA
100 ms
EtOH (80 mM)
CTRL
B
I_h (% of control)
EtOH, mM
C
I_h (% of control)
EtOH, mM
D
CTRL
EtOH (20 mM)
EtOH (80 mM)
CTRL
E
Rise time (% change)
EtOH, mM
F
EtOH (20 mM)
EtOH (60 mM)
I_h (% of control)
mV

Figure 4. Ethanol's actions on I_h in rat CA3 pyramidal neurons.

A. Representative traces of I_h recorded from single CA3 pyramidal neuron under voltage clamp conditions, with one hyperpolarizing step (-115 mV) without (black trace) and with EtOH (violet trace). Effect of EtOH (20 mM) and EtOH (80 mM) when perfused in the bath . **B.** Scatter plot reported the effect of different concentrations of EtOH on the modulation of I_h. Data are expressed as percentage of variation ± SEM induced by 15 min of EtOH perfusion compared with I_h during ACSF perfusion only. *** $P < 0.001$. n=83. **C.** Bar graph reported the dose-response relation at different concentrations of EtOH. Data are expressed as percentage of control ± SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. **D.** Representative traces of rise time (tau) component of current deactivation in the absence (CTRL) and presence of EtOH at -115 mV. **E.** Bar graph reported the variation of the tau component of I_h during the application of EtOH 20 or 80 mM. Data are expressed as percentage of variation ±SEM n=25. * $P<0.05$, Column statistics. **F.** Graph reported the values of I/V relationship during bath perfusion of EtOH (20 mM) and (60 mM). Data are as the mean of absolute values ±SEM. n=10.

A

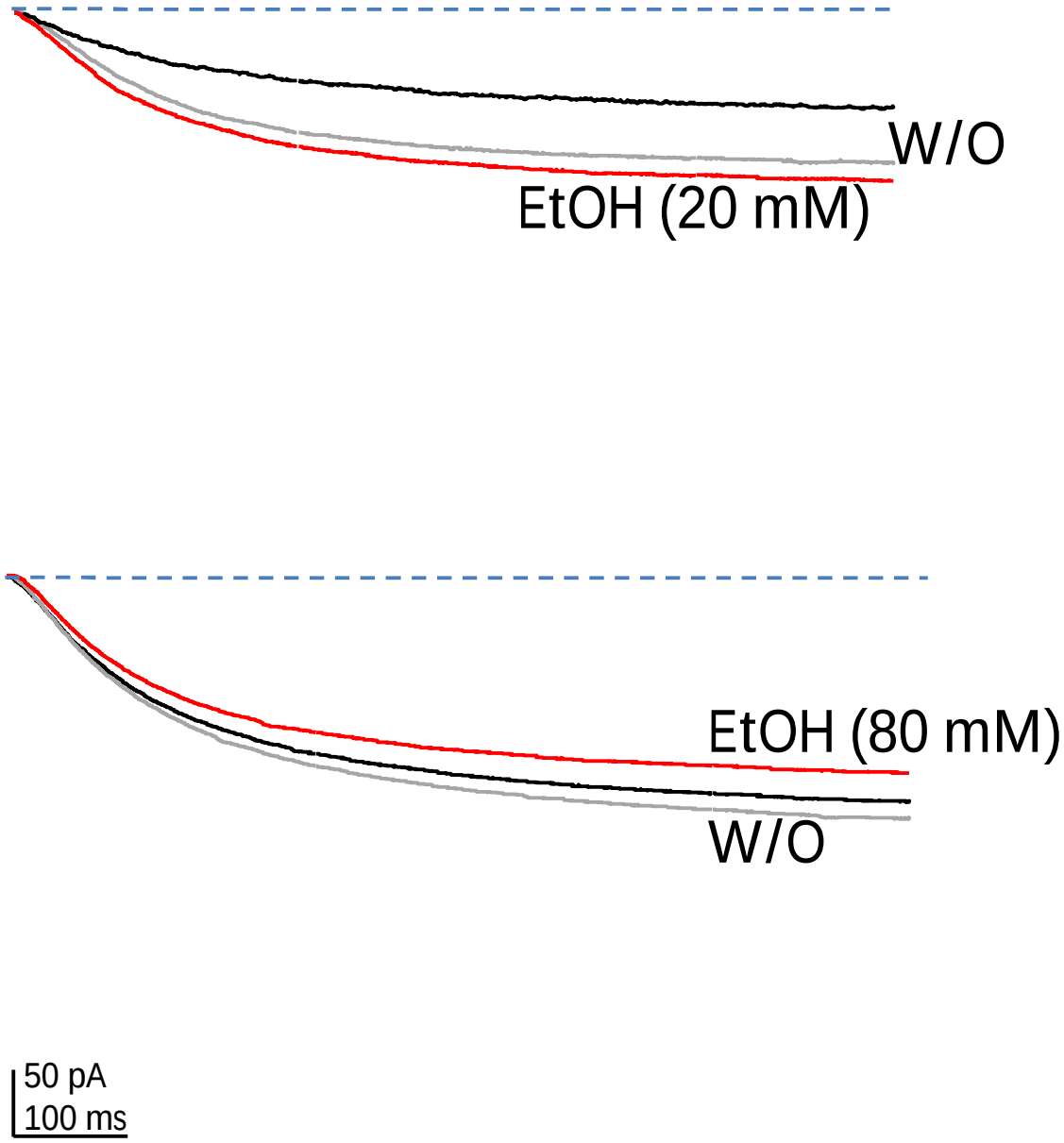


Figure 5. Washout EtOH's actions on I_h in rat CA3 pyramidal neurons.

A. Representative traces of I_h amplitude during bath perfusion and washout of EtOH at low and high concentrations.Control (black trace), EtOH (red trace), and washout (grey trace).

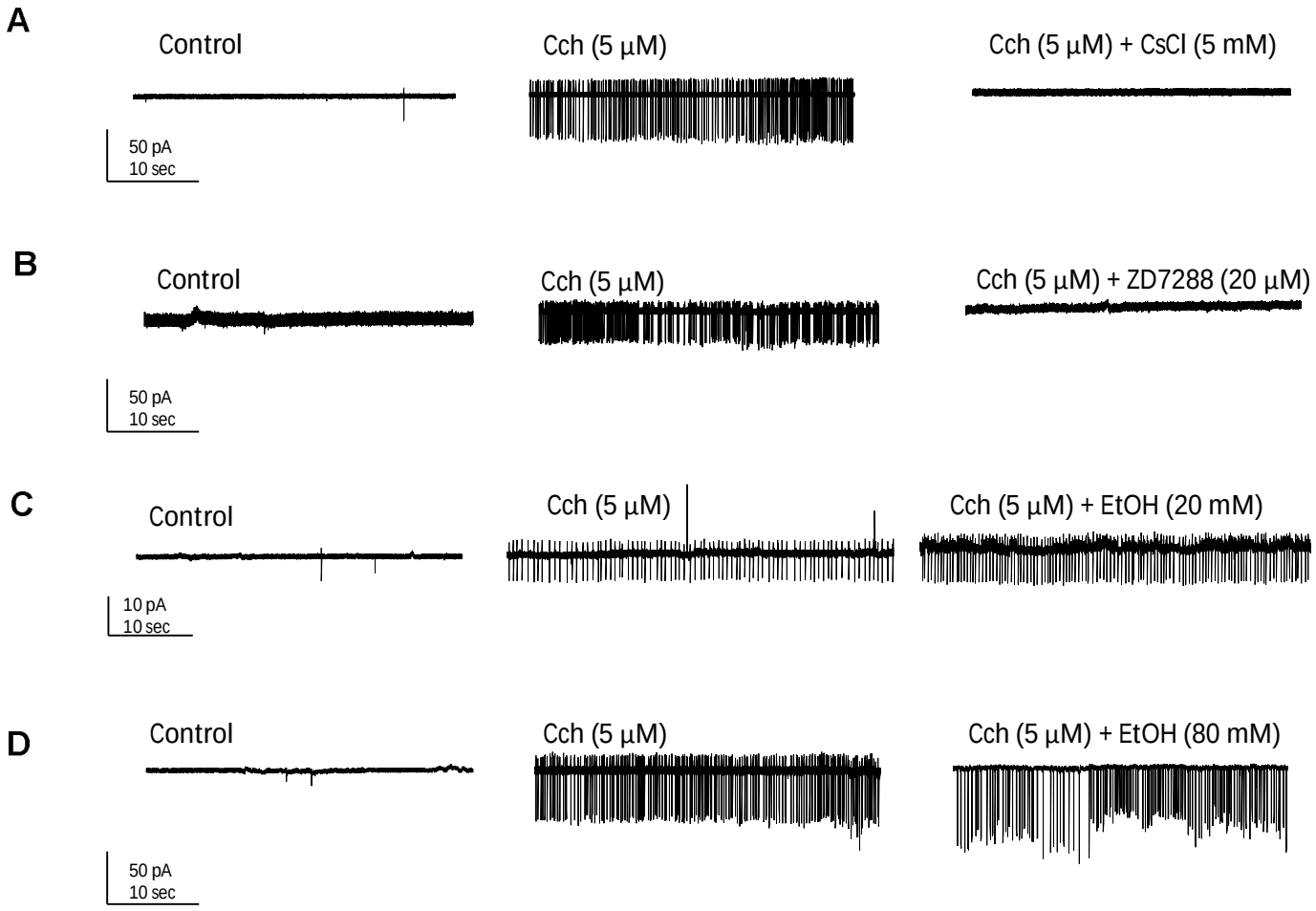


Figure 6. Ethanol and I_h antagonists modulate the AP firing rate of CA3 pyramidal neurons.

A. Sample traces showing the effect of the slice perfusion with the muscarinic receptor agonist charbacol on AP firing rate recorded in cell attached CA3 pyramidal neurons. After a stable effect with carbachol was reached the HCN non selective antagonist CsCl (5 mM) was applied. **B.** ZD-7288 (20 μM) decrease the firing frequency induced by carbachol in CA3 pyramidal neurons in reversible manner. **C.** Perfusion of EtOH (20 mM) modulates in positive manner the firing frequency induced by carbachol. **D.** Carbachol-induced increase of firing frequency is modulated in negative manner during perfusion of EtOH (80 mM).

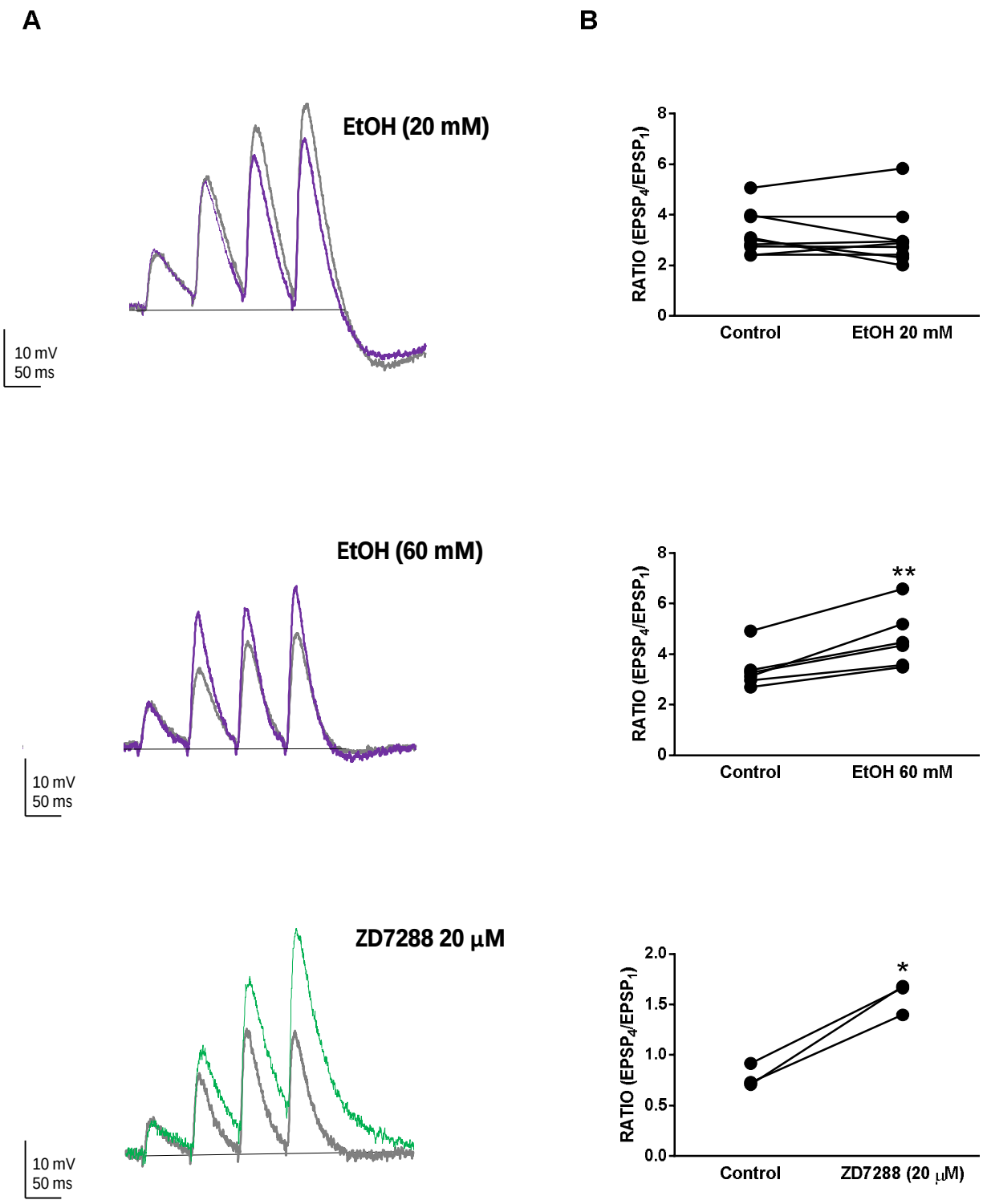


Figure 7. Ethanol's actions on temporal summation.

A. Representative traces of dendritic eEPSP summation recorded in presence of EtOH (20 mM) and (80 mM), and during bath perfusion of selective blocker of HCN channels, ZD-7288 (20 µM). **B.** Scatter plots reporting the quantitative effects of EtOH concentrations and ZD-7288 on temporal summation. Data are expressed as ratio between EPSP4 and EPSP1. * P<0.05; ** P<0.001, Paired t-tests.

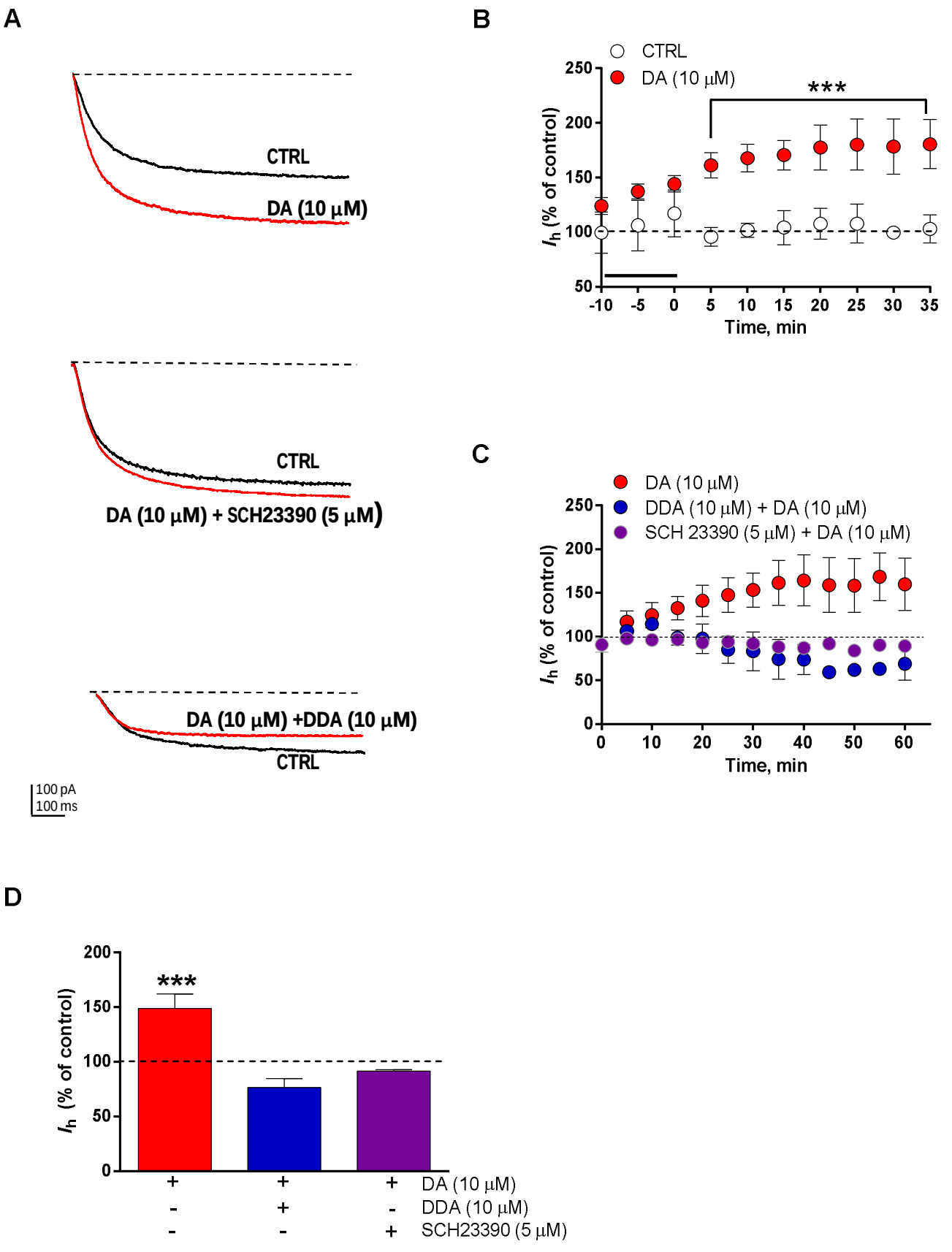
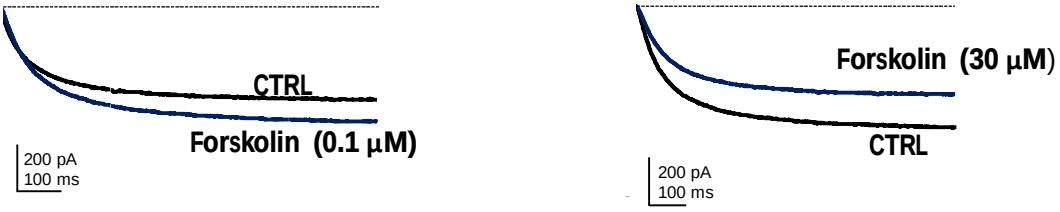

A
CTRL
DA (10 μM)
CTRL
DA (10 μM) + SCH23390 (5 μM)
DA (10 μM) +DDA (10 μM)
CTRL
100 pA
100 ms
B
CTRL
DA (10 μM)
***
I_h (% of control)
Time, min
C
DA (10 μM)
DDA (10 μM) + DA (10 μM)
SCH 23390 (5 μM) + DA (10 μM)
I_h (% of control)
Time, min
D
***
I_h (% of control)
DA (10 μM)
DDA (10 μM)
SCH23390 (5 μM)

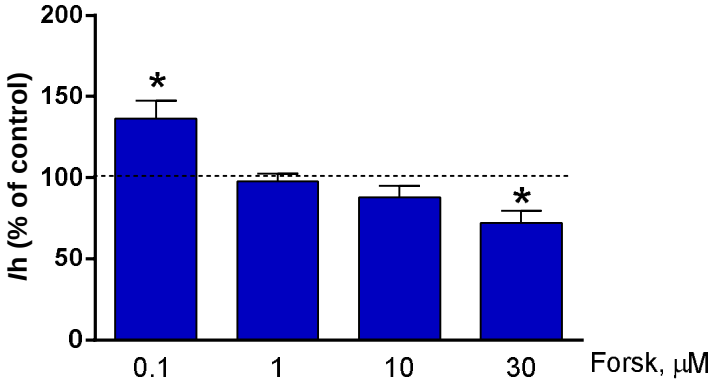
Figure 8. Dopamine induced a positive modulation of I_h in CA3 pyramidal neurons.

A. Representative traces of I_h recorded at -115 mV during bath perfusion of dopamine (DA) 10 μM (upper panel), or during co-application of DA with the selective D1 receptor SCH23390 5 (μM) (middle panel) or the adenylate cyclise inhibitor DDA (10 μM) (lower panel). **B.** Graph reported the effects of DA 10 μM after 5 min bath perfusion on HCN-channels activity. Data are expressed as percentage of control. ***P<0.001. n=5. **C.** Graph reported the DA activity on HCN channels during bath-perfusion of DA, DA plus DDA, and DA plus SCH23390. Data are expressed as percentage of control. n=9. **D.** Graph bar summarized the DA effects on HCN channels compared to activity in presence of DDA and SCH23390, respectively an inhibitor of AC and selective antagonist of D_1Rs.

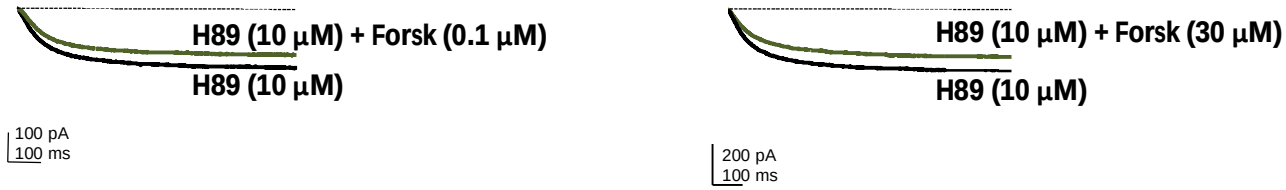
A


CTRL
Forskolin (0.1 μM)
200 pA
100 ms
Forskolin (30 μM)
CTRL
200 pA
100 ms

B


200
150
100
50
0
Ih (% of control)
*
*
0.1
1
10
30
Forsk, μM

C


H89 (10 μM) + Forsk (0.1 μM)
H89 (10 μM)
100 pA
100 ms
H89 (10 μM) + Forsk (30 μM)
H89 (10 μM)
200 pA
100 ms

D

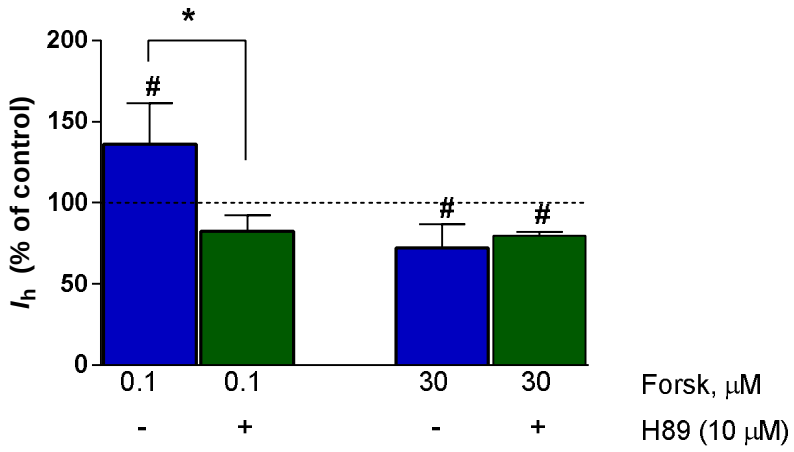

200
150
100
50
0
Ih (% of control)
*
#
#
#
0.1
0.1
30
30
-
+
-
+
Forsk, μM
H89 (10 μM)

Figure 9. Modulation of I_h by forskolin in CA3 pyramidal neurons.

A. Representative traces of I_h amplitude during bath perfusion of an activator of adenylyl cyclase, forskolin at low (left trace) and high concentrations (right trace). **B.** Bar graph reported the effects of forskolin dose response assay on HCN-channels activity. Effects are visible after 20 minutes of bath perfusion. Data are expressed as percentage pf control ±SEM. n=19. *P<0.05, Column statistics test. **C.** Traces reporting the modulatory effects of forskolin on I_h amplitude during co-application of protein kinase A inhibitor. **D.** Bar graph reported the modulatory activity of forskolin in presence of H89. Data are expressed as percentage of control ±SEM. n=19. *P<0.05; $^{\#}$P<0.001.

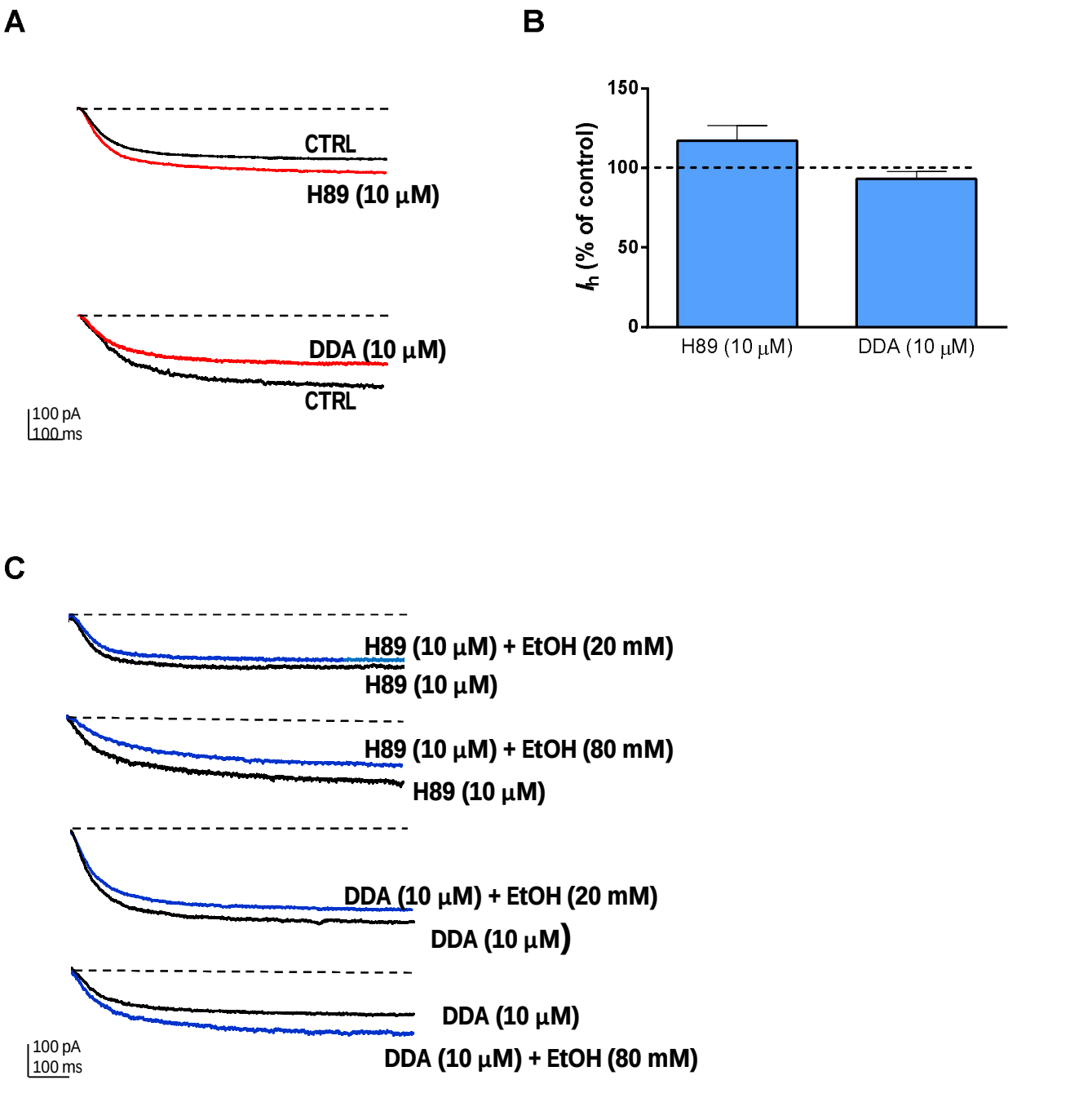
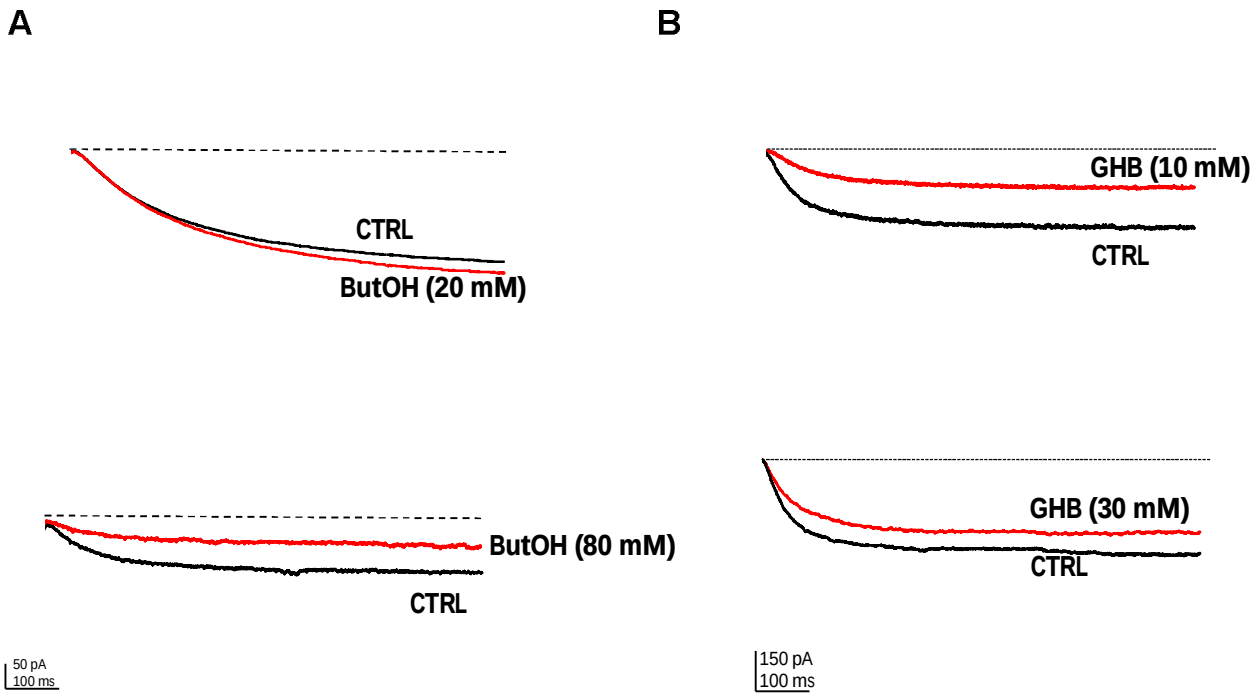
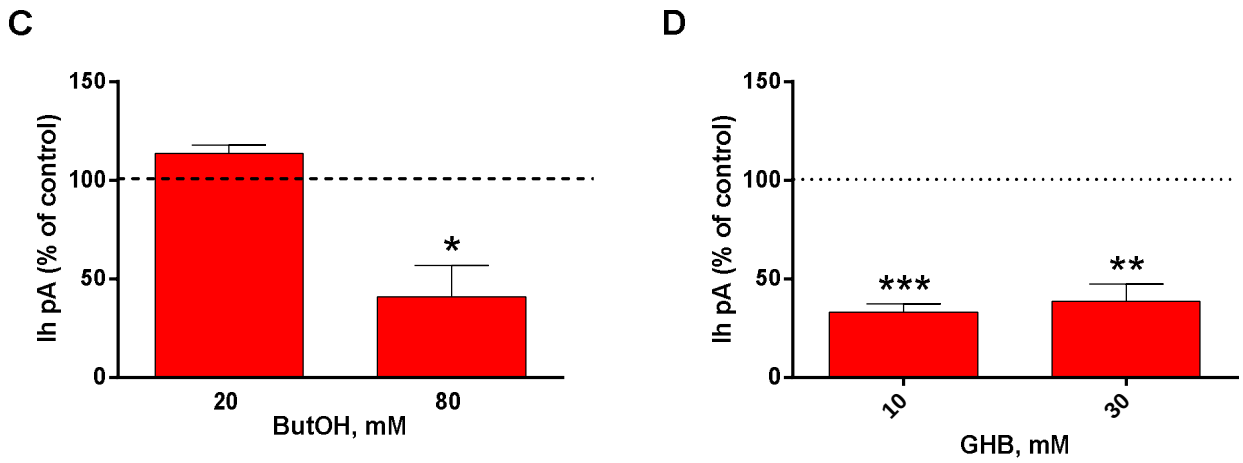

A
CTRL
H89 (10 μM)
DDA (10 μM)
CTRL
100 pA
100 ms
B
150
100
50
0
I_h (% of control)
H89 (10 μM)
DDA (10 μM)
C
H89 (10 μM) + EtOH (20 mM)
H89 (10 μM)
H89 (10 μM) + EtOH (80 mM)
H89 (10 μM)
DDA (10 μM) + EtOH (20 mM)
DDA (10 μM)
DDA (10 μM)
DDA (10 μM) + EtOH (80 mM)
100 pA
100 ms
D
150
100
50
0
I_h (% of control)
*
**
20 80 20 80 20 80
EtOH, mM
DDA (10 μM)
H89 (10 μM)

Figure 10. Modulatory action of EtOH on AC/cAMP/PKA intracellular pathway.

A. Traces reporting effects of selective inhibitor of PKA, H89 (10 μM) (upper trace), and inhibitor of adenylyl cyclase, DDA (10 μM) (lower trace) on I_h amplitude. **B.** Bar graph reported the modulatory activity of H89 and DDA on HCN channels activity. **C.** Representative traces reporting the biphasic modulatory activity of EtOH on I_h amplitude during co-application of H89 and DDA (10 μM). **D.** Bar graph summarized the EtOH activity on HCN-channels when the AC/cAMP/PKA intracellular pathway is pharmacological blocked compared with the EtOH activity during control conditions. *P<0.005; **P<0.001.


A
CTRL
ButOH (20 mM)
ButOH (80 mM)
CTRL
50 pA
100 ms
B
GHB (10 mM)
CTRL
GHB (30 mM)
CTRL
150 pA
100 ms


C
Ih pA (% of control)
150
100
50
0
*
20
80
ButOH, mM
D
Ih pA (% of control)
150
100
50
0
***
**
10
30
GHB, mM

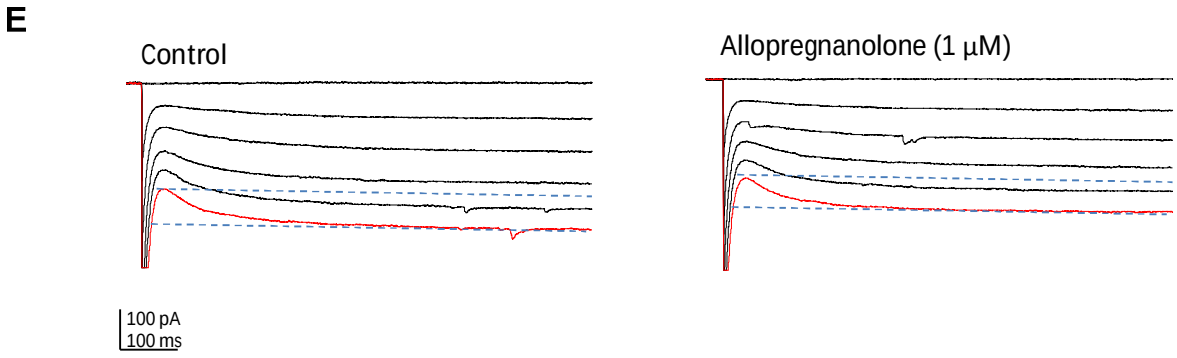

E
Control
Allopregnanolone (1 µM)
100 pA
100 ms

Figure 11. Modulation of HCN channels activity by other molecules.

A. Traces of I_h amplitude recorded in slices of hippocampal CA3 neurons in presence of ButOH (10 mM) (upper) and (80 mM) (lower). **B.** Representative traces showing the modulatory activity of GHB at low (upper) and high (lower) concentrations during bath perfusion for 15 min **C.** Bar graph reported the modulatory effects of ButOH on I_h amplitude during its bath perfusion for 15 min as made for EtOH. Data are expressed as percentage of control. *P<0.05, Column statistics test. **D.** Bar graph compared the biphasic effect of GHB on I_h when perfused in the slice. Data are expressed as percentage of control. ***P<0.001; **P<0.01 *P<0.05, Column statistics test. **E.** Representative traces reporting the I_h amplitude recorded before (left) and after (right) bath perfusion of Allopregnanolone (1 μM) in hippocampal slices.

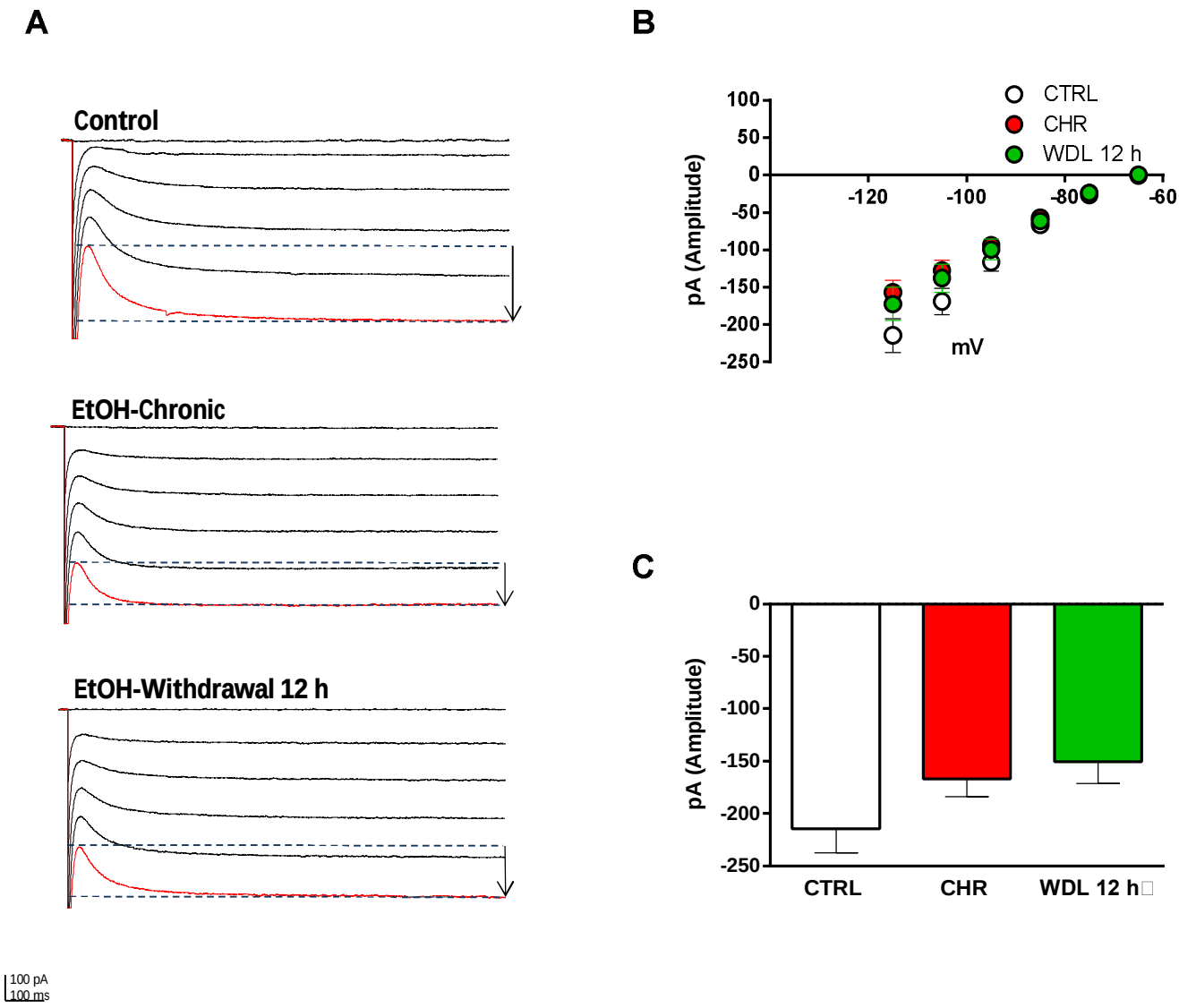
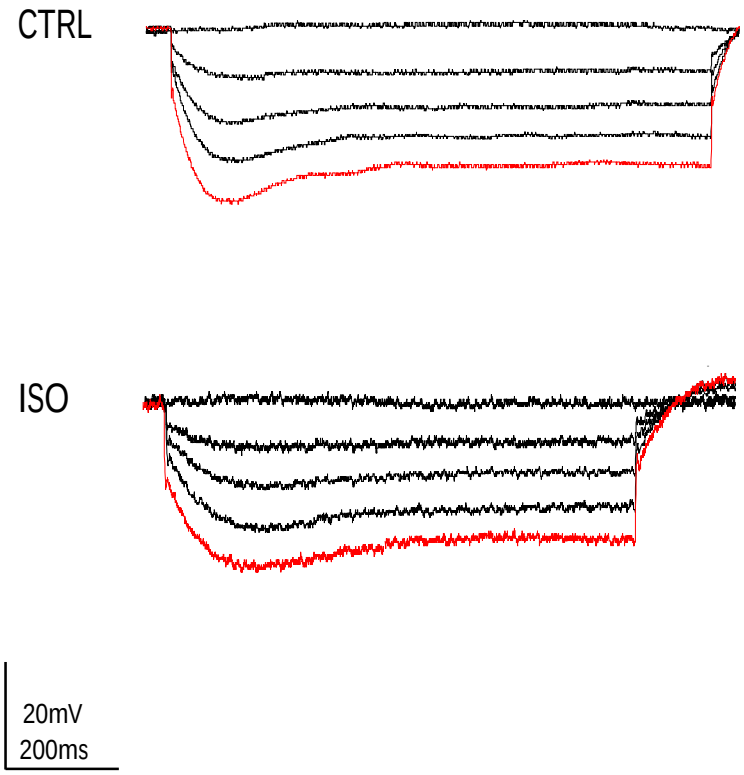

A
Control
EtOH-Chronic
EtOH-Withdrawal 12 h
100 pA
100 ms
B
CTRL
CHR
WDL 12 h
pA (Amplitude)
mV
C
pA (Amplitude)
CTRL
CHR
WDL 12 h

Figure 12. Effects of chronic EtOH exposure on HCN channels activity in CA3 pyramidal neurons.

A. Representative traces of I_h evoked in different experimental groups: control, chronic and withdrawal. **B.** Graph reporting the I_h amplitude at different hyperpolarizing steps from -65 to -115 mV of the different experimental groups. Data are expressed as the mean of absolute values. **C.** Bar graph summarized the HCN channels activity recorded at -115 mV. Data are expressed as the mean of absolute values.

A


CTRL
ISO
20mV
200ms

B

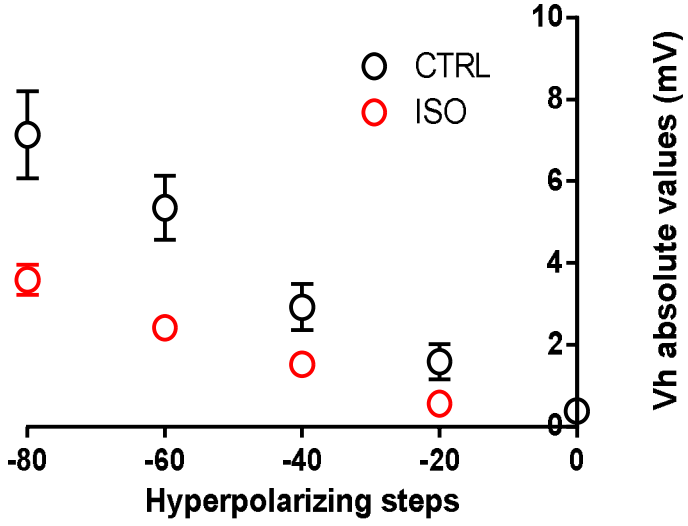

CTRL
ISO
10
8
6
4
2
0
-80
-60
-40
-20
0
Hyperpolarizing steps
Vh absolute values (mV)

Figure 13. HCN channels activity in hippocampal slices of control and isolated C57/BL6J male mice.

A. Representative traces of I_h amplitude recorded in hippocampal slices of control, isolated animals through current clamp protocol. **B**. Graph showed the I_h amplitude in the different experimental groups. Data are expressed as the mean of absolute values of voltages recorded during hyperpolarizing currents steps from -80 pA to 0 pA.

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