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Electrical Pacing Inhibits Gap Junction-Mediated Cardiac Cell-to-Cell Communication by Promoting Cx43 Acetylation

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ABSTRACT

Communication among cardiomyocytes depends upon Gap Junction (GJ) protein expression and conductance. Previous studies demonstrated that electrical stimulation can induce GJ remodeling and evidences from neurons also indicate that electrical pacing modifies Lysine acetylase (HAT) and deacetylases (HDAC) activities. Aim of the present work was to establish whether electrical stimulation modulates GJ-mediated cardiac cell-cell communication by acetylation dependent mechanisms.

Methods and results: Neonatal rat cardiomyocytes (NRCM; n=3) and in HL-1 atrial cells (n=20) were exposed to electrical field stimulation for 24 hours (0.5 Hz, 20 V, 0.5 msec pulses). Connexin 43 (Cx43) expression decreased almost 50% in NRCM and 40 % in HL-1; in contrast Cx40 and Cx45 expression was unchanged. Further, confocal microscopy revealed that electrical stimulation induced Cx43 accumulation in the cytoplasm of HL-1 cells. Electrical stimulation significatly down-regulated HDAC activity up to the 30% (n=3), whereas HAT activity was not modified; the net effect was a general increase of cell protein acetylation, confirmed by western blot analysis. Specifically, the pacing-dependent acetylation of Cx43 was proven by immunoprecipitation assay (n=5). Interestingly, our model mimicked the action of the HDAC pan-inhibitors TSA and SAHA on Cx43 expression and intracellular distribution, although we did not observe Cx43 mRNA significant reduction in electrically stimulated cells. In agreement, MG132 proteasome inhibitor (10 μM) restored Cx43 expression level. Finally, also the treatment of paced cells with the HAT inhibitor Anacardic Acid (AA, 0.5 μM) was able to rescue Cx43 level (n=4). Intriguingly, preliminary results also indicate lateralization and increased acetylation of Cx43 in the left ventricles of dogs with pacing-induced dilated cardiomyopathy (n=4).

Conclusions:*In vitro* electrical stimulation of cardiac cells promotes Cx43 acetylation, which results in Cx43 down-modulation and intracellular relocalization, confirmed also *in vivo*. Our findings suggest that electrical activity-dependent increase in Cx43 acetylation might be a novel mechanism for the regulation of cardiomyocyte communication and, thus, represent a new tool for rhythm disturbance regulation.

INTRODUCTION

GAP JUNCTIONS

A hallmark of multicellularity is the coordinate response of groups of cells against external stimuli, thereby adapting more rapidly to the surrounding environment. Moreover, cells undergoing deleterious changes need to be isolated from the majority to preserve the integrity of the group. In either case, a system is required to allow cells to control and shape the functional state of their neighbors by exchanging signaling molecules. Such a system of communication must also be rapidly modulated to continuously adapt to the immediate needs of the group of coupled cells. These features are met by specialized structures, the intercellular channels, usually collected in distinct regions of the plasma membrane called gap junctions (GJ). The terms 'gap junction channel' and 'intercellular channel' are, therefore, equivalent and define the same structure. These channels control a unique form of communication in that the exchange of molecules is direct and doesn't involve secretion into the extracellular space. Intercellular channels appear to be present in virtually all multicellular organisms, from mesozoa to humans (Revel, 1988). The presence of intercellular channels may be determined by recording the passage of current between coupled cells which permits a precise quantitation of junctional conductance. Intercellular channels participate in the regulation of diverse functions, including contraction of cardiac and smooth muscle, transmission of neuronal signals at electrotonic synapses and metabolic cooperation in development and avascular organs. The remarkable functional conservation of direct cell-to-cell coupling throughout the animal kingdom, however, is not matched at the molecular level of the structural protein components. Intercellular channels in vertebrates are made of connexins, a family of highly related proteins (Kanno et al., 1995).Connexins, as a large family of transmembrane proteins involved in essential cell functions, are expressed in almost all tissues, except for differentiated skeletal muscle, erythrocytes and mature sperm cells (Rackauskas et al., 2010) (Table 1).

Human Connexins	Mouse Connexins	GJ (pS)	Expression patterns of connexins in different tissues
Cx23	Cx23	ND	Human and mouse genomes. Transcription and translation have not been demonstrated in humans
Cx25		ND	Human genome
Cx26	Cx26	115–150	Breast; skin; cochlea; liver; endometrium; glial cells; airway epithelium; somniferous tubules;pancreas
Cx30	Cx30	160	Skin; brain; cochlea; airway epithelium; exocrine gland
31.3	Cx29	ND	Oligodendrocytes; skeletal muscle; liver; pancreas; kidney
Cx30.3	Cx30.3	ND	Skin
Cx31	Cx31	85-15	Skin; airway epithelium; cochlea; placenta
Cx31.1	Cx31.1	ND	Skin
Cx31.9	Cx30.2	15	Mouse heart; mouse brain
Cx32	Cx32	58-70	Liver; skin; Schwann cells; oligodendrocytes; endometrium; gland cells
	Cx33		Testes
Cx36	Cx36	5-15	Retina; pancreatic beta cells; neurons throughout the central nervous system
Cx37	Cx37	219-300	Vascular smooth muscle; endothelium; ovaries; skin

Cx40	Cx40	158-198	Skin; nervous system; endothelium; heart
Cx40.1	Cx39	ND	Human genome; developing muscle of mouse
Cx43	Cx43	90-110	Most widely expressed connexin, present in at least 34 tissues and 46 cell types
Cx45	Cx45	30	Human pancreatic ductal epithelial cells ; SA and AV nodes of the heart; neurons; oligodendrocytes; astrocytes; vascular system skin; osteoblasts; retina; uterus
Cx46	Cx46	140-152	Lens; alveolar epithelium
Cx47	Cx47	55	Brain; spinal cord; oligodendrocytes
Cx50	Cx50	212	Lens
Cx58		ND	Human genome
Cx62	Cx57	57	Mouse oocytes; horizontal cells of the retina

Table1: Connexins distribution in different tissues (adapted from Rackauskas et al., 2010).

Permeability of intercellular channels to second messengers may also regulate secretion by both the exocrine and endocrine pancreas and plays a critical role in pattern formation during development , oncogenic transformation and control of cell. Recent work has shaken two of the postulates on which our understanding of intercellular channels was based:

a) intercellular channels are no longer to be considered as passive conduits allowing the free passage of ions and small molecules in nonspecific fashion;

b) the establishment of communication between cells has proved to be neither passive nor promiscuous but a process that is controlled by the compatibility among connexins.

The GJ is a highly specialized organelle consisting of clustered channels whose unique design permits the direct intercellular exchange of ions and molecules through central aqueous pores. Each cell contributes one half of the channel, called the connexon. Two connexons interact in the extracellular space to form the complete GJ channel, allowing for direct communication between the cytoplasm of the participating cells. Like other membrane channels, each connexon is formed by the oligomerization of the structural protein subunits, termed connexins. Six molecules of connexin leaving a central pore form a hemichannel or connexon, which can be made up by the same connexin isoform (homomeric connexons) or by different isoforms (heteromeric connexons, Figure 1).

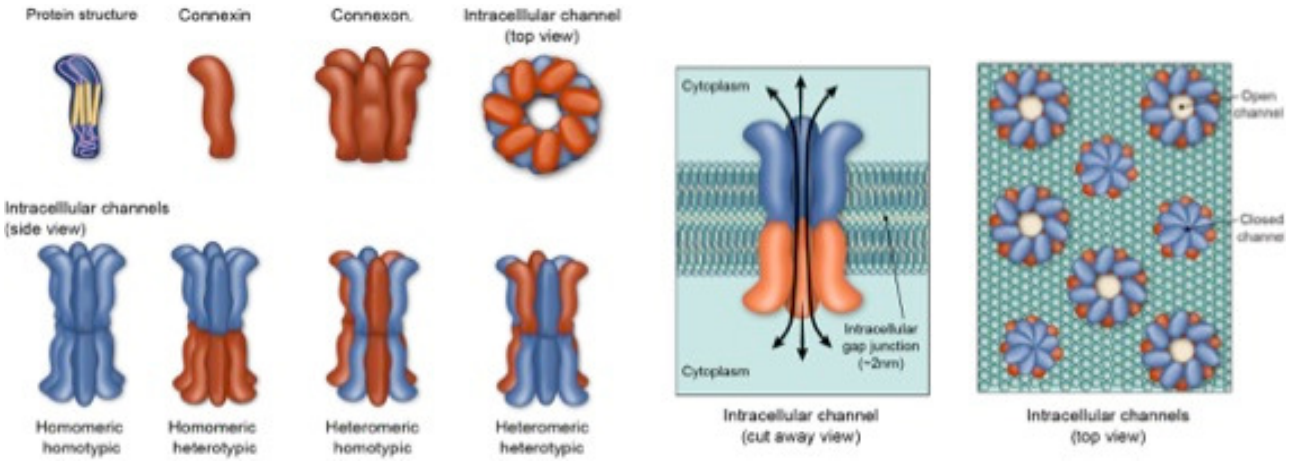

Figure 1: Structure of gap junction intracellular channels (adapted from Mathias et al., 2010).

Such intercellular channels synchronize responses in multicellular organisms allowing the passage of molecules with molecular mass up to 1.2 KDa such as ions, small metabolites and other second messenger molecules between the cytoplasms of adjacent cells (van Veen et al., 2001). Both electrical and metabolic coupling are essential for many tissues. Connexins (Cx) constitute a large family of sarcolemmal proteins sharing a common structure, consisting in four transmembrane α-helices, two extracellular and one intracellular loop, and amino and carboxy-terminal domains

located at the cytoplasmic side of the membrane (Harris, 2001, Figure 2). Moreover, the discovery of connexin mutations in human disease and the targeting of specific connexin genes is illustrating a new spectrum of functional implications for intercellular channels. Several articles have reviewed in great detail the early anatomical, biochemical, physiological and pharmacological characterization of intercellular channels. Up to present, 21 different connexin isoforms , distinguished by their theoretical molecular weight (in KDa), have been described in humans, although not all of them are expressed in all tissues. Some cell types, as keratinocytes, express a wide variety of connexin isoforms, including Cx43, Cxs 26, 30, 31, 40 and 45, whereas others, as hepatocytes, express a limited diversity of connexin isoforms, including only Cx 32 and 26 (Rackauskas et al., 2010). In the other extreme, Cx 57 is the only isoform that has been described in horizontal retinal cells, although other unknown connexin might be also expressed there, as Cx 57-null mice do not have apparent sight or behaviour defects (Hombach et al., 2004), have been described in humans (Sohl and Willecke, 2003). Differences between these connexin isoforms are mostly due to variations in amino acid sequence at the carboxy-terminal domain, although differences in the cytoplasmic loop have been also described (van Veen et al., 2001).

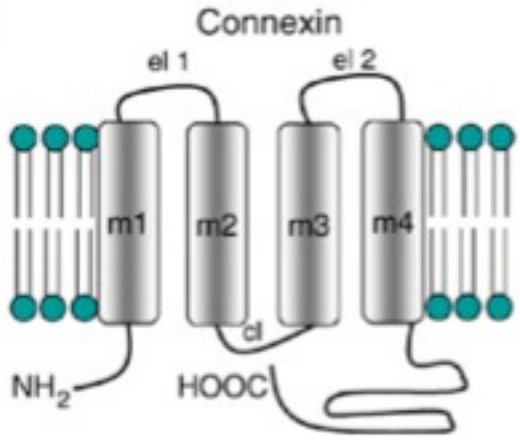

Figure 2: Connexin structure.The connexin protein creates four membrane domains (m1–m4) with two extracellular loops (el 1+2), one cytoplasmic loop (cl), and the N and C termini exposed to the cytoplasm (adapted from Wagner, 2008).

Interaction between the extracellular domains of two hemichannels from adjacent cells originates an intercellular channel, which tend to aggregate in plaques, known as GJ (van Veen et al., 2001). In the process of docking of two hemichannels, are important three conserved cysteines residues located in each extracellular loop of each connexin molecule. Disulfide bonds between these cysteines are needed to create the β-sheet conformation required for the interaction between the two opposing hemichannels. Gap junctional plaques are formed in areas in which membranes of neighbouring cells are in close contact, known as intercalated disks, and that in cardiomyocytes are located at the cell poles, perpendicular to the longitudinal axis of the cells (Shaw et al., 2007) . However, Cx43 is by far the most widely expressed connexin isoform, present at least in 34 tissues and 46 different cell types, including cardiomyocytes and astrocytes (Laird, 2006). Although four connexin isoforms (Cx43, Cx40, 45 and 30.2 in mice, and 31.9 in humans) have been described in the heart, only Cx43 and connexin 40 are mostly expressed in the working myocardium, with Cx43 being by far the most abundant. In fact, GJ formed by both isoforms are present in atrial cardiomyocytes whereas only Cx43 forms GJ in ventricular myocardium (Lin et al., 2010). In the heart, GJ mediate synchronized cardiac contraction (Jalife et al., 1999), whereas in other tissues they are important for cell growth and differentiation, embryonic development and tissue homeostasis (Kjenseth et al., 2010). Connexins are synthesized in the endoplasmic reticulum, but they oligomerize into hemichannels in the Golgi complex. Connexons are then transported into vesicles to the cell membrane, in a process in which microtubules and actin filaments are essential (Kjenseth et al., 2010). Although this process is not completely understood, recent evidences indicate that hemichannels might be directly delivered to the intercalated disks (Shaw et al., 2007). Intercalated disks can be seen, in fact, as an "organelle" involved in maintaining synchrony within cell populations, in which desmosomes and adherens junctions give mechanical continuity, whereas GJ provide a pathway for transfer of ions and small molecules between cells. In this way, connexins located at this "organelle" interact with a number of other proteins, either junctionals or non-junctionals, including sodium channels, microtubules, and proteins from mechanical junctions, as cadherins, plakophilin or plakoglobin. Some of these proteins might be important for GJ

function. In fact, formation of functional GJ requires pre-formation of mechanical junctions (Saffitz, 2006; Rohr, 2007). In this sense, inhibition of cadherin expression has been described to disrupt GJ assembly (Wei et al., 2004). Moreover, Zonula Occludens -1 (ZO-1), which links cadherin to the actin cytoskeleton, may mediate localization of both cadherins and GJ at the intercalated disks (Palatinus et al., 2011), and a direct or indirect cross-talk between the carboxy-terminal domain of connexins and ZO-1 has been suggested to regulate GJ assembly (Chakraborty et al., 2010). Turnover of connexins is rapid, with some connexin isoforms having very short half lifes (1.5-5 hours). Ubiquitination is an important regulatory mechanism of connexins degradation, which can rapidly modify the number of GJ present at the cell membrane. After ubiquitination, connexins are removed from GJ by endocytosis in one of the cells, forming a double-membrane vacuole called annular GJ, which is then transformed into an endosome. In this state, connexins might be recycled and transported again to the plasma membrane forming hemichannels, or may be degraded in lysosomes (Kjenseth et al., 2010). The knowledge of the molecular mechanisms involved in connexins biosynthesis, GJ assembly and their degradation is important to find indirect methods able to modify their abundance. In addition to their presence at intercalated disks, connexins are also located in other regions of the plasma membrane as single hemichannels. In this way, they may allow the exchange of molecules and ions between the extracellular space and the cytoplasm (Rodriguez-Sinovas et al., 2012). First, it was thought that these hemichannels should be kept closed to maintain cellular homeostasis, and that they opened only under pathological conditions. However, it is now well known that they are strictly regulated, and that their opening controls important functions (Saez et al., 2010). Extracellular cation concentrations, intracellular pH, transmembrane potential and mechanical stimulation, among others, have been found to modulate hemichannel gating. As most of these factors are altered during ischemia, hemichannel opening has been proposed to play a role in ischemia-reperfusion injury by increasing intracellular Ca2+ overload and edema (Shintani-Ishida et al., 2007). Although plasma membrane is the main final destination for most of connexin molecules, localizations other than cell membrane have been also reported for some isoforms. In fact, the entire Cx43 protein (Huang et

al., 1998) or its carboxy-terminal domain (Dang et al., 2003) have been found in the nucleus of human brain glioblastoma tumor cells, and in cardiomyocytes and HeLa cells, respectively. There, connexins may directly affect gene transcription. Studies in Cx43-deficient cells have demonstrated altered expression of several apoptotic genes, as those coding for some caspases (Vinken et al., 2012). Furthermore, Cx43 overexpression in neonatal rat cardiomyocytes has been shown to decrease DNA synthesis, by mechanisms independent of GJ intercellular communication (Doble et al., 2004). Moreover, several lines of evidence have supported that only the carboxy-terminal domain of some connexin isoforms is responsible for their regulatory effect on cell growth, as this effect is lost after truncation of the carboxy-terminal tail (Omori and Yamasaki, 1999). This finding is also supported by the fact that mutations in the extracellular loop of Cx43 abolished GJ formation, but did not modify the inhibitory effect on cell proliferation (Olbina and Eckhart, 2003). As occurred with Cx43, other connexin isoforms, as connexin 45.6, have been described to promote lens cell differentiation, through a mechanism independent of GJ communication (Gu et al., 2003). Interestingly, the presence of Cx43 in mitochondria from endothelial cells (Li et al., 2002b) and cardiomyocytes (Boengler et al., 2005; Rodriguez-Sinovas et al., 2012) has been recently described.

GJ in cardiovascular system

The most abundantly expressed connexins in the cardiovascular system are Cx37, Cx40, Cx43, and Cx45. The expression of these proteins displays a large heterogeneity throughout the circulatory system (i.e. arteries, veins, lymphatic vessels, and the heart). In the vessels of healthy subjects, endothelial cells (ECs) of large conduit arteries express Cx37 and Cx40, whereas Cx43 and (to a much lower extent) Cx45 are mostly expressed by vascular smooth muscle cells (VSMCs)(van Kempen M.J.,2002). The level and pattern of connexin expression in conduit arteries changes with age and during atherogenesis (YEh H.I., 2000), suggesting that connexins may be contributing to the latter process. In the apolipoprotein-E-deficient (*ApoE*$^{-/-}$) mouse model for atherosclerosis, it

has indeed been shown that Cx37 and Cx40 display atheroprotective properties (Chadjichristos C.E., 2010), whereas Cx43 appeared pro-atherogenic (Kwak et al., 2003). Recently, both Cx37 and Cx40 have been described to interact with endothelial nitric oxide synthase (eNOS) in aortic ECs (Pfenniger A., 2010). This suggests that both Cx37 and Cx40 may regulate release of eNOS-derived nitric oxide (NO). Interestingly, ECs and VSMCs of small muscular resistance arteries express Cx37, Cx40, and Cx43 (Looft-Wilson et al., 2011; Sandow et al., 2012). In these arteries, connexins are involved in the spreading of endothelium-dependent vasodilatation and relaxation induced by endothelium-derived hyperpolarizing factor(s) (Sandow et al., 2002). ECs of large veins have been described to express Cx37, Cx40, and Cx43 (Inai et al., 2009), although Cx40 and Cx37 expression levels seem lower than those of the aorta (Chang et al., 2010). Finally, lymphatic ECs express Cx37, Cx43, and Cx47 (Kanady et al., 2011), and recently Cx37 has been shown to be of crucial importance for the formation of lymphatic valves (Sabine et al., 2012); i.e. *Cx37*$^{-/-}$ mice lack lymphatic valves and therefore display reduced lymph flow. More recently, Cx37 has been reported to be critically involved in the formation of venous valves as well (Munger et al., 2013). Classically, connexins expressed by the working myocardium contribute to synchronization of cardiac contractions by electrically coupling cardiomyocytes. However, cardiac connexins can also play a deleterious role, e.g. after periods of ischaemia by passing so-called 'death signals' from one cardiomyocyte to the next (Severs et al., 2008). Healthy ventricular myocardium mainly expresses Cx43 and, to a lesser extent, Cx45, and healthy atrial myocardium expresses high levels of both Cx40 and Cx43 in addition to small amounts of Cx45 (Severs et al., 2008). Cells that form the electrical conduction system responsible for the rapid spread of electrical signals from the sinoatrial node towards the ventricles have been described to express a variety of connexins (i.e. Cx45, Cx43, Cx40, and Cx30). The particular set of connexins expressed by these cells is strongly dependent on their location (Van Kempen et al., 1995). In summary, multiple connexins are expressed in the cardiovascular system that not only experience the different biomechanical forces within this system, but may also act as effector proteins in co-ordinating responses within groups of cells towards these forces.

Connexins Regulation

Voltage changes

GJ-channels are regulated by voltage changes, depending on theconnexin isoforms that are expressed. Some of them are influenced by membrane voltage (Vm) and by transjunctional voltage (Vj), whereas others only by Vj. The exact mechanisms of voltage gating are not well known, but it has been suggested that each connexin hemichannel may have at least two different gates: whereas the fast gate depends only on Vj, the slow mechanism may depend both on Vj and Vm. These two gates should be located at different structural areas of connexins, as they can be modified by mutations in different regions of the protein (Gonzalez et al., 2007). As mentioned before, the fast Vj-gating closes the channel from the fully-open state to a residual state at either voltage polarity. Depending on the connexin isoform, some hemichannels will close under negative or positive potentials on their cytoplasmic face. For instance, connexin 26, 30 and 50 close at positive voltages, but connexin 31, 32, 37, 40, 43, 45 and 57, at negatives. Interestingly, Cx46 is able to close on both, negative and positive potentials, with different gating mechanisms (Rackauskas et al., 2010). First evidences supported that voltage polarity of the fast Vj-gating mechanism was regulated by charged residues located at the amino-terminal domain, acting as voltage sensors (Bukauskas et al., 2002). However, more recent studies have reported abolition of the fast Vj-gating after truncation of the carboxy-terminal domain of different connexin isoforms (Gonzalez et al., 2007). Thus, although it cannot be excluded that the amino-terminal domain plays a role, the most accepted mechanism for fast Vj-gating is that the flexible carboxyterminal domain binds to the cytoplasmic loop, partially closing the pore ("chain and ball" model) (Gonzalez et al., 2007). The slow Vj-gating mechanism, that mediates transitions from/to the residual state to/from the fully closed channel, always closes under relative negative potentials into the cell. Moreover, this mechanism has been also associated with chemical gating, as Ca^{2+}, lipophiles and lowered pH have been described to fully close slow Vj-sensitive gates from the residual substate (Bukauskas and Peracchia, 1997; Peracchia, 2004). On the other hand, depolarization of Vm

increases the incidence of slow transitions that lead to a fully closed channel state (Bukauskas and Weingart, 1994). Mutational studies of Cx43 have suggested that the Vm sensor is located in a different region of the carboxy-terminal domain than that involved in fast Vj-gating. However, interactions between both gating sensors cannot be discarded (Revilla et al., 2000).

pH

Cytoplasm acidification has been shown to reduce GJ intercellular communication. However, pH sensitivity is different depending on the connexin isoform that is expressed. Therefore, an slight reduction in intracellular pH is able to partially close channels formed by Cx43, but not by Cx32, which need higher intracellular acidification levels to achieve the same degree of GJ closure (Morley et al., 1996). In fact, pH sensitivity has been analyzed for a wide variety of connexin isoforms expressed in oocyte pairs showing the following decreasing order: Cx 50>46>45>26>37>43>40>32 (Stergiopoulos et al., 1999). Sensitivity to intracellular pH seems to depend on the carboxy-terminal region of the protein, as truncation of that region abolishes pH regulation of Cx43 (Liu et al., 1993). This was later confirmed for other connexin isoforms, as Cx 37, 40 and 50, but not for Cx 45 (Stergiopoulos et al., 1999). Interestingly, these studies also observed that the carboxyterminal domain of connexin 40 is able to regulate channels formed by truncated-Cx43 and vice versa (Stergiopoulos et al., 1999). Furthermore, ablation of the carboxyterminal domain has been observed to eliminate the residual state of GJ channels, whereas it can be restored after co-expression of the carboxy-terminal region as a separate protein (Anumonwo et al., 2001; Moreno et al., 2002). These findings made some authors to suggest that pH/chemical gating can be dependent on a "ball and chain" mechanism, similar to that previously described for fast Vj-gating (Delmar et al., 2004). However, there is still some controversy whether pH gating can be mediated by direct protonation of some histidine residues in the carboxy-terminal domain (Trexler et al.,1999), or by indirect mechanisms through protonation of endogenous aminosulfonates, such as taurine in intact cells (Harris, 2001).

Ca^{2+} regulation

Regulation of GJ channels by Ca^{2+} has been widely analyzed (Peracchia, 2004; Lurtz and Louis, 2007). Early studies demonstrated that intracellular injections of Ca^{2+} induced electrical uncoupling (Rose and Loewenstein, 1975; De Mello, 1975). Sensitivity of GJ channels to intracellular Ca^{2+} concentrations depends not only on the connexin isoform, but also on the cell type. However, it is still unclear whether the effects of Ca^{2+} are direct or mediated through intracellular messengers. In this sense, several evidence indicates that Ca^{2+} probably induces GJ closure by activation of calmodulin, which may act directly as a gating particle (Peracchia et al., 2000; Peracchia, 2004). Calmodulin, a soluble acidic protein, forms complexes with Ca^{2+}, which then bind to basic amphiphilic α-helix domains. Interestingly, calmodulin has been shown to colocalize with connexins (Sotkis et al., 2001), and the interaction between several connexin isoforms and calmodulin has been previously demonstrated (Rackauskas et al., 2010), including connexin 38, 32, 37, 43, 44 and 50. Closure of GJ channels by intracellular Ca^{2+} during ischemia may play an essential role in protecting intact cells from membrane depolarization, and leakage of metabolites through GJ, by disconnecting them from damaged cells in the so-called "healing-over" process (Rackauskas et al., 2010). However, the counterpart would be an increased incidence of ventricular arrhythmias (Jalife et al., 1999).

Post-translational modifications

Although it is complex and incompletely understood, phosphorylation is an important regulatory mechanism of connexins, that not only modifies channel molecular structure, open probability and mean open-time, but also alters connexin intracellular trafficking and assembly into gap junctional plaques. Depending on the connexin isoform, the phosphorylation site and the biochemical environment, the effect of phosphorylation can be different (van Veen et al., 2001). The carboxy-terminal domain is full of serine, threonine and tyrosine residues that can be phosphorylated by multiple protein kinases. For example, 19 phosphorylation sites have been described for Cx 43 , the most abundant connexin in nature. Particularly, some of these serine residues able to be

phosphorylated are: 364, phosphorylated by protein kinase A (PKA), 368 by PKC, and 255, 279 and 282 by mitogen activated protein kinases (MAPK) (Lampe and Lau, 2004, Figure 3).

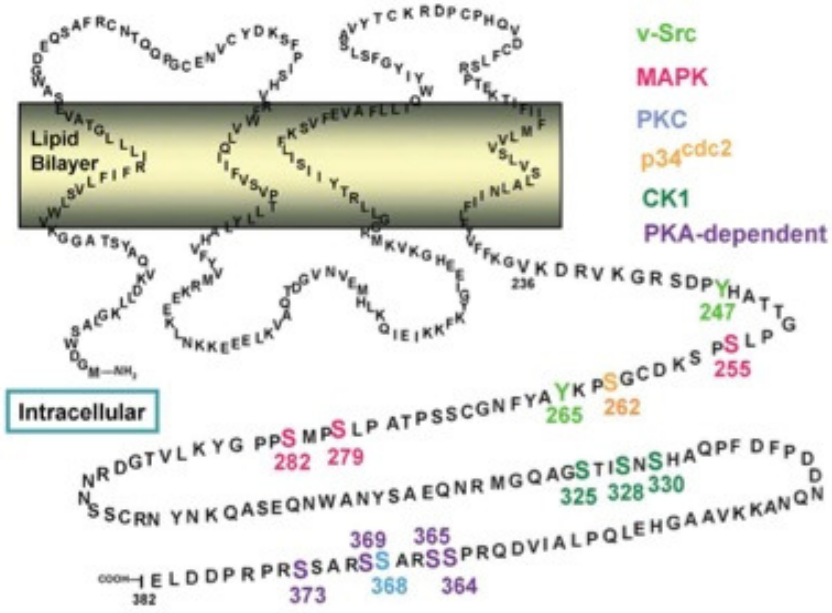

Figure 3: Cx43 most common phosporylation sites (adapted from Lampe and Lau, 2004).

In addition, Cx 36 and 56 can be phosphorylated on the cytoplasmic loop (Rackauskas et al., 2010). Phosphorylation may modify the net charge of the protein, which, in turn, may also alter voltage or pH sensitivity. Cx43 regulation by phosphorylation has been widely analyzed. Initial phosphorylation of Cx43 occurs within 15 min of synthesis so some phosphorylation events likely can occur prior to Cx43 arrival at the plasma membrane. Enhanced gap junction assembly occurs upon activation of cAMP-dependent protein kinase (PKA) via increased Cx43 trafficking to the plasma membrane (Lampe, 1994). The development of specific monoclonal antibodies which are able to recognize primarily non-phosphorylated Cx43 show that the transition of Cx43 from the cytoplasm to the plasma membrane involves phosphorylation, likely on S365 (Sosinsky et al., 2007). Subsequently, casein kinase 1 (CK1) phosphorylates Cx43 at S325/328/330 , sites involved in the movement of Cx43 from the plasma membrane into the gap junction (Lampe et al., 2002), while activation of PKC can halt assembly of new junctions. It has been described that phosphorylation of Cx43 by PKA increases cell-to-cell communication and channel permeability, whereas phosphorylation by PKG and MAPK reduces GJ intercellular communication in

cardiomyocytes. More controversial is the effect of phosphorylation mediated by PKC isoforms α and ε on Cx43. On one hand, it reduces single channel conductance and cell-to-cell permeability, but on the other hand, it enhances electrical conductance between paired cardiomyocytes (Kwak and Jongsma, 1996; Schulz and Heusch, 2004). In addition, PKCε activation may induce Cx43 hyperphosphorylation, which has been shown to accelerate Cx43 proteolytic degradation, resulting in decreased number of GJ channels and conduction impairment (Lin et al., 2006). Reduced GJ intercellular communication has been also observed after protein tyrosine kinase phosphorylation of Cx43 at residue 265. Moreover, Cx43 phosphorylation of serine residues 325 and 330 by casein kinase in rat kidney cells has been shown to reduce membrane GJ channels, thus decreasing GJ conductance (Schulz and Heusch, 2004). On the other hand, dephosphorylation of Cx43 by protein phosphatases has been demonstrated to increase single channel conductance in rat cardiomyocytes. Nevertheless, the phosphorylation state of connexins is not reduced to the balance of all these kinases and phosphatases. In fact, kinases and phosphatases may interact between them, thus making regulation by phosphorylation even more complex (Schulz and Heusch, 2004). Analysis of specific phosphorylation sites in mouse heart have shown that Cx43 is heavily phosphorylated on casein kinase 1 (CK1) sites that promote gap junction assembly (Lampe et al., 2006) but upon ischemia, these CK1 sites are dephosphorylated (Axelsenetal., 2006) while phosphorylation on the PKC site at S368 is increased (Hund et al., 2007). In addition, rapid (5 min) dephosphorylation of S365 occurs in response to ischemia (Sosinsky et al., 2008), consistent with the "gatekeeper" concept where S365 and S368 phosphorylation events are inversely related in vivo (Axelsen etal., 2006). However, works in rat and rabbit hearts showed that S368 is phosphorylated under baseline conditions and actually decreases upon ischemia, indicating PKC interaction with Cx43 may show differences between species (Jozwiak and Dhein, 2008). Despite phosporylation is still the most known and studied post-translational modification related to Cx43 expression, in the last years also acetylation has been involved in GJ regulation. Specificately, in dystrophic hearts, the total HAT activity was significantly increased and paralleled by a relatively high level of PCAF and p300 expression. This alteration was fully corrected by an antioxidant

treatment with Anacardic Acid (AA). The authors demonstrated that Cx43 distribution and function was regulated by N^{ε}-lysine acetylation in the presence of an altered HDAC/HAT balance, which apperared also as associated respectively with PCAF, HDAC3 and HDAC4 (Colussi et al., 2011).

EPIGENETICS

Nucleosome organization and regulation

Epigenetic changes in general refer to stable and heritable modifications of chromatin, the DNA and its associated histone proteins, that are independent of the underlying DNA sequence and that help to determine the phenotypic traits of cells during development (Berger et al., 2009). The core unit of chromatin is the nucleosome, which consists of 147 bp of DNA folded around histone octamers containing two each of the histone proteins H2A, H2B, H3 and H4 (Berger, 2007). Changes in chromatin structure allow (or forbid) specific multi-protein transcriptional regulator complexes to access DNA sequences. Such changes in chromatin structure are achieved essentially by three distinct mechanisms: DNA methylation, histone modifications and ATP-dependent chromatin remodeling (Kouzarides, 2007). Epigenetic modifications of chromatin have generally been considered to be both stable and heritable. However it has been demonstrated which, in post-mitotic cells such as fully differentiated neurons, epigenetic modifications might be highly dynamic (Borrelli et al., 2008). Histones are covalently modified at their N-terminal tails to regulate transcriptional activation and repression. The most studied histone tail modifications are acetylation and methylation, even if histones can undergo numerous other post-translational modifications, such as phosphorylation, ADP-ribosylation, sumoylation, ubiquitylation and proline isomerization (Turner, 2007). In addition to that, histones are often concurrently modified on several residues. Covalently modified histone residues, either alone or in combination, generate distinct docking sites for the recruitment of multiprotein nuclear complexes that influence the structure and function of chromatin (Turner, 2007).The nuclear enzymes that regulate the known post-translational modifications of histone have been identified, including those responsible for histone acetylation and deacetylation, arginine methylation, lysine methylation and demethylation, histone phosphorylation and ubiquitylation (Kouzarides, 2007). The recent discovery of histone deimination, a process that counteracts arginine methylation by converting arginine to citrulline, suggests that the known epigenetic histone modifications can in principle be regulated

dynamically, and thus may contribute to fast adaptive transcriptional responses to extracellular hints. It is conceivable that most, if not all, chromatin-modifying enzymes are targeted by signaling pathways that directly link environmental cues to gene expression.

The Acetylation/Deacetylation balance

Several protein properties are regulated through lysine acetylation, including DNA-protein interaction, subcellular localization, transcriptional activity, stability, and involvement in signaling pathways. Besides, the dynamic state of post-translational protein acetylation is intimately linked to aging and to several major diseases such as cancer, retroviral pathogenesis, neurodegenerative disorders and cardiovascular diseases.At the chromatin level, it has been widely demonstrated that the balance between acetylation and deacetylation of histone and non-histone proteins plays a pivotal role in the regulation of gene expression. The general model of transcription is based on the interaction among RNA Pol II, general transcription factors, coactivators, corepressors, and sequence-specific DNA-binding proteins (DBPs), which confer tissue and signal-dependent specificity. Coactivator and corepressor complexes contain a varietyof chromatin-modifying enzymes, including Histone Acetyltranferases (HATs) and Histone Deacetylases (HDACs). HATs are classified into two different groups, HAT A and HAT B, depending on the mechanism of catalysis and on cellular localization (Table 2).

Mammalian HATs					
Class	Subclass	Homology to yeast	Mammalian members	Mechanism of catalysis	Cell localization
A	GNAT family	Gcn5	GCN5L PCAF	Transfer of acetyl group from acetyl-CoA to ε-NH_2 group of histone N-tails after the assembly into nucleosomes	Nucleus
	MYST-family	Esa1; Sas2; Sas3	Tip60 HBOI MORF MOZ CLOCK NCOAT MOF		
	Others	HAT1; Elp3; Hpa2; Nut1	p300/CBP TFIIIC complex ACTR/SRC-1 ATF-2		
B		Hat1	HAT1	Transfer of acetyl group from acetyl-CoA to ε-NH_2 group of free histones prior to their deposition on DNA	Cytoplasm

Table 2: Mammalian members of HAT family (adapted from Gregoretti et al., 2004).

The members of the HAT A family are found in the nucleus, where they transfer the acetyl group from Acetyl-CoA to anε-NH2 group of histone N-tails after the assembly into nucleosomes. The HAT A family can be further divided into three subclasses depending on the homology with yeast proteins. Conversely, the members of the HAT B family act in the cytoplasm and transfer the acetyl group from Acetyl-CoA to anε-NH2 group of free histones prior to their deposition on the DNA.

HDACs can be grouped into four classes in relation to their phylogenetic conservation(Gregoretti et al., 2004, Table 3). Class I, class II, and class IV, which are related to the yeast Rpd3, Had1, and Hos3 proteins, respectively, encompass the classical family of zinc-dependent HDACs, while class III consists of the NAD+-dependent yeast Sir2 homologues, which comprise the sirtuin family (De Ruijter et al., 2003).

Mammalian HDACs				
Class	Homology to yeast	Mammalian members	Mechanism of catalysis	Cell localization
I	Rpd3	HDAC1 HDAC2 HDAC3 HDAC8	Zn^{2+} ion dependent	Ubiquitous
II	Hda1	HDAC4 HDAC5 HDAC7 HDAC9 HDAC6 HDAC10	Zn^{2+} ion dependent	Shuttle between nucleus and cytoplasm
III	Sir2	SIRT1 SIRT2 SIRT3 SIRT4 SIRT5 SIRT6 SIRT7	NAD^{+} dependent	Nucleus Cytoplasm Mitochondria Mitochondria Mitochondria Nucleus Nucleus
IV	HOS3	HDAC11	Zn^{2+} ion dependent	Nucleus

Table 3: Mammalian members of HDAC family (adapted from Gregoretti et al., 2004).

In eukaryotes, HATs and HDACs are involved in several aspects of cellular homeostasis. For example, in yeast, the HAT Gcn5 is required for the regulation of various cellular processes such as cell response to stress, meiosis, and DNA replication (Xue-Franzen et al., 2010). In mammals, the HAT p300/CBP plays a pivotal role in cell growth, myotube differentiation, and apoptosis (Simone et al., 2004). Additionally, PCAF, anHAT enzyme originally identified as a p300/CBP-binding

protein, is known to play a key role in regulating myofilament contractile activity, the myogenic program, and adipocyte proliferation (Gao et al., 2010). The G1-S phase progression in the cell cycle is mediated by class I HDACs; homologous recombination involves members of the sirtuin family; members of HDACs are found in complexes with transcriptional repressors in multipotent neural progenitor; HDACs play a role in the prevention of cytoxicity arising from protein aggregation in neural cells (Hageman et al., 2010; Uhm et al., 2010).Under normal conditions, the availability and enzymatic activities of HATs and HDACs are maintained in a balance, with the degree of chromatin acetylation contributing to either transcriptional repression or transcriptional activation. It has generally been assumed that the activation of HATs and the activation of HDACs have mutually exclusive effects on gene expression. However, a recent genome-wide analysis of promotor occupancy in human lymphocytes has revealed that both types of enzyme are found at transcriptionally active genes associated with acetylated histones and phosphorylated RNA polymerase II (PolII) (Wang et al., 2009). Genes that were transcriptionally inactive but were poised for activation (bearing the epigenetic H3K4 histone methylation mark) were maintained in an inactive state by dynamic and transient cycles of histone acetylation and deacetylation. Pharmacological inhibition of HDAC activity correlated with increased binding of PolII to gene promoters, suggesting that the main mechanism by which HDACs maintain poised genes in an inactive state might be by inhibiting the recruitment of Pol II. By contrast, silent genes, which had little or no H3K4 methylation, were not bound by HDACs and HATs and remained repressed when exposed to extracellular stimuli. These findings thus suggest that HATs and HDACs can collaborate in certain scenarios, and also that stimulus-dependent activation of one class of enzyme does not necessarily need to be coupled with the inhibition of its functional counterparts. These data give further complexity to the general model of gene expression, suggesting that the dynamic cycle of acetylation and deacetylation by the transient binding of HATs and HDACs may set up primed genes for future activation. Histone modifications, together with factors responsible for adding, interpreting, and removing epigenetic marks, regulate specific responses of the eukaryote genome, and this represents the basis of the "histone code hypothesis." Indeed, epigenetic marks are sites

of recognition for specific readers and effectors. In the case of acetylation marks, certain modified lysines represent specific binding surfaces for bromodomain-containing proteins, which are part of large complexes controlling chromatin architecture.The cellular and physiological functions of lysine acetylation are not limited to the regulation of gene expression. Lysine acetylation assumes a wider significance in many physiological processes, as it also targets non-histone proteins. Interestingly, more than 500 acetylated unique proteins with multiple acetylation sites were categorized as being involved in chromatin-templated processes. Following the identification of additional localizations of HATs and HDACsin other cell compartments, a search for new targets has begun with the aim of determining potential novel biologic functions of these enzymes. As observed for histone acetylation/deacetylation, the dynamic balance in the acetylation of nonhistone proteins seems to bemaintained by a physical and functional interplay between HAT and HDAC activities. The maintenance of an undifferentiated state requires that chromatin architecture sustains the silencing of target genes involved in lineage progression. This implies an acetylation balance strongly shifted towards deacetylation. The opposite occurs during lineage progression, when these genes need to be activated, and thus the balance must be weighted towards acetylation. Any modification of this equilibrium will interfere with the proper execution of the proliferating/differentiating program and may contribute to thedevelopment of a pathologic condition. Hence, HATs and HDACs play a pivotal role in the differentiation/proliferation balance of several cells and tissues. Several studies conducted to decipher histone acetylation and deacetylation dynamics suggested that the simultaneous presence of HATs and HDACs and their physical interaction play a key role in the regulationof the acetylation balance(Yamagoe et al., 2003, Figure 4).

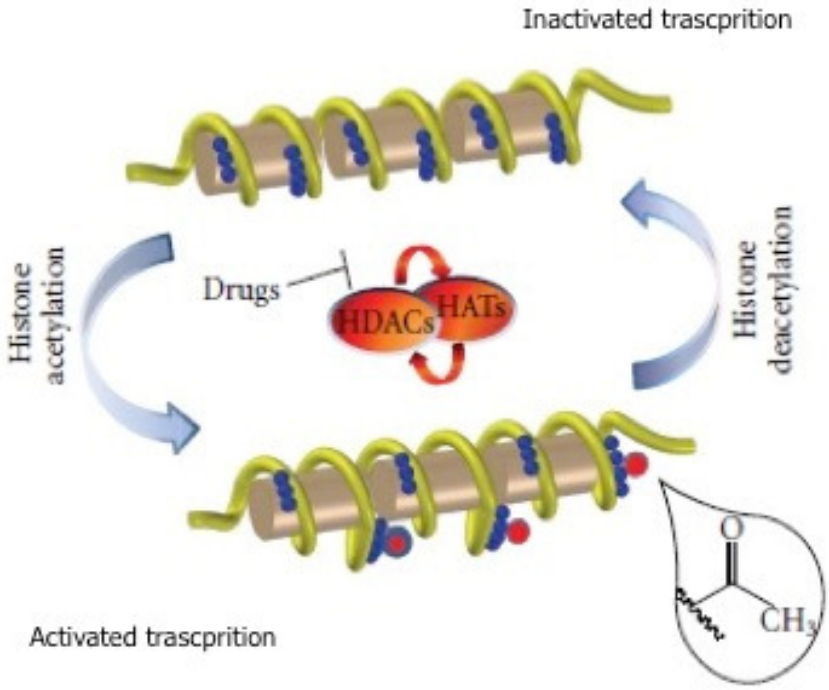

Figure 4: Physical and Functional interaction between HATs and HDACs regulates gene expression (adapted from Yamagoe et al., 2003).

Increasing evidences indicate that the interaction between HATsand HDACs occurs in a dynamic fashion depending on the physiological conditions of the cell. Thus, acetylation homeostasis has to be considered intimately linked to cell homeostasis, and global changes in epigenetic modulators are important in the genetic reprogramming during cell proliferation or differentiation. Several studies shed a new light on the importance of HATs and HDACs balance, in fact has been shown that this balance could control the stability of various proteins such as p53 (Luo et al., 2000), *β*-catenin (Ge et al., 2009), and SMAD7 (Simonsson et al, 2005), thereby modulating the signaling pathways in which these proteins are involved (Sadoul et al., 2008). In addition, it has been demonstrated that HAT/HDAC balance plays a key role in DNA replication, recombination, and repair directly regulating the stability of WRN, a multifunctional protein responsible for these processes; moreover, it regulates proteins involved in nucleocytoplasmic shuttling, such as Importin *α*, or in translocation to the nucleus (Bannister et al., 2000) and finally it regulates protein misfolding and aggregation associated with cytoxicity (Boyault et al., 2007). The interplay

between HDACs and HATs is also linked to adipocyte differentiation. Downregulation of HDAC1 activity results in preferential histone hyperacetylation at the promoter regions of adipocyte marker genes. Specifically, HDAC1 directly interacts with PPAR*γ*, themaster adipogenic factor, and represses its transcriptional activity. Thus, the downregulation of HDAC1 promotes PPAR*γ*activity by relieving it from repression. A very similar mechanism occurs during osteoblast differentiation suggesting that the modulation of HDAC expression and activity may be a general way of regulating cell differentiation (Eung et al., 2006). Additionally, a recent work showed that neuronal outgrowth is driven by intrinsic and extrinsic factors ultimately affecting the balance between HAT and HDAC activities. Indeed, the addition of TSA leads to hyperacetylation of specific pro-neuronal outgrowth gene promoters. This suggests the presence of a positive feedback loop initiated by the relative increase in acetyltransferase activity through HDAC inhibition. This leads to histone hyperacetylation and activation of the CBP, p300, and PCAF promoters. p300/CBP and PCAF in turn promote p53 acetylation which plays a key role in neuronal outgrowth (Gaub et al., 2010). The genetic reprogramming driving neuronal and ologodendrocyte lineage progression depends on the interplay between pluripotency-associated factors and epigenetic modulators. Thus, the acetylation balance plays a pivotal role in this process together with the histone trimethylation pattern. Several works showed that adult multipotent neural progenitor cells differentiated predominantly into neurons in the presence of the HDAC inhibitor valproic acid (VPA). VPA treatment also actively suppressed glial differentiation, even in conditions favoring lineage-specific differentiation (Hsieh et al., 2004). Moreover, the progressive restriction of cell lineage during differentiation from multipotent neural stem cells to oligodendrocyte progenitors (OPCs) is characterized by the progressive decrease of genes such as Sox2 (pluripotencyassociatedfactor) and chromatin modifications on astrocytic and neuronal genes that are initiated by the activity of HDACs and are antagonized by Brca1 and Brm (Liu et al., 2007). The alteration of the HAT/HDAC balance may revert committed progenitors to multipotent cells displaying Sox2 expression (Shen et al., 2008). The equilibrium between HATs and HDACs is also a nodal point in cell proliferation processes. It is well known that p300/CBP are involved in cell cycle control by regulating the

transition from the G1 to the S phase. Indeed, cells lacking p300 activity display proliferation defects(Ait-Si-Ali et al., 2000).

AIM OF THE STUDY

During the last decades connexins have been assumed an emerging and crucial role in maintaining the correct functionality of cardiac muscle. Indeed, heart could be briefly represented as a mechanical pump in which electric stimuli interact with mechanical ones resulting overall in the contraction of the muscle, allowing thus blood to flow onto each district of the organism and viceversa. Moreover, numerous evidences indicate that exposure to electric fields (EFs) have an impact on different cell functions. It is in fact well recognized that processes such as cell migration (Guo et al., 2010), development (McGaigh and Zhao, 1997), wound healing, nerve growth, angiogenesis (Song et al., 2004) and metastases (Robinson and Messerli, 2003) are modulated by bioelectric signals. Recent reports also indicate that electrical activity can have a direct impact on transcriptional response of excitable cells. Activity dependent regulation of chromatin modifying enzymes such as HATs and HDACs has been described for neurons, where depolarizing stimuli can lead to the activation of the HAT CBP by increasing intracellular Ca^{2+} concentration via the activation of L-type Ca^{2+} channels (Riccio,2010; Hardinghamet al., 1999).

From the point of view of cardiac cell function, cell pacing can promote the transcriptional regulation of genes such as GATA4, Adss1 and (adenylosuccinate synthetase 1)(Xia et al., 2000),NFAT3. Further, high frequency stimulation has been shown to induce the hypertrophic growth (McDonoug and Glembotsky, 1992; Ivesteret al., 1993) of cultured cardiomyocytes (CMs).

Of note, altered expression and/or distribution of Cx43, which is the major component of GJs connecting working CMs (Lin et al., 2010), has been described in case of cardiac rhythm disturbances, such as atrial fibrillation (AF) and tachycardia-associated remodeling (Molica et al., 2014). Therefore, being able to promote Gap Junctions (Nakashimaet al., 2014) and electrical remodeling (Brundelet al., 2004), tachypacing of cultured CMs has also been used as a model of *in vitro* AF (Zhanget al., 2014), although prolonged stimulation time (8 – 24 hours) might induce calcium overload and consequent cell damage (Brundel et al., 2004).

Importantly, one recent work has shown that tachypacing causes CM loss of function and electrical remodeling partly through HDAC6 activation and subsequent deacetylation-induced depolymerization of alpha-Tubulin. Nevertheless, the activation of epigenetic enzymesfollowing

electrical stimulation, and more specifically their action on cytoplasmic substrates, is a phenomenon yet poorly investigated. Recent works have shown that HDAC4 and PCAF play a role in the acetylation-dependent regulation of cardiac myofilament contraction (Gupta et al., 2008). Further, lysine acetylation alters GJ-protein Cx43 expression and intracellular distribution, thus possibly impacting cell to cell communication and cardiac function (Colussiet al., 2011).

In this context, aim of the present work was to assess whether electrical stimulation could impact GJ remodeling and function through acetylation/deacetylation based mechanisms.

METHODS

HL-1 cardiomyocyte culture

HL-1 mouse atrial cardiomyocytes (Claycomb et al., 1998) were kindly donated by William Claycomb (Louisiana State University, New Orleans) and cultured in Claycomb medium (all from Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 4 mM L-Glutamine, 100 U/mL Penicillin, 100 mg/mL Streptomycin, 0.3 mM Ascorbic Acid and 10 mM Norepinephrine as previously described (Claycomb et al., 1998). Cells were plated onto gelatin/fibronectin-coated 35 mm Petri dishes on a density of 10000 cells/cm^2. After 48 hours cells reached approximately 90% confluence and were used for subsequent pacing experiments.

Pacing Conditions

HL-1 cells were stimulated at 0.5 Hz for 24 hours in with a C-Pace EP equipped with a C-Dish able to accommodate six 35 mm Petri dishes (IonOptix Corp). A biphasic square-wave stimulus was chosen in order to minimize electrolysis at the electrodes (Berger et al., 1994). Pulse duration and width were set at 5 msec and 20V respectively, as with this combination it was possible to capture all beating areas evident by microscopic inspection.

Administration of Verapamil (Ver, 10 μM, Sigma-Aldrich), Anacardic Acid (AA, 0.5 μM, Sigma-Aldrich) and MG132 (10 μM, Sigma-Aldrich) was performed once just before starting electric stimulation. Not stimulated cells (NS) and stimulated cells (St) with respective treatments (NS+Ver, NS+AA and NS+MG132) were considered as controls.

Whole Cell I-Clamp

Spontaneous action potentials (APs) were recorded in single HL-1 cell using the patch-clamp technique in perforated-patch configuration (Amphotericin -B method(Cerbai et al., 1994). Cells were placed in an experimental bath on the stage of an inverted microscope (Nikon Eclipse Ti) and superfused with normal Tyrode's solution containing in mM: 132 NaCl, 4 KCl, 1.2 $MgCl_2$ 10 HEPES, 1.8 $CaCl_2$ and 11 Glucose (pH 7.35, NaOH). APs were measured with a patch amplifier (Multipatch 700B; Molecular Devices Inc.), signals were digitized via a DAC/ADC interface (Digidata 1550; Molecular Devices Inc.) and data acquired by means of pCLAMP 10.1 software (Molecular Devices

Inc.).Patch-clamp pipettes, prepared from glass capillary tubes by means of a horizontal puller (Flaming / Brown micropipette puller, model P-1000; Sutter Instrument) had a resistance of 2.5-3.5 MΩ when filled with the internal solution containing (in mM): 115 K Methanesulfonate, 25 KCl, 10 HEPES, 3 $MgCl_2$, 0.13 Amphotericin B (pH 7.00 KOH). Temperature was maintained at 36.5 ± 0.5°C throughout the experiment.

Calcium transient recording

HL-1 cells were loaded with 5μM Fluo-4-AM (Molecular Probes) at room temperature for 15 minutes. Calcium transient were recorded by imaging-video acquisition system, by using the same epifluorescence microscope used for APs recordings equipped with a 75 W Xenon lamp and connected to a CCD camera (Cool SnapTM EZ Photometrics). Cells were excited at 488 nm wavelength and fluorescence emission was measured at 520 nm and recorded by means of MetaFluor Software (Molecular Devices).Imaging was scanned repeatedly at 80 ms intervals. Cells were superfused with normal Tyrode's solution and temperature was maintained at 36.5 ± 0.5°C throughout the experiment.

V-FITC and Propidium Iodide Assay

The apoptotic effectof chronic pacing on HL-1 cells was evaluated using annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (Annexin V/PI)assay (R&D Biosciences). HL-1 cells were seeded onto six-well plates at a density of approximately 5×10^5 cells per well and allowed to attach for 24 h. The cells were then electrically stimulatedfor 24 hours. Paced cells were collected, washed twice with ice-cold PBS, and re-suspended in 100 μL of 1× binding buffer before being incubated with 5 μL Annexin V-FITC (Signalway Antibody) according to the manufacturer's protocol. After 15 min of incubation at RT in the dark, 10 μL of 20 μg/mL PI was added to each tube, and the cells were gently re-suspended. Next, 400 μL PBS was added to each tube. The number of stained cells was quantified using a flow cytometer.

Neonatal Rat Ventricular Cardiomyocyte (NRCM) Isolation

48 hours neonatal rats were anesthesized by inhalation of isoflourane (99.9% from Vedco, St. Joseph, MO 64507), hearts were collected and put into ice-cold PBS, where atriums were removed and residual ventricles were cut into small pieces. Heart fragments were then digested four times, 20 min each at 37 °C, using 30 ml of enzyme digestion solution containing 136.8 U/ml collagenase CLS I (Worthington), 0.6 mg/ml pancreatin (Sigma-Aldrich) in ADS buffer (116.4 mM NaCl, 5.4 mM KCl, 5.5 mM Glucose, 1 mM NaH_2PO_4 H_2O, 0.8 mM $MgSO_4$ H_2O, 20 mM Hepes, pH 7.4). NRCMs were purified by using a Percoll (Sigma-Aldrich) step gradient and centrifuging at 3000 rpm for 30 min at room temperature in a swinging bucket rotor, switching-off the centrifuge brakes. Fibroblast cells, in the upper phase,were removed by aspiration and NRCMs were collected and washed in ADS buffer. The myocytes were finally resuspended in plating medium (4:1 DMEM:M199, 10% horse serum, 5% fetal bovine serum, 1% Sodium Pyruvate, 10000 U/ml Penicillin (Invitrogen), 10000 μg/ml Streptomycin (Invitrogen) and 20 mM L-Glutamine (Sigma-Aldrich) and plated at a density of 80000 cells/cm^2 on dishes coated with 0.1% gelatin. The day after, the plating medium was substituted by the maintenance medium (4:1 DMEM:M199, 5% horse serum, 5% fetal bovine serum, 1% Sodium Pyruvate, 10 mM cytosine arabinoside (Ara-C, Sigma-Aldrich), 10000 U/ml Pennicilin (Invitrogen), 10000 ug/ml Streptomycin (Invitrogen) and 20 mM L-Glutamine (Sigma-Aldrich). The purity of cultured NRCMs was approximately 90% when the number of cell nuclei (DAPI)-positive) was compared with the number of myocytes (Troponin-C-positive).

Adult Human Stromal Cell (hCStC) Isolation

Human auricle fragments were obtained from patients that underwent either coronary bypass or valve substitution following Local Ethic Committee approval and signed informed consent in accordance with the declaration of Helsinki. Fragments were minced and incubated for 45 min at 37 °C with a PBS solution Ca^{++} and Mg^{++} free containing 1 mg/ml Collagenase II (Worthington, Biochemical Corporation, Lakewood, USA) and 0.2% Bovine Serum Albumin (BSA, Sigma-Aldrich,

Milan, Italy). After digestion, the solution was passed trough nylon filter (70 µm, BDBiosciences, Milan, Italy) and centrifuged for 10 min at 1300 rpm. The pellet was suspended in culture medium and cells placed in an humidified incubator gassed with 5% CO_2 at 37 °C. After 2–3 h, adherent cells were mainly composed by fibroblasts and non-adherent cells were removed. Cardiac fibroblasts (cFbs) were cultured either with complete Endothelial Growth Medium-2 (EGM-2, Cambrex, Milan, Italy) or Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Milan, Italy): F12 (Invitrogen) 1:1 containing 10% Fetal Bovin Serum (FBS, HyClone, Logan, USA), supplemented with 10.000 U/ml Penicillin, 10.000 µg/ml Streptomycin (Invitrogen) and 20 mM l-Glutamine (Sigma-Aldrich).

Western Blot Analysis

Whole cells lysates were obtained harvesting cells after stimulation and treatments with Laemmli buffer containing the phosphatase inhibitors NaF (10 mM) and Na_3VO_4 (0.4 mM) and a protease inhibitor cocktail (all from Sigma-Aldrich). The protein concentration was determined using the Bio-Rad protein assay reagent, following manufacturer's instructions. Subsequently, 30 µg of protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare) in 20 mM Tris-HCl (pH 8.0), containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% non-fat dry milk in 1× PBS containing 0.05% Tween 20 (PBS-T) for 1 hour at room temperature and incubated overnight in the same solution at 4°C with antibodies against total Cx43 (1:5000, Abcam), pS368Cx43 (1:500, Santa Cruz), anti-acetyl lysine (pan-Ac-K) (1:1000, Abcam), Ac-α-tubulin (1:1000, Abcam), α-tubulin (1:2000, Sigma-Aldrich), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000 Sigma-Aldrich), anti-Histone H3 (H3, 1:2000, Abcam) and β-actin (1:5000, Sigma-Aldrich). Anti-pS373Cx43 was produced as previously described (Lampe et al., 2004). Membranes were washed three times in 1× PBS-Tween buffer, followed by the incubation with the appropriate HRP-linked IgG for 1h at room temperature. Specific proteins were then visualized using the enhanced chemiluminescence (ECL) detection kit (Supersignal West Dura Extended Duration Substrate, ThermoScientific). Results of Western

blotting were quantified either by ImageJ v1.28 or Uvitec analyzer softwares. Optical density values were internally normalized on α-tubulin, GAPDH, H3 or β-actin expression.

Real Time RT-PCR

For gene expression analysis, total RNA was extracted using TRIzol reagent and 1 µg of RNA was reversely transcribed using SuperScript® VILO cDNA Synthesis Master Mix (Invitrogen). cDNA was amplified by SYBR-GREEN quantitative PCR on CFX96™ Real-Time PCR Detection System (Bio-Rad). Five candidate housekeeping genes, among several commonly used ones, were investigated in order to select the most stable in HL-1 cell model: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-Actin (ACTB), hypoxanthine guanine phosphoribosyl transferase (HPRT), TATA box binding protein (TBP) and β-2 microglobulin (B2M). NormFinder software (Andersen et al., 2004) was used to identify the optimal normalization gene among the selected candidates and TBP resulted as the most suitable housekeeping gene for Connexin-43 (Cx43) expression study. The following primers for mouse Cx43 and TBP were used:

TBP fw	5′-TCAAACCCAGAATTGTTCTCC-3′
TBP rev	5′-AACTATGTGGTCTTCCTGAATCC-3′
Cx-43 fw	5′-ACTCCTGTACTTGGCTCACGT -3′
Cx-43 rev	5′-CCTTGCCGTGTTCTTCAATCC -3

Raw expression intensities of Cx43 were normalized to the Ct value of TBP, chosen as internal control. Relative quantitation was performed using the ΔΔCt method. Fold changes in gene expression were estimated as $2^{(-\Delta\Delta Ct)}$ (Livakand Schmittgen, 2001).

Cell Immunofluorescence

HL-1 cells were fixed in 4% PFA for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min at room temperatire. After blocking for 1 h at room temperature with PBS containing 5% goat serum, cells were incubated overnight at 4°C with a rabbit polyclonal anti connexin-43antibody (AbCam) (dilution 1:400) in PBS/2% goat serum. The day after, cells were washed three times with PBS and then incubated for 1 h at 37°C with the FITC-conjugated goat anti-rabbit secondary antibody (dilution 1:200) diluted in PBS/2%goat serum. Nuclei were counterstained with Hoescht 33342 (Sigma-Aldrich). Epifluorescence images were obtained using a Zeiss confocal microscope equipped with a 40X oil objective. Image acquisition was carried out at Room Temperature using the Zen software. Brightness/contrast adjustment was performed in Adobe Photoshop.

HAT & HDAC activity assays

The HAT activity in the nuclear extract was quantified using the colorimetric HAT activity assay kit (BioVision) according to the manufacturer's instructions. Briefly, 50 µg of nuclear extract was incubated with HAT substrates I and II and NADH-generating enzyme in HAT assay buffer for 2 h at 37 °C. Absorbance was determined at 440 nm in an ELISA plate reader. HAT activity was expressed as relative OD values per µg of protein sample.
HDAC activity assay were perfomed using kit from BioVision (BioVision Research Products, Mountain View, CA, USA) according to manufacturer's instructions. Biefly, 50 µg of whole cellular extracts were diluted in 85 µL of ddH_2O; then, 10 µL of 10× HDAC assay buffer were added followed by addition of 5 µL of the colorimetric substrate; samples were incubated at 37° for 1 h. Subsequently, the reaction was stopped by adding 10 µL of lysine developer and left for additional 30 min at 37°C. Samples were then read in an ELISA plate reader at 400 nm. Both the kits contained negative and positive controls.

Dye Transfer assays

Cell-to-cell gap junction mediated communication was determined by two independent fluorescent dye transfer assays:

a) Calcein-DilC12 transfer:

As described previously with minor modifications (Colussi et al., 2011), two independent populations of HL-1 cardiomyocytes were subjected to 24 hours electrical pacing as described above. 4 hours prior the end of the experiment, one cell population was incubated 1h at 37 °C with the DilC12 red fluorescent tracer (25 µg/mL). The second cell preparation was incubated with the Calcein AM green dye (1 h at 37 °C; 1 µM). Green cells were then trypsinized and plated on red cells at a ratio of 1:10.Cells were then allowed to settle and establish cell-to-cell communication under electrical stimulation. Dye transfer was evaluated 6 hours later by confocal microscopy counting the number of double-stained fluorescent cell (yellow) in 20 randomly chosen fields. Experiments were repeated three times in duplicate;

b) Lucifer Yellow transfer:

For each experiments, an equal number of control, electrically stimulated and electrically stimulated plus AA (20nM) dishes were prepared. Lucifer yellow (2 mM in a standard intracellular solution composed by KAsp 122 mM, KCl 20 mM, $MgCl_2$ 1 mM ,HEPES 10 mM, EGTA 5 mM, $CaCl_2$ 1.6 mM, pH 7.3) was injected by a whole cell patch clamp technique (Axopatch Multiclamp 700B amplifier from Axon Instruments, Foster City, California) into clustered HL-1 cells perfused with an extracellular solution containing : NaCl 135 mM, KCl 4.5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1.8 mM, NaH_2PO_4 0.4 mM, HEPES 10 mM, Glucose 10 mM, pH 7.4. A patch was maintained on the target cell for 15 minutes to allow the internal pipette solution to diffuse into the patched and surrounding connected cells. Cell-to-cell transfer was monitored using an inverted microscope (NIKON TS100) equipped with fluorescent illumination and imaged were acquired by a digital camera (Infinity 1, Lumenera Corporation). The extent of coupled cells was determined by counting the number of adjacent cells containing the tracer and the time dependence of the spreading of the dye into the recipient cell (ImageJ, v1.28 software).

Canine Model

Heart failure was induced in dog as previously described (Mitacchioneet al., 2014). Briefly, eight adult, male, mongrel dogs (25-27 kg) were anesthetized with propofol (6 mg/kg i.v.) and maintained with 1.5-2% isofluorane during 40% oxygen/60% air ventilation. A thoracotomywas then performed in the left fifth intercostal space, a catheterwas placed in the descending thoracic aorta, a solid-state pressure gauge (P6.5; KonigsbergInstruments) was inserted into the left ventricle through the apex, a Doppler flow transducer (Craig Hartley) was placed around theleft circumflex coronary artery and a pair of pacing leads was fixed on the left ventricular (LV) freewall. Wires and catheters were run subcutaneously to the intrascapularregion, the chest was closed in layers and the pneumothorax wasreduced. Antibiotics were given after surgery and the dogs wereallowed to fully recover. After 7-10 days of recovery from surgery, dogs were trained to lie quietly on the laboratory table. Heart failure (HF) was then induced in four dogs by pacing the left ventricle (LV) at 210 beats/min for 3 weeks and at 240 beats/min for an additional week. Dogs were considered in congestive HF when LV end-diastolic pressure was < 25 mmHg, reflected by clinical signs such as dyspnea and ascites. Four chronically instrumented dogs were used as normal control. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Temple University and conform to the guiding principles for the care and use of laboratory animals published by the National Institutes of Health.

Confocal Histological analysis

Cryosections from control and tachypacing-treated dogs were used for immunofluorescence analysis. After fixation with 4% paraformaldehyde for 10 min, sections were washed with PBS and incubated for 1h with 10% BSA/PBS to block non-specific protein-binding sites and overnight at 4°C with anti Cx43 antibody (1:400, Abcam). After a brief rinse, sections were incubated with FITC-conjugated secondary antibody (1:200, Jackson). Images were acquired with a confocal laser scanning system (TCS-SP2, Leica Microsystems) equipped with an Ar/ArKr laser for 488-nm excitation and HeNe laser for 543-nm excitation. DAPI staining were imaged during two-photon

excitation (740 nm, <140 fs, 90 MHz) performed with an ultrafast, tunable, mode-locked Ti: Sapphire laser (Chameleon, Coherent, Inc.).

Co-Immunoprecipitation assays

HL-1 protein extract for immunoprecipitation were obtained after lysis in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Igepal CA630, 1% sodium deoxycholate (DOC), 0.1% SDS and 1% Glycerol supplemented with protease/phosphatase inhibitor mix. Dog tissue proteins were extracted in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2mM $MgCl_2$ and 1% DOC supplemented with 1 mM PMSF and protease inhibitor mix). Identification of acetylated Cx43 was performed by immunoprecipitation using the antibody anti-pan-Ac-K (4 µg for 500 µg of total proteins, Abcam) and anti-Cx43 (1:1000, Abcam). The Ademtech's Bioadembeads paramagnetic beads system was used to immunoprecipitate the specific proteins according to the manufacturer's instructions. Negative controls were performed with the same amount of protein extract immunoprecipitated with the corresponding purified IgG antisera (SantaCruz) in absence of primary antibody. Immunoprecipitated samples were resolved by SDS–PAGE, transferred onto nitrocellulose membrane (GE Healthcare) and western blot performed as described above.

Statistical analysis

Data represent the means of at least three independent experiments ± SEM. Two-tailed Student t test was used to calculate the significance (GraphPad Prism 5.0). A P-value <0.05 was considered statistically significant.

RESULTS

Validation of HL-1 cell pacing model

The spontaneous activation frequency of HL-1 cells at the density used in this series of experiments (Figure 5) was approximately 0.5 Hz as measured using whole cell I-clamp (Figure 5A) and as seen by spontaneous calcium transients (Figure 5A). After 24 hours of field stimulation at 0.5 Hz HL-1 cells did not show morphological changes (Figure 5B) nor evident signs of sufferance; moreover, cells did not exhibit viability loss (Figure 5C). Higher stimulation frequencies, although closer to *in vivo* mouse cardiac frequency, were not chosen in the present work as viability significantly decreased in HL-1 cells exposed to 5 Hz field stimulation for 24 hours (data not shown), likely because of myolisis induced by calcium overload (Brundelet al., 2004).

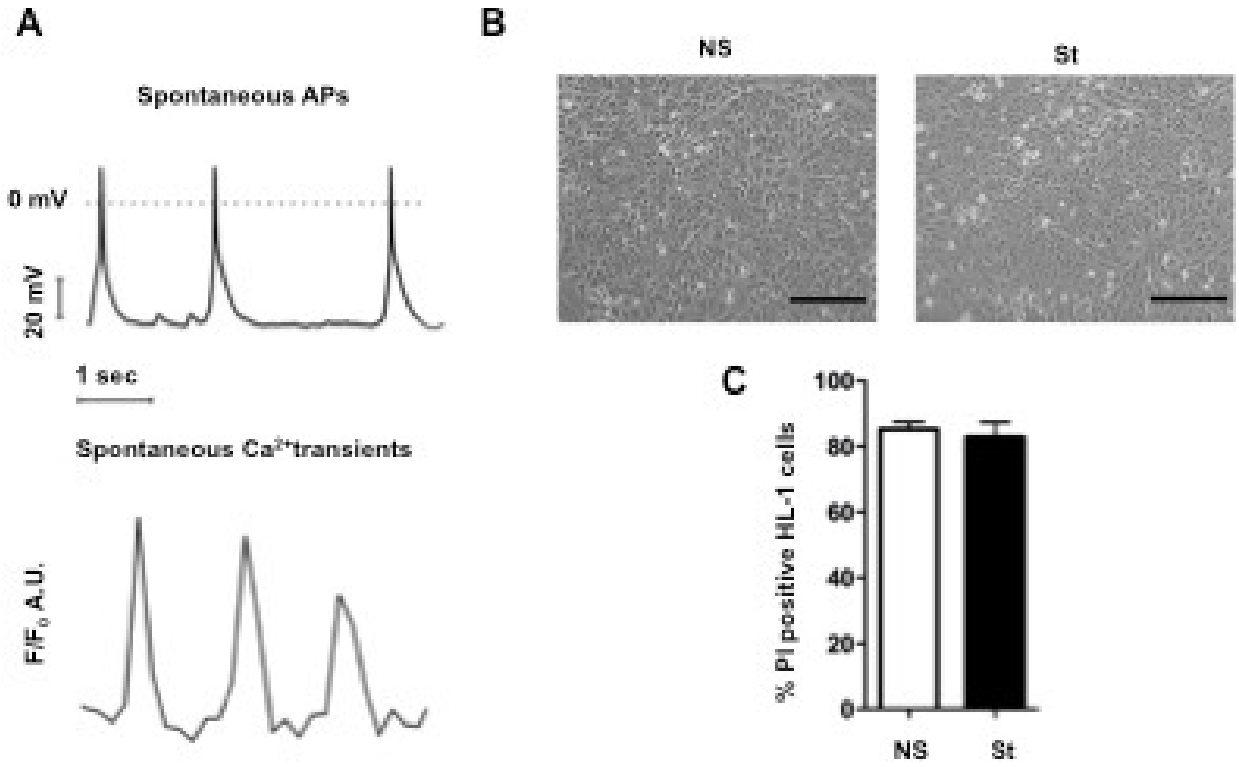

Figure 5: Effects of chronic pacing on HL-1 cells.**A:** Spontaneous Action Potentials measured by perforated-patch technique in single HL-1 cell and spontaneous calcium transient measured by fluorescence intensity oscillations in Fluo-4 AM-loaded HL-1 cells. **B:** Electrical stimulation did not show any effect on HL-1 cells morphology (scale bars: 50 μM). **C:** PI staining on NS and St HL-1 cells did not reveal any change on cell viability after pacing.

Cx43 expression and intracellular localization was altered by electrical stimulation

Pacing of HL-1 cells at their intrinsic frequency caused a significant reduction in the expression of Cx43 protein (Figure 6A). The expression of Cx40, Cx45 and of TroponinT-c (TnT-c)cardiac marker remained unchanged (Figure 6A). Significant Cx43 reduction was also observed in neonatal rat cardiomyocytes (NRCMs) exposed to the same experimental conditions, but not in human cardiac fibroblasts (hCStCs) (Figure 6B). NRCMs were captured by 0.5 Hz stimulation and exhibited normal contractile behavior during and after 24 hour treatment. Of note, electrical pacing did not induce cell death nor evident morphological change neither in NRCMs (Figure 6C) nor in hCStCs (data not shown).Confocal analysis confirmed Cx43 down-modulation in stimulated cells (Figure 6D), revealing that Cx43 also delocalized from cell membrane and accumulated in the cytoplasm mainly in the peri-nuclear region. The pacing induced reduction of Cx43 expression was prevented when the L-Type Ca^{2+} blocker Verapamil was added to HL-1 cardiomyocytes during electrical stimulation (Figure 6E).

Treatment of HL-1 cells with the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) and Trichostatin A (TSA) mimicked the effect of electrical stimulation on Cx43 protein expression (Figure7A) and localization (Figure 7B and Colussi et al., 2011), thus suggesting the hypothesis that electrical stimulation could promote Cx43 acetylation.

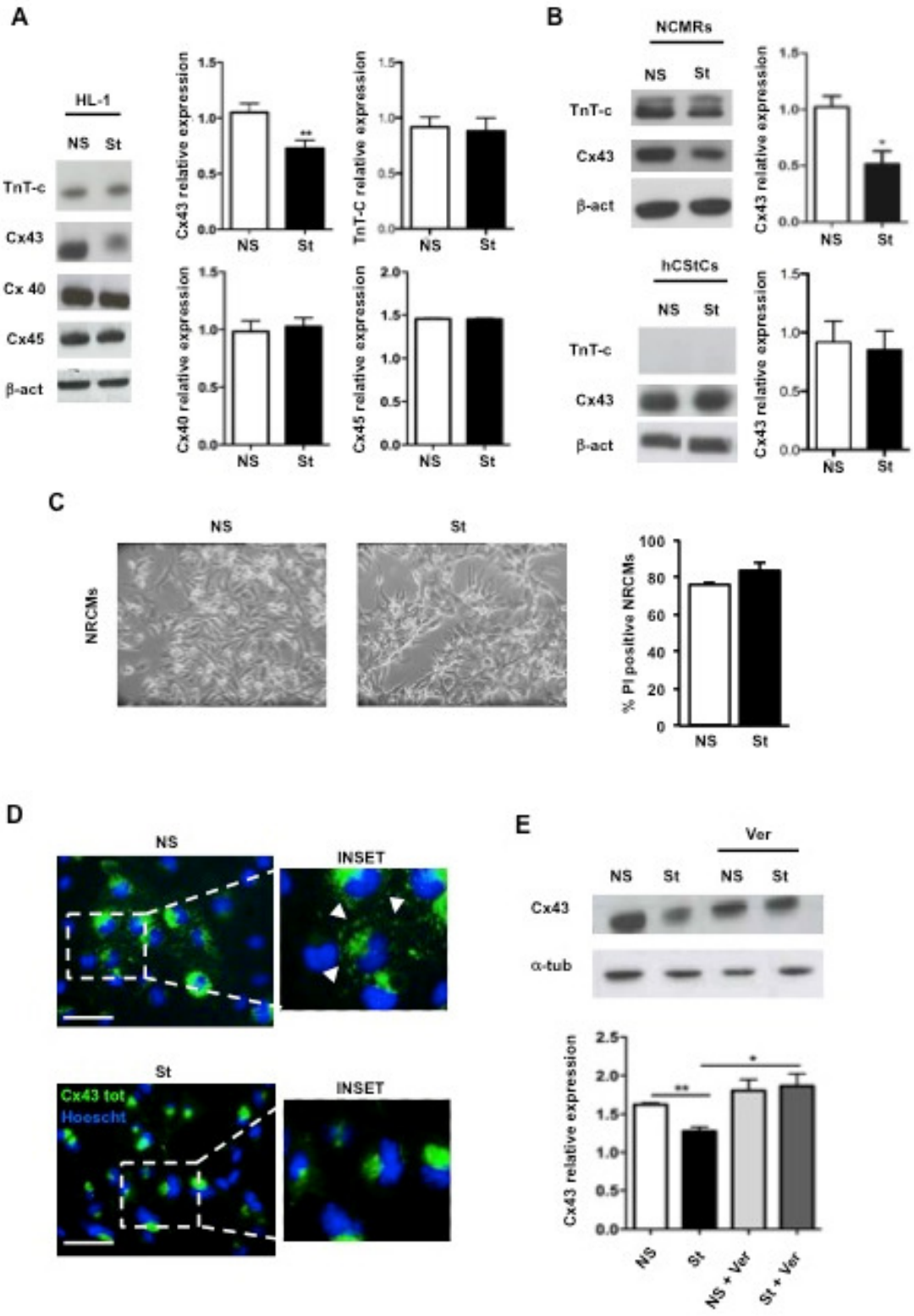

Figure 6: Effects of chronic pacing on Cx43 expression and localization.**A:** Western Blot analysis of NS and St HL-1 cellsshowed significant reduction of Cx43 expression (n=20) while Cx45 and Cx40 remained stable, as well as TnT-c protein (n=3). **B:** Western Blot analysisconfirmed the net decrease of Cx43 expression in NCRMs after stimulation, while remained stable in hCSTC non-excitable cells (n=3).**C:** Electrical stimulation did not show any significant variation on morphology and mortality of NCRMS after stimulation (scale bars: 50 μM). **D:** Immunofluorescence assays (n=3) performed on HL-1 cells revealed in St cells re-localization of Cx43 from intercalated disks, accumulating in the cytoplasm mainly in the peri-nuclear region (stimulation (scale bars: 50 μM). **E:** Western Blot analysis of NS and St HL-1 cells treated with Verapamil showed rescue of Cx43 expression in St+Ver cells (n=4).

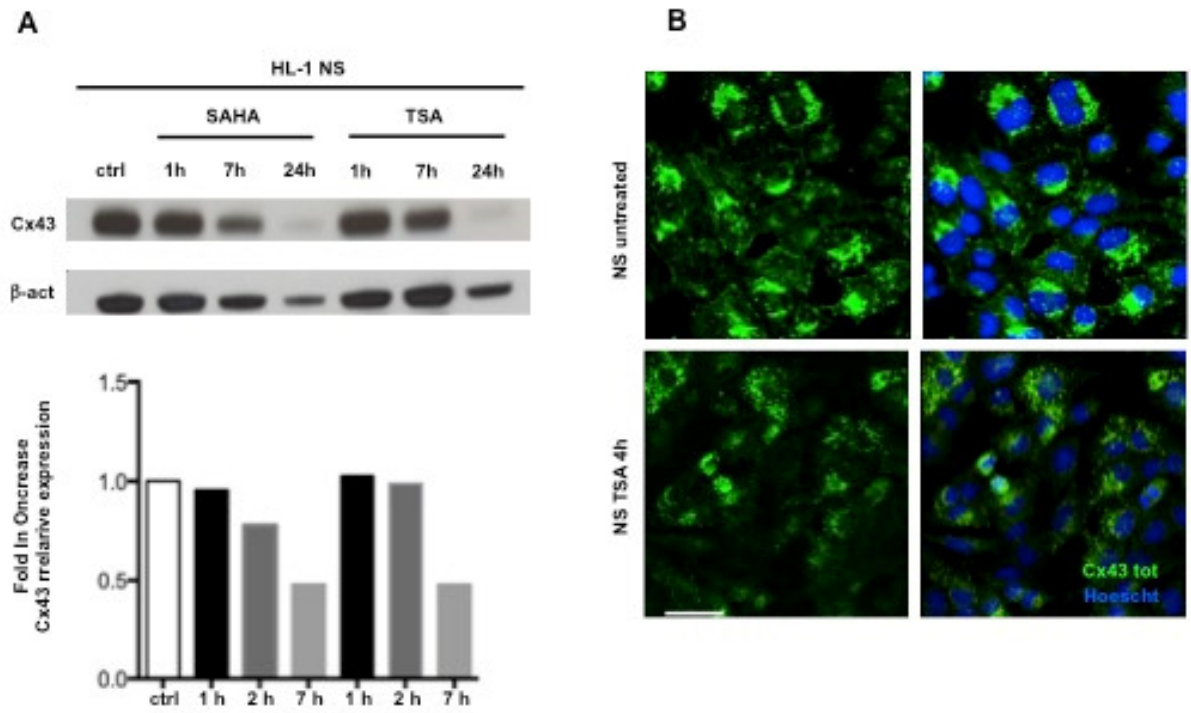

Figure 7: Treatment of HL-1 cells with the HDAC inhibitors SAHA and TSA affected Cx43 expression and localization. **A:** Western Blot analysis of HL-cells treated TSA and mM of SAHA at different time points showed decrease on Cx43 expression (n=3). **B:** Immunofluorescence assays performed on HL-1 cells treated for 4 hours TSA mimicked the effect of the electrical stimulation on Cx43 localization(n=3, scale bars: 50 μM).

Electrical stimulation caused an alteration of the acetylation/deacetylation balance resulting in Cx43 direct acetylation

Electrical stimulation significantly down-regulated HDAC activity up to the 30%, whereas HAT activity was not modified (Figure 8A); the net effect was a general increase of the acetylation of proteins with a molecular mass comprised between 25 and 130 kDa, confirmed by western blot analysis of Ac-α–tubulin (Figure 8B) and pan-Ac-K (Figure 8C). The pacing-dependent acetylation of Cx43 was proven by immunoprecipitation assay (Figure 8D). Notably, Real Time RT-PCR showed that Cx43 mRNA expression was not influenced by electrical pacing (Figure 8E), while observed protein reduction was rescued after the treatment with the proteasome inhibitor MG132 (Figure 8F). Taken together these observations confirmed that pacing-induced Cx43 down-modulation depends on the activation of post-translational mechanisms.

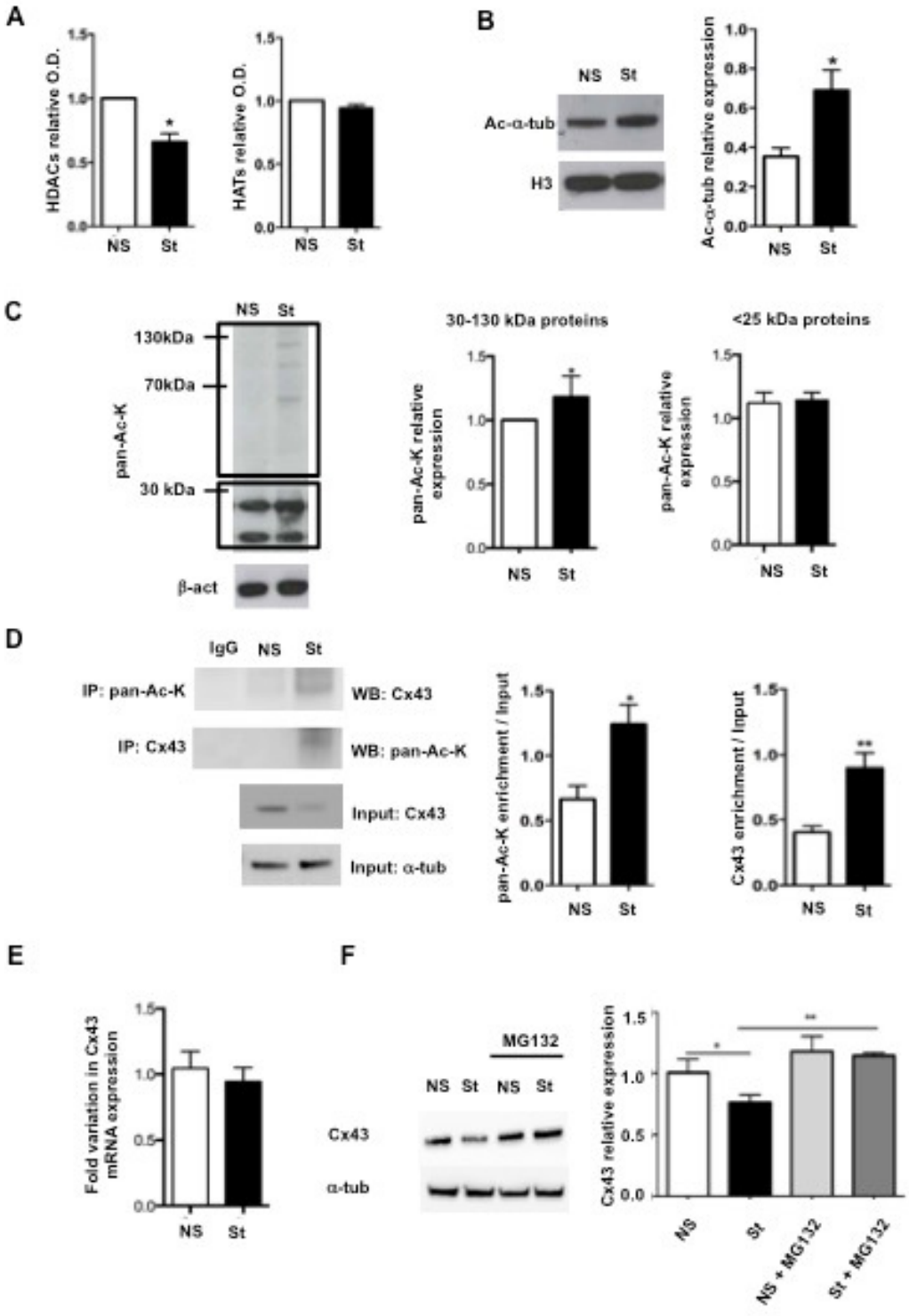

Figure 8: Chronic pacing directly influenced cell acetylation/deacetylation balance and triggered Cx43 specific acetylation. **A:** Schematics of HAT and HDAC activity assays performed on NS and St HL-1 revealed reduction on deacetylases activity (n=3). **B:** Western blot analysis of NS and St HL-1 cells showed an increase of pan-Ac-K only in cytoplasmic proteins (from 25 to 130 kDa) while nuclear proteins (below 25 kDa) expression remained stable (n=4) **C:** Western blot analysis of of NS and St HL-1 cells for ac-a-tub confirmed pro-acetylated trend after chronic pacing. **D:** Immunoprecipitation and western blot analysis of Cx43 expression and acetylation on NS and St HL-1 cells revealed Cx43 acetylation due to electrical stimulation (n=5). **E:** Real Time RT-PCR analysis did not reveal any variation in Cx43 mRNA expression in NS and St HL-1 cells. **F:** Western Blot analysis of HL-1 cells treated with MG132 showed trescue of Cx43 expression in St+MG132 cells (n=4).

Cell Pacing caused impaired cell-cell communication, rescued by AA treatment

Cell-cell communication was first assessed by a fluorescent dye-transfer method (Colussi et al., 2011) based on the use of two cell population labeled by two different dyes, GJ permeant Calcein (green) and Lipophilic and non-GJ diffusible DilC12 tracer (red). After electrical stimulation, the percentage of double positive stained HL-1 cardiomyocytes here used as an index of intercellular signal transmission, was reduced of approximately the 50% (Figure 9A). Comparable results were obtained when the extent of dye coupling between cells was assessed by intracellular microinjection of Lucifer yellow. Also in this case the percentage of coupling, defined as the percentage of injections resulting in efficient dye transfer to the neighboring cell, was significantly reduced in electrically stimulated HL-1 cardiomyocytes (Figure 9B) although the kinetic of the process did not show any variation. Consistently with a global effect of reduced communication GJ-mediated, electrical stimulation also enhanced the net amount of Cx43 phosphorylation at the inhibitory residue S368 (Solanand Lampe, 2007). In parallel, phosphorylation levels of residues S373, which is associated with GJ function up-regulation (Lampe et al., 2004), remained unchanged (Figure 9C).

Importantly, the treatment of stimulated cells with the general HAT inhibitor AA was able to rescue both Cx43 expression and functional GJ-mediated cell coupling, as evident by the recovery of coupling percentage in Lucifer Yellow microinjected cell clusters (Figure 9D).

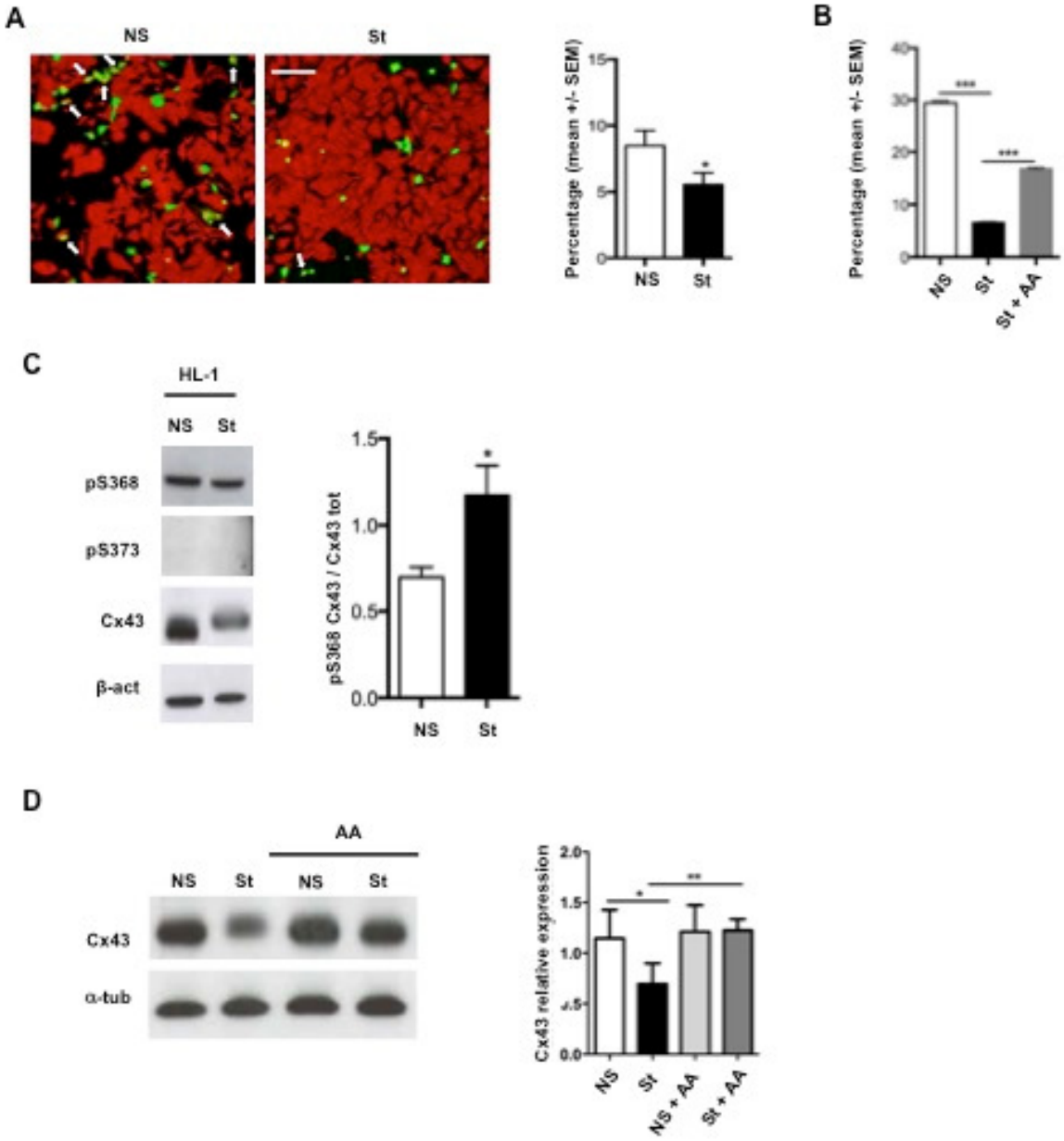

Figure 9: Electrical stimulation led to a reversibile reduction of cell-cell communication. **A:** Evaluation of Calcein AM diffusion through GJs revealed reduction of cell-cell communication in St Hl-1 cells compared with NS. Arrows indicate dye diffusion (yellow) from donor (Calcein AM/green) to recipient cells (DilC12/red). The graph shows the average number of Calcein-AM and DilC12 double-positive cells per field (n=5, scale bars: 50 μM). **B:**Intracellular microinjection of Lucifer yellow confirmed reduction of cell-cell communication due to chronic pacing and rescue of coupling between cells after AA treatment. The graph shows the number of adjacent cells containing the tracer and the time dependence of the spreading of the dye into the recipient cell (n=5). **C:** Western Blot analysis of NS and St HL-1 cells revealed a significant increase of Cx43 phosporylation on the specific inhibitory residue of Ser368 (n=6) after stimulation, while phosporylation on ser373 (n=2) did not occur. **D:** Western Blot analysis of NS and St HL-1 cells treated with 0.5 mM of HAT inhibitor AA showed rescue of Cx43 expression in St+AA cells (n=4).

Cx43 shows acetylation and lateralization in a dog model of tachypacing induced heart failure

In order to assess whether electrical pacing could promote Cx43 acetylation also *in vivo*, we chose to test a canine model of tachycardia-induced cardiomyopathy. Although Western Blot analysis showed that Cx43 expression remained unchanged (Figure 10B), confocal analysis of left ventricle thin sections demonstrated that tachypacing was associated with extensive Cx43 dissociation from intercalated disks and extensive lateralization (Figure 10A). Immunoprecipitation experiments confirmed that Cx43 acetylation increased in dogs with heart failure induced by tachypacing (Figure 10C).

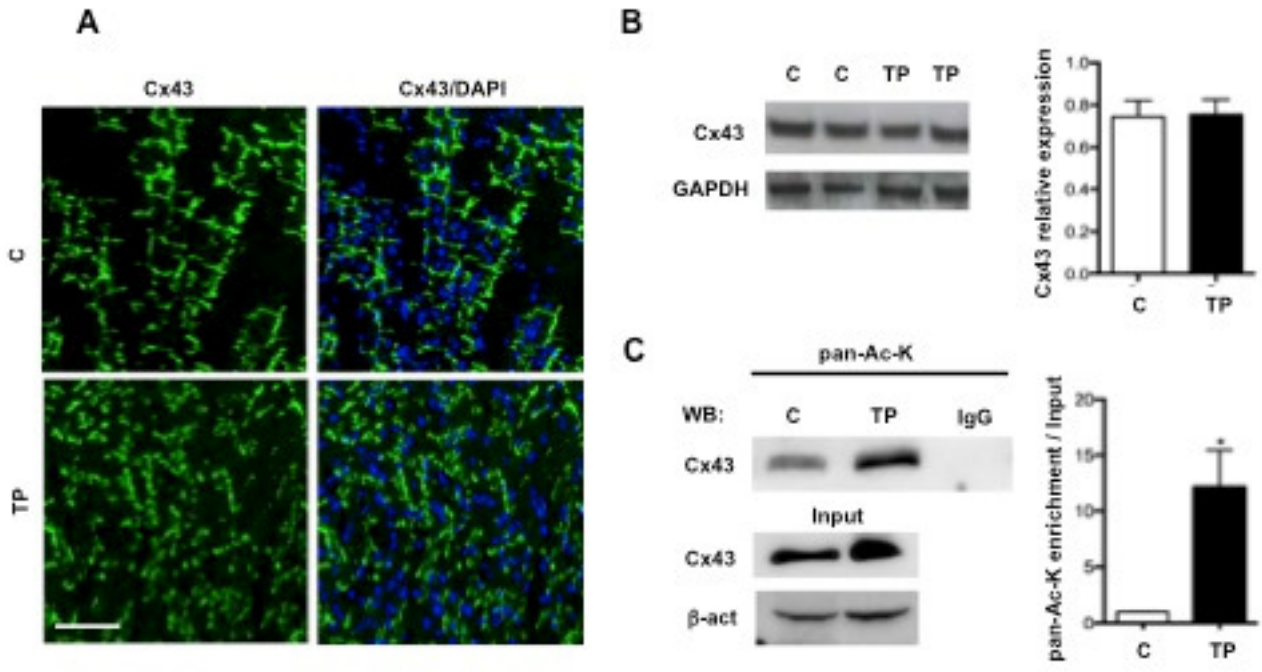

Figure 10: Tachypaced hearts showed Cx43 acetylation. **A:** Immunofluorescence assays performed on hearts sections collected from canine model of tachycardia-induced cardiomyopathy revealed Cx43 dissociation from intercalated disks and lateralization (n=4, scale bars: 50 μM). **B:** Western Blot analysis of hearts sections collected from canine model of tachycardia-induced cardiomyopathy did not reveal any change in Cx43 expression (n=4). **C:** Immunoprecipitationand western blot analysis of Cx43 expression and acetylation on hearts sections collected from canine model of tachycardia-induced cardiomyopathy revealed Cx43 acetylation due to tachypacing (n=4).

DISCUSSION

The present study describes a model of cultured HL-1 atrial cardiomyocytes paced at their intrinsic frequency for 24 hours, resulting in an acetylation-dependent reduction of Cx43 expression and, consequently, of cell-to-cell communication. HL-1 cells have been chosen as the primary cell model in our study due to the fact that they represent an easy-culture and commonly accepted model of cardiomyocytes (Zhanget al., 2014; Jiang et al., 2014; Colussiet al., 2011). Nevertheless, pacing dependent Cx43 reduction was also evident in NRCM but not in human fibroblasts, suggesting that the observed effect depends on mechanisms only present in excitable cells. This hypothesis is supported by the evidence that Verapamil, an inhibitor of L-type Ca^{2+} channels (ICa,L) (Penget al., 2010) suppressed the pacing-induced reduction of Cx43 expression and it's in line with the observation that electrical activity-dependent epigenetic response induced in neurons is related to calcium entry (Riccio et al., 2010). Intriguingly, the effect that we observed in paced cell is only partially equal to that induced by the pan-class II HDAC SAHA and TSA. In fact, it was demonstrated here and in previous work (Colussiet al., 2011) that SAHA treatment is able to induce Cx43 protein down-modulation and lateralization. However, Veenstra and coworkers also showed that SAHA induced Cx43 mRNA reduction in neonatal mouse cardiomyocytes, consistently with the decreased association of Cx43 promoter with RNA Pol II and the increased association with HDAC I (Xuet al, 2013). In our model the acetylation increase was only evident for proteins with molecular weights ranging from 25 to 130 kDa, while the signals for anti-Ac-K remained stable at lower molecular weights compatible with histones (below 25 kDa). This observation, along with the fact that Cx43 reduction is rescued by the treatment with the proteasome inhibitor, suggests that electrical pacing might not significantly affect histone acetylation status and exerts its action on Cx43 mainly through post-translational mechanisms. Nevertheless, at this stage it is not possible to exclude other general effects on specific histone modifications (e.g AcK9H3, AcK14H3). Further experiments are needed to elucidate this important point, which is beyond the aim of the present work. Importantly, the fact that the net increase of Cx43 acetylation correlates with its increased degradation through the proteasome machinery is not surprising. It is in fact recognized that lysine acetylation can either preventing or promoting polyubiquitination and

lysosomal degradation of different substrates (Mateo et al., 2009; Robertet al., 2011; van Loosdregt et al., 2010) or can serve as a direct signal for proteasomal degradation (Qianet al., 2013). More specifically, lysine acetylation was recently identified as an endogenous regulator of proteolytic activity in the heart, suggesting that inhibitors of cardiac proteasomal function can be regulated might represent a new promising class of pharmacological agents. (Wanget al., 2013). Of note, although in the past other authors have reported that electrical stimulation can affect cell conduction properties by increasing the expression of Cx43, usually these reports refer to higher frequency (3 Hz) and shorter stimulation time condition, e.g. from 60 (Nakashimaet al., 2014) to 120 minutes (Inoueat al., 2004), thus making it difficult to compare previous results with the results of the present work. Importantly, different papers demonstrated that Cx43 is lateralized and its expression is decreased during the development of heart failure induced by tachypacing (Akaret al., 2007; Yanget al., 2007). Despite we could not detect a significant reduction of Cx43 expression in our ventricle samples obtained from dogs with pacing-induced cardiomiopathy, our data clearly showed a significant delocalization of Cx43 from the intercalated disks. This was associated with an increase of Cx43 acetylation, already known for being responsible of Cx43 dissociation from plasma membranes in a mdx mouse model of dystrophic hearts (Colussiet al., 2011). Thus, in the present work a link is provided for the first time connecting GJ remodeling already described *in vivo* following altered ventricular activation (Patelet al., 2001; Akaret al., 2007; Yang et al., 2007) to the acetylation/deacetylation balance of cardiomyocytes. Although the histone acetylase P300/CBP associated factor (PCAF) seemed to be responsible for Cx43 acetylation in (Colussi et al., 2001) further experiments are required to understand whether the same enzyme play a major role in the context of electrical activity dependent Cx43 acetylation. In conclusion, the present work provides evidences that electrical stimulation can promote Cx43 acetylation in cardiac cells both*in vivo* and *in vitro*, resulting in Cx43 down-modulation and intracellular relocalization. Our findings suggest that activity-dependent alteration of acetylation/deacetylation balance might be a novel mechanism for the regulation of cardiomyocyte

communication and, thus, sustain the hypothesis that HAT/HDAC modulators represent a new tool for rhythm disturbance regulation.

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