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S. Mahapatra[†], C. Calorio [†], D. Vandael, A. Marcantoni, V. Carabelli, E. Carbone

Department of Neuroscience, NIS Center, University of Torino, 10125 Torino, Italy

Correspondence to: Emilio Carbone

[†] These authors contributed equally to this work

Key words: LVA and HVA calcium channels, Ca^{2+}-secretion coupling, vesicle secretion, catecholamines release, endocytosis, adrenal medulla

Acknowledgments: The authors thank the Marie Curie Research Training Network "*CavNET*", Italian M.I.U.R., Regione Piemonte, Università di Torino, Compagnia di San Paolo di Torino.

Abstract

Voltage gated Ca^{2+} channels are effective voltage sensors of plasma membrane which convert cell depolarizations into Ca^{2+} signaling. The chromaffin cells of the adrenal medulla utilize a large number of Ca^{2+} channel types to drive the Ca^{2+}-dependent release of catecholamines into blood circulation, during normal or stress-induced conditions. Some of the Ca^{2+} channels expressed in chromaffin cells (L, N, P/Q, R and T), however, do not control only vesicle fusion and catecholamine release. They also subserve a variety of key activities which are vital for the physiological and pathological functioning of the cell, like: *i*) shaping the action potentials of electrical oscillations driven either spontaneously or by ACh stimulation, *ii*) controlling the action potential frequency of tonic or bursts firing, *iii*) regulating the compensatory and excess endocytosis following robust exocytosis and *iv*) driving the remodeling of Ca^{2+} signaling which occurs during stressors stimulation. Here, we will briefly review the well-established properties of voltage-gated Ca^{2+} channels accumulated over the past three decades focusing on the most recent discoveries on the role that L- (Cav1.2, Cav1.3) and T-type (Cav3.2) channels play in the control of excitability, exocytosis and endocytosis of chromaffin cells in normal and stress-mimicking conditions.

Introduction

Catecholamine release in adrenal chromaffin cells is driven by Ca^{2+} signaling primarily controlled by voltage-gated Ca^{2+} channels (Cav) which open during membrane depolarization in forms of single spikes, trains of action potentials or sustained resting depolarizations. Despite this apparently simple duty, chromaffin cells are endowed with a large number of Ca^{2+} channel isoforms. So far, there is evidence for the functional expression of two L-types (Cav1,2, Cav1.3), two T-types (Cav3.1, Cav3.2), one N-type (Cav2.1), one P/Q-type (Cav2.2) and one R-type (Cav2.3) channels [1- 3], but it is likely that functional splice variants of these channel isoforms exist and will be soon identified in mammalian chromaffin cells. Studies in the past 30 years have brought clear evidence that each channel type possess distinct gating properties, expression densities and intracellular regulatory mechanisms which can be associated to specific events of chromaffin cells activities (cell firing, vesicle fusion, fast and slow catecholamine release, rapid and late vesicles retrieval) during normal or pathophysiological stress conditions (for reviews see [4-7]). Understanding into more details these critical

differences would be beneficial to clarify the multiple pathways through which Ca^{2+}-signaling regulates normal and stress-modified chromaffin cells function.

Here, besides briefly summarizing the well-established properties of Ca^{2+} channels controlling catecholamine secretion that are nicely discussed in recent reviews [4-6,8], we will survey in more detail some recent findings concerning specific functions of voltage-gated Ca^{2+} channels. In particular, we will focus on: *i)* the newly uncovered role that L-type channels (LTCCs) play in shaping the action potential and setting the pacemaker currents responsible for repetitive firing [7,9], *ii)* the specific role that LTCCs play in regulating the Ca^{2+}-dependent fast endocytosis during prolonged Ca^{2+}-entry [10,11], *iii*) the way in which T-type channels (TTCCs) contribute to the inborn oxygen chemosensitivity of chromaffin cells to regulate the non-neurogenic release of catecholamine in newborns [12-13] and *iv*) the TTCCs remodeling which occurs when chromaffin cells are subjected to short or long-lasting forms of stress-mimicking conditions (elevated cAMP levels, β-adrenergic modulation, chronic hypoxia, PACAP and high-frequency sympathetic stimulations) [14-17], and *v)* how TTCCs are able to control the "low-threshold" exocytosis of adult chromaffin cells [18-19]. These new entries widen the number of Ca^{2+}-signaling pathways controlled by voltage-gated Ca^{2+} channels and open the possibility for the search of new Ca^{2+} channel blockers and therapies that could be beneficial for the treatment of neurological and cardiovascular diseases associated to chronic-stress disorders.

1. The voltage-gated Ca^{2+} channels of chromaffin cell: crucial players of cell excitability, catecholamine secretion and vesicle retrieval

Voltage-gated Ca^{2+} channels are protein complexes which comprise a main pore-forming α_1-subunit of 190–250 kDa in association with auxiliary β-, $\alpha_2\delta$- and γ-subunits. The α_1-subunit is composed of four membrane-spanning domains (I–IV) linked together in a single polypeptide chain. Each domain contains six putative transmembrane segments (S1–S6) plus a "P" loop that dips partially into the pore to form the pore lining [20]. The cytoplasmic loops linking the four domains are the sites of interactions with β-subunits, second messengers, membrane-binding proteins and channel gating. Presently, ten α1-subunits have been cloned with specialized functions and distributions. Four members belong to the Cav1 group (L-types), three to the Cav2 group (N, P/Q and R-types) and three to the Cav3 group (T-types). Based on the threshold of activation, the

Cav1 and Cav2 channels are also indicated as "high-voltage-activated" (HVA) channels, while the Cav3 are "low-voltage-activated" (LVA) channels. This classification, however, is only indicative since L-type Cav1 subunits (Cav1.3) that activate at very negative voltages, close to the values of Cav3 (T-type) channels, are found to exist [21-24].

The adrenal chromaffin cells of mammalian species express all types of voltage-gated Ca^{2+} channels described above [4,7,25]. Their expression density remarkably changes among animal species, although their function remains surprisingly similar. The Ca^{2+} channels of chromaffin cells shape action potential waveforms, control catecholamine secretion and vesicle retrieval and regulate Ca^{2+}-dependent events that originate near the plasma membrane. An obvious question is: why chromaffin cells, which are round shaped cells with no neuronal-like morphology and specialized pre- and post-synaptic domains needs so many Ca^{2+} channel types (L, N, P/Q, R, T) to perform few simple basal functions? There is not a clear answer to this question, but there is increasing evidence that their gating properties and regulation by external stimuli and intracellular second messengers assign to each of them specific functions in the control of cell excitability, action potential firing, catecholamine secretion and vesicle retrieval. We will now focus on the more relevant aspects that each Ca^{2+} channel group (Cav1, Cav2 and Cav3) plays in the physiology of chromaffin cells during normal functioning and under stress conditions.

2. The Cav1 (L-type) channels

L-type Ca^{2+} channels (LTCCs) are widely expressed in many tissues and control a number of Ca^{2+}-dependent responses in excitable cells. Of the four identified α1-subunits (Cav1.1, Cav1.2, Cav1.3, Cav1.4), chromaffin cells express only the Cav1.2 and Cav1.3 isoforms [2,3,9,26], which are both highly sensitive to 1,4-dihydropyridines blockers (Ca^{2+} antagonists) or activators (Ca^{2+} agonists). Cav1.2 and Cav1.3 are largely expressed in rat (RCCs) and mouse (MCCs), where they contribute up to 40-50 % of the total Ca^{2+} current, while are less expressed in human (HCCs) and bovine chromaffin cells (BCCs) [4].

2.1. L-type channels and catecholamine secretion – Looking at the data reported in the past 25 years on Ca^{2+} channels-secretion coupling in chromaffin cells it appears evident that LTCCs are those that mostly contribute to secretion when long lasting stimuli are applied. Either prolonged depolarization with high KCl solutions

[27], sustained applications of ACh [28,29] or repeated sympathetic stimulation [30] in isolated cells or intact adrenal glands of bovine, rat, mouse and cat underline a predominance of LTCCs in regulating catecholamine release (see ref. [4,31] for recent reviews). This occurs independently of LTCCs expression densities and it is associated with two gating properties of these channels: *i)* the slow voltage- and Ca^{2+}-dependent inactivation of Cav1.2 and Cav1.3 that allows sustained Ca^{2+} fluxes during prolonged (or repeated) depolarizations and *ii)* the low steady-state inactivation of both channels which favors their availability near physiological resting potentials (-50, -60 mV). However, when evaluated using capacitance changes on single isolated cells, the contribution of LTCCs appears more limited and proportional to the quantity of Ca^{2+} charges carried. Compared to Cav2 channels, LTCCs possess the same Ca^{2+}-efficiency and contributes proportionally to their density of expression [32-34]. An exception is the adrenal mouse slice in which secretion is dominated by R-type channels [35].

A correct evaluation of the efficacy of Ca^{2+} channels to secretion is based on the estimate of the maximal rate of vesicle release using square pulses of increasing duration (from 10 to 200 ms) [34,36,37]. In rat chromaffin cells the maximal rate of release calculated during very low Ca^{2+} influx (i.e., for pulse durations comparable to the action potential width) is ~650 fF/s [36] when all Ca^{2+} channels are available and is reduced to 450 fF/s when only L- and R-type channels are functioning [18,34]. Assuming a single vesicle capacitance of 1.3 fF [38] the maximal rate of release for chromaffin cells is estimated as 400-500 vesicles/s while in presynaptic terminals the rate of release can be as high as 300 vesicles/ms [39], i.e., nearly 3 orders of magnitude higher. These remarkable differences can be attributed to a constitutive diverse geometrical arrangement of secretory vesicles and Ca^{2+} channels in the two systems, which reflects the different time scale in which the two systems respond to a stimuli. In one case, the rapid rise of Ca^{2+} near the presynaptic active zone is achieved through a high co-localization of vesicles and Ca^{2+} channels (N- and P/Q) in microdomains. Alternatively, in the case of chromaffin cells, the slower rise of Ca^{2+} near the secretory granules is achieved by the activation of multiple channels (N, P/Q, R and L) distributed over large areas, covering tens of square micrometers (see below).

2.2. L-type channels and vesicle retrieval - Another interesting property of LTCCs is their direct participation to the control of vesicle endocytosis in bovine chromaffin cells [10]. Interestingly, despite their modest contribution (20%) to the total Ca^{2+} current in bovine chromaffin cells, several lines of evidence indicate that

LTCCs are preferentially coupled to endocytosis and functionally co-localized with clathrin and dynamin proteins [40]. A first indication comes from the block of LTCC by nifedipine, which reduces exocytosis by only 20% while inhibits endocytosis by 90%. There is not yet a clear explanation to this phenomenon but it seems evident that endocytosis is favored by Ca^{2+} channels that are able to maintain prolonged Ca^{2+} entries during sustained depolarizations like the L-type [40], and that sphingosine plays a permissive role in the regulation of Ca^{2+}-dependent endocytosis [11]. In addition, when using the double-pulse protocol to estimate the pool of vesicles ready for release (RRP), paradoxically nifedipine transforms the usually reduced exocytotic response elicited by the 2^{nd} pulse into a facilitated one [41]. This surprising finding suggests the existence of an uncovered fast endocytotic process (kiss and run?) controlled by LTTCs. Block of LTTCs by nifedipine makes available, within the short interpulse interval (100 ms), an increased number of vesicles which do not undergo fast endocytosis and thus may contribute to the increased exocytosis during the 2^{nd} pulse. Following these new findings, it would be extremely interesting to determine whether Cav1.2 or Cav1.3 have any specific role on vesicle retrieval. An argument in favor of Cav1.3 is its slower and less complete time dependent inactivation with respect to Cav1.2 [9,21,22]. The delayed inactivation of Cav1.3 could be physiologically relevant for sustaining prolonged Ca^{2+} influxes that support normal endocytosis [42], but this issues needs to be tested in future work.

2.3. The voltage-independent autocrine modulation of LTCCs **-** Since chromaffin cells constantly release catecholamines during low and sustained sympathetic stimulation [43], Ca^{2+} channels experience a basal and a stimulus-induced autocrine modulation which alters their activation-inactivation time course and quantity of Ca^{2+} entry during activity (see [5,25,31]). This effect is mediated by adrenergic, opiodergic and purinergic signaling through G-protein coupled receptors (GPCRs) and occurs both in isolated cells [44,45] and adrenal gland slices [46]. In the case of LTCCs the modulation is dual: they can either down- or up-regulate the currents. The down modulation is fast, mediated by PTX-sensitive G-proteins [44,46] and limited to membrane micro-domains [47], while the up-regulation is slow, remote and mediated by the cAMP/PKA phosphorylation pathway [45-48]. Given that LTCCs can also be down-regulated by the NO/cGMP/PKG phosphorylation pathway [49], it may occur that depending on cell conditions (high cAMP/low cGMP versus low cAMP/high cGMP), chromaffin cells LTCCs can undergo extreme up- or down-modulations which would change Ca^{2+}

currents by one order of magnitude and therefore may strongly affect catecholamine release. The rationale of these opposing synergistic regulations of LTCCs is that PKA and PKG act independently on both Cav1.2 and Cav1.3 channel isoforms, producing cumulative effects on their open channel probability (Mahapatra, Marcantoni, Carabelli, Carbone; unpublished results).

2.4. L-type as pacemaker channels - Cav1.3 and, to a minor degree, Cav1.2 play also a key role in pacemaking mouse chromaffin cells near resting conditions [7,9,24,48], making them even more strategic for chromaffin cell physiology at rest and during stressful stimulations. Fig. 1 shows two examples of how nifedipine reduces the frequency or block the spontaneous firing and alter the shape of APs. The C-terminal "long" form of Cav1.3 α_1-subunit ($Cav1.3_{42}$), is highly expressed in mouse chromaffin cells [9] and activates at about 9 and 24 mV more negative voltages than Cav1.2 and Nav1.7 channels, respectively (Fig. 2A) [24]. In addition, Cav1.3 inactivates slowly and only partially during pulses of 0.5 to 1 s in wild-type MCCs, compared to the fast inactivating Cav1.2 available in $Ca1.3^{-/-}$ KO MCCs (see Fig. 2B). Thus, Cav1.3 is a suitable channel for pacemaking chromaffin cells with spontaneous firing frequencies of 0.5-2 Hz and interpulse potentials of -50 mV. Removal of Cav1.3 in KO mice causes a marked decrease of L-type pacemaking current (see Fig. 2C) and a drastic reduction in the percentage of spontaneously firing cells in 4 mM external KCl [9]. The percentage of firing cells can change markedly if MCCs are maintained at more depolarized resting potentials using higher KCl concentrations [24]. These non-physiological conditions tend to overestimate the contribution of Cav1.2 channels (which activate at potentials more positive than Cav1.3) and may lead to inaccurate conclusions on the role of LTCCs on pacemaking [50].

3. The Cav2 (N, P/Q, R-type) channels

N-, P/Q- and R-type channels are highly expressed in the nervous system, where they conduct the presynaptic Ca^{2+} currents that initiate synaptic transmission. The efficiency of neurotransmitter release is steeply dependent on the 3rd to 4th power of Ca^{2+} entry through these voltage-gated channels making them an important target of synaptic regulation. Cav2.1 channels carrying P/Q-type Ca^{2+} currents and Cav2.2 channels carrying N-type Ca^{2+} currents are the predominant pathways through which Ca^{2+} initiates the rapid release of glutamate, ACh and GABA. Extensive studies indicate that Cav2.1 and Cav2.2 contain a synprint region [20],

suggesting that these channels may localize to regions of the membrane where the SNARE complex has formed, thus allowing their interaction with syntaxin, SNAP-25, and synaptotagmin. Their functioning is also critically regulated by many G protein subunits, which form the basis of Ca^{2+}- and voltage-dependent signal transduction at the synaptic terminal [20].

3.1. Any specific regulatory role of Cav2 channels on catecholamine secretion? - In chromaffin cells Cav2.1 and Cav2.2 are expressed at different densities in all animal species [4] while Cav2.3 is preferentially expressed in MCCs and RCCs [9,35,45,46]. Cav2.1 and Cav2.2 are effectively coupled to secretion but the Ca^{2+}-dependence is nearly linear and comparable to the Ca^{2+}-dependence experienced by the other Ca^{2+} channels expressed in chromaffin cells [15,18,34,36,40,51,52]. This indicates functional "loose coupling" of Cav2 channels to the secretory vesicles ready for release, which is consistent with the idea that Ca^{2+} channels and release sites are not tightly co-localized as at the presynaptic terminals [53] but distributed within an average distance of 200-300 nm and assembled in specialized regions of the surface membrane with dimensions of several micrometers [54,55]. In this way, the dominant Ca^{2+} signal regulating vesicle release derives from the activation of multiple channels distributed over areas covering tens of square micrometers, rather than from Ca^{2+} channel clusters localized in microdomains [56].

Concerning the regulatory role of Cav2.1, Cav2.2 channels in secretion, electrophysiological studies are quite homogeneous and support the idea that both channel subtypes are functionally coupled to the secretory apparatus, without any preferential coupling [6,32,34,51,57]. Nevertheless, a predominant role of P/Q-type channels in regulating the fast release of vesicles from the immediately releasable pool (IRP) has been recently proposed. [58]. To the purpose, square pulses of 10 ms to 0 mV from -80 mV were applied to specifically recruit vesicles from the IRP. The authors found that P/Q-type channels control most of the IRP and Cav2.1 KO mice display very little exocytosis during these short stimuli. Along this line, the role of different Ca^{2+} channel types on exocytosis has been recently investigated in bovine chromaffin cells using amperometry and 10 s pulses of 70 mM KCl as secretory stimuli [59]. Under these conditions, L-, N- and P/Q-type channels contributed similarly to cytosolic Ca^{2+} increases, but they differently regulated the fusion pore expansion and the frequency of exocytic events. The P/Q-type channels accelerated the final steps of exocytosis, whereas the N-type slowed down the process. Finally, it is worth noticing that different stimuli, like APs or square pulse

depolarizations, may differently affect the contribution of Ca^{2+} channel types to the overall Ca^{2+} current and consequently to the secretory responses. In this view, Cav2.1 channels apparently play a prominent role on secretion when using trains of triangular APs rather than square depolarizing pulses [60]. This is likely due to the rapid activation of Cav2.1 with respect to the other Ca^{2+} channels which is more evident during stimuli of short duration since less affected by fast channel inactivation. This suggests that channel gating and the type of stimuli applied, rather than the specific co-localization of the secretory apparatus with Ca^{2+} channels, critically regulate the exocytosis in chromaffin cells. In relation to this, it would be more appropriate to use APs stimuli previously recorded from the secreting cell (see Fig. 1) rather than APs of *ad hoc* shape.

3.2. The voltage-dependent modulation of Ca2.1 and Ca2.2 channels - At variance with LTCCs, which control the subthreshold current regulating AP firing in RCCs and MCCs, the Cav2 channels contribute mostly to the upstroke and falling phase of APs. They are also uniquely modulated in a voltage-dependent manner by G protein coupled pathways [61]. In chromaffin cells, Cav2.1 and Cav2.2 are autocrinally inhibited by ATP and opioids that are released during cell activity [62-65]. The inhibition occurs in membrane microdomains, without the involvement of diffusible second messengers, and is manifested by a long delay of channel openings (slow activation) at low potentials [66,67]. This effect, however, can be reversed by voltage. The normal fast activation is recovered during strong depolarizations [68,69] or when a strong pre-pulse anticipates a low voltage depolarization [70]. This phenomenon is called "voltage-dependent facilitation" and is attributed to a protein-protein interaction between the receptor activated Gβγ subunit and the II-III loop of Cav2 channels, [71-73].

Interestingly, this Cav2.1 and Cav2.2 down-modulation can be partially reverted during short depolarizations repeated at high-frequency [74] as it occurs during high-frequency AP trains delivered under stress conditions. The final result is that the attenuated Ca^{2+} influx through Cav2.1 and Cav2.2 at rest, due to the down-regulation induced by the released ATP and opioids, can be partially reverted during sustained cell activity. This phenomenon is particularly evident in clusters of bovine chromaffin cells [75] and in slices of mouse adrenal glands [46] where the packed organization of the cells is well preserved and the released neurotransmitters (ATP and opioids) can accumulate in the extracellular space to exert a basal tonic inhibition on cell firing and catecholamine secretion. Finally, it is worth mentioning that the neuronal calcium sensor-1 (NCS-1) exert a role

in the tonic inhibition of Cav2.1 channels, particularly in the voltage-independent type of modulation. NCS-1 acts through an autocrine, purinergic receptor mediated pathway, thus requiring exocytosis and the activation of PTX-insensitive G-proteins. To complete the mechanism of Ca^{2+} channel inhibition in chromaffin cells, it is also necessary the phosphorylation of Cav2.1 α1-subunit at the tyrosine residues by a Src-like kinase, strictly controlling Ca^{2+}-dependent exocytosis and cell functioning [76]. Besides this, NCS-1 is also shown to alter secretion by acting on signaling pathways rather than directly on the secretory machinery. More specifically, NCS-1 regulates chromaffin cells secretion by favoring PIP2 production, leading to IP3 increases and Ca^{2+} release from intracellular stores [77]. In addition, also the Ca^{2+}-dependent inactivation of Cav2.1 and Cav2.2 channels by Ca^{2+}/calmodulin may differently regulate Ca^{2+} currents, thus affecting catecholamine release [78].

4. The Cav3 (T-type) channels

T-type Ca^{2+} channels (TTCCs) are transient, low-voltage activated (LVA) Ca^{2+} channels that control Ca^{2+} entry during mild depolarizations near resting potential. Due to their widespread localization and biophysical properties, TTCCs control key functions like: low-threshold spikes, oscillatory cell activity, muscle contraction, hormone release, cell growth, differentiation and proliferation [8,79]. Given this, they are now proposed as therapeutic targets for a variety of diseases like: hypertension, angina pectoris, heart failure, atrial fibrillation, neuropathic pain, epilepsy, sleep disorders, obesity and cancer [80].

TTCCs possess unique gating properties that facilitate their electrophysiological identification: *i)* activation is strictly voltage-dependent and channels open at very low voltages (~ -50 mV), *ii)* inactivation is voltage-dependent and complete within tens of milliseconds above 0 mV, *iii)* deactivation is slow at potentials near rest (-60, -50 mV), *iv)* Ca^{2+} and Ba^{2+} are carried equally well and single channel conductance is 3 to 4-fold lower than that of Cav1 and Cav2, and *v)* activation and steady-state inactivation overlap at potentials near resting giving rise to a sizeable inward 'window current'. Molecular cloning of TTCCs has provided evidence for the existence of three different pore forming α1 subunits (Cav3.1, Cav3.2, Cav3.3) with only 25% amino acid homology but similar pore structure organization to Cav1 and Cav2 [79].

4.1. Weak expression of TTCCs in chromaffin cells of adult animals - Despite their widespread distribution in most tissues (neuronal, muscular, endocrine), TTCCs are weakly expressed or absent in adult chromaffin cells.

In adult BCCs, the mRNA encoding for Cav3.1 and Cav3.2 is clearly expressed [26], but functional TTCCs have not been detected [67,81-83], except in one case [84]. TTCCs are on the contrary functionally expressed in embryonic and neonatal RCCs [12,13,85] and are available in a small percentage of adult RCCs [14,16,86]. TTCCs are also not normally expressed in adult MCCs [9,48,75] although there is clear evidence that MCCs display the typical "low-threshold I/V shoulder" associated with TTCCs during ramp commands (see Fig. 4 in [87]. These low-threshold currents are also visible in Cav1.3 KO MCCs (V. Navarro-Tableros, V. Carabelli, E. Carbone, unpublished observation), excluding the possibility that they may derive from the C-terminus "short" splice variant of Cav1.3 α_1-subunit ($Cav1.3_{42a}$) that activates at very negative potentials and inactivates quickly in a Ca^{2+} dependent manner [23]. It is thus very likely that availability of TTCCs may critically depend on chromaffin cell conditions or stimulations, as in the case of applied chronic stressors [14- 16,19] which up-regulate Cav3.2 channels in rat and mouse chromaffin cells (see figure 3A,B and below).

4.2. TTCCs as controllers of low-threshold exocytosis - When expressed, TTCCs are shown to be effectively coupled to the secretory vesicles and control a sizeable amount of "low-threshold" catecholamine release in chromaffin cells [5,15-18]. The Ca^{2+}-dependence of TTCCs-controlled secretion is linear and strikingly similar to that of LTCCs [18], which is comparable to that of P/Q and N-type channels [34]. Independently of the low voltage range of activation, TTCCs are equally distributed near the docked secretory vesicles as the HVA channels. Thus, TTCCs lower the threshold of AP generation and ensure a broader interval of voltage control on catecholamine secretion, which may be relevant when chromaffin cells depolarize steadily or fire at very high sympathetic stimulation during stress conditions.

4.3. TTCCs channel remodeling in response to stressors - As for the nicotinic receptors and gap-junction channels [88] also TTCCs undergo functional remodeling during stress [8]. This is particularly evident in chromaffin cells of adults animals as an adaptive response to stress conditions like chronic (CH) and intermittent hypoxia (IH) [13,16], β-AR and high-frequency sympathetic stimulation [14,19] and as during the transition from intra- to extra-uterine life [12], when newborns experience episodic hypoxia conditions and catecholamine secretion switches from a non-neurogenic to a neurogenic control mode [89].

A common molecular mechanism in response to the indicated stressors is a marked recruitment of functioning Cav3.2 channels. Up-regulation of Cav3.2 channels lowers the threshold of AP firing [14],

increases the amount of catecholamines secreted at low-voltages [15,16] (figure 3C) and mobilizes a RRP equivalent to that of L-type channels [18]. An increased density of T-type channels is also expected to decrease the resting potential and increase the firing frequency, as it occurs in spontaneously firing rat chromaffin cells in the adrenal-gland slices of stressed animals [90]. Indeed, an increased density of functioning T-type channels is a remodeling mechanism that many cells and tissues develop in response to stressors. Table 1 lists a number of stressors and stress-induced conditions that lead to the up-regulation of T-type channels in a variety of tissues and cells. Notice how neuronal, muscular and endocrine cells all respond to stressors by increasing the density of Ca3.1 and Cav3.2 channels, reinforcing the view that T-type are "stress-induced channels" [8,19].

4.4. Signaling pathways of TTCCs up-regulation during hypoxia – As listed in Table 1, there are multiple ways through which Cav3.2 channels are up-regulated by stressors in chromaffin cells. They act either acutely within minutes (sustained sympathetic stimulation, PACAP) or slowly, requiring hours or days (chronic and intermittent hypoxia, β_1-AR stimulation, VIP, aldosterone, ACTH). The one rapidly developing (minutes) is most evident in mouse innervated adrenal gland slices [19]. This pathway is activated by PACAP (released during high-frequency splanchnic stimulation) and mediated by cAMP-activated exchange proteins (EPAC) that stimulates PLC and PKC through a non-canonical cAMP-dependent pathway. The PKC target is the phosphorylation of the Na^+/Ca^{2+} exchanger which causes a small depolarization near resting potential, sufficient to activate a robust low-threshold current carried by Cav3.2 channels (see ref. [17] for more details).

The second pathway, slowly developing, is a long lasting mechanism that requires hours or days to fully progress. This is the case of chronic/intermittent hypoxia and cell exposure to β_1-AR agonists (isoprenaline), which both act by increasing the levels of CACNA1H mRNA (Cav3.2 gene) and their action is prevented by protein synthesis inhibitors [13-16,18], suggesting net recruitment of newly synthesized Cav3.2 channels. For the case of β_1-AR stimulation, Cav3.2 recruitment is mediated by a cAMP-dependent (PKA-independent) EPAC pathway which leads to the activation of transcription factors [14], most likely through the phosphorylation of ERK (see [91]).

Concerning chronic and intermittent hypoxia, recent findings indicate the involvement of hypoxia-inducible factors (HIFs). HIF-2α is involved in the up-regulation of Cav3.2 in PC12 cells exposed to chronic hypoxia [92] and overnight incubations with the unspecific HIF activator desferrioxamine (DFX), mimics the

effects of hypoxia in PC12 cells [92] and RCCs [16]. In addition to HIFs, also NADHP-oxidases (NOX2, NOX4) and reactive-oxygen species (ROS) appear to play a main role in the recruitment of Cav3.2 in neonatal RCCs subjected to intermittent hypoxia (IH). An hypothetical scheme in which NOX, ROS and HIF are linked together is reported in Fig. 4. The scheme is derived from ref. [93] in which ROS is shown to activate PLC, DAG and PKC, favoring the stabilization and translocation of HIF-1α to the nucleus to start transcription and gene expression.

4.5. Splanchnic innervation (or denervation) set the expression level of TTCCs in neonatal chromaffin cells

- Chromaffin cells possess an inborn chemosensitivity to O_2 levels during fetal life, when the control of hormone release from the adrenal medulla is mostly non-neurogenic [89] and effectively regulated by the gap-junction coupling existing between neighboring cells [94]. During this developmental phase, chromaffin cells respond to acute hypoxia with a robust catecholamine release, mostly controlled by Cav3.2 channels [12]. High densities of T-type channels and enhanced electrical coupling facilitate action potential generation and propagation, as well as the synchronous release of catecholamines from cell populations of the adrenal medulla, with the outcome of an increased amount of hormone release that helps sustaining the increased cardiovascular response to hypoxia [88]. Of great significance, however, is the recent observation that cholinergic innervation of the adrenal gland, following postnatal development [95], causes a loss of functional Cav3.2 channels [12] paralleled by an increased density of α_7-built nAChRs [96]. But even more interesting, is the observation that O_2-sensitivity and Cav3.2 channel expression is reestablished in chromaffin cells after adrenal gland denervation, suggesting an opposing correlation between nicotinic AChRs expression and Cav3.2 channels down-regulation or vice versa [12]. This apparent coupling between AChRs and Cav3.2 is worth being investigated since T-type channels are shown to co-localize with Kv4 and HCN1 channels [97,98] and are effectively down-regulated by closely associated neuronal TRPV1 channels [99].

Concluding remarks

The importance of voltage-gated Ca^{2+} channels in the control of chromaffin cell firing, catecholamine secretion and vesicle retrieval is now well documented and supported by an increasing number of unquestionable findings. Cav1.3 and Cav1.2 contribute to shape the AP waveform and supporting the pacemaker current which

set the frequency of spontaneously firing cells. The two LTCC isoforms are also clearly involved in the regulation of fast vesicle retrieval following prolonged Ca^{2+} loadings. At variance with LTCCs, Cav2.1 and Cav2.2 contribute little to the pacemaker current but more to sustain the AP upstroke and appear linked to the fast release of vesicles belonging to the IRP. Both group of channels (Cav1 and Cav2) are also effectively modulated by the same neurotransmitters released by chromaffin cells (autocrine modulation) and all together, may possibly act as a feedback to remove resting inhibition and potentiate the massive release of neurotransmitters during sustained cell activity. Cav3.2 channels play a specific role in Ca^{2+} channel remodeling when chromaffin cells increase their activity in response to stressors. The increased expression (or availability) of Cav3.2 and Cav3.1 lowers the threshold of cell-excitability, increases the firing frequency and broaden the voltage range of catecholamine secretion toward more negative potentials. In this way, T-type channels support a "low-threshold" exocytosis which could be of relevance during hypoxia, β-adrenergic and PACAP-mediated stimulation. In conclusion, all Ca^{2+} channels of chromaffin cells play key roles in controlling specific functions of chromaffin cells but all appears to contribute, depending on their expression density, to the Ca^{2+} signaling regulating the bulk release of catecholamine during sustained depolarizations.

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Figure legends

Figure 1 – LTCCs control the frequency of spontaneously firing mouse chromaffin cells. In the upper panel nifedipine (3μM) is shown to slow the spontaneous firing of the cell and change the shape of the AP (red trace). It is evident the increased amplitude of the overshoot, the broadening of the AP width and the reduction of the after-hyperpolarization due to a reduced activation of BK channels coupled to LTCCs [7]. In the second case (lower panel) the cell is first exposed to 3μM rolipram (a selective blocker of PDE4) that elevates the level of intracellular cAMP and up-regulates the LTCCs. Nifedipine (3μM) causes the full block of AP firing accompanied by a slight hyperpolarization (adapted from ref. [48]).

Figure 2 - Cav1.3 possesses the right biophysical properties to sustain the slow pacemaker current underlying the spontaneous firing of chromaffin cells. ***A)*** Voltage-dependence of Cav1.3, Cav1.2 and Nav1.7 channel activation. The curves are derived from wild-type (WT) and Cav1.3$^{-/-}$ KO MCCs as described in ref. [24]. ***B)*** The L-type current at +10 mV (green trace) of WT MCCs (carried by Cav1.3 and Cav1.2) inactivates more slowly than that of Cav1.3$^{-/-}$ KO MCCs carried only by Cav1.2 channels. ***C)*** Ca^{2+} currents in WT and Cav1.3$^{-/-}$ KO MCCs during an AP-clamp using the command voltage indicated on top (cyan trace). Notice the robust slow inward current recorded during the interspikes interval (black trace) and its nearly complete block by nifedipine (3 μM, red trace) in WT MCCs. In Cav1.3$^{-/-}$ KO MCCs the same current is much reduced and only the addition of BayK-8644 (1 μM, blue trace) is able to reveal it (adapted from ref. [9]).

Figure 3 - Stress stimuli induce *de novo* expression of Cav3.2 (T-type) channels and "low-threshold" exocytosis in rat chromaffin cells. A) I-V curves of R- and T-type channels expressed in control (black trace) and cAMP-treated cells after exposure to 200 μM pCPT-cAMP (red trace). Both cells were pretreated with ω-toxins and bathed in solutions containing 1 μM nifedipine to minimize N-, P/Q and L-type Ca^{2+} currents. Cav3.2 channels are responsible for the low-threshold early peak of current at about -22 mV in cAMP-treated cells. The high-threshold peak at about + 12 mV is mainly due to R-type channels (adapted from ref. [14]). **B)** T-type currents recorded in isolation from a RCC cell incubated with 3.2 μM ω-CTx-GVIA, 2 μM ω-Aga-IVA, 1 μM SNX-482 and bathed with 1 μM nifedipine to nearly block all HVA channels (adapted from ref. [14]). **C)** Ca^{2+} currents and membrane capacitance changes at −30 mV (10 mM external Ca^{2+}) in control, cAMP-treated and hypoxic RCCs. Notice the potent block of Ca^{2+} currents and secretion induced by 50 μM Ni^{2+} in the hypoxic RCC (red trace) in which N-, P/Q- and L-type channels were blocked by ω-toxins and nifedipine (adapted from ref. [15,16]).

Figure 4 - Cell signaling pathways leading to CACNA1H gene expression and Cav3.2 channels recruitment during hypoxia. The final results of chronic/intermittent hypoxia are: *i)* the activation of transcription factors (HIF, CREB, etc…) and CACNA1N gene expression that lead to Cav3.2 channels recruitment (*upper panel*) and *ii)* the detection of "low-threshold" amperometric spikes associated with

catecholamine release during mild depolarization (4 mM KCl), which are not detectable in control conditions (*lower panel*). The schematic pathway induced by hypoxia (NOX, ROS, PLC, PKG, HIF) is taken from ref. [93].

Table

Table 1 – Type of stressors or stressor-like stimuli that induce recruitment or up-regulation of Cav3 (T-type) channels in different cells and tissues

Stressor	Tissue	Cell type	Cav3 type	Reference
Chronic hypoxia	Adrenal gland	PC12	Cav3.2	[91]
Long-term β-AR exposure	Adrenal gland	Adult RCCs	Cav3.2	[14]
Chronic hypoxia	Adrenal gland	Adult RCCs	Cav3.2	[16]
Adrenal gland splanchnic denervation	Adrenal gland	Neonatal RCCs	Cav3.2	[12]
Intermittent hypoxia	Adrenal gland	Neonatal RCCs	Cav3.1, Cav3.2	[13]
Acute high-frequency sympathetic stimulation	Adrenal gland	Adult MCCs (adrenal gland slices)	Cav3.2	[19]
Acute PACAP-mediated stimulation	Adrenal gland	Adult MCCs (adrenal gland slices)	Cav3.2	[18]
Long-term ACTH exposure	Adrenal gland	Bovine zonafasciculata cells	Cav3.2	[100,101]
Long-term VIP exposure	Adrenal gland	Bovine zonafasciculata cells	Cav3 (?)	[100]
Chronic hypoxia	Heart (Ventricular myocytes)	Neonatal rat ventricular myocytes	Cav3.2	[102]
Pulmonary hypertension	Heart (Ventricular myocytes)	Rat ventricular myocytes	Cav3 (?)	[103]
Cardiomyopathic heart	Heart (Ventricular myocytes)	Hamster ventricular myocytes	Cav3 (?)	[104]
Hypertrophic heart	Heart (Ventricular myocytes)	Feline ventricular myocytes	Cav3 (?)	[105]
Long-term aldosterone exposure	Heart (Ventricular myocytes)	Neonatal rat ventricular myocytes	Cav3.2	[106]
Glucocorticoids	Heart (Ventricular myocytes)	Neonatal rat ventricular myocytes	Cav 3.2	[107]
IGF-1	Smooth muscle	Pulmonary artery smooth muscle cells	Cav 3.1	[108]
Long-term aldosterone exposure	Cancer cells	Human adrenocarcinoma	Cav3.2	[109]
Cell proliferation	Cancer cells	Human cancer cells	Cav3.1, Cav3.2	[110,111]
Diabetic neuropathic pain	CNS	Rat hindpaws sensory neurons	Cav3.2	[112]
Diabetic neuropathic pain	CNS	Rat lumbar DRG neurons	Cav 3.2	[113]
Chronic visceral pain	CNS	Rat colonic sensory neurons	Cav3.2	[114]
Single episode of status epilepticus	CNS	Hippocampal neurons	Cav 3.2	[115]
17-β estradiol (E2)	CNS	Hypothalamus/pituitary neurons	Cav 3.1	[116]

Figure 1

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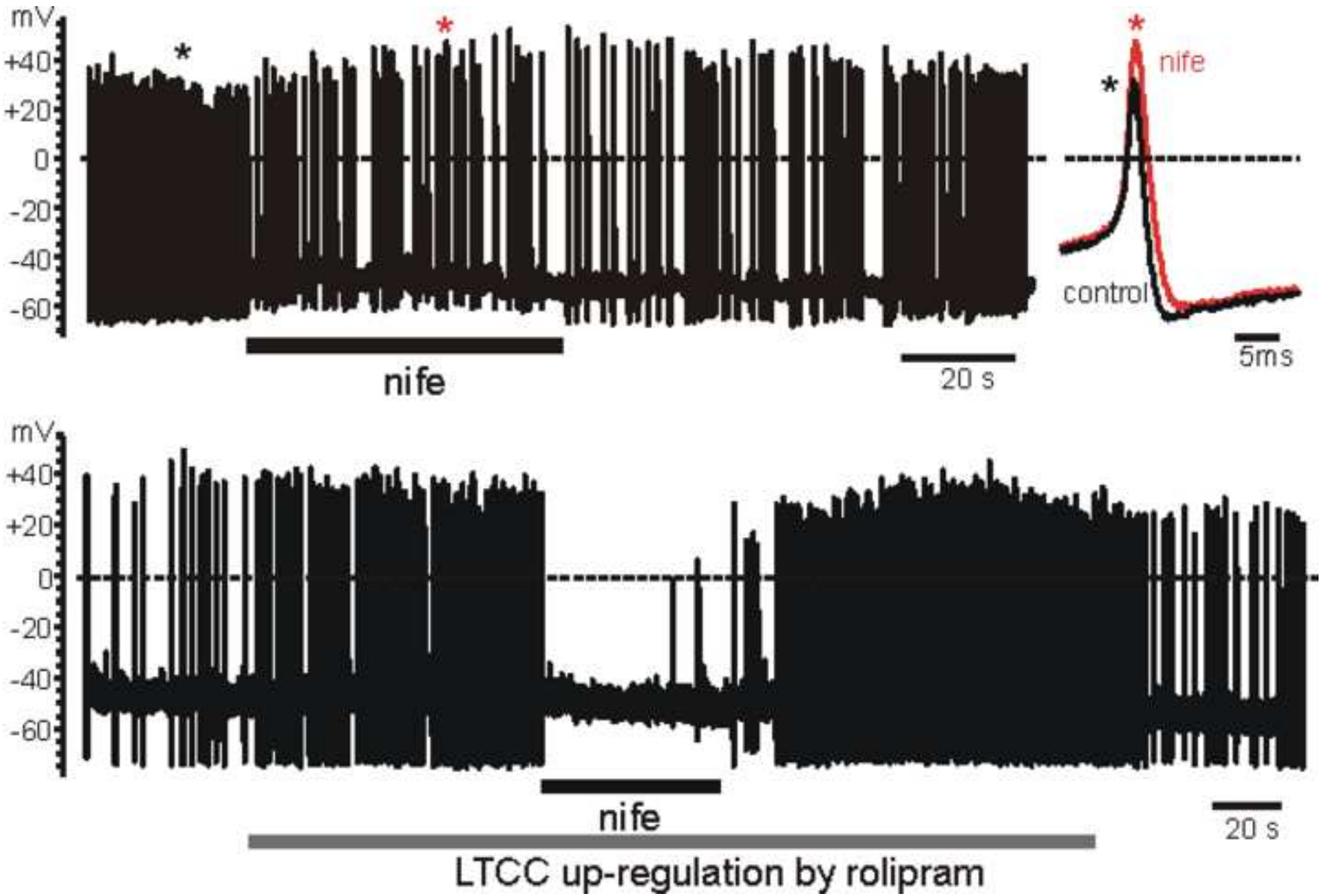

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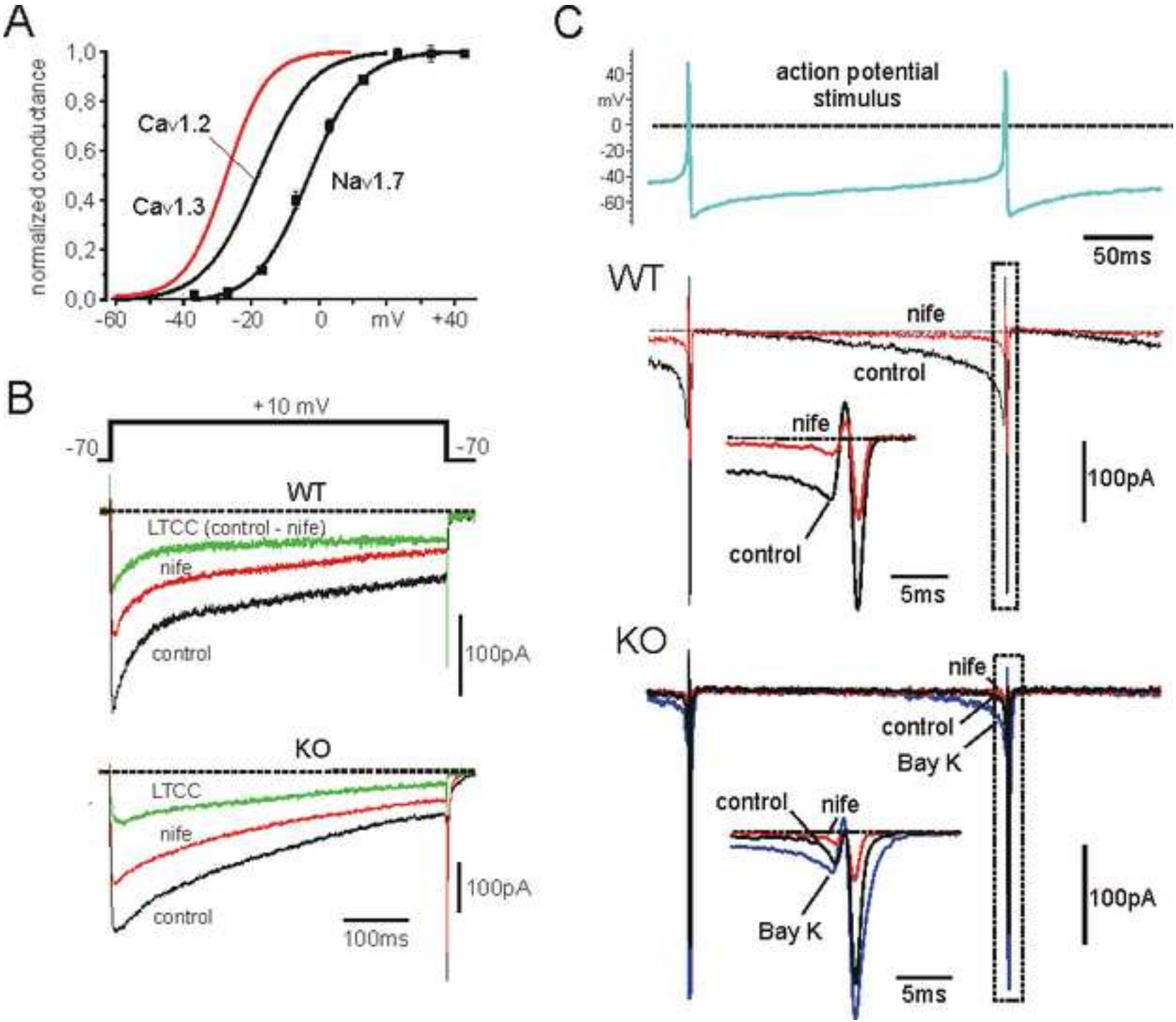

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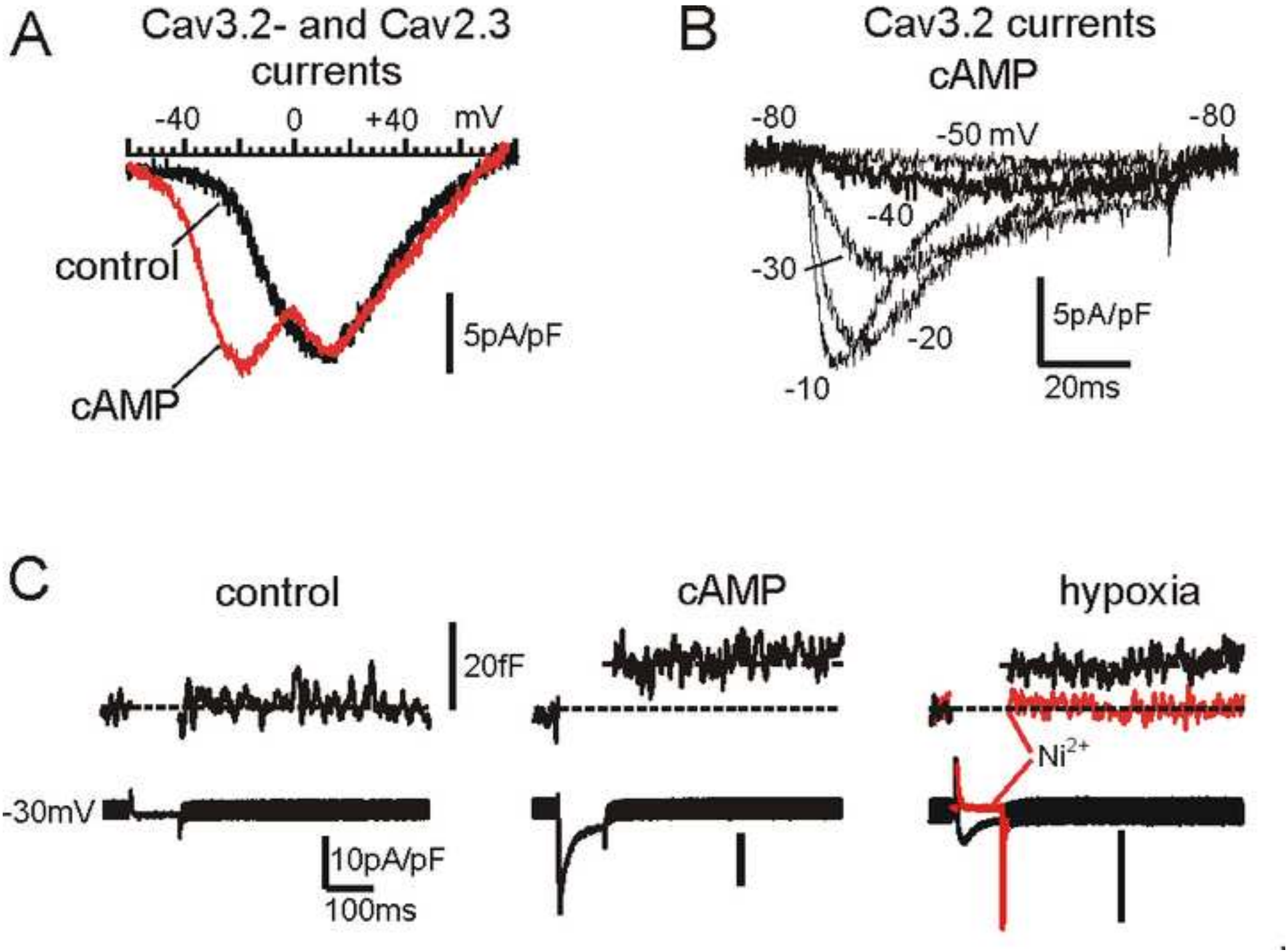

Figure 4

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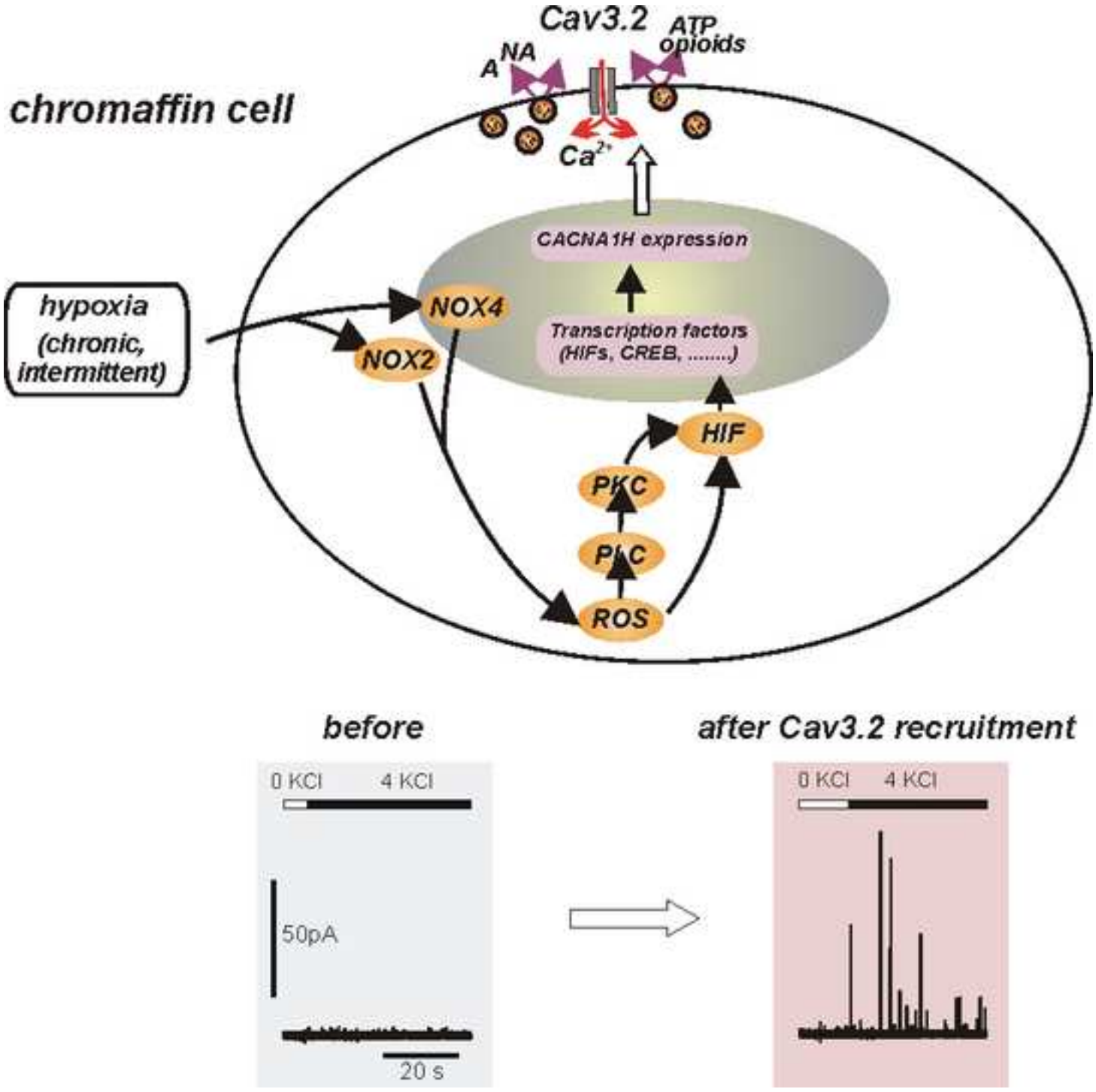

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.
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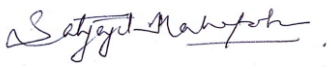
SATYAJIT MAHAPATRA	satyajit.mahapatra@unito.it
CHIARA CALORIO	chiara.calorio@unito.it
DAVID VANDAEL	davidhenrif.vandael@unito.it
ANDREA MARCANTONI	andrea.marcantoni@unito.it
VALENTINA CARABELLI	valentina.carabelli@unito.it
EMILIO CARBONE	emilio.carbone@unito.it

SATYAJIT MAHAPATRA	22-12-2011	
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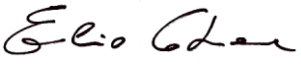
DAVID VANDAEL	22-12-2011	
ANDREA MARCANTONI	22-12-2011	
VALENTINA CARABELLI	22-12-2011	
EMILIO CARBONE	22-12-2011	

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CHIARA CALORIO 22-12-2011 

DAVID VANDAEL 22-12-2011 

ANDREA MARCANTONI 22-12-2011 

VALENTINA CARABELLI 22-12-2011 

EMILIO CARBONE 22-12-2011