

**Functional chromaffin cell plasticity in response to stress:
focus on nicotinic, gap junction and voltage-gated Ca²⁺ channels**

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Abstract

An increase in circulating catecholamines constitutes one of the mechanisms whereby human body responds to stress. In response to chronic stressful situations, the adrenal medullary tissue exhibits crucial morphological and functional changes that are consistent with an improvement of chromaffin cell stimulus-secretion coupling efficiency. Stimulus-secretion coupling encompasses multiple intracellular (chromaffin cell excitability, Ca^{2+} signaling, exocytosis, endocytosis) and intercellular pathways (splanchnic nerve-mediated synaptic transmission, paracrine and endocrine communication, gap junctional coupling), each of them being potentially subjected to functional remodeling upon stress. This review focuses on three chromaffin cell incontrovertible actors, the cholinergic nicotinic receptors and the voltage-dependent T-type Ca^{2+} channels that are directly involved in Ca^{2+} -dependent events controlling catecholamine secretion and electrical activity, and the gap junctional communication involved in the modulation of catecholamine secretion. We show here that these three actors react differently to various stressors, sometimes independently, sometimes in concert or in opposition.

Introduction

In mammals, catecholamine secretion from adrenal chromaffin cells represents an ubiquitous mechanism helping the organism to cope with stressful situations. In response to stress, catecholamines are among the first mediators to be released. Once delivered into the blood circulation, these stress hormones exert multiple actions in particular on the cardiovascular system, leading to appropriate adjustments of blood pressure and cardiac rhythm, and of the energy metabolism, enabling the organism to respond with a threat for survival. To ensure appropriate catecholamine secretion, whose needs strongly differ from basal to stressful conditions, stimulus-secretion coupling of adrenal chromaffin cells undergoes continuous

adaptation. This is achieved by transient or sustained remodeling of pivotal molecular, cellular and tissular adrenal medullary determinants. Among them, synaptic cholinergic neurotransmission between splanchnic nerve endings and chromaffin cells, gap junction-mediated intercellular communication between chromaffin cells and voltage-gated calcium channels at the membrane of chromaffin cells, appear to play substantial roles. For each of these three stimulus-secretion coupling components, this review will describe their contribution to catecholamine exocytosis in basal resting conditions when the hormonal need is low, and how they remodel in response to stressors when the catecholamine demand is high. We will show that the initial stress experienced at birth occurs in the absence of effective acetylcholine neurotransmission and that response of the adrenal medulla involves a non-neurogenic catecholamine secretion in which both gap junctions and low-voltage-activated T-type channels are key actors. Subsequently, the development of the neurogenic control down-regulates the contribution of gap junctions and T-type channels and devotes the predominant control of catecholamine secretion to acetylcholine receptors and high-voltage activated calcium channels (L, N, P/Q, R). In response to chronic stress in adulthood, concerted remodeling of cholinergic synaptic transmission, gap junctions and calcium channels occurs, so as to optimize the excitation-secretion coupling to the new body conditions.

1- Nicotinic acetylcholine receptor channels: the primary target of stimulus-secretion coupling

The primary stimulus of catecholamine secretion from the adrenal medulla comes from the activation of the splanchnic nerve, which releases acetylcholine from its axon terminals and stimulates postsynaptic cholinergic receptors located on chromaffin cells (Douglas, 1968; Kidokoro and Ritchie, 1980; Wakade, 1981). Although cholinergic muscarinic receptors can also participate in chromaffin cell stimulus-secretion coupling (Barbara et al., 1998; Olivos

and Artalejo, 2008), we focus here exclusively on nicotinic receptors (nAChRs). Indeed, while nAChRs ensure a rapid transmission of preganglionic impulses, muscarinic receptors are considered to play a more subsidiary role mostly by facilitating the nicotinic responses. In particular, they are proposed to contribute to long-lasting excitation of chromaffin cells by regulating the catecholamine secretory response upon graded preganglionic stimulation and prolonged periods of time (Olivos and Artalejo, 2008).

1.1. Chromaffin cell nicotinic acetylcholine receptors under resting conditions

The classical view is that activation of nAChRs evokes a membrane depolarization, which in turn triggers electrical activity and calcium influx from voltage-dependant calcium channels (Kilpatrick et al., 1982). This massive calcium influx, potentiated by a calcium-induced calcium release from the endoplasmic reticulum via the ryanodine receptors (Alonso et al., 1999; Inoue et al., 2003; Wu et al., 2010), would then be responsible for triggering exocytosis of catecholamine-containing granules. Additionally, calcium influx through the nAChR channel itself may directly contribute to intracellular calcium elevation and to catecholamine release. Although challenged recently (Arnaiz-Cot et al., 2008), this hypothesis is strongly supported by the fact that activation of nAChRs triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization (Mollard et al., 1995).

Chromaffin cell nAChRs belong to the neuronal nAChR family (Gotti et al., 2006; Sala et al., 2008). They are composed of five principal transmembrane subunits of the α and β families. Since calcium permeability and duration of pore opening largely depend on the subunit composition (Albuquerque et al., 2009), it is worth studying this composition and its putative regulation during physiological (development, aging,...) and/or physiopathological (stress, hypertension,...) situations. Heteromeric $\alpha 3\beta 4$ receptors are considered the main nAChRs expressed in bovine (Criado et al., 1992; Campos-Caro et al., 1997) or rat (Di Angelantonio et al., 2003; Colomer et al., 2010) chromaffin cells, although $\alpha 5$ subunit

transcript has also been detected in bovine chromaffin cells (Campos-Caro et al., 1999). $\alpha 3\beta 4$ nAChRs belong to the α -bungarotoxin insensitive receptor class and they are believed to account for most of the acetylcholine-triggered catecholamine release in control conditions, which is mainly insensitive to this toxin (Kilpatrick et al., 1981; Sala et al., 2008). In addition to this dominant form, α -bungarotoxin sensitive homomeric $\alpha 7$ - and $\alpha 9/\alpha 10$ -built nAChRs are also expressed in bovine (Garcia-Guzman et al., 1995) and rat (Martin et al., 2003; Colomer et al., 2010) chromaffin cells, but their role in catecholamine secretion is less clear. Using specific toxins, Lopez and collaborators (Lopez et al., 1998) have demonstrated that $\alpha 7$ nAChRs do contribute to acetylcholine-induced inward current and to catecholamine secretion from bovine chromaffin cells. However, it remains that catecholamine release is insensitive to α -bungarotoxin in many preparations (Sala et al., 2008), shading doubts on the ubiquitous contribution of this receptor to catecholamine exocytosis. Regarding $\alpha 9$ -containing nAChRs, they contribute to spontaneous excitatory postsynaptic and acetylcholine-evoked inward currents (Colomer et al., 2010), but their contribution to catecholamine exocytosis remains to be investigated. Human chromaffin cells express both homomeric and heteromeric nAChRs, as evidenced by the identification of $\alpha 7$ (Perez-Alvarez et al., 2011c) and $\alpha 6\beta 4$ -containing channels (Perez-Alvarez et al., 2011a). From a functional point of view, these nAChRs differentially contribute to catecholamine exocytosis. Ca^{2+} entry through $\alpha 7$ nAChRs is unable to evoke exocytosis by itself, but these receptors contribute to fast-onset membrane depolarization and subsequently to hormone secretion (Perez-Alvarez et al., 2011c). By contrast, $\alpha 6\beta 4$ -containing nAChRs are directly involved in the exocytotic process (Perez-Alvarez et al., 2011a).

1.2. Modulation in response to stressful situations

Direct sensitivity to hypoxia is the mean by which chromaffin cells release catecholamines during fetus expulsion, when the transition from the intra-uterine cocoon to the airborne

outside induce a lack of oxygen and threatens all organs. The neonate copes with this stressful situation by triggering a catecholamine surge. By stimulating the cardiovascular and respiratory systems, catecholamines contribute to the ability of the organism to withstand acute oxygen deprivation and to protect organs from severe damages. The striking features of catecholamine secretion occurring in neonates are that i) it occurs before the complete maturation of splanchnic nerve synapses, adrenal medullary innervation being non-functional until the end of the first postnatal week (Slotkin, 1986; Parker et al., 1988) and ii) it is triggered directly by the propensity of some chromaffin cells to sense oxygen decrease and to respond by catecholamine release (Seidler and Slotkin, 1985; Thompson et al., 1997; Garcia-Fernandez et al., 2007) and reviewed in (Colomer et al., 2011). Maturation of splanchnic nerve synapses during the first postnatal week allows the neurogenic control of catecholamine release to take place and sets a tonic inhibition on the direct sensitivity of chromaffin cells to hypoxia (Thompson et al., 1997; Garcia-Fernandez et al., 2007; Nurse et al., 2009). This negative control is indeed mediated by acetylcholine and by the contribution of the $\alpha 7$ -built nAChRs (Buttigieg et al., 2009). Further support for this contribution comes from the observation that long-term hypoxia, supposed to stimulate the direct sensitivity of chromaffin cells to hypoxia, decreases the expression of $\alpha 7$ subunit in ovine fetus adrenal gland (Ducsay et al., 2007). Recently, it has been reported that the expression of $\alpha 9$ -containing nAChRs (measured both at the messenger and protein levels) is specifically upregulated by cold exposure-evoked stress (Colomer et al., 2010). Although their contribution to catecholamine release has not been evaluated yet, in view of their high calcium permeability as compared to $\alpha 3$ -containing nAChRs (Jagger et al., 2000; Fucile, 2004; Fucile et al., 2006), it is highly probable that $\alpha 9$ -containing nAChRs significantly contribute to the high catecholamine secretion needed during chronic stressful situations. All these findings strengthen an important contribution of nAChRs in adaptive response to stress.

As an attempt to schematize the respective role of nAChRs in the adrenal medullary tissue, we can state that the $\alpha 3\beta 4$ -built receptor is the pillar on which acetylcholine-evoked catecholamine release lays primarily. Homomeric $\alpha 7$ - and $\alpha 9$ -containing receptors, although probably contributing to this process, seem mainly involved in adjoining but crucial long-term regulatory processes. Indeed, cholinergic innervation of the adrenal medulla does not only trigger secretion, it also maintains a negative control on two specific properties of chromaffin cells: their specific activation by hypoxia ($\alpha 7$ -built nAChRs, see above) and their degree of gap junction-mediated intercellular communication ($\alpha 9$ -built nAChRs, see chapter 3).

In summary, a global view would be that, in control conditions, heteromeric $\alpha 3\beta 4$ receptors carry out the main part of catecholamine secretion while other cholinergic functions, involving long-term regulations of chromaffin cells, would be taken in charge by homomeric $\alpha 7$ and/or $\alpha 9/\alpha 10$ nAChRs. In response to the threat of chronic stress (long-term hypoxia, cold exposure, ...), up- or down-regulation of these homomeric channels would allow chromaffin cells to recover some of their inhibited functions and to be better armed to respond and protect the individual. Consistent with this hypothesis, $\alpha 7$ - but not $\alpha 4\beta 2$ -built nAChRs have been described as the main nAChR subtype responsible for the attenuation of lipopolysaccharide-induced neuroinflammation in the brain (Tyagi et al., 2010). A further support for the important role of homomeric nAChRs in the stress response comes from the finding that $\alpha 7$ nAChRs are strongly regulated by chronic stress and corticosteroids/glucocorticoids (Carrasco-Serrano and Criado, 2004), especially in hippocampus (Hunter et al., 2010), a brain region involved in stress reactivity. It is noteworthy that beside the adrenal medullary tissue, stress also induces remodeling of nAChRs in other sympathetic ganglia, as recently reported in sympathetic neurons in response to hyperglycemia (Campanucci et al., 2010). Altogether and in addition to numerous studies performed in brain of stressed animals, neuronal nAChRs appear to be crucial targets in the response to stress.

2- Gap junction channels: a positive modulator of catecholamine secretion

As mentioned above, adrenal catecholamine secretion is under a dual regulation involving an incoming initial cholinergic command from the splanchnic nerve terminals synapsing onto chromaffin cells and a local modulation through gap junctions expressed by chromaffin cells (Colomer et al., 2009, 2011 for two recent reviews). The cholinergic control of catecholamine secretion is known for several decades (Douglas et al., 1967; Douglas, 1968; Axelrod, 1971; Douglas, 1975). By contrast, the involvement of gap junctional coupling between chromaffin cells in catecholamine release has been recently reported (Martin et al., 2001), although it was suspected for a long time (Cena et al., 1983).

Gap junction channels are built of the apposition of two hemichannels in adjacent cells. Each hemichannel is composed of six protein subunits called connexins in vertebrates. Gap junction channels connect the cytoplasm of two contiguous cells, therefore providing a direct route for electrical and metabolic signaling between coupled cells. By regulating the passage of ions and small molecules, gap junction channels are critically important in many biological activities, including hormone secretion from endocrine/neuroendocrine cells (Caton et al., 2002; Colomer et al., 2009; Jain and Lammert, 2009; Stojilkovic et al.; Bosco et al., 2011).

2.1. Connexin expression in the adrenal medullary tissue

The presence of intercellular junctions in the adrenal medullary tissue was initially uncovered by freeze-fracture (Grynszpan-Wynograd and Nicolas, 1980). Gap junction plaques were identified in various species, including mouse, guinea-pig, hamster and rabbit, but not in rat (Grynszpan-Wynograd and Nicolas, 1980). Regarding protein subunit expression, six connexins (Cx26, Cx29, Cx32, Cx36, Cx43 and Cx50) have been unambiguously found in the adrenal medullary tissue to date (recently reviewed in (Colomer et al., 2009; Colomer et al., 2011)). Table 1 summarises the main connexins expressed in the mammalian adrenal medulla, both in normal and tumoral tissue. The presence of connexin-

built gap junctions has been described in several adrenal medullary cell types, including both secreting and non-secreting cells.

To date, three connexins (Cx36, Cx43 and Cx50) have been identified in chromaffin cells (figure 1), with a differential expression between species. While rat neuroendocrine chromaffin cells exhibit both Cx36 and Cx43 (Meda et al., 1993; Martin et al., 2001; Colomer et al., 2008b), mouse chromaffin cells appear to be dominantly coupled by Cx36-built gap junctions, Cx43 being weakly expressed (Murray and Pharrams, 1997; Degen et al., 2004; Li et al., 2004), personal observations). In the human adrenal medulla, the main connexin expressed in chromaffin cells is Cx50 (Willenberg et al., 2006). In addition, the expression of connexins also depends on the normal or tumoral state of the tissue, as reported in human. Indeed, in pheochromocytomas, tumors arising from catecholamine-secreting cells, expression of Cx26 and Cx32, which are not present in the normal tissue, has been detected (Willenberg et al., 2006; Colomer et al., 2011 for review) (table 1).

Regarding non-secreting cells in the adrenal medullary tissue, Cx29 is expressed in S100-positive cells (Eiberger et al., 2006), targeting likely the glial-like sustentacular cell population (Cocchia and Michetti, 1981; Lloyd et al., 1985) (figure 1A). This is consistent with previous studies illustrating the presence and functionality of gap junction channels between glial-like non-endocrine pituitary folliculostellate cells (Morand et al., 1996; Fauquier et al., 2001). Cx29 is also expressed in cells surrounding the preganglionic sympathetic nerve fibers that innervate the medulla (Eiberger et al., 2006).

2.2. Possible functions of gap junction channels in the adrenal medulla

2.2.1. Under resting conditions: a moderate contribution to catecholamine release

The involvement of intercellular junction-mediated electrocoupling process in adrenal catecholamine release has been initially proposed by Ceña and colleagues (Cena et al., 1983). Consistent with this assumption, a junctional communication mediated by a low-conductance

electrical coupling between chromaffin cells has been recorded in mouse acute slices (Moser, 1998), thus opening the possibility that depolarization of one cell may trigger action potentials in adjacent cells and that electrical coupling may ensure activation of several, if not all cells within a cluster. The contribution of gap junctions to the propagation of electrical (and ensuing Ca^{2+}) signals between chromaffin cells has been unambiguously demonstrated in rat adrenal slices in the beginning of the 2000s, by the work of Martin and collaborators (Martin et al., 2001). It is noteworthy that under basal conditions, most of the coupled cells exhibit a weak electrical coupling, both in females (Martin et al., 2001) and males (Colomer et al., 2008b). This results in the propagation of a small depolarizing waveform in response to action potentials evoked in the stimulated cells (figure 2A). However, when the membrane potential of the coupled cell is close to action potential threshold, a firing activity can be evoked (figure 2B). This gap junction-mediated electrical propagation is physiologically relevant, as evidenced by the detection of exocytotic signals in coupled cells in response to stimulation (depolarization-evoked action potentials or iontophoretic application of nicotine) of a single chromaffin cell (Martin et al., 2001). The current hypothesis is that, under basal conditions corresponding to low frequency splanchnic nerve discharge, few synaptic boutons are stimulated and the propagation of secretory events (electrical and Ca^{2+} signals) through gap junctions represents an efficient complement to synaptic transmission to amplify catecholamine release without mobilizing a large number of synapses.

2.2.2. In response to stressful situations: a significant contribution to catecholamine release

Stressful situations are associated with a massive and robust release of catecholamines, and as proposed by Ceña and collaborators (Cena et al., 1983), an amplifying signal is likely required. Because gap junctions enable propagation of secretory signals between adjacent chromaffin cells, we previously hypothesized that they can be plausible candidates involved in the amplification process. Several findings support the proposal of an up-regulated gap junctional coupling between chromaffin cells in stressed animals (cold exposure): i) Cx36 and

Cx43 expression increases in stressed rats (Colomer et al., 2008b) (figure 3A), ii) the connexin-permeant fluorescent tracer Lucifer yellow more widely spreads within the medullary tissue of stressed rats (Colomer et al., 2008a) (figure 3B) and iii) half of coupled chromaffin cell pairs in stressed rats exhibit a robust electrical coupling that allows the transmission of unattenuated action potentials between cells (Colomer et al., 2008b). Consistently, the cytosolic Ca^{2+} concentration rise triggered by iontophoretic application of nicotine in a single chromaffin cell simultaneously occurs in a larger number of cells when recorded in adrenal slices from stressed rats (Colomer et al., 2008b) (figure 4). Although the direct contribution of increased gap junctional coupling to increased catecholamine secretion has not been evidenced in stressed rats yet, all these findings are consistent with. Altogether, this indicates that gap junction-driven intercellular communication between chromaffin cells is an important determinant of the regulation of catecholamine secretion from the adrenal medullary tissue.

2.3. Interaction between synaptic transmission and gap junctional coupling

2.3.1. Under resting conditions: a negative regulatory control

As mentioned above, both cholinergic synaptic neurotransmission and gap junctional coupling between chromaffin cells are involved in adrenal catecholamine secretion. Interestingly, these two intercellular communication pathways do not function independently but are tightly co-regulated and continuously interact with each other. Indeed, cholinergic innervation enters the play during early postnatal life when synapses mature and begin to exert a negative control on gap junctions (Martin et al., 2005). This tonic inhibitory control persists in adults, as evidenced by the increased gap junctional communication between chromaffin cells in response to the pharmacological blockade of postsynaptic nAChRs (Martin et al., 2003; Colomer et al., 2010). In addition, the junctional coupling between chromaffin cells can also be re-activated by sectioning the splanchnic nerve (Martin et al.,

2003). Conversely, it is inhibited by a prolonged stimulation of nAChRs (Colomer et al., 2011). In summary, in all cases in which the cholinergic synaptic transmission is not competent, junctional coupling between chromaffin cells is significantly enhanced and relays neurotransmission in the control of catecholamine secretion. To date, the cellular mechanisms targeted by nAChR signaling and involved in the inhibitory control of synaptic transmission on gap junctions are largely unknown. The trafficking intracellular machinery is the only identified mediator (Martin et al., 2003) but most of the characterization remains to be performed.

2.3.2. In response to stressful situations: a synergistic action

In response to chronic stress, gap junctional and synaptic communications undergo profound anatomical and functional remodeling, resulting in increased synaptic inputs on highly coupled chromaffin cells (Colomer et al., 2008a; Colomer et al., 2008b). One remarkable feature of this plasticity is the abolition of the tonic inhibitory control exerted by synaptic transmission on junctional coupling. By acting in synergy, the two pathways likely allow the adrenal medulla to respond to an increased catecholamine demand.

Thus, it is clear that, in the adrenal medullary tissue, gap junctional communication between chromaffin cells and synaptic cholinergic neurotransmission undergo parallel or opposite regulations. In control conditions, cholinergic innervation down-regulates gap junctional coupling but, in stressful situations, this inhibition is abolished and an increase of both communication pathways is observed. To date, the mechanisms shutting down the inhibitory control are unknown. In a finely tuned coordination with synaptic transmission, gap junctional channels contribute to the adrenal chromaffin cell function, not only at rest when the hormonal need is weak but also in response to a huge catecholamine demand. Our proposal is that in basal conditions, the firing nerve discharge is low and chromaffin cells exhibit an action potential firing matching the sympathetic tone and release catecholamines at a modest rate (Fulop and Smith, 2007). Under these conditions, gap junction-mediated

coupling may help chromaffin cells to coordinate their electrical and ensuing calcium activities and may therefore represent an efficient complement to synaptic transmission to amplify catecholamine release. In response to an acute stress occurring with a sudden increase in splanchnic nerve discharge frequency, gap junctional intercellular communication would also facilitate catecholamine release. By contrast, when the firing discharge of the splanchnic nerve is high and sustained, as observed in response to a chronic stress, increased strength of gap junctional coupling could either have a synergistic effect with synaptic transmission, or exert a shunting effect preventing over-secretion of catecholamines as recently proposed (Colomer et al., 2009).

3- Voltage-gated Ca^{2+} channels: crucial players of chromaffin cell excitability and catecholamine secretion

Voltage-gated Ca^{2+} channels are protein complexes which encompass 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 membrane to form the pore lining (Catterall, 2011). 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 α_1 -subunits have been cloned with specialized functions and distributions. Four members belong to the Cav1 group (L-type), three to the Cav2 group (N, P/Q and R-types) and three to the Cav3 group (T-type). Based on the activation threshold, 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) open at voltages very close to the negative values of Cav3 (T-type) channel

activation (Lipscombe et al., 2004; Singh et al., 2008; Marcantoni et al., 2010; Mahapatra et al., 2011).

3.1. Chromaffin cell Ca²⁺ channels under resting conditions

The adrenal chromaffin cells of mammalian species express all types of voltage-gated Ca²⁺ channels described above (Carbone et al., 2001; Carbone et al., 2006; Garcia et al., 2006; Marcantoni et al., 2008; Vandael et al., 2010). Their expression density changes remarkably among animal species although their function remains surprisingly identical. Ca²⁺ channels shape action potential waveforms, control catecholamine secretion and vesicle retrieval and regulate Ca²⁺-dependent events that originate near the membrane. An obvious question is: why chromaffin cells, which are round-shaped with no synaptic contacts and neuronal-like morphology, need so many Ca²⁺ channel types (L, N, P/Q, R, T) to perform few simple basal functions? There is no clear answer to this question, but increasing evidence indicates that Ca²⁺ channels in chromaffin cells play roles similar to those in other tissues. Before focusing on the more relevant aspects of Ca²⁺ channel remodeling during stress, we will briefly summarize here the function that each Ca²⁺ channel group (Cav1, Cav2 and Cav3) plays in the physiology of chromaffin cells during normal functioning.

3.1.1. The Cav1 (L-type) channels

L-type Ca²⁺ channels (LTCCs) are widely expressed in many tissues and control a number of Ca²⁺-dependent responses in excitable cells. Of the four identified α 1-subunits (Cav1.1, Cav1.2, Cav1.3 and Cav1.4), chromaffin cells express only the Cav1.2 and Cav1.3 isoforms (Garcia-Palomero et al., 2000; Baldelli et al., 2004; Benavides et al., 2004; Marcantoni et al., 2010), which are both highly sensitive to 1,4-dihydropyridines blockers (Ca²⁺ antagonists) or activators (Ca²⁺ agonists). Cav1.2 and Cav1.3 are highly expressed in rat (RCCs), mouse (MCCs) and human chromaffin cells (HCCs) and they control a large fraction of catecholamine release induced by different stimuli such as: sustained splanchnic nerve

stimulation (Lopez et al., 1992), KCl- and ACh-induced depolarization (Cena et al., 1983; Lopez et al., 1994) and voltage-clamp pulses (Horrigan and Bookman, 1994; Carabelli et al., 2003; Garcia et al., 2006; Marcantoni et al., 2008).

Since chromaffin cells constantly release catecholamines, during low and sustained sympathetic stimulation (Chan and Smith, 2001), LTCCs and the other Ca^{2+} channels experience a basal and a stimulus-induced autocrine modulation, which alters their activation/inactivation time course and the amount of Ca^{2+} entering the cell during activity (Carbone et al., 2001; Marcantoni et al., 2007; Marcantoni et al., 2008). This effect is mediated by adrenergic, opioidergic and purinergic G protein-coupled receptors (GPCRs) and occurs in both isolated cells (Hernandez-Guijo et al., 1999; Cesetti et al., 2003) and adrenal gland slices (Hernandez et al., 2011). In the case of Cav1.2 and Cav1.3, the modulation is dual: it can either down- or up-regulate LTCC currents. The down-modulation is fast, mediated by PTX-sensitive G proteins (Hernandez-Guijo et al., 1999; Hernandez et al., 2011) and limited to membrane micro-domains (Carabelli et al., 2001), while the up-regulation is slow, remote and mediated by the cAMP/PKA phosphorylation pathway (Carabelli et al., 2001; Cesetti et al., 2003; Marcantoni et al., 2009; Hernandez et al., 2011). Given that LTCCs can also be down-regulated by the NO/cGMP/PKG phosphorylation pathway (Carabelli et al., 2002), all this suggests that, depending on cell conditions (high cAMP/low cGMP *versus* low cAMP/high cGMP), chromaffin cell LTCCs can undergo extreme up- or down-modulations which may change Ca^{2+} currents and catecholamine release by one order of magnitude (Mahapatra, Marcantoni, Carabelli and Carbone, unpublished results).

LTCCs also possess two further key properties that make them even more strategic for chromaffin cell physiology at rest and during stressful stimulations. One is related to the role that Cav1.3 (and Cav1.2) plays in pacemaking mouse chromaffin cells near resting conditions (Marcantoni et al., 2010; Vandael et al., 2010) and another relates to the strict control that LTCCs exert on endocytosis following secretion (Rosa et al., 2007; Rosa et al., 2010; Rosa et

al., 2011). The long form of Cav1.3 (Cav1.3₄₉) is highly expressed in mouse chromaffin cells (Marcantoni et al., 2010) and activates at about 9 and 24 mV more negative voltages than Cav1.2 and Nav1.7 channels (half activation at -27 mV in 2 mM Ca²⁺ (Mahapatra et al., 2011). Furthermore, Cav1.3₄₉ inactivates slowly and only partially during pulses of 0.5 to 1 s. Thus, this channel is suitable for pacemaking chromaffin cells with spontaneous firing frequencies of 0.5-2 Hz and interpulse potential of -50 mV. Indeed, removal of Cav1.3 in Cav1.3^{-/-} KO mice causes a dramatic decrease of L-type pacemaking currents and a drastic reduction in the number of spontaneously firing cells in an external medium containing 4 mM KCl (Marcantoni et al., 2010). This estimate can be overlooked if MCCs are kept at more depolarized resting potentials using higher KCl concentrations (Mahapatra et al., 2011; Perez-Alvarez et al., 2011b).

LTCCs do also participate to the control of vesicle endocytosis in bovine chromaffin cells (Rosa et al., 2007). Block of LTCCs by dihydropyridines has little effects on the fast exocytosis but largely prevents both compensatory and excess endocytosis, thus causing an increased slow exocytosis during prolonged Ca²⁺ entries that stimulate vesicle retrieval. There is not yet a clear explanation to this phenomenon but it seems evident that endocytosis is favored by Ca²⁺ channels, like the L-type, that are able to maintain prolonged Ca²⁺ entries during sustained depolarizations (Rosa et al., 2011) and that sphingosine plays a permissive role in the regulation of Ca²⁺-dependent endocytosis (Rosa et al., 2010).

3.1.2. 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 Ca²⁺ currents that initiate synaptic transmission. The efficiency of neurotransmitter release is steeply dependent on the 3rd to 4th power of Ca²⁺ entry through these voltage-gated channels making them an important target of synaptic regulation. Cav2.1 channels carrying P/Q-type Ca²⁺ currents and Cav2.2 channels carrying N-type Ca²⁺ currents are the predominant pathways through which Ca²⁺ initiates the rapid release of

neurotransmitters (glutamate, acetylcholine, GABA). Extensive studies indicate that Cav2.1 and Cav2.2 functioning is critically regulated by many different protein interactions (SNARE complex and G protein subunits) with their intracellular domains, which form the basis of Ca^{2+} - and voltage-dependent signal transduction at the synaptic terminal (Catterall, 2011).

In chromaffin cells, Cav2.1 and Cav2.2 are expressed at different densities in all animal species (Garcia et al., 2006) while Cav2.3 seems to be preferentially expressed in MCCs and RCCs (Albillos et al., 2000; Marcantoni et al., 2010). 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 calcium channels expressed in chromaffin cells (Horrigan and Bookman, 1994; Kim et al., 1995; Carabelli et al., 2003; Thiagarajan et al., 2004; Giaccipoli et al., 2006; Carabelli et al., 2007a; Rosa et al., 2011). 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 (Chow et al., 1992), but distributed within an average distance of 200-300 nm and assembled in specialized regions of the surface membrane with dimensions of several micrometers (Klingauf and Neher, 1997; Neher, 2006). In this way, the dominant Ca^{2+} signal regulating vesicle release derives from the activation of multiple channels operating over distances of many micrometers rather than from Ca^{2+} channel clusters localized in microdomains (Wu et al., 2009).

At variance with LTCCs, which control the subthreshold current regulating action potential firing in RCCs and MCCs, the Cav2 channels contribute mostly to the upstroke and falling phase of action potentials. They are also differently modulated in a voltage-dependent manner by G protein-coupled pathways (Ikeda and Dunlap, 1999). In chromaffin cells, Cav2.1 and Cav2.2 are autocrinally inhibited by ATP and opioids that are released during cell activity (Gandia et al., 1993; Albillos et al., 1996a; Albillos et al., 1996b; Currie and Fox, 1996). 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 negative potentials (Carabelli et al., 1996; Carabelli et al., 1998). This effect, however, can be reversed by depolarization. The normal fast activation is recovered during strong depolarizations (Marchetti et al., 1986; Bean, 1989) or when a strong pre-pulse anticipates a weak depolarization (Elmslie et al., 1990). This phenomenon is called “voltage-dependent facilitation” and is attributed to a protein-protein interaction between the receptor activated G $\beta\gamma$ subunit and the II-III loop of Cav2 channels (Ikeda, 1996; Herlitze et al., 1997; Zamponi et al., 1997). Interestingly, this Cav2.1 and Cav2.2 down-modulation can be partially reverted during short depolarizations repeated at high frequency (Currie and Fox, 2002) mimicking high frequency action potential trains that occur under stress conditions. The final result is that the attenuated Ca²⁺ 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 (Hernandez-Guijo et al., 1998b) and slices of mouse adrenal glands (Hernandez et al., 2011) where the packed organization of the cells is well preserved and the released neurotransmitters (ATP and opioids) can accumulate in the extracellular space and exert a basal tonic inhibition on cell firing and catecholamine secretion.

3.1.3. The Cav3 (T-type) channels

T-type Ca²⁺ channels (TTCCs) are transient, low-voltage activated Ca²⁺ channels that control Ca²⁺ entry during mild depolarizations near resting potential. Studies in the past 25 years have clarified their functional role in controlling: low-threshold spikes, oscillatory cell activity, muscle contraction, hormone release, cell growth, differentiation and proliferation (Perez-Reyes, 2003; Carbone et al., 2006). Due to their widespread localization and function, TTCCs are 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 (Giordanetto et al., 2011).

TTCCs possess unique properties, which facilitate their biophysical identification: (1) activation is sharply voltage-dependent and channels open at hyperpolarized potentials (-50 mV in 5 mM Ca^{2+}); (2) inactivation is voltage-dependent, independent of Ca^{2+} and complete within tens of milliseconds at positive potentials; (3) deactivation is slow at potentials near rest; (4) Ca^{2+} and Ba^{2+} are carried with equal efficacy and single channel conductance is a factor 3- to 4-fold lower than that of Cav1 and Cav2; (5) activation and steady-state inactivation (channel availability) overlap near resting potential, providing a 3-20 pA inward 'window current'. Molecular cloning of TTCCs has provided evidence for the existence of three different pore forming $\alpha 1$ subunits (Cav3.1, Cav3.2 and Cav3.3), which possess only 25% amino acid homology but similar pore structure organization to Cav1 and Cav2 (Perez-Reyes, 2003).

Despite their widespread distribution in most tissues (neuronal, muscular, endocrine), TTCCs are either absent or weakly expressed in adult chromaffin cells. In adult bovine chromaffin cells, the mRNA encoding Cav3.1 and Cav3.2 is expressed (Garcia-Palomero et al., 2001), but functional TTCCs have not been detected (Cena et al., 1983; Artalejo et al., 1991; Albillos et al., 1993; Carabelli et al., 1998), except in one case (Diverse-Pierluissi et al., 1991). On the contrary, TTCCs are functionally expressed in embryonic and neonatal RCCs (Bournaud et al., 2001; Levitsky and Lopez-Barneo, 2009; Souvannakitti et al., 2010) and are available in a small percentage of adult RCCs (Hollins and Ikeda, 1996; Novara et al., 2004; Carabelli et al., 2007b). TTCCs channels are also not expressed in adult MCCs (Hernandez-Guijo et al., 1998a; Marcantoni et al., 2010), but these cells sometimes display the typical "low-threshold shoulder" on the I/V relationship of ramp commands, which is the fingerprint of TTCCs (see Fig. 4 in (Gosso et al., 2011)). These low-threshold currents are also present in Cav1.3 KO MCCs (Navarro-Tableros, Carabelli and Carbone, unpublished observation), excluding the possibility that they derive from the C-terminus "short" splice variant of Cav1.3 $\alpha 1$ -subunit (Cav1.3_{42a}), which activates at very negative potentials and inactivates quickly in a

Ca²⁺ dependent manner (Singh et al., 2008). It is thus very likely that availability of TTCCs may depend critically on chromaffin cell conditions or stimulations, as in the case of applied chronic stressors (Novara et al., 2004; Giaccipoli et al., 2006; Carabelli et al., 2007b) or acute sympatho-adrenal stress stimuli (Hill et al., 2011), which up-regulate Cav3.2 channels in RCCs and MCCs (figures 5A and 5B).

When expressed in chromaffin cells, TTCCs are shown to control a sizeable “low-threshold” catecholamine release that is absent in cells possessing only HVA channels (Giaccipoli et al., 2006; Carabelli et al., 2007a; Carabelli et al., 2007b; Marcantoni et al., 2008). Of relevance is the effective coupling existing between TTCCs and the pool of readily releasable vesicles. The Ca²⁺-dependence of TTCCs-controlled secretion is linear and strikingly similar to that of LTCCs (Carabelli et al., 2007a), which in turn is comparable to that of P/Q and N-type channels (Carabelli et al., 2003). Thus, although preserving a low-voltage range of activation, TTCCs appear similarly distributed around the docked vesicles as the HVA channels. In conclusion, TTCCs lower the threshold of action potential generation and ensure a broader interval of voltage control on catecholamine secretion, which may be significant when chromaffin cells depolarize steadily or fire at high frequencies (15-20 Hz) during stress conditions.

3.2. Chromaffin cell Ca²⁺ channel remodeling in response to stressful situations

As for the nicotinic receptors and gap junction channels (Guerineau and Desarmenien, 2010), voltage-gated Ca²⁺ channels undergo functional remodeling during stress (Carbone et al., 2006). This is particularly evident in chromaffin cells of adult animals as an adaptation response to stressful situations including chronic and intermittent hypoxia (Carabelli et al., 2007b; Souvannakitti et al., 2010), β -adrenergic receptor (AR) and high-frequency sympathetic stimulation (Novara et al., 2004; Hill et al., 2011) and hypoxia occurring during the transition from intrauterine to air-breathing life (Levitsky and Lopez-Barneo, 2009).

A common molecular mechanism in response to the indicated stressors is a marked recruitment of functioning Cav3.2 channels that: i) lowers the threshold of action potential firing (Novara et al., 2004) (figure 5C), ii) increases the amount of secreted catecholamines at low potentials (-30, -40 mV) (Giancippoli et al., 2006; Carabelli et al., 2007b) (figures 6A and 6B) and iii) mobilizes a pool of readily releasable vesicles equivalent to that recruited by L-type channels (Carabelli et al., 2007a). Figures 5C and figures 6A and 6B summarize these properties associated with Cav3.2 channels specifically up-regulated by stressors. Most likely, an increased density of T-type channels is also the cause of depolarized resting potential and increased firing frequency occurring in spontaneously firing RCCs in the adrenal gland slices of stressed animals (Colomer et al., 2008a). An increased density of functioning T-type channels during stressful stimuli, appears also to be a more general remodeling mechanism that many cell types and tissues experience in response to hormones (ACTH, aldosterone), cell proliferation and cell conditions associated to stressors. Table 2 lists a number of stressors and stress-induced conditions which lead to an up-regulation or recruitment of newly available Cav3.2 T-type channels in a variety of cells. Notice how neuronal, cardiac and endocrine tissues all respond to stressors with an increased density of Cav3.2 (and Ca3.1) channels, justifying the informal terminology that T-type channels are “stress-induced channels” (Carbone et al., 2006; Hill et al., 2011).

3.2.1. The signaling pathways underlying T-type channels up-regulation in chromaffin cells

Table 2 illustrates the multiple pathways through which Cav3.2 channels are up-regulated by stressors. Some act acutely within minutes (PACAP and high-frequency sympathetic stimulation) while the majority act more slowly, requiring days for full development (hypoxia, β_1 -AR stimulation, VIP, aldosterone and ACTH exposure). In addition, the action of stressors is mediated by different intracellular messengers and transcription factors, suggesting the existence of multiple pathways converging on a common target: the activation, up-regulation or recruitment of Cav3.2 channels.

Regarding the stress response of chromaffin cells, there is now consisting evidence in favor of two well distinct pathways in which Cav3.2 are either activated or up-regulated by stressors. One pathway develops rapidly (minutes) and is most evident in mouse adrenal gland slices. The cascade of events 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 (Hill et al., 2011). The PKC target is the phosphorylation of the Na⁺/Ca²⁺ exchanger which causes a small depolarization near resting potential, sufficient to activate a robust low-threshold current carried by Cav3.2 channels, which are also up-regulated by the stimulation (Kuri et al., 2009) for a schematic model). Significantly different, although involving similar molecular components, are the long-lasting mechanisms by which hypoxia and cell exposure to β_1 -AR agonists (isoprenaline) act on Cav3.2 channels. Both systems require hours or days to fully develop. They cause increased levels of CACNA1H mRNA (Cav3.2 gene) and their action is prevented by specific inhibitors of protein synthesis (Novara et al., 2004; Giancippoli et al., 2006; Carabelli et al., 2007a; Carabelli et al., 2007b; Souvannakitti et al., 2010), suggesting a net recruitment of newly available Cav3.2 channels (figure 7). For the case of β_1 -AR stimulation, Cav3.2 recruitment is mediated by a cAMP-dependent Epac pathway which leads to the activation of transcription factors (Novara et al., 2004), possibly through the phosphorylation of ERK, as reported for other Epac-regulated cell signaling pathways (Borland et al., 2009).

For the case of hypoxia (figure 7), the present findings indicate the involvement of hypoxia-inducible factors (HIF-1 α , HIF-2 α), although a direct proof of their participation is still missing. HIF-2 α appears to be involved in the up-regulation of Cav3.2 in PC12 cells exposed to chronic hypoxia (Del Toro et al., 2003) and overnight incubation with the unspecific HIF activator desferrioxamine (DFX) mimics the effects of hypoxia in PC12 cells (Del Toro et al., 2003) and RCCs (Carabelli et al., 2007b). In addition to HIFs, NADPH-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. A hypothetical scheme in which NOX, ROS and HIF are linked together is reported in figure 7. The scheme is derived from the work of Prabakhar's group in which ROS are shown to activate PLC, DAG and PKC, favoring the stabilization and translocation of HIF-1 α to the nucleus to start transcription and gene expression (Yuan et al., 2008).

In conclusion, several issues remain to be clarified about the molecular pathways regulating the stress-induced response of chromaffin cells, but it seems very likely that with some adjustments, the two pathways illustrated in figure 7 could cover a large number of stress-induced effects observed in different cells and tissues (see for instance (Liu et al., 2010). Additional pathways cannot be excluded and further work will possibly highlight alternative ones.

3.2.2. T-type channel remodeling in chromaffin cells during intrauterine-neonate transition and cholinergic splanchnic denervation

A unique property of chromaffin cells is their high chemosensitivity to hypoxia during fetal life, when these cells respond to acute lowering of O₂ levels with a marked catecholamine release, mostly controlled by Ca²⁺ influx through Cav3.2 channels (Levitsky and Lopez-Barneo, 2009). As discussed previously, in neonatal animals (<7 days), the control of hormone release from the adrenal medulla is mostly non-neurogenic (Slotkin, 1986) and strongly linked to the robust gap junction coupling existing between neighboring cells (Martin et al., 2003; Guerineau and Desarmenien, 2010). Tight electrical coupling and high density of T-type channels facilitate action potential generation and propagation, as well as synchronous release of catecholamines from extended cell populations of the adrenal medulla, resulting in an increased amount of hormone release (Colomer et al., 2009; Guerineau and Desarmenien, 2010). It is however interesting that the T-type channels expressed in the chromaffin cells of newborn rats, not only are responsible for the catecholamine release during acute hypoxia, but

are also up-regulated during intermittent hypoxia (Souvannakitti et al., 2010). As intermittent hypoxia mimics the physiological conditions experienced by the fetus during delivery, the up-regulation of T-type channels underscores the specific role that these channels play in sustaining the O₂-sensitivity of the adrenal medulla under stressful conditions (hypoxia and hypoglycemia), during adaptation to the extra-uterine life.

Of relevance is also the observation that cholinergic innervation of the adrenal gland, which follows postnatal development (Seidler and Slotkin, 1985), causes a loss of functional Cav3.2 channels (Levitsky and Lopez-Barneo, 2009) paralleled by an increased sensitivity to nicotine (Nurse et al., 2009) and an increased density of available α_7 -built nAChRs (Buttigieg et al., 2009). The close correlation between chromaffin cell innervation and O₂ sensitivity is further strengthened by the finding that both O₂ sensitivity of chromaffin cells and expression of Cav3.2 channels re-appear after adrenal gland denervation. This suggests that: i) Cav3.2 are the Ca²⁺ channels mainly responsible for chromaffin cell response to hypoxia and ii) cholinergic splanchnic innervation of the adrenal medulla down-regulates the expression of Cav3.2 channels through an unknown mechanism (Levitsky and Lopez-Barneo, 2009).

An interesting question is whether there is a link between the lowering of functional Cav3.2 channels and the increased expression of nAChRs. In sensory neurons, T-type channels can undergo down-regulations following intracellular Ca²⁺ overloading (Carbone and Lux, 1987). If this is a possible mechanism, a way for Ca²⁺ to massively enter the cells is through the activation of ion channel receptors which are permeable to Na⁺ and Ca²⁺, like TRPV1 and nAChRs (Chung et al., 2008; Albuquerque et al., 2009). Recent studies in rat sensory neurons have shown that Cav3.2 channels are effectively down-regulated if large Ca²⁺ fluxes are passed through capsaicin-activated TRPV1 channels located close to T-type channels (Comunanza et al., 2011). The same may occur in developing chromaffin cells if, for instance, increasing densities of nAChRs are repeatedly activated and large Ca²⁺ inward currents flow through the activated nAChRs. Explaining whether and how up-regulation of nAChRs and

down-regulation of T-type channels are linked together in developing chromaffin cells is an intriguing issue that future work will certainly help to clarify.

Concluding remarks

The continuous and coherent remodeling of the adrenal stimulus-secretion coupling is essential to the achievement of appropriate catecholamine secretion in response to the body demand. It is now established that gap junctional communication, nAChRs and voltage-gated T-type channels play major roles in chromaffin cell plasticity. Noteworthy, the up-regulation of one partner is associated with the up-, or down-regulation of the other two. Much remains to be done and future studies will address the molecular mechanisms regulating the cross-talks among these membrane channels and the identification of other molecular determinants that undergo an effective remodeling during stress. Concerning this latter, the C-terminus slowly inactivating “long form” of Cav1.3 (L-type) channels is shown to be down-regulated and replaced by the fast-inactivating Cav1.3 “short variant” in chromaffin cells of spontaneously hypertensive rats (Segura-Chama et al., 2011). Since similar remodeling may also occur for other components regulating chromaffin cells excitability (K⁺ channels, G proteins-coupled receptors, ion pumps and ion exchangers), it is obvious that a complete picture of chromaffin cells plasticity during stress might include many more actors, whose identification would be beneficial for a full understanding of the chromaffin cell role in regulating stress response.

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Figure legends

Figure 1: Cellular distribution of connexins within the mammalian adrenal medullary tissue

A. Neuroendocrine chromaffin cells are coupled by three connexins (Cx36, Cx43 and Cx50), while non-secreting sustentacular cells express a unique connexin (Cx29). In addition, note that Cx29 is also present in cells surrounding preganglionic sympathetic nerve fibers innervating the medulla. **B.** Representative example of Cx43 immunolabeling in the rat adrenal medulla (polyclonal rabbit anti-Cx43 from Zymed Laboratories).

Figure 2: Weak electrical coupling between a chromaffin cell pair in a control rat

Two adjacent cells were recorded in the current-clamp mode using the dual patch-clamp technique. A depolarizing current was applied in cell 1 (cell 1*) and the subsequent membrane potential changes simultaneously monitored in both cell 1* and cell 2. **A.** Cells 1* and 2 were maintained at a membrane potential of -70 mV. Chart recording illustrating that emission of an action potential in cell 1* results in a small membrane depolarization in cell 2. **B.** Cell 1* was current-clamped at -80 mV and cell 2 was slightly depolarized to -50 mV, a membrane potential close to action potential threshold. The amplitude of the depolarizing wave transmitted to cell 2 in response to a weak depolarization of cell 1* (not robust enough to generate action potentials in the stimulated cell), is sufficient to trigger an action potential. These findings show that, even if weak, an electrical coupling between chromaffin cells can have physiologically relevant consequences.

Figure 3: Up-regulation of gap junctional communication in stressed rat

Histogram showing that protein expression of both Cx36 and Cx43 is enhanced in cold stressed rats (3- and 2-fold, respectively). This parallels an increased expression of Cx36 mRNA (2.5-fold) but not Cx43 mRNA. **B.** As a functional consequence, an increased

diffusion of Lucifer yellow within the adrenal medulla is observed. Lucifer yellow was introduced into the adrenal gland by scrape-loading. Pictures illustrate raw data (focus on the medullary tissue) and the surface plot analysis (pseudo-colored wireframe plot) shows an extended Lucifer yellow diffusion in the medulla of a cold stressed rat as compared to an unstressed rat (modified from (Colomer et al., 2008a, figure 3).

Figure 4: Extended nicotine-induced simultaneous $[Ca^{2+}]_i$ rises in cold stressed rat chromaffin cells

Adrenal slices were loaded with the Ca^{2+} -sensitive fluorescent probe Oregon Green 488 BAPTA-1. Nicotine (200 mM) was iontophoretically applied on cell 1* through a sharp microelectrode leading to focal stimulation of a single cell (the onset of the nicotinic stimulation is indicated by an arrow) and $[Ca^{2+}]_i$ changes were imaged using real-time confocal microscopy. In a control rat (panel A), the $[Ca^{2+}]_i$ increase originating in the stimulated cell was simultaneously detected in only one adjacent cell while in a stressed rat (panel B), a simultaneous $[Ca^{2+}]_i$ increase was recorded into up to 6 adjacent cells.

Figure 5: cAMP-pre-treatment induces *de novo* expression of Cav3.2 (T-type) channels in rat chromaffin cells

A. I-V curves of R- and T-type channels expressed in control and cAMP-treated cells after exposure to 200 μ M pCPT-cAMP. Both cells were pretreated with ω -toxins and bathed in solutions containing 1 μ M 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. **B.** T-type currents recorded in isolation from a rat chromaffin cell incubated with 3.2 μ M ω -CTx-GVIA, 2 μ M ω -Aga-IVA, 1 μ M SNX-482 (10 min) and bathed with 1 μ M nifedipine to nearly block all HVA channels. **C.** cAMP-recruited T-type channels lower the

threshold of action potential firing in rat chromaffin cells. On the upper and lower panels are shown action potentials recorded from a control (upper traces) and a cAMP-treated rat chromaffin cell (lower traces) during current-clamp stimulations of increasing amplitude (5-15 pA). Cells were maintained at -60 mV by injecting -2 to -3 pA. Note that in the cAMP-treated cell, 5 pA is sufficient to elicit a spike, while in control the minimal current is significantly higher (8 pA) (adapted from Novara et al., 2004).

Figure 6: Cav3.2 channels control “low-threshold” exocytosis in rat chromaffin cells exposed to hypoxic conditions or cAMP

A. Ca²⁺ currents and membrane capacitance changes at -30 mV (10 mM external Ca²⁺) in control, cAMP-treated and hypoxic cells. Notice the potent block of Ca²⁺ currents and secretion induced by 50 μM Ni²⁺ in an hypoxic cell (red trace) in which N-, P/Q- and L-type channels were blocked by ω-toxins and nifedipine. **B.** Amperometric spikes evoked by small membrane depolarizations using 15 mM KCl, recorded from a hypoxic cell expressing T-type channels. Addition of 50 μM Ni²⁺ caused a marked depression of amperometric spikes frequency. The cell was pre-incubated with ω-toxins and perfused with solutions containing 1 μM nifedipine (adapted from (Carabelli et al., 2007b)).

Figure 7: Schematic drawings of cell signaling pathways leading to CACNA1H gene expression and Cav3.2 channels incorporation during stressor stimulation

The final result of β₁-adrenergic receptor stimulation and chronic/intermittent hypoxia is the activation of transcription factors (HIF, CREB, ...) and CACNA1H gene expression that lead to Cav3.2 channel incorporation into the plasma membrane. The pathway driven by β₁-adrenergic receptor stimulation (isoprenaline, forskolin, pCPT-cAMP) is derived from Novara et al. (2004). The pathway induced by chronic/intermittent hypoxia (NOX, ROS, PLC, PKG, HIF) is taken from Yuan et al. (2008).

Table 1: Summary of the main connexins expressed in the normal and tumoral adrenal medullary tissue from various species

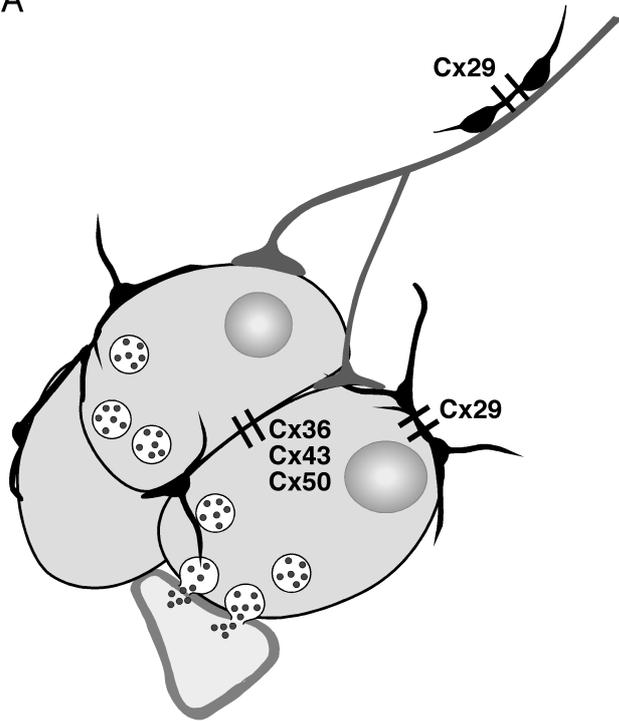
Data collected from the combination of western blot, immunostaining, single-cell PCR, real-time PCR and β -galactosidase assay techniques. (+/-, weakly expressed; +, robustly expressed)

	MOUSE	RAT		GUINEA-PIG	HUMAN		
		normal tissue	PC-12 cells		normal tissue	benign pheochromocytoma	malignant pheochromocytoma
Cx26						+ (Willenberg et al., 2006)	+ (Willenberg et al., 2006)
Cx29	+ (Eiberger et al., 2006)						
Cx32						+/- (Willenberg et al., 2006)	+/- (Willenberg et al., 2006)
Cx36	+ (Degen et al., 2004 ; Li et al., 2004)	+ (Martin et al., 2001 ; Colomer et al., 2008b)	+ (Lu et al., 2007)				
Cx43	+/- (Murray and Pharrams, 1997)	+ (Meda et al., 1993 ; Martin et al., 2001 ; Colomer et al., 2008b)		+/- (Murray and Pharrams, 1997)		+/- (Willenberg et al., 2006)	+/- (Willenberg et al., 2006)
Cx50					+ (Willenberg et al., 2006)	+ (Willenberg et al., 2006)	+/- (Willenberg et al., 2006)

Table 2: Type of stressor or stressor-like stimuli that induce recruitment or up-regulation of Cav3 (T-type) channels in different cells and tissues

Stressors	Cell type, tissue	Cav3 type	Reference
Chronic hypoxia	PC12	Cav3.2	Del Toro et al., 2003
Long-term β-AR exposure	Adult RCCs	Cav3.2	Novara et al., 2004
Chronic hypoxia	Adult RCCs	Cav3.2	Carabelli et al., 2007b
Adrenal gland splanchnic denervation	Neonatal RCCs	Cav3.2	Levitsky & Lopez-Barneo, 2009
Intermittent hypoxia	Neonatal RCCs	Cav3.1, Cav3.2	Souvannakitti et al., 2010
Acute high-frequency sympathetic stimulation	Adult MCCs (adrenal gland slices)	Cav3.2	Hill et al., 2011
Acute PACAP-mediated stimulation	Adult MCCs (adrenal gland slices)	Cav3.2	Kuri et al., 2009
Long-term ACTH exposure	Bovine zona fasciculata cells	Cav3.2	Barbara & Takeda, 1995 Liu et al., 2010
Long-term VIP exposure	Bovine zona fasciculata cells	Cav3 (?)	Barbara & Takeda, 1995
Chronic hypoxia	Neonatal rat ventricular myocytes	Cav3.2	Pluteanu & Cribbs, 2009
Pulmonary hypertension	Rat ventricular myocytes	Cav3 (?)	Takebayashi et al., 2006
Cardiomyopathic heart	Hamster ventricular myocytes	Cav3 (?)	Sen & Smith, 1994
Hypertrophic heart	Feline ventricular myocytes	Cav3 (?)	Nuss & Hauser, 1993
Long-term aldosterone exposure	Neonatal rat ventricular myocytes	Cav3.2	Lalévée et al., 2005
Long-term aldosterone exposure	Human adrenocarcinoma	Cav3.2	Lesouhaitier et al., 2001
Cell proliferation	Human cancer cells	Cav3.1, Cav3.2	Mariot et al., 2002 Panner & Wurster, 2006
Diabetic neuropathic pain	Rat hindpaws sensory neurons	Cav3.2	Jagodic et al., 2007
Chronic visceral pain	Rat colonic sensory neurons	Cav3.2	Marger et al., 2011

A



B Cx43 immunostaining

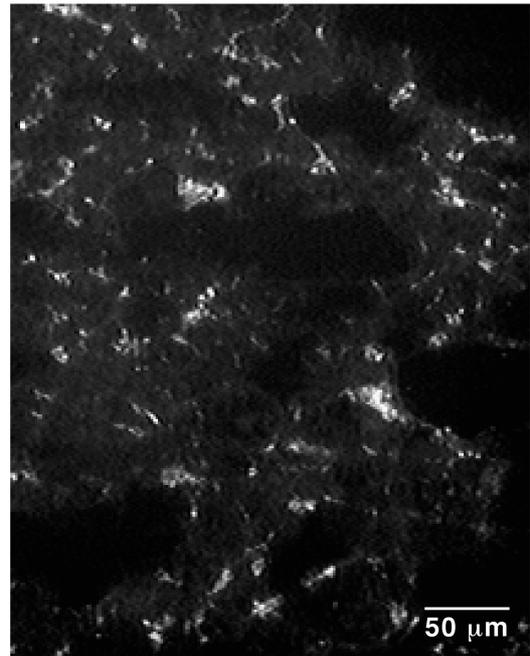


Fig. 1

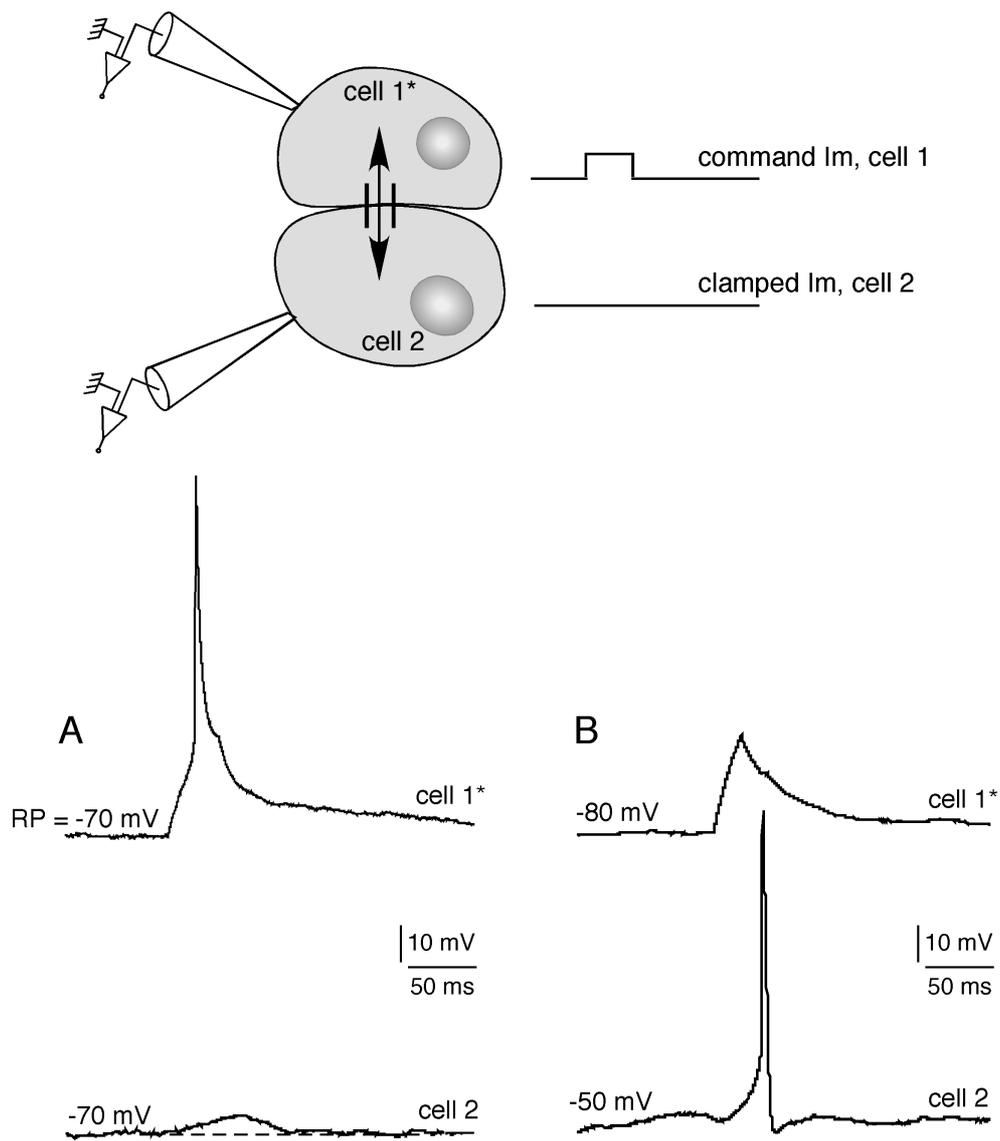


Fig. 2

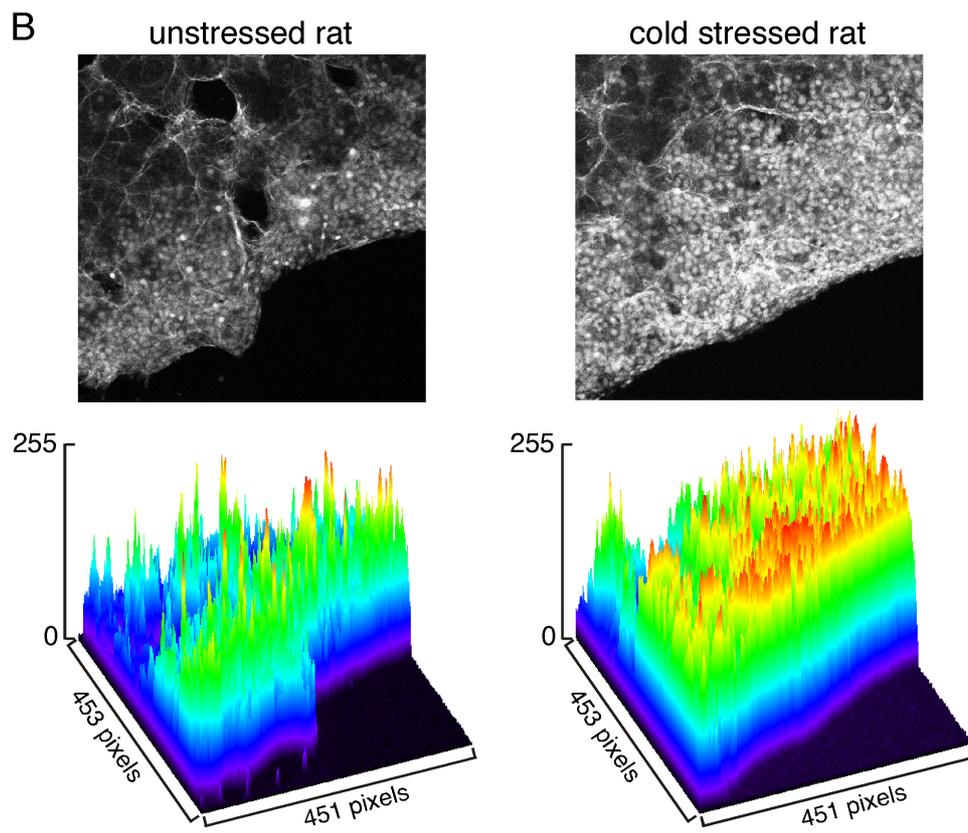
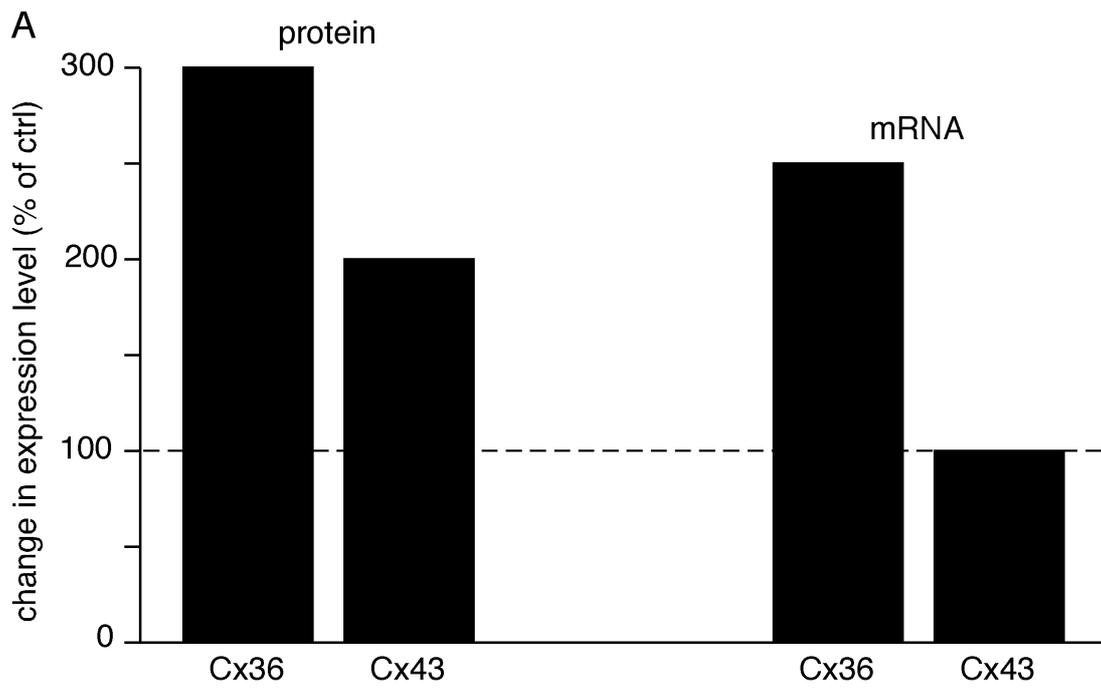


Fig. 3

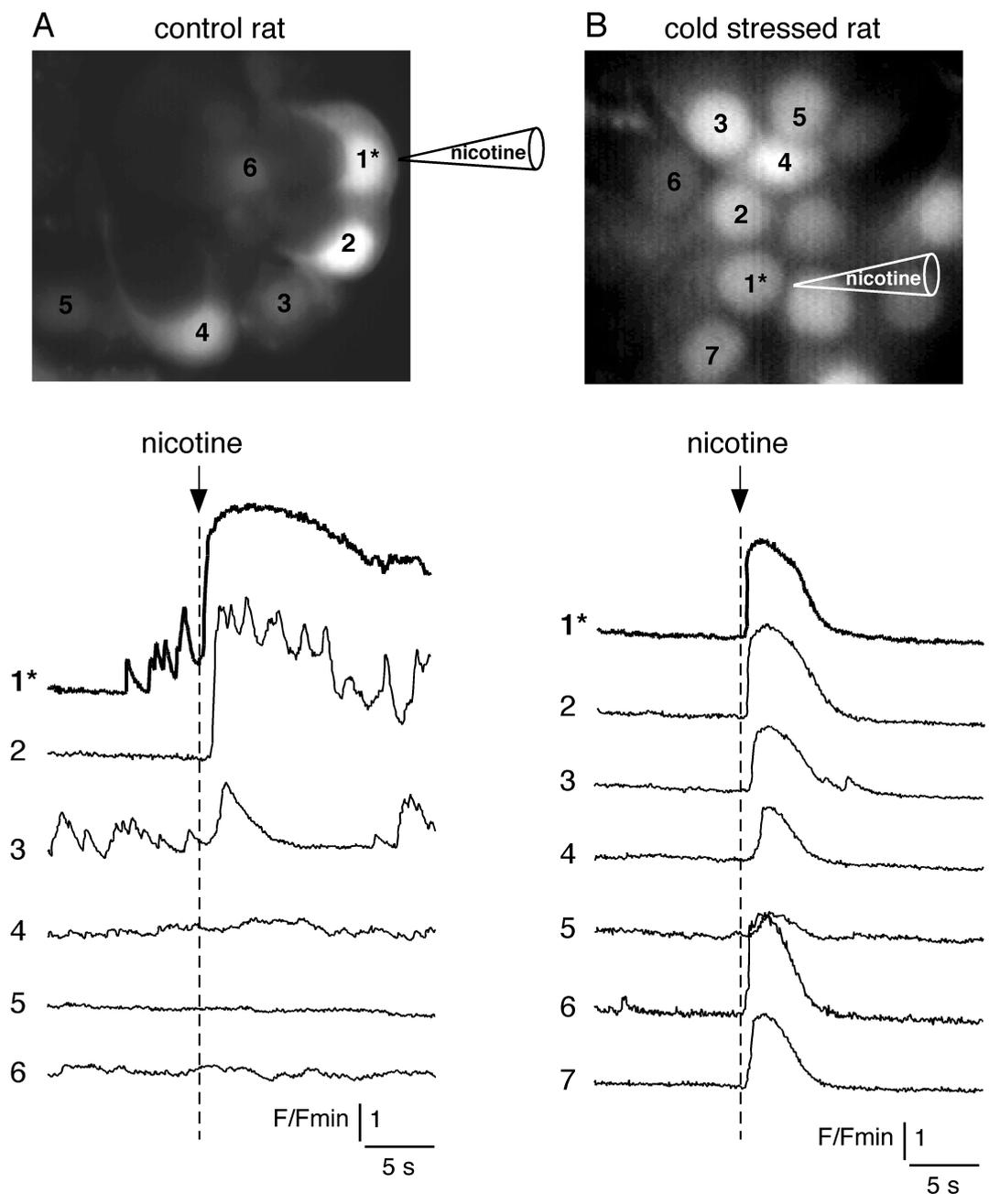


Fig. 4

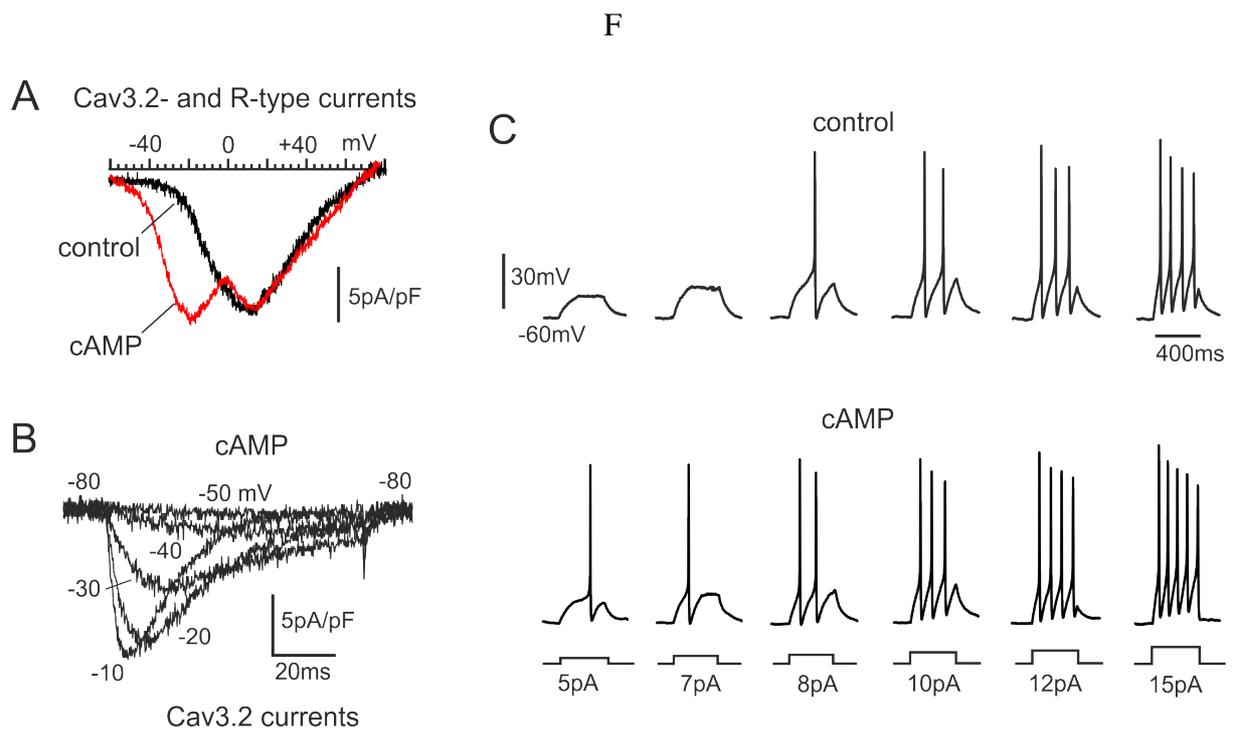


Fig. 5

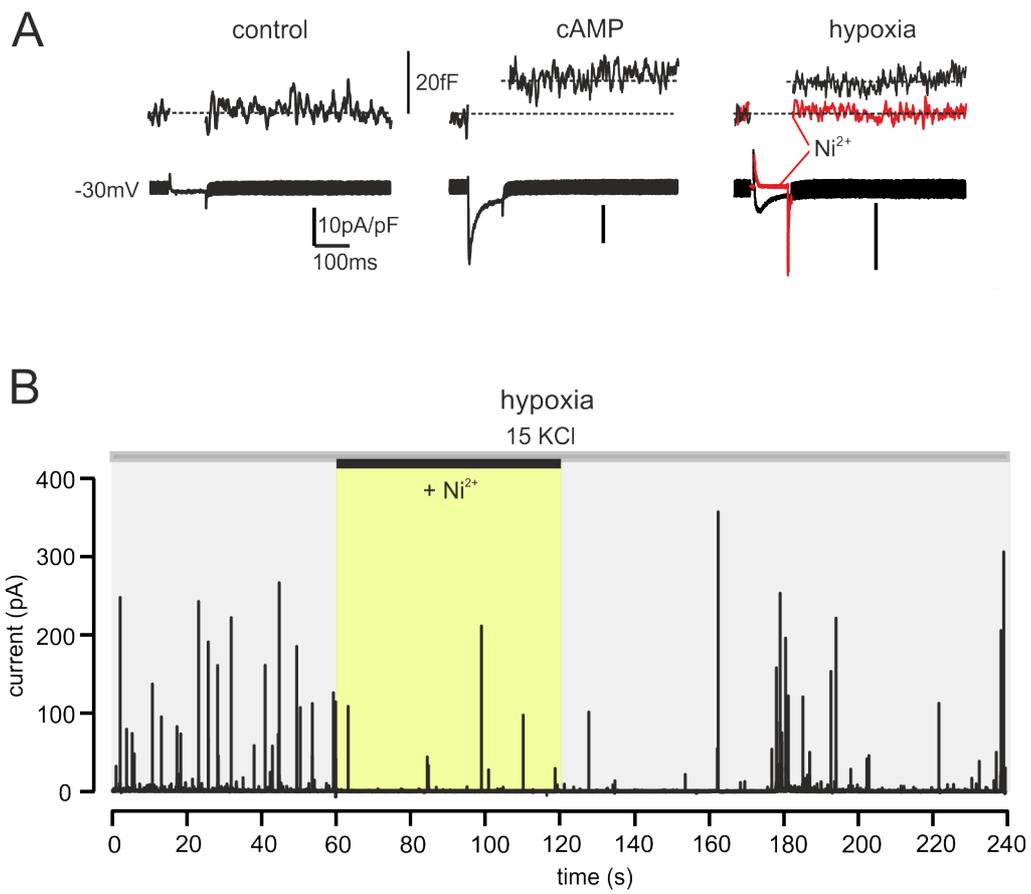


Fig. 6

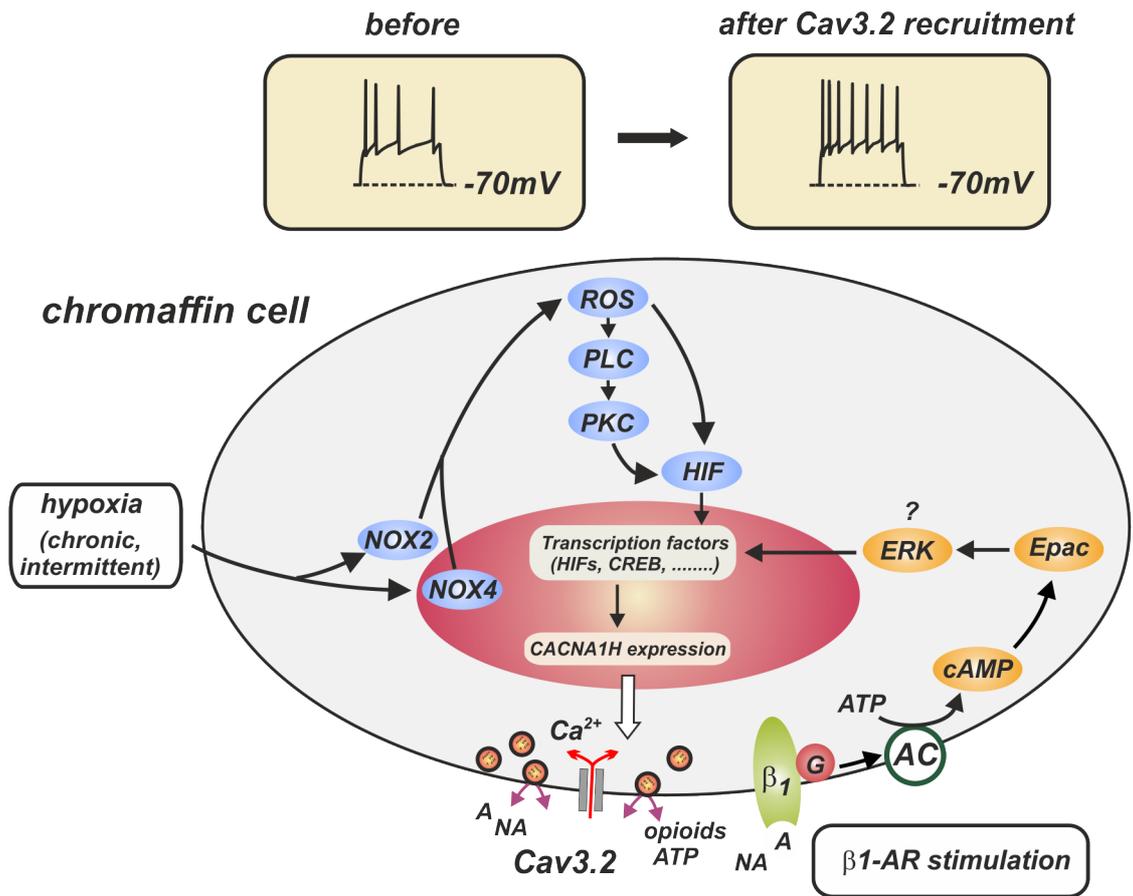


Fig. 7