

# Ca<sub>v</sub>1.3 Channels as Key Regulators of Neuron-Like Firings and Catecholamine Release in Chromaffin Cells

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**Abstract:** Neuronal and neuroendocrine L-type calcium channels (Cav1.2, Cav1.3) open readily at relatively low membrane potentials and allow Ca<sup>2+</sup> to enter the cells near resting potentials. In this way, Cav1.2 and Cav1.3 shape the action potential waveform, contribute to gene expression, synaptic plasticity, neuronal differentiation, hormone secretion and pacemaker activity. In the chromaffin cells (CCs) of the adrenal medulla, Cav1.3 is highly expressed and is shown to support most of the pacemaking current that sustains action potential (AP) firings and part of the catecholamine secretion. Cav1.3 forms Ca<sup>2+</sup>-nanodomains with the fast inactivating BK channels and drives the resting SK currents. These latter set the inter-spike interval duration between consecutive spikes during spontaneous firing and the rate of spike adaptation during sustained depolarizations. Cav1.3 plays also a primary role in the switch from “tonic” to “burst” firing that occurs in mouse CCs when either the availability of voltage-gated Na channels (Nav) is reduced or the β<sub>2</sub> subunit featuring the fast inactivating BK channels is deleted. Here, we discuss the functional role of these “neuron-like” firing modes in CCs and how Cav1.3 contributes to them. The open issue is to understand how these novel firing patterns are adapted to regulate the quantity of circulating catecholamines during resting condition or in response to acute and chronic stress.

**Keywords:** BK and SK currents, exocytosis, L-type channels, Nav1.3 and Nav1.7 channels, tonic and burst firing.

## INTRODUCTION

The chromaffin cells (CCs) of the adrenal medulla are the major source of circulating adrenaline that mobilize the body response to situations of fear, stress and danger [1]. In adult animals the release of adrenaline from CCs is strictly controlled by the sympathetic nervous system (neurogenic control) [2]. Upon splanchnic nerve discharge, repeated quantal release of acetylcholine (ACh) leads to the activation of nicotinic and muscarinic receptors, sustained CC depolarizations and increased firing frequency [3-5]. This causes voltage-gated Ca<sup>2+</sup> channel openings, increased Ca<sup>2+</sup>-entry and robust exocytosis of catecholamine-containing vesicles [5-8]. Adrenaline release is finely regulated by all types of the voltage-gated Ca<sup>2+</sup> channels (Cav) that are expressed in CCs (Cav1, Cav2 and Cav3; L-, N-, P/Q-, R- and T-type). These channels open readily during membrane depolarization and warrant rapid Ca<sup>2+</sup>-fluxes of variable size and time course in response to the broad range of membrane potentials (-50 to +40 mV) spanned during an action potential (AP) waveform.

Given the strong Ca<sup>2+</sup>-dependence of catecholamines secretion and the effective voltage-sensitivity of Cav

channels it is obvious that CCs activity is strictly conditioned by the effective AP firing patterns driving the cell. CCs fire at frequencies that change from 0.2-1 Hz at rest to 20-30 Hz during stress-mimicking conditions. In order to do so, CCs are endowed with a broad array of Na<sup>+</sup>, Ca<sup>2+</sup> and K<sup>+</sup> channels that warrant suitably shaped APs and carefully adjusted Ca<sup>2+</sup> influx depending on the cell needs [9]. Although neurogenically controlled, a variable fraction of bovine, rat, mouse, and human adrenal chromaffin cells fire spontaneously when cultured *in vitro* [10-20] or prepared in slices of the adrenal medulla [21-23]. Firing occurs either in form of “tonic irregular” sequences of single action potentials [9] or in form of burst firings where a series of APs ride on top of a depolarizing slow-wave plateau potential [16, 21, 22, 24, 25]. In this latter case, slightly depolarized plateau potentials of 100-300 ms can drive large Ca<sup>2+</sup> influx. This in turn produces enhanced catecholamine release, as proved in isolated rat CCs (RCCs) where secretion induced by repeated bursts is remarkably enhanced if compared to the release induced by single APs of increasing frequency [26]. In this view, there is strong parallelism between stimulus-secretion coupling of CCs and neurons possessing comparable slow firing rates (0.2-2 Hz) such as midbrain dopaminergic neurons [9, 27, 28]. In these neurons burst firing is twice as potent in triggering secretion as compared to trains of regularly spaced APs, possessing the same average frequency [29]. Switching from tonic to burst firing is thus a common mechanism that neurons and

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neuroendocrine cells adopt to boost stimulus-secretion coupling when required.

The strong dependence of neurotransmitter release on AP-shape, frequency and firing patterns (tonic vs. bursts) demands for a detailed analysis of the gating properties of the different ion channel types that contribute to the size and time course of pacemaking currents. Past and recent studies in mouse and rat CCs have highlighted the orchestrated role that  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{K}^{+}$  channels play in shaping the pacemaker current and sustaining the firing modes of CCs under different physiological conditions. Variations of one or more of these components may explain the firing changes that occur under extreme physiological conditions when, for instance, the levels of released neurotransmitters, extracellular pH, extracellular  $\text{K}^{+}$  concentration, NO and hormones are significantly altered. This would be even more critical in neonatal CCs where the control of catecholamine release is mainly “non-neurogenic” and sensitive to hypoxia [30, 31].

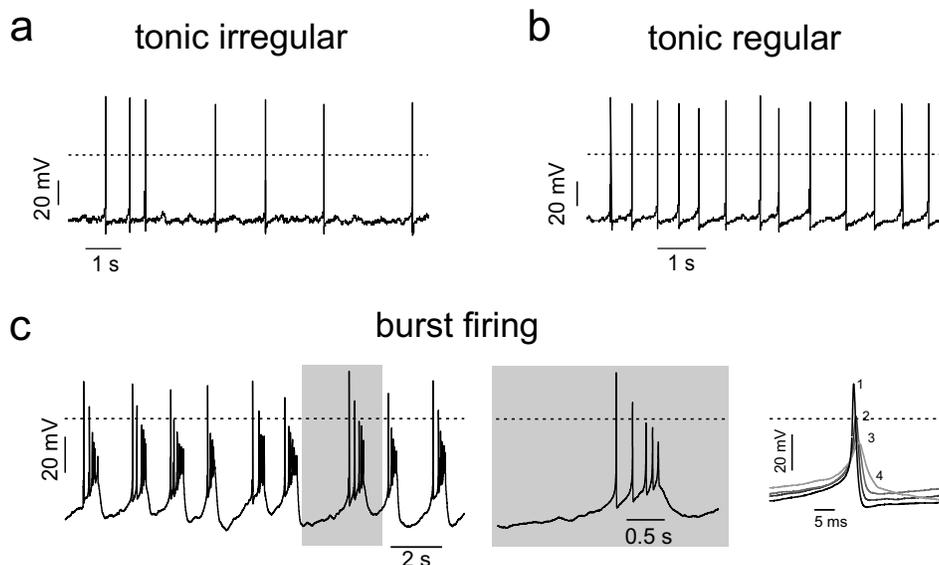
In this review of the special issue celebrating the 50<sup>th</sup> anniversary of  $\text{Ca}^{2+}$  channel discovery by Harald Reuter [32], we will focus on the role that L-type Cav1.3 channels play in regulating spontaneous and electrically-evoked AP firing in CCs. We will review the peculiar way in which Cav1.3 regulates the pacemaking of CCs in concert with SK [20] and “fast-inactivating” BK channels [9, 19] that shape APs and set the frequency of firing at rest and during stimulation. We will also review the newly uncovered burst firing patterns of CCs, and how this firing mode may be triggered by reducing Nav channels and “fast-inactivating” BK channel availability [22, 24]. All this appears uniquely linked to the degree of expression and gating properties of Cav1.3 channels that regulate both the neuron-like firing modes and the  $\text{Ca}^{2+}$ -dependent release of catecholamines from the adrenal medulla.

## TONIC VERSUS BURSTS FIRING MODE IN CHROMAFFIN CELLS: BASIC REQUIREMENTS

In neurons and neuroendocrine cells, spontaneous firing takes place when sufficient inward current is driven by small voltage perturbations near resting potential. Critical for triggering spontaneous AP oscillations is the net balance between inward and outward currents (leak,  $\text{Na}^{+}$ ,  $\text{Ca}^{2+}$  and  $\text{K}^{+}$ ) near resting potential. When the inward depolarizing current outweighs the outward current, a more positive unstable potential is reached at the start of the “negative conductance” region of the IV characteristics [33, 34]. At this unstable potential, any small depolarization causes a tiny inward current that further depolarizes the cell and triggers an AP train. Low-threshold spontaneous membrane potential oscillations occur easily if the cell possesses high input resistance (2-5 G $\Omega$ ) and is equipped with sufficiently large densities of Nav and Cav channels that activate readily at low voltages like chromaffin cells [11, 35, 36]. An additional fundamental requirement for driving spontaneous firing in slow spiking cells is the presence of a slowly inactivating current that sustains the slow pacemaker potential.

Spontaneous firing is rather variable in CCs regardless of whether the cells are isolated [9-11, 16, 18-20, 24] or in slices of the adrenal gland [21-23]. In many CCs the firing is “tonic irregular”, *i.e.*, spikes occur at variable frequency (Fig. 1a). In mouse CCs (MCCs), the degree of regularity is negatively correlated with spike frequencies recorded at rest. Fast spiking cells are typically more regular and display “tonic regular” firings (Fig. 1b).

As in many neurons, a fraction of CCs can undergo also spontaneous bursts at normal physiological conditions [22, 24]. Burst firing of APs occurs as a train of APs on top of a sustained plateau potential of variable duration (Fig. 1c). The burst terminates with a marked afterhyperpolarization (AHP) whose deepness sets the duration of the inter-bursts interval



**Fig. (1).** Different firing patterns of spontaneously active RCCs and MCCs. **a, b** Spontaneous AP traces recorded from two different RCCs displaying typical tonic irregular and tonic regular firing patterns, respectively. **c** Representative trace of spontaneous AP trains fired in a burst-like mode recorded from a MCC. To the right are shown a single burst at an expanded time scale (grey window) and the overlap of consecutive APs within a burst. Numbers indicate the position/sequence in the burst (adapted from ref. [24]).

(IBI): deeper AHPs generate longer IBIs. The origin of this “neuron-like” bursting mode in CCs is completely unknown but one can speculate that it derives from accumulated  $K^+$  channel inactivation and from the presence of sizeable slowly inactivating inward currents ( $Ca^{2+}$  or  $Na^+$ ) that sustain the plateau potential and part of the spikes amplitude. Any reduction of the  $K^+$  currents activated during the first AP attenuates the AHP to more positive values (plateau potential) where Cav and Nav channels can be activated to sustain the quickly repeated APs of the bursts. As several ion channels contribute to set the plateau potentials and spike amplitudes, the key issue is then to uncover how their degree of expression, gating properties and sensitivity to endogenous and exogenous modulators may alter the equilibrium between tonic and burst firing modes in CCs. Below we will review the biophysical features of Cav1.3, BK, SK and Nav channels that in concert set the firing of CCs.

### THE Cav CHANNELS OF CHROMAFFIN CELLS

Mammalian adrenal chromaffin cells express all types of Cav channels (L, N, P/Q, R and T-type) [5, 8, 9, 37, 38]. Their expression density changes remarkably among animal species, cell conditions and development. In adult animals L, N, P/Q and R-type channels are the dominant species [5, 8, 10, 39]. They shape action potential waveforms, control catecholamine secretion and vesicle retrieval. Despite the many reports, convincing proof of a specific co-localization of either one of the expressed Cav channel types with the secretory apparatus is still lacking. Each channel type control catecholamine release with the same  $Ca^{2+}$ -dependence [6, 40-48]. The question is thus, why chromaffin cells which are spherically shaped cells and do not possess neuron-like morphology and specialized pre-post microdomains need so many different  $Ca^{2+}$  channel types for their function? A possibility is that depending on their different sensitivity to external signaling and intracellular second messengers, the many Cav channel types warrant compensatory paracrine and autocrine responses to different CCs functioning conditions. Along this view, Cav2.1 and Cav2.2 channels can be effectively down-regulated by released ATP and opioids that are co-released with adrenaline and noradrenaline [49-54]. Conversely, Cav1.2 and Cav1.3 L-type channels could be both strongly up-regulated by elevating the cAMP/PKA levels [55-57] or drastically down-regulated by cGMP/PKG mediated pathways [57]. Cav channels can also undergo drastic up-regulation during cell re-modeling, as in the case of Cav3.2 T-type channels [6, 39]. Cav3 channels are usually not expressed in adult CCs but are functionally active in embryonic CCs when innervation of the adrenal medulla has not reached complete maturation [30, 58, 59]. At this developmental stage, CCs respond only to non-neurogenic stimuli. Cav3.2 channels are *de-novo* recruited in adult CCs to sustain “low-threshold” exocytosis during stress mimicking conditions such as during  $\beta$ -adrenergic receptor stimulation [60] or chronic hypoxia [61]. Recent evidence indicates that exogenous expression of Cav3.2 channels in a chromaffin cell line induces “low-threshold” release of catecholamines, which can be blocked by coexpressing a peptide corresponding to the syntaxin-1A interaction domain of the channel [62].

### Cav1.3 AS PACEMAKER CHANNEL: A COMPLEX GROWING CONCEPT

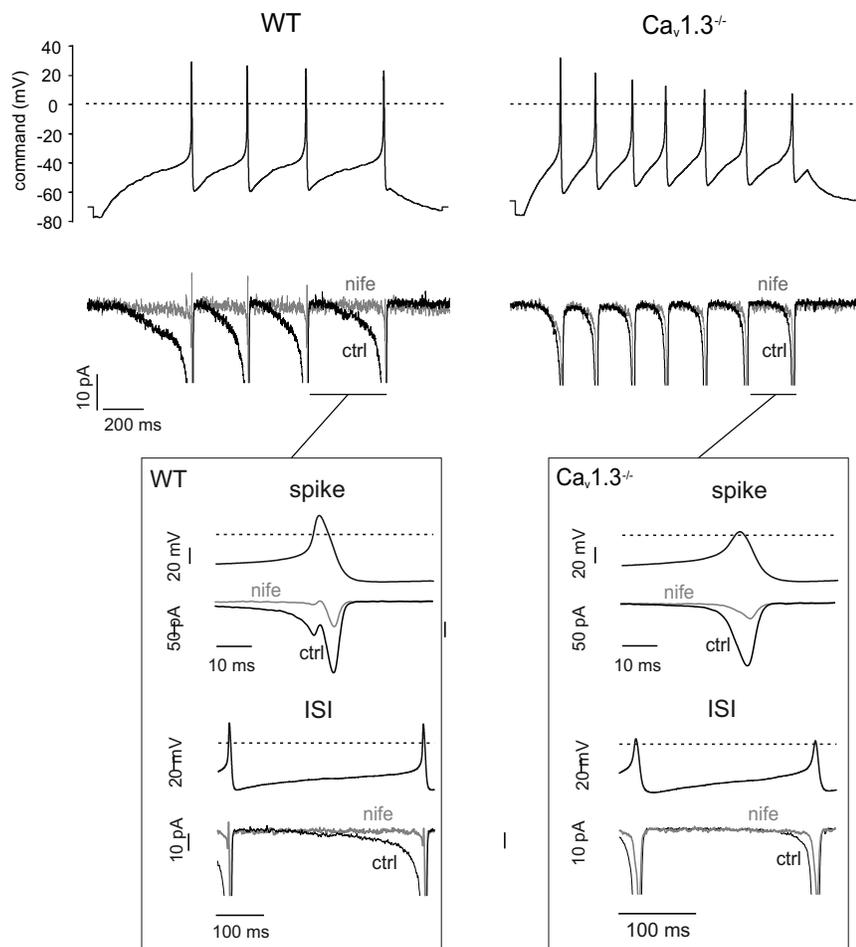
CCs express mainly the two neuronal Cav1.2 and Cav1.3 channel isoforms, that have strong amino acid homologies. They both contribute to about 50% of the total current in cat, rat and mouse CCs [5, 7, 11, 56]. In humans, L-type channels are reported to contribute to 30% of the total [63], but this is most likely an underestimate since the DHP blocking assay was done with cells clamped at rather negative  $V_h$  (-80 mV) using low doses of nifedipine (1  $\mu$ M).

Cav1.2 and Cav1.3 are also directly involved in the control of vesicle endocytosis [64, 65] and pacemaking of spontaneous [10, 11] and evoked AP firings [9]. The last observation is a relatively new concept that gains increasing evidence. Many reports on neurons, cardiac myocytes and neuroendocrine cells suggest the involvements of L-type channels in pacemaking [9]. The question is thus, are both channels suitable for pacemaking or one of them is optimal for triggering auto-rhythmicity? Looking at their gating properties, it is evident that Cav1.3 possesses the characteristics to sustain subthreshold currents that may contribute to sustain spontaneous low frequency firing patterns (0,2 to 2 Hz) typical of chromaffin cells [10, 11, 19], substantia nigra pars compact neurons [28, 66-68] and sinoatrial node (SAN) “pacemaker cells” [69].

Data derived from heterologously expressed channels indicate that: *i*) Cav1.3 has similar single-channel conductance to Cav1.2 [70] but activates with steep voltage-dependence at more negative voltages [71, 72] and, *ii*) Cav1.3 has faster activation but slower and less complete voltage-dependent inactivation as compared to Cav1.2 [71]. In addition, Cav1.3 is less sensitive to DHPs as compared with Cav1.2, requiring about 10-fold more DHPs for full block at normal  $V_h$  (-70 mV) [73]. Cav1.3 sensitivity toward DHPs increases markedly at less negative  $V_h$  (-50 mV) reaching comparable  $IC_{50}$  values of Cav1.2 [71]. Thus, at -50 mV saturating concentrations of DHPs that block Cav1.2 ensure also full block of Cav1.3. This is crucial when assaying the role of Cav1.3 during spontaneous AP recordings in firing cells that spend most of their time at the interspike potential around -50 mV (see ref. [36]).

Despite a recent report indicates selective block of reconstituted Cav1.3 channels [74], contradicting findings [75, 76] suggest there are not yet available specific blockers for this Cav1 isoform. Due to this, the exact role of Cav1.3 in cellular auto-rhythmicity can be assayed only indirectly using the Cav1.3 deficient mouse (Cav1.3<sup>-/-</sup>) [77]. This mouse model has a strong phenotype characterized by deafness and bradycardia. Deafness derives from the complete absence of Cav1.3 L-type currents in cochlear inner hair cells and degeneration of outer and inner hair cells [77]. Bradycardia derives from the abolition of the major component of the Cav1.3 L-type current activating at low voltages in the SAN “pacemaker cells” controlling the heart rhythm and rate under physiological conditions [69].

A specific role of L-type channels in CC pacemaking was first reported in RCCs [10]. Increasing doses of nifedipine could either decrease the firing frequency or block the spontaneous firing. In these experiments, a tight coupling of



**Fig. (2).** Deletion of Cav1.3 causes a marked reduction of the pacemaking L-type current flowing during the interspike interval. Top, representative AP trains from a WT (left) and  $Ca_v1.3^{-/-}$  MCC (right) recorded after a 15 pA (700 ms) current step starting from  $V_h = -70$  mV. The voltage traces were used as voltage-clamp command to reveal the interspike specific apamin-sensitive currents. Middle,  $Ca^{2+}$  currents of WT (left) and  $Ca_v1.3^{-/-}$  MCCs (right) recorded in control condition (black trace, ctrl) and in the presence of 3  $\mu$ M nifedipine (grey, nife). External solution contained 135 mM TEA, 300 nM TTX and 2 mM  $Ca^{2+}$ . Bottom insets,  $Ca^{2+}$  currents flowing during the spike and the interspike interval of WT and  $Ca_v1.3^{-/-}$  MCCs shown at an expanded time scale (adapted from ref. [20]).

L-type channels to the fast-inactivating BK channels of RCCs was also noticed, in good agreement with previous reports by Chris Lingle's lab [78, 79]. An L-type pacemaker current of 10 to 20 pA amplitude was first reported in AP-clamp experiments in WT MCCs [11]. A subthreshold inward  $Ca^{2+}$  current of this size passing through the high input resistance of chromaffin cells (2-5 G $\Omega$ ) [11, 19, 35] is sufficient to generate pacemaker potentials of 10-20 mV that drive the cell from inter-pulse potentials of -50 mV to the threshold of the AP up-stroke (-30 mV) [11]. Deletion of Cav1.3 reduces drastically the amplitude of this pacemaker current and the fraction of firing MCCs [19]. An example is shown in Fig. 2 where the inward  $Ca^{2+}$  currents of WT and  $Ca_v1.3^{-/-}$  MCCs flowing during the inter-pulse interval (ISI) are compared. Loss of Cav1.3: i) decreases markedly the level of the nifedipine-sensitive currents driving spike generation, ii) raises the rheobase (from 4 to 6.6 pA) and iii) decreases the extent of spike frequency adaptation during sustained current injections [20].

As pacemaker channel, Cav1.3 appears quite unique if compared with other neuronal channels that generate spike

firings and carry mostly  $Na^+$  currents, like the hyperpolarization-activated cation channels (HCN) [80], the persistent [81] and resurgent [82]  $Na^+$  channels and background channels [83]. The inward  $Ca^{2+}$  current generated by Cav1.3 is always accompanied by outward  $K^+$  currents associated with  $Ca^{2+}$ -activated BK and SK channels that are highly expressed in neurons [84] and neuroendocrine cells [85, 86]. Thus, activation of Cav1.3 generates net currents that might be inward or outward depending on the degree of coupling and expression density of BK and SK channels. In the case of BK, it depends also on membrane potential since these channels are also steeply voltage-dependent [84].

#### THE Cav1.3-BK CHANNEL NANODOMAINS REGULATE AP SHAPE AND CELL FIRING MODES

As in many brain regions, BK channels are highly expressed in RCCs [10, 86], MCCs [9, 19, 22] and bovine CCs (BCCs) [85, 87]. To activate during physiological depolarization, BK channels need an intracellular  $Ca^{2+}$  concentration of at least 10  $\mu$ M [84]. Such high

concentrations occur only within “Ca<sup>2+</sup>-nanodomains” in the close vicinity of the Ca<sup>2+</sup> source [88]. For this purpose, the neuronal BK channels are often co-localized to either the P/Q- [89], N- [90] or L-type [91] channels. RCCs and MCCs express two different BK channels that are predominantly coupled to L-type Cav1 channels [9, 79] and can be distinguished according to their inactivation kinetics: a fast-inactivating (BK<sub>i</sub>) and a slowly inactivating channel type (BK<sub>s</sub>) [9, 19, 78]. The BK<sub>i</sub> channel is typically expressed in CCs and gives rise to slowly adapting cell firing. The BK<sub>s</sub> channel has gating properties similar to the central neurons and smooth muscle BK channels and causes fast adapting cell firings [78]. Due to their strong voltage-dependence, BK channels contribute to the repolarization phase of the AP (AHP) [84, 86], influence the refractory period and regulate the firing rate of CCs. In MCCs, block of BK channels by paxilline significantly augments the firing frequency by delaying AP repolarization and slightly reducing the early phase of the AHP [19]. Notice that due to the tight Cav1-BK coupling also nifedipine, like paxilline, delays AP repolarization and reduces the early phase of the AHP in MCCs [9, 19] and RCCs [10, 78] (see Fig. 4 in ref [9]). However, due to the block of Cav1.3, the DHP either blocks or slows down the firing rate.

Given the critical role of Cav1.3 in pacemaking MCCs, the Cav1-BK channel coupling is a key determinant for setting the AP shape and the firing frequency of CCs. In addition, the coupling is drastically altered by deletion of Cav1.3 in a way that loss of Cav1.3 prevents the expression of BK<sub>i</sub> channels [19]. This property appears to be an adaptive phenomenon first discovered in cochlear inner hair cells [91]. In MCCs this is evident by comparing the BK channel types expressed in WT and Cav1.3<sup>-/-</sup> MCCs using standard protocols of Ca<sup>2+</sup> pre-loading which maximally activate all available BK channels (Fig. 3a). WT MCCs are shown to express a predominance of fast-inactivating BK currents while Cav1.3<sup>-/-</sup> MCCs display typically slowly inactivating BK currents. Using fast (BAPTA) and slow (EGTA) Ca<sup>2+</sup> buffers it is also possible to prove that the coupling between BKs and Cav1.2 channels in Cav1.3<sup>-/-</sup> MCCs is BAPTA- and EGTA-sensitive suggesting that the two channels are separated by an average distance of more than 160 nm. Conversely, the coupling between Cav1.3 and BK<sub>i</sub> channels in WT-MCCs is BAPTA-sensitive but EGTA-insensitive, suggesting an average distance of 50 to 160 nm between the two channels. In other words, BK<sub>i</sub> and Cav1.3 channels form functional “Ca<sup>2+</sup>-nanodomains” that regulate the steepness of the AHP phase and thus set the-shape and frequency of AP trains.

Because BK<sub>i</sub> channel inactivation is associated with the presence of a specific β2 subunit that shifts channel activation toward more negative voltages at a given Ca<sup>2+</sup> [92, 93], these findings suggest that loss of Cav1.3 channels may lead to the loss of BK-β2 subunits. This could also indicate that β2 is the critical element regulating the close coupling between Cav1.3 and BK<sub>i</sub> channels. In the absence of β2 the coupling between BK and Cav1.3 (or Cav1.2) channels is weaker and less critical for cell firing. That β2 may be a key element of BK channels that helps regulating MCCs firing is suggested by recent findings showing that deletion of the β2 subunit produces rather drastic changes to MCCs firing

patterns [22]. The knock-out of BK-β2 subunits in fact slows AP repolarization and, during constant current injection, decreases AP firing. This clearly supports the idea that the β2-mediated shift of the BK channel activation range affects repetitive firing and AP properties. Unexpectedly, CCs from BK-β2 deficient mice show an increased tendency toward spontaneous slow-wave burst firings, suggesting that the peculiar activation properties of BK channels in the absence of β2 subunits may predispose CCs to burst firing and, possibly, to an associated increased secretion of catecholamines. It is worth noticing that burst firing occurs also in BK deficient (BK<sup>-/-</sup>) MCCs during L-type current activation by BayK 8644 [9, 19] and in WT MCCs when Nav channel availability is reduced by either slowly inactivating or blocking them (see below).

### THE “FUNCTIONAL” Cav1.3-SK CHANNEL COUPLING REGULATE CELL FIRING FREQUENCY AND FIRING ADAPTATION

SK channels (K<sub>Ca2.1</sub>, K<sub>Ca2.2</sub>, K<sub>Ca2.3</sub>) are widely expressed throughout the central and peripheral nervous system where they act as key modulators of neuronal excitability [94, 95]. They control intrinsic excitability and pacemaking, dendritic integration, postsynaptic responses as well as synaptic plasticity [84]. Besides affecting firing frequencies, SK channels are involved in switches from single spiking into burst firing patterns and can drive spike frequency adaptation and accommodation [96-98]. SK currents contribute to the AHP phase of single or burst of APs, slowing-down the pacemaker cycle. The high Ca<sup>2+</sup>-sensitivity of SK channels is at the basis of the prevailing current view that SK channels do not have to be necessarily close to a specific Ca<sup>2+</sup> source to get activated [84]. Several studies nevertheless demonstrated a specific coupling of SK channels to Cav channels or neurotransmitter receptors [90, 99-102].

In chromaffin cells, SK channels were first biophysically identified in BCCs [85] and RCCs [86]. Experiments on cat adrenal glands showed that secretion of catecholamines induced by electrical stimulation or acetylcholine application could be potently stimulated by SK block with apamin [103]. In RCCs, the SK channels mediate the biphasic effect of muscarine on AP firing, which consists in a transient hyperpolarization followed by a sustained depolarization [4, 104]. The hyperpolarization is induced by an IP<sub>3</sub>-mediated increase of intracellular Ca<sup>2+</sup> via M1 receptors activation [4] while the depolarization is associated with the block of an M-type K<sup>+</sup> channel [12].

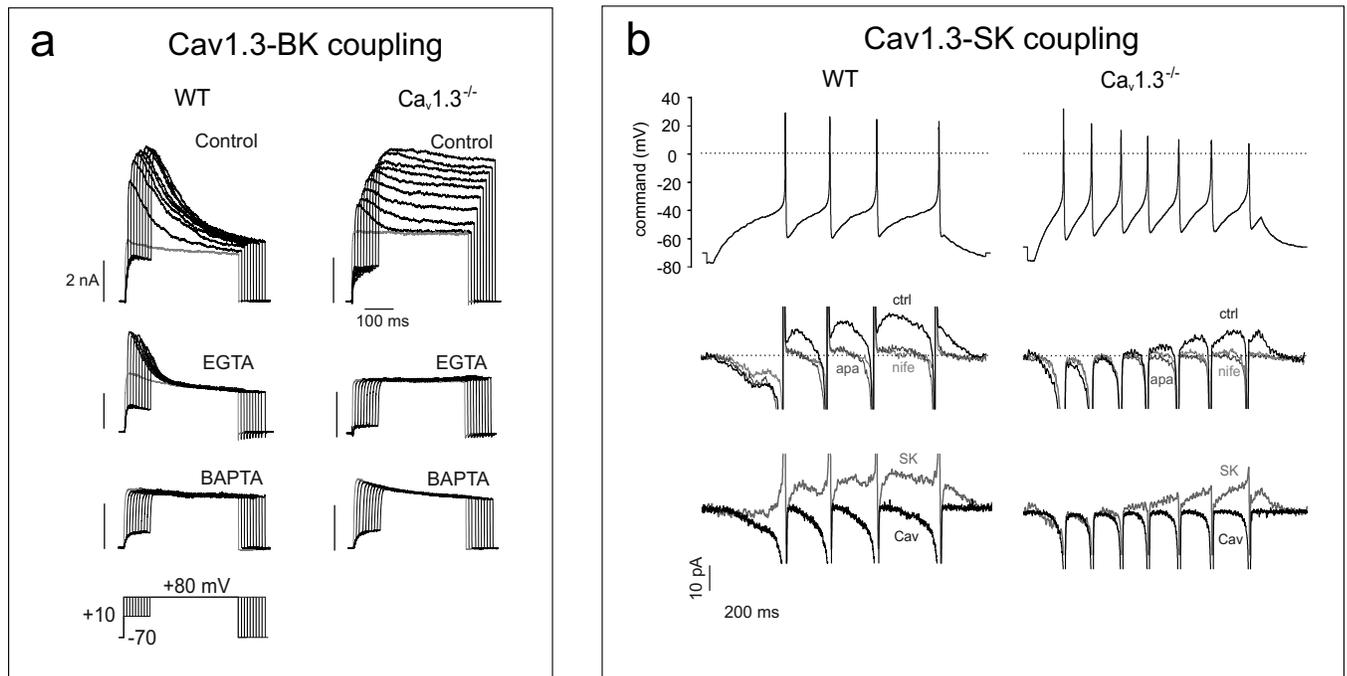
SK channels are of extreme importance for the control of MCCs firing [20]. By comparing WT and Cav1.3<sup>-/-</sup> MCCs firing it has been possible to uncover a “functional” coupling between Cav1.3 and SK channels that slows down MCC's basal firing rate and leads to spike frequency adaptation upon sustained current injections [20]. This is not surprising, since in MCCs, as in most neurons, the SK channels are equally driven by L, N, P/Q and R-type channels (see Fig. 9 in ref. [20]). As Cav1.3 drives most of the slow pacemaking current, it is evident that the SK currents at rest are mainly driven by these L-type channels. Thus, during spontaneous firing the “Ca<sub>v</sub>1.3-SK coupling” builds-up robust subthreshold SK currents that slows-down the pacemaking

and guarantees the effective recovery of  $\text{Na}_v$  channels required to maintain stable the AP firings. A critical role of SK currents on MCC firings is evident by observing that: i) the MCC firings is slow and irregular at control and becomes faster and more regular by blocking the SK channels with apamin, ii) the frequency of spontaneously firing MCCs increases nearly 3-fold upon addition of apamin and, iii) exists a negative correlation between the basal firing frequency and the percentage of frequency increase induced by apamin. A corollary of this is that the presence of SK channels decreases the precision of firing in MCCs, which is in contrast to most central neurons where robust SK currents increase the regularity and precision of firing [97, 98, 105, 106]. The increased precision of firing is due to an enhanced  $\text{Na}_v$  channel availability sustained by SK channels that is required for pacemaking these cells.

The “functional” Cav1.3-SK channel coupling has a similar critical role on the response to sustained depolarization. It allows the fast adaptation of the initial high-frequency to a low sustained frequency during prolonged stimulations. Loss of Cav1.3 induces a nearly two-fold reduction of the total  $\text{Ca}^{2+}$  charge entering the cell during pacemaking with consequent reduction of SK currents (Fig. 3b). In this case, the induced AP-firing adapts more slowly and generates AP trains of higher frequency and lower amplitude. The “functional” and “not localized” coupling to SK channels thus accounts for the inability of Cav1.3<sup>-/-</sup> MCCs to adapt their firing frequency.

As a consequence, the Cav1.3-SK coupling confers to Cav1.3 the dual role of “drive” and “brake” of MCC's excitability that is crucial when controlling sustained catecholamine release from the adrenal gland during stressful stimuli [5]. The braking action of SK channels is particularly crucial to prevent over-excitation that could damage cells that fire slowly (~1 Hz) and rely on “ $\text{Ca}^{2+}$ -dependent pacemakers” such as chromaffin cells [19], dopaminergic and histaminergic neurons [67, 107].

The fast adapting response to sustained depolarization due to the Cav1.3-SK coupling has clear-cut implications on the overall organization of catecholamine release in the adrenal medulla. The first is that increasing the frequency of stimulation does not necessarily lead to a linear increase of catecholamine secretion. The catecholamine release induced by trains of electrical stimulations (0.1 to 30 Hz), saturates between 3 and 10 Hz and then declines in rat and cat chromaffin cells [3, 103, 108]. The second implication is that, similarly to certain neuronal networks [109], the phasic response regulated by the Cav1.3-SK coupling may help the firing synchronization in the adrenal medulla where chromaffin cells are electrically coupled [39]. Synchronization followed by adaptation to low firing frequencies in extended areas of the adrenal medulla may optimize the release of catecholamines during prolonged stressful stimulation, preventing excessive accumulation of undesired levels of circulating catecholamines.



**Fig. (3).** Deletion of Cav1.3 prevents the activation of fast inactivating BK channels and reduces the size of SK currents during spike frequency adaptation. **a)** The BK channels of Cav1.3<sup>-/-</sup> MCCs are mainly non-inactivating [19]. Membrane-permeable EGTA-AM is unable to prevent the coupling in WT MCCs but is effective on Cav1.3<sup>-/-</sup> MCCs. BAPTA-AM is effective on both WT and Cav1.3<sup>-/-</sup> MCCs (adapted from ref. [9]). The pulse protocol is shown at the bottom left. **b)** Top, evoked AP trains from a representative WT and Cav1.3<sup>-/-</sup> MCC used as voltage-clamp command to reveal the  $\text{Ca}^{2+}$  and SK currents of the same cells (Fig. 2). Middle,  $\text{Ca}^{2+}$  and SK currents recorded in the presence of a Tyrode standard (2 mM  $\text{Ca}^{2+}$ , 4 mM  $\text{K}^+$ ) containing 300 nM TTX and 1  $\mu\text{M}$  paxilline in control (black traces) and in the presence of either 3  $\mu\text{M}$  nifedipine (light grey) or 200 nM apamin (dark grey). Notice how nifedipine is equivalent to apamin in blocking the outward SK currents. Bottom, SK currents (grey traces) obtained after subtraction of the apamin trace from the control trace of the middle panel overlapped with the  $\text{Ca}^{2+}$  currents of (Fig. 2) (black traces) (adapted from ref. [20]).

### Nav1.3 AND Nav1.7 CHANNELS DO NOT CONTRIBUTE TO PACEMAKER CURRENT

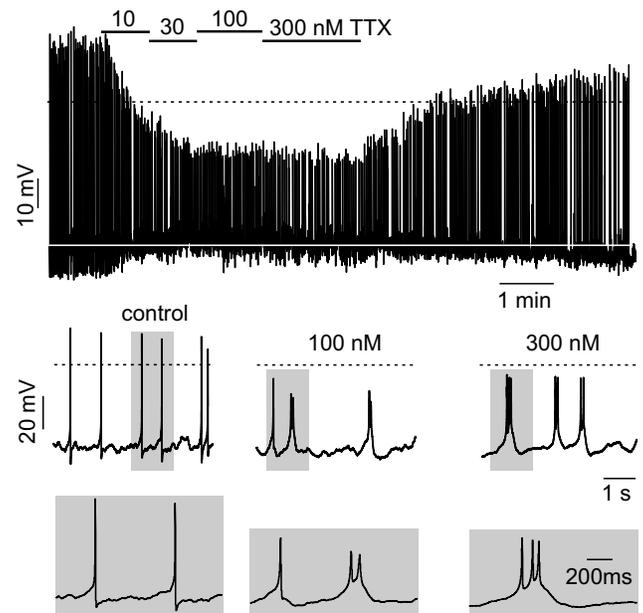
As in most brain neurons [33, 110], voltage-gated Na<sup>+</sup> channels (Nav) are highly expressed in the CCs of all animal species [14, 15, 17, 21, 24, 35, 36, 111-114]. Nav channels generate and shape the AP waveform and thus directly contribute to CCs excitability. Early studies have shown the existence of a background Na<sup>+</sup> conductance that could drive Na<sup>+</sup>-influx at rest in rat and gerbil CCs [13, 14]. TTX application or Na<sup>+</sup> removal from the extracellular medium reduces, but does not block rat CCs firing [111]. In MCCs, Nav channel block by TTX preserves AP firing [21, 24, 36], suggesting that Nav channels play a different role in CC excitability than L-type channels [9]. Furthermore, Nav channels in MCCs activate at 24 mV more positive potentials than Cav1.3, suggesting a minor contribution to sub-threshold pacemaker currents [36], even considering that Nav channels are expressed at much higher densities with respect to Cav channels in CCs [35]. Taken all together these observations raise two key questions: do Nav channels contribute to the pacemaking of CCs? How critical is their availability to trigger CCs firing modes?

Concerning the first issue, the answer is clearly linked to the type of Nav channels expressed in CCs. Presently, Nav1.7 is thought to be the main voltage-gated Nav channel expressed in bovine and human adrenal tissues [115, 116]. In the rat adrenal gland, mRNA encoding Nav1.7 is expressed only in the medulla [117], with a plausible localization in CCs, as also reported in PC12 cells [118]. A recent report by our group shows that cultured MCCs express Nav1.7 (scn9A) and Nav1.3 (scn3A) with a 7.3-fold higher expression level for the scn3A transcript [24]. In conclusion, all the available functional studies on CC Nav channels show convincingly that these channels are TTX-sensitive and fast-inactivating [24, 35, 36, 113, 114]. Fast inactivating Nav channels could partly contribute to pacemaking in fast-spiking neurons [27, 119] with firing frequencies of 40-100 Hz but could hardly sustain pacemaking currents in slow firing cells with inter-pulse intervals of 0.3 to 2 s duration, such as in CCs. All this, however, cannot exclude that CCs could express "persistent" or "resurgent" Na<sup>+</sup> currents that partly contribute to slow pacemaking. This possibility has been recently investigated using traditional whole-cell and perforated micro-vesicle single channel recordings in MCCs [24]. It is concluded that the Nav1.3/Nav1.7 fast inactivating channels expressed in MCCs are fully inactivated after step depolarization of 200-1000 ms. The steady-state net currents at these time intervals are <1-2 pA and comparable to the background current noise. It is thus unlikely that Nav1.3 and Nav1.7 contribute to the pacemaker current in MCCs. In addition, MCCs do not possess "persistent" Nav currents which are typically associated with Nav1.6 (scn8a) [82]. Persistent Na<sup>+</sup> currents activate at potentials as negative as -70 mV [120] and sustain tonic firing at elevated frequencies (20-100Hz) in several neurons [121-124]. MCCs do not fire at elevated frequencies and lack scn8a [24]. In addition, the auxiliary  $\beta$  subunit scn4b (Nav $\beta$ 4) is linked to resurgent Na<sup>+</sup> currents [125]. Neither scn4b nor "resurgent" Na<sup>+</sup> currents are revealed in MCCs [24].

### Nav1.3/Nav1.7 CHANNELS AVAILABILITY REGULATE THE Cav1.3-MEDIATED SWITCH FROM TONIC TO BURST FIRING

Concerning the second question it is worth recalling that as in most neurons, Nav channels availability is critical for shaping the AP waveform in MCCs [20, 36]. Subtle changes in spike shape can lead to drastic changes in Cav channel recruitment and in AP-induced Ca<sup>2+</sup> transients [126]. AP broadening is indeed a common way to optimize presynaptic Ca<sup>2+</sup> influx at central presynaptic terminals [127]. Neuronal Nav channel availability is drastically reduced by depolarizing the resting membrane potential [128, 129]. The same is true in MCCs where the reduced Nav channel availability drastically alters L-type channel recruitment and, paradoxically, boosts catecholamine secretion [24].

As shown in Vandael *et al.*, 2015 [24], Nav channel availability can be reduced by either blocking Nav1.3/Nav1.7 channels by TTX or by lowering the resting membrane potential from physiological (-50 mV) to steady depolarized potentials (-40 mV). In the latter case, the slow and partial recovery from fast inactivation and the fast entry into the closed-state inactivation at -40 mV, reduces Nav channel availability by nearly 50%. This leads to a reduced AP peak and less K<sub>V</sub> and BK channel activation that attenuates the fast afterhyperpolarization phase. This in turn slows the return to baseline potentials followed by a switch from net outward to net inward currents after AP termination. When Nav channel availability is reduced up to 20% of total either by TTX block or steady depolarization the conversion from outward to inward current is more drastic and a switch from tonic into burst firing is observed. Figure 4 shows an example of switch from tonic to burst firing in the presence of saturating doses of TTX.

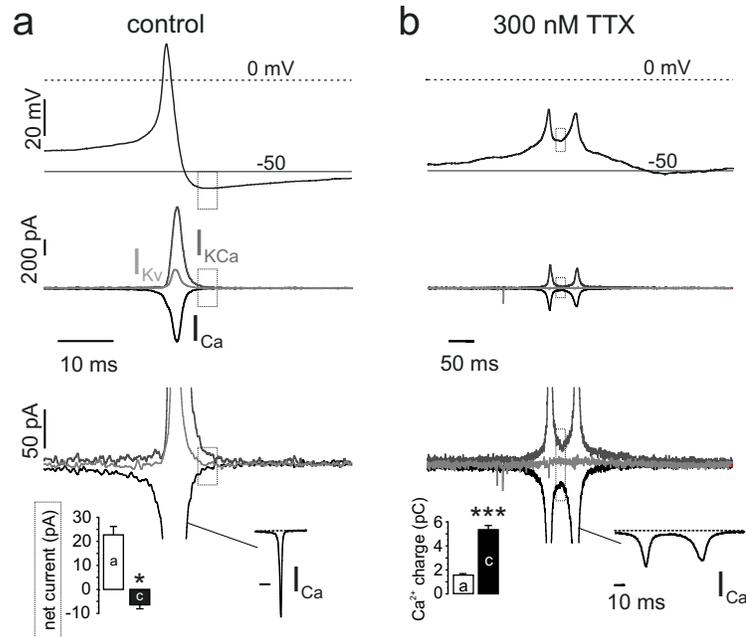


**Fig. (4).** TTX leads to a dose-dependent switch into burst firing of MCCs. Top, representative current-clamp trace showing the action of increasing TTX concentration. Middle and bottom, better view of the effects of increasing TTX concentrations on AP waveforms and firing patterns at slower and faster (grey windows) time scales. Periods of activity were taken from the top panel (adapted from ref. [24]).

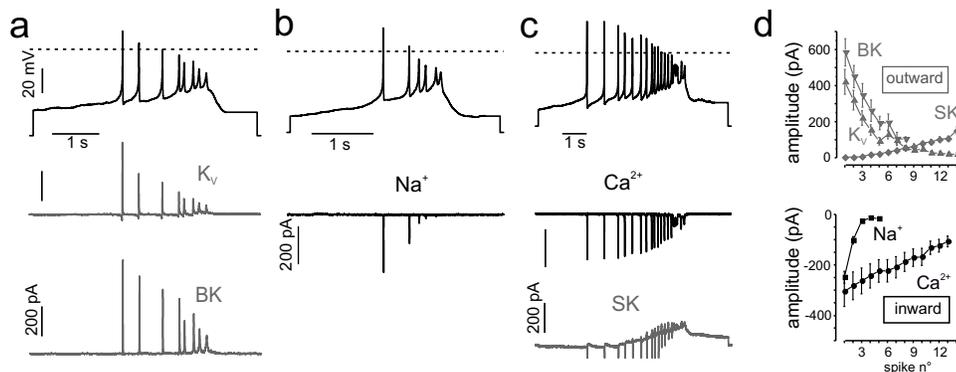
During burst firing, brief periods of high frequency firing are separated by relatively long gaps of no activity. Spikes fire in bursts on top of a slow-wave depolarization plateau that lasts for the whole burst duration. AP-clamp experiments show clearly that while the net current during the AHP of single control spikes is outward, the current during the intraburst interval is net inward and carried by  $\text{Ca}^{2+}$  (dashed rectangles in Fig. 5a). Broadening of APs during cell depolarization and bursts of APs in the presence of TTX causes a ten-fold prolongation of  $\text{Ca}^{2+}$  currents (bottom inset in Fig. 5b) that, despite the lower amplitudes, carries about four-fold more  $\text{Ca}^{2+}$  charges inside the cell [24], demonstrating that  $\text{Ca}^{2+}$  currents are the main source of the

slow depolarization plateau. In conclusion, the incoming  $\text{Ca}^{2+}$ , together with a lack of sufficient outward  $\text{K}^+$  current is the triggering event of burst firing when  $\text{Nav}$  channels availability is strongly attenuated.

Spontaneous burst firing is also observed in a minority of MCCs (14%) (Fig. 1c and 6). A train of high frequency spikes emerge on a significantly longer slow-wave depolarization plateau with respect to those induced by TTX (200-500 ms vs. 80-200 ms, respectively). Due to the  $\text{Nav}$  channel availability, the first spike amplitude is always larger as compared to the subsequent APs that are gradually broader, of lower overshoot and reduced AHP amplitude (Fig. 6).  $\text{Nav}$ ,  $\text{Kv}$  and  $\text{BK}$  currents decline rapidly during the burst while  $\text{Cav}$  currents



**Fig. (5).** Reducing  $\text{Nav}$  availability by TTX inverts net current during the AHP from outward to inward. **a)** Top, AP-clamp experiment showing representative control spike. Middle,  $\text{Kv}$  currents (light grey traces) were measured in a Tyrode standard solution with TTX (300 nM) and  $\text{Cd}^{2+}$  (200  $\mu\text{M}$ ).  $\text{Ca}^{2+}$  currents (black) were measured in the presence of TTX (300 nM) and high extracellular TEA (135 mM).  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents (dark grey) were obtained by subtracting from a control recording in Tyrode standard with 300 nM TTX the  $\text{Kv}$  and the  $\text{Ca}^{2+}$  current. Bottom, close up of the middle panel. The dashed rectangles indicate the AHP phase and the respective currents that sustain it. **b)** same as for a, using a spike doublet fired after complete block of  $\text{Nav}$  currents with 300 nM TTX. Bottom-left inset in a: net current amplitudes measured during the AHP phase indicated by the dashed rectangles. Bottom-left inset in b:  $\text{Ca}^{2+}$  charge entering the cell during the AHP phase calculated by integrating the corresponding  $\text{Ca}^{2+}$  inward current ( $I_{\text{Ca}}$ ) shown in full to the right (adapted from ref. [24]).



**Fig. (6).** Time courses of  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents during a slow-wave burst in different MCCs. **a)** AP-clamp recordings of  $\text{Kv}$  and  $\text{BK}$  currents. Top shows the burst recorded and used as voltage command (black trace),  $\text{Kv}$  and  $\text{BK}$  currents are shown in grey. **b, c)** as for panel a but currents isolated were  $\text{Ca}^{2+}$  (black),  $\text{SK}$  (grey) and  $\text{Na}^+$  (black). **d).** Top,  $\text{BK}$ ,  $\text{SK}$  and  $\text{Kv}$  outward current amplitudes versus the spike number of the burst. Bottom, same as top but for the  $\text{Na}^+$  and  $\text{Ca}^{2+}$  inward currents (adapted from ref. [24]).

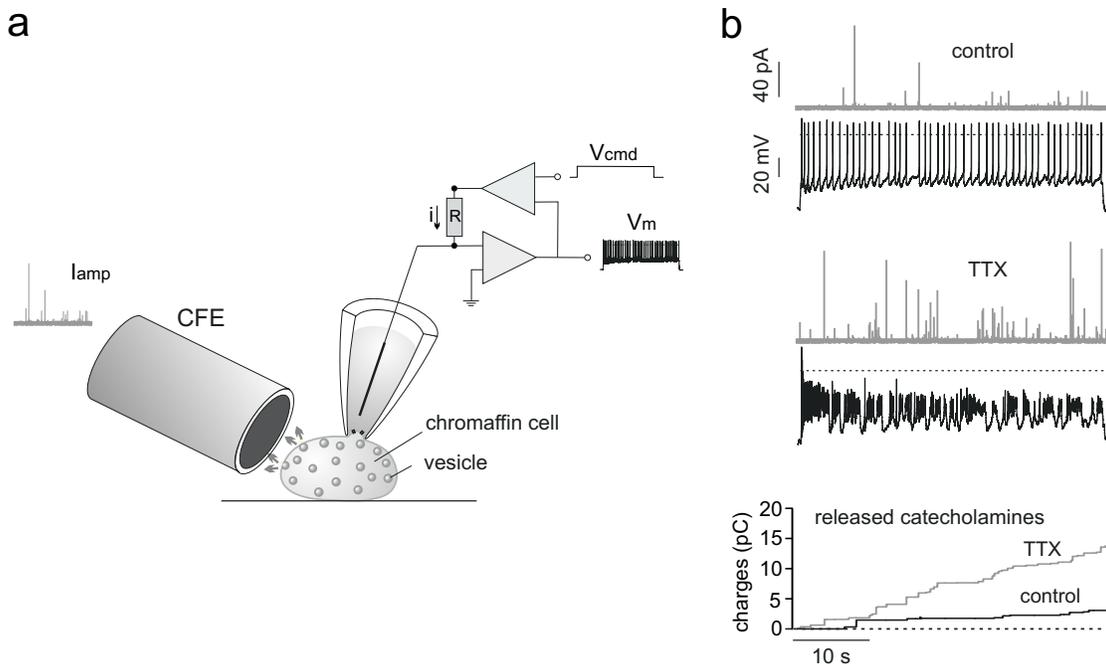
decline more slowly. Conversely, SK currents build up rapidly to terminate the burst. Thus, burst firings originating from plateau potentials lasting for hundreds of milliseconds give origin to large  $\text{Ca}^{2+}$  influxes that may boost the  $\text{Ca}^{2+}$ -dependent secretion of catecholamines. In this view, burst firing represents a way by which CCs rapidly increase the release of adrenaline and noradrenaline upon requirement.

Figure 7 shows that this indeed is the case if current-clamped MCCs are first hyperpolarized to stop their spontaneous activity and prevent  $\text{Ca}^{2+}$  accumulation at the onset of the recording. Control cells and cells pre-treated with TTX are then stimulated for 40 s with a constant depolarizing pulse to induce secretion. The fast quantal release of catecholamines revealed with carbon fibre amperometry [11, 61] (Fig. 7a) shows clearly that the increased AP frequency during bursts in the presence of TTX enhances significantly the quantity of released catecholamines (Fig. 7b, bottom). Thus, block of Nav channels with TTX to induce burst firing paradoxically boosts  $\text{Ca}^{2+}$ -entry and release of catecholamines. Although not shown, it is likely that most of the exocytosis is sustained by Cav1.3 since is the dominant pacemaking channel supporting prolonged depolarizations in MCCs.

#### TONIC VERSUS BURST FIRING IN CHROMAFFIN CELLS: A “NEURON-LIKE” WAY OF SIGNALLING TO BOOST EXOCYTOSIS

The present findings show that CCs possess intrinsic “neuron-like” firing properties typical of some central

neurons that undergo burst firing [27]. CCs spontaneously fire at rest in an irregular tonic manner and change into burst firing depending on the expression density and gating conditions of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  channels that control the firing. Recent evidence supports the view that this occurs when the net current passing during the AP repolarization phase turns from outward to inward, *i.e.*, when inward  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents prevail on outward  $\text{K}^+$  currents. Thus, burst firing is apparent in MCCs when  $\text{Kv}$  channels are blocked by TEA [21] or when the  $\beta_2$  subunit controlling the fast inactivation rate of Slo1 BK channels is deleted [22]. Burst firing occurs also in a minority of MCCs under physiological conditions [22, 24], most likely because of a critical balance between functioning  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  channels. In RCCs and BCCs, burst firing appears when either the ERG  $\text{K}^+$  channel is blocked by the antiarrhythmic drug WAY or when an M-current is blocked by an histamine-mediated pathway [16, 18]. In this view, any modulatory mechanism that leads to a depression of  $\text{K}^+$  outward current or to a potentiation (or maintenance) of  $\text{Ca}^{2+}$  currents is potentially able to induce burst firing in CCs. Indeed, burst firing occurs also when the availability of functional fast-inactivating Nav1.3/Nav1.7 channels is strongly attenuated by block with TTX or by accelerating the Nav channel entry into closed-state inactivation at more depolarized resting potentials. This latter, represents a novel modulatory pathway by which “fast inactivating” Nav channels control burst firing in spontaneously firing cells [24]. A role typically played by “persistent” and “resurgent” neuronal Nav channels.



**Fig. (7).** Burst firing associated with Nav channel block boosts MCC exocytosis. **a**) Experimental arrangement to record catecholamine secretion using a carbon fibre microelectrode (CFE) from a current-clamped MCC. The cell is constantly depolarized to evoke an AP train ( $V_m$ ) by passing a constant current ( $i$ ) through the glass pipette using a square pulse voltage command ( $V_{cmd}$ ). The quantal release of adrenaline and noradrenaline is detected in forms of amperometric currents by the CFE ( $I_{amp}$ ) placed very close to the CC. **b**) Top, example of combined recording of APs (black traces) and amperometric currents (grey traces) by CFE amperometry in control. Middle, combined recording of AP bursts and amperometric events during TTX application to the same cell of the top. Bottom, overlap of cumulative secretion plots obtained by integrating the amperometric recordings shown in **a** and **b** for control (black trace) and TTX application (grey trace) (adapted from ref. [24]).

Burst firing in CCs is in all cases sustained by the L-type Cav1.3 currents which activate at relatively low voltages and inactivate slowly at potentials near rest. Cav1.3 sets and regulates the firing cycle by sustaining the slow pacemaking current and by triggering BK and SK currents. In CCs the slow-wave bursts enhance the quantity of Ca<sup>2+</sup>-entry inside the cell and thus boost exocytosis of catecholamines. In altered physiological conditions, this may occur in various ways: i) by any mechanism that dampens Kv and BK channel gating, ii) by regulatory pathways which up-regulate L-type channels [56, 57] or, iii) by modulatory pathways that cause steady CC depolarizations and drastically reduce Nav1.3/Nav1.7 channel availability. This latter might be applicable to conditions such as plasma hyperkalemia [36], acidosis [130] or increased histamine levels that induce sustained depolarizations, burst firing [18] and enhanced circulating catecholamines [131]. Of particular interest in CC biology is the potential role of muscarinic receptors, whose activation causes prolonged membrane depolarizations, increased cell firing and sustained catecholamines release [4, 21]. The action is mediated by the M1 muscarinic receptor [132] and proceeds through the inhibition of TASK1-like channels [130]. As muscarine activates the PKC pathway in RCCs [4, 133], it is likely that Nav channels may undergo the well-known PKC-dependent down-regulation mediated by muscarinic receptors [134]. This, together with the 30% reduced Nav channels availability at depolarized V<sub>h</sub>, would critically increase the probability of MCCs to switch into burst firing patterns, boosting catecholamine release during ACh-driven acute stress responses.

An open question is what could be the clinical impact of CC burst firing and increased catecholamine release following Nav channel block. Nav channel blockers are widely used in therapy and thus, it would be interesting, for instance, to establish whether increased levels of catecholamines may occur in patients with ventricular tachycardia treated with Nav1.5 channel blockers [135]. Obviously, an increased level of circulating catecholamines will reverse the beneficial effects of Nav1.5 blockers. To this specific issue we can observe that cardiac Nav1.5 channels are structurally and functionally different from neuronal Nav1.3/Nav1.7 channels [110]. Nav1.5 channels are TTX-insensitive and carry large "late" sodium currents (I<sub>NaL</sub>) in myocardial pathologies [136] while Nav1.3 and Nav1.7 are TTX-sensitive and fast-inactivating [24, 36]. In addition, several used Nav1.5 blockers are many-fold more potent in inhibiting I<sub>NaL</sub> than the peak I<sub>Na</sub> [137]. For instance, the piperazine derivative ranolazine exerts its therapeutic effects at ~10 μM with minimal or no effect on peak I<sub>Na</sub>. Given this, it seems unlikely that a Nav1.5 blocker could reduce the peak of fast-inactivating Nav1.3/Nav1.7 currents of CCs to less than 20% to induce burst firing and increased circulating catecholamines that would enhance cardiac arrhythmias in hearts treated with I<sub>NaL</sub> blockers [136].

We conclude by observing that in physiological, pathological or therapeutical conditions, the density of functional Nav1 channels is a key element for driving Cav1.3 channels availability and CCs function. For this, is very important to identify all possible causes that down-regulate Nav1.3/Nav1.7 channels availability and shift CCs firing from tonic to bursts, including potential Nav channel blockers used in therapy [138].

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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