Molecular and functional characterization of the mouse intracardiac nervous system

Short title: mouse intracardiac nervous system characterization

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Abstract

Background

The intracardiac nervous system (ICNS) refers to clusters of neurons, located within the heart, that participate to the neuronal regulation of cardiac functions and are involved in the initiation of cardiac arrhythmias. Therefore, deciphering the role of the ICNS in cardiac physiology and physiopathology is mandatory. Whereas transgenic mouse models represent powerful tools to reach this goal, the mouse ICNS is still poorly characterized.

Objective

The objective of the present study was to provide a phenotypic, electrophysiological and pharmacological characterization of the mouse ICNS.

Methods

Global cardiac innervation and phenotypic diversity was investigated by performing immunohistochemistry on cleared murine heart and on tissue sections. Patch clamp technique was used for electrophysiological and pharmacological characterization of isolated mouse intracardiac neurons.

Results

We identified the expression of 7 distinct neuronal markers within mouse intracardiac neurons demonstrating the neurochemical diversity of this network. Of note, we described for the first time in mouse, the existence of neuron expressing the calcium binding protein calbindin, the neuropeptide Y (NPY) and the cocain and amphetamine regulated transcript (CART) peptide. Electrophysiological studies also revealed the existence of two different neuronal population based on their electrical behavior. Finally, we demonstrated that these neurons can be modulated by several neuromodulators.

Conclusion

This study demonstrated that mouse ICNS shares similar molecular and functional complexity to that of other species and therefore is a suitable model to decipher the role of individual neuronal subtypes in the modulation of cardiac function and in the initiation of cardiac arrhythmias.

Keywords: Intrinsic cardiac ganglia, Autonomic nervous system, Heart, cardiac innervation, cardiac electrophysiology.

Introduction

Neural control of the heart involves central and peripheral neurons that act interdependently to modulate cardiac parameters such as heart rate, conduction velocity or contractility. As part of this cardiac neuronal regulation, the intrinsic cardiac nervous system (ICNS) is receiving growing attention. Indeed, whereas they were initially considered as simple parasympathetic postganglionic neurons, studies conducted over the past 30 years suggested a more complex organization of intracardiac neurons, involving the existence of sensory, local regulatory and motor neurons within intracardiac ganglia, leading to the concept of "little brain on the heart"[1].

Phenotypic studies have been conducted in many species to try to identify several putative functional neuronal subpopulations. Beside cholinergic phenotype, the presence of catecholaminergic, glutamatergic and nitregic phenotypes have been described within intracardiac neurons[2–10]. This neurochemical diversity have also been illustrated by the expression of several neuropeptides such as neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide, somatotastin (SST), or cocaine and amphetamine regulated transcript (CART) peptide[3,6,10–13].

From a functional point of view, several studies mainly conducted in rat and guinea pig have also identified various types of cardiac neurons based on their electrical membrane properties[14–17]. In the guinea pig, electrophysiological experiments combined to a morphological characterization of intracardiac neurons gave rise to the identification of three distinct types of neurons including a putative sensory one[16]. However, we are still lacking evidence demonstrating that this neuronal diversity is associated with a functional specialization of neurons. Such a characterization would bring essential information to assess the impact of cardiac neuronal network in cardiac physiology. This would be even more important since ICNS have also been implicated in cardiac arrhythmias. For example, atrial fibrillation (AF) have been correlated with excessive activity of intracardiac neurons[18] and specific stimulation of cardiac ganglia has been able to trigger AF[19]. Moreover, ablation of ganglionated plexus has been shown to reduce AF and is now one of the strategies used in therapy[20]. Deciphering the ICNS

modulations of cardiac function through the role of individual neuronal subtypes is therefore essential but was for a long time limited by technical approaches available.

Recent advances in genetic engineering have opened novel opportunities to improve our comprehension of the ICNS and its pathophysiological involvement in arrhythmias. For example, cre-lox systems combined with optogenetic or DREADDs approaches represent a powerful tool to address more precisely the function of one particular subtypes of intracardiac neurons. However, because these technologies are almost only available in mouse models, a better understanding of the mouse ICNS is required.

To date, the few existing studies on mouse cardiac neurons seem to confirm the phenotypic heterogeneity observed in other models. Indeed, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) and neuronal nitric oxyde synthase (nNOS) have been reported in these neurons[2,7]. However, to our knowledge, the expression of other neuronal markers has not been yet investigated. Moreover, very little is known regarding the electrical and pharmacological properties of mouse cardiac neurons with only one study investigating this aspect[21].

This study was therefore designed in an effort to better characterize the phenotypic, electrophysiological and pharmacological properties of the mouse ICNS. Immunohistochemical experiments were conducted on cleared whole murine hearts and on tissue sections allowing us to (1) quantify global autonomic cardiac innervation and (2) investigate the phenotypic heterogeneity of mouse intracardiac neurons. This examination was further proceed with the characterization of passive and active electrical properties as well as pharmacological responses of isolated mouse cardiac neurons using the patch clamp technique.

Methods

Animals

Experimental procedures were performed using adult C57/BL6 mice (8-20 weeks) of either gender in accordance with the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. The protocol was approved by the local ethics committee "COMETHEA".

iDISCO heart clearing

Mouse hearts were stained and cleared using a modified iDISCO+ protocol[22,23]. Images were acquired with the ALICE's custom-built mesoSPIM microscope at Wyss Center, Geneva. (See supplement).

Immunohistochemistry

Immunohistochemistry was performed on 40–50 µm heart sections and acquired using a confocal laser scanning microscope (FV3000 Olympus) (See supplement).

Neuron dissociation

Ganglia were enzymatically digested with 2mL HBSS containing 3mg/mL collagenase typeII (Worthington), 7.5mg/mL dispaseII and 0.25 mg/mL DNaseI (30mn at 37°C). This was followed by an incubation in 2mL trypsin-EDTA 0.25% supplemented with 0.25 mg/mL DNaseI (35mn at 37°C). Finally, cells were gently triturated with fire-polished Pasteur pipettes coated with SVF and plated on laminin-coated 35mm Petri dishes.

Electrophysiology

Electrical membrane properties of isolated cardiac neurons were determined using the whole-cell configuration of the patch clamp technique. Recordings were carried out at room temperature within 30 hours following isolation. Data acquisition and analysis were performed using pClamp software (v11, Molecular devices, San Jose, California, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (San Diego, California, USA). Data are presented as mean±SEM. Mann-Whitney test was used for statistical comparison.

Results

Cholinergic and catecholaminergic innervation of mouse heart

Neural control of the heart involved a combination of peripheral and intrinsic neural structures[1]. In order to investigate global cardiac innervation in mice, we performed immunodetection of cholinergic (ChAT-IR) and catecholaminergic (TH-IR) structures on cleared murine hearts.

This approach allowed us to appreciate the dense network of TH-IR nerves innervating mouse hearts (supplementary movie). Large bundles of nerves accessed the heart through the heart hilum, at the base of the heart, and extended toward the dorsal and the ventral side of both ventricles (fig.1a). Ventricular innervation was mainly located at the epicardial surface even if thinner fibers could be seen deeper through the heart wall, especially coursing along the interventricular septum (supplementary movie).

ChAT-IR was used to identify intrinsic cardiac ganglia. As seen in figure 1, ganglia were exclusively located in the dorsal side of the heart, in close proximity to the pulmonary veins. The mean number of ganglia per heart were 18±3 ranging from 13 to 23 (n=3, fig.1d). Most of them were characterized by TH-IR puncta allowing us to see individual neurons (fig.1a insets 1 and 2). Unlike TH, ChAT-IR fibers were almost exclusively located around ganglionated plexus where they interconnected multiple ganglia.

Global cardiac innervation was further studied by developing a volumetric quantification approach using Imaris. Based on ChAT staining, the volume of each individual ganglion was estimated resulting in a total ganglion volume of $1.95\pm0.17\ 10^7\ \mu m^3$ (n=3; fig.1b,e) per heart. We also developed an automatic pipeline in ImageJ to quantify the total length of TH-IR nerve fibers innervating both ventricles and found a total length of $1.68\pm0.21\ 10^5\ \mu m$ (n=3; fig.1c,f).

Neurochemical phenotype of mouse intracardiac neurons

Immunodetection of the cholinergic marker ChAT confirmed the prominent cholinergic phenotype of intrinsic cardiac neurons (98.7%). This marker also labeled intra and interganglionic nervers fibers as

well as varicose terminals surrounding neurons. Immunoreactivity for TH was detected in approximately 30% of cardiac neurons and in numerous nerves fibers (fig.2a-c).

Except the widely established cholinergic and catecholaminergic markers we were able to observe a large proportion of neurons (46%) expressing the calcium binding protein calbindin D-28k (calbindin). In neuronal somata, calbindin-IR was always observed in cytoplasm and was often accompanied by a strong nucleus staining (fig.2d-f). Calbindin-IR was also present in multiple intra and inter-ganglionic nerve fibers and occasionally in pericellular baskets surrounding neuronal somata (fig.2d inset). Calbindin neurons were always co stained by ChAT and 11.8% of cardiac neurons showed immunoreactivity for calbindin, ChAT and TH (Table1).

A small population of neurons stained with nNOS, the neuronal enzyme responsible for the synthesis of NO, was also present in cardiac ganglia. These neurons were not present in all ganglia and account for only 2% of total neurons (fig.2g-i). The majority but not all nNOS-IR somata coexpressed ChAT.

In very rare occasions, we were able to observe immunoreactivity for the vesicular glutamate transporter 2 (VGLUT2) (fig.2j-l). However, no somata appeared stained by the other vesicular glutamate transporter VGLUT1. Finally, we were unable to observe any GABAergic nor serotoninergic phenotype despite the use of different markers (Gad67, GABA, TPH2).

Intracardiac neurons have been shown to express several neuropeptides in various species, especially in rodents[6,11]. In mouse hearts, we observed a large proportion of neuronal somata immunoreactive for NPY (67%) and CART peptides (61%), however, due to antibody crossreactions, we didn't quantify the number of neurons coexpressing both peptides. In somata, these two staining appeared as granular with a perinuclear localization suggesting a localization in the endoplasmic reticulum and the Golgi apparatus (fig.3). Numerous intra and interganglionic nerve fibers were also labeled by these two peptides.
Calcitonin Gene Related Peptide-IR (CGRP) and Substance P-IR (SP), two peptides characteristic of sensory neurons, were also observed in nerve fibers but never in neuronal somata (fig.3a-f). Both peptides were frequently colocalized although SP-IR nerves fibers were far less numerous. IR for SST and VIP was also tested, however we were unable to see any labeling for these neuropeptides.

Electrophysiological properties of mouse intracardiac neurons

After isolation, cardiac neurons were identified as cells with rounded cell body with numerous processes extended from it (fig.4a). The majority of neurons appeared as multipolar, even if some uni and bipolar neurons were also observed.

Passive and active electrical membrane properties of isolated mouse cardiac neurons were determined using the patch clamp technique in current clamp mode. At rest, isolated cardiac neurons were defined by a membrane potential of -61.3±0.8mV (n=42), an input resistance of 1420.1±70.8 MΩ (n=36) and a membrane capacitance of 33.4±1.4pF (n=39) (Supplementary table2).

In our conditions, no spontaneous firing activity was observed. Investigation of discharge characteristics revealed two distinct profiles. 81.4% of neurons showed limited firing activity (phasic neurons) characterized by a maximum of 1 to 3 action potentials (AP) at the onset of the stimulus while 18.6% of neurons exhibited adapting firing behavior (fig.4b-c). We also determined that adapting neurons were defined by a lower rheobase (37.1±2.9pA; n=7) compared to phasic neurons *(*66.3±3.9pA; n=35; $p<0.0005$, fig.4d). Moreover, adapting neurons displayed a shorter (71.5±6.4ms (*adapting*) vs 151.6±22.2ms (*phasic*); $p<0.05$, fig.4e) and smaller (5.6±0.3mV (*adapting*) vs 9.2±0.6mV (*phasic*); $p<0.05$, fig.4f) afterhyperpolarization (AHP).

Upon brief injection of suprathreshold depolarizing current, AP exhibited an amplitude of 134.2 ±1.7mV and a half duration of 1.84±0.04ms (n=38). Approximately half of APs were followed by AHP with an amplitude of 8.3±2.6mV while the other neurons did not exhibit any AHP (fig.5a-e). Electrophysiological properties of mouse intracardiac neurons are summarized in supplementary table2.

Pharmacological response of mouse intracardiac neurons

Since the ICNS is under the dependence of several modulators[24], we investigated the pharmacological response of isolated mouse cardiac neurons to known peripheral neuromodulators.

All tested neurons (n=21) exhibited membrane voltage and current response to acetylcholine (Ach) perfusion and the resulting inward current were sufficient to trigger AP (fig.6a). The superfusion of Ach resulted in the development of a large inward current that showed a decay in amplitude before the end of the drug application, probably due to channel desensitization.

The expression of several purinergic receptors have been described in rat cardiac neurons[25] and adenosine triphosphate (ATP) and other purinergic compounds have been reported to modulate canine intracardiac neurons[24]. In our study, exogenous application of ATP was accompanied by a strong membrane depolarization and AP in all tested neurons (n=4) (fig.5). In voltage clamp (HP=-50mV), ATP evoked a rapidly activating, sustained inward current, characteristic of P2X receptors activation.
In our study, perfusion of bradykinin (BK) was able to slowly depolarize mouse cardiac neurons without eliciting AP (0.2µM: 8.4±1.1mV (n=7) ; 0.5µM: 13.0±2.4mV (n=8)). This was confirmed by the observation of a very slow activating inward current of small amplitude after BK exposure in voltage clamp experiments (fig.6c).

Discussion

In this study, by using multi-technique approaches, we provide for the first time a detailed report of anatomic, phenotypic, electrophysiological as well as pharmacological properties of the mouse ICNS.

Cholinergic and catecholaminergic innervation of mouse heart

For the past 30 years, cardiac innervation and ICNS have been studied in a variety of species using tissue sections or whole-mount approaches. Although these techniques have brought a lot of information regarding the cardiac autonomic innervation, they suffer from some limitations as they imply loss of sample integrity or only report superficial information. The recent development of optical clearing techniques combined to advances in 3D imaging of large-scale specimen have overcome these limitations, opening new opportunities to study global cardiac innervation.

We took advantage of the iDISCO clearing method to visualize and quantify in three dimensions the sympathetic and parasympathetic innervation of the mouse heart. By using this approach, we have been able to determine the exact location and number of cardiac ganglia in the whole non-sectioned murine heart. Our results are in accordance with Rysevaite et al.[26] who reported a mean number of 19±3 ganglia. Since three dimensions analysis of ganglia was missing in the literature, we employed a volumetric approach enabling the quantification of ganglion size and found that cumulative ganglia occupied a volume of 0.02mm^3. We also developed an automatic pipeline in ImageJ to quantify the total length of

nerve fibers innervating the heart. Our approach is complementary to the remarkable work done by Rajendran et al.[27] who developed a clearing-imaging-analysis pipeline to assess diameter and orientation of nerve fibers innervating the mouse heart. This anatomical description of neuronal cardiac circuits will bring useful information for the comprehension of the autonomic cardiac innervation as well as for the identification of abnormal cardiac innervation in pathological states as it has been described in myocardial infarction or in cardiac autonomic neuropathy[28,29].

Neurochemical phenotype of mouse intracardiac neurons

So far, very little information was known about the neurochemical diversity of intracardiac neurons in mouse, with only 3 different neuronal markers (ChAT, TH and nNOS) identified[2,7,8]. Our results confirmed that almost all intracardiac neurons expressed the cholinergic marker ChAT and that almost 30% of them co-expressed ChAT and TH[7,8]. We also found a small population of nitrergic neurons which is consistent with previous investigations that have reported such a phenotype in rat, guinea pig, rabbit and human[3–5].

We described for the first time the expression of 4 additional markers within somata of mouse intracardiac neurons demonstrating that this species is also characterized by a relative phenotypic diversity as it has been reported in others mammals[3,5,10]. Indeed, we emphasized the existence of neurons expressing the calbindin, NPY and CART peptides and to a lesser extent glutamatergic neurons. A large proportion (46%) of mouse intracardiac neurons express the calcium binding protein calbindin. To date, only one study identified such neurons in rat heart with a proportion of only 7% of total neurons[3]. The expression of this protein has been reported in central and peripheral neurons and is often used as a marker to discriminate different functional subpopulation of neurons such as sensory neurons[30]. We also observed a lot of intra- and inter-ganglionic fibers as well as terminals surrounding cell bodies that were immunoreactive for calbindin, suggesting that calbindin-expressing neurons may be crucial components of local reflexes. In the future, it would be interesting to investigate whether the expression of this protein is associated with any specialized function.

Recently, glutamatergic neurons immunoreactive for VGLUT1, VGLUT2 and glutaminase, the synthetic enzyme for glutamate, have been found within the ICNS of rat[9]. In our study, we occasionally observed neurons immunoreactive for VGLUT2 but we failed to detect any VGLUT1 immunoreactivity. Therefore we suggest that in mouse, glutamatergic intracardiac neurons may exist but does not represent a significant neuronal population.

The expression of a variety of neuropeptides also account for the neurochemical diversity of intracardiac neurons, especially in rodents[6,11]. However, except the description of sensory fibers immunoreactive for CGRP and SP, the expression of other neuropeptides has not been investigated in mouse[7]. Here, we identified the expression of two neuropeptides, NPY and CART, within mouse intracardiac neurons. The expression of NPY within these neurons was not surprising since it appeared to be widely distributed in the autonomic nervous system and its cardiovascular effects have been well documented[31].

CART peptide expression has been found to concern only a small number of cardiac neurons in the guinea pig while it has been observed in 46% of neuron in the rat[11,12]. This proportion is even more important in mouse, with 61% of neurons showing immunoreactivity for CART. This peptide has been extensively studied in the enteric nervous system where it appeared to be expressed in many neurons but experimental evidence elucidating its biological function are still lacking[32]. Further studies should try to clarify its function within the cardiac context.

Electrophysiological and pharmacological properties of mouse intracardiac neurons

The complex organization of the ICNS has been further supported by the examination of its electrophysiological properties. Indeed, based on their electrical behavior, different subtypes of neurons have been identified within mammals, demonstrating that intracardiac neurons were not forming a homogeneous population[14,16,17]. Here, we report the first detailed investigation of passive and active electrical properties of mouse intracardiac neurons. These neurons appeared to have uniform resting membrane and AP properties. However, by studying their firing activity we identified two distinct neuronal populations as observed previously[15,17,21]. Indeed, while the majority of neurons were classified as phasic due to their limited firing activity, a small proportion of neurons were able to discharge more

APs and were defined as adapting neurons. Phasic neurons had a significant higher rheobase as well as higher AHP amplitude and duration compared to the adapting one, which confirm the existence of two types of neurons displaying different functional characteristic in terms of excitability. Little information is known about the different ionic channels expressed by intracardiac neurons, especially in the mouse, hence, it would be interesting to closely investigate the molecular determinants of these two types of electrical behaviors.

A significant number of studies have revealed the diversity of neuromodulatory sources of intracardiac neurons which further suggest that they can act as integrative centers. In the dog, neuronal activity have been found to be regulated by mechanical as well as chemical stimuli[27]. Similarly, a variety of substances have been reported to modulate excitability of intracardiac neurons in rat and guinea pig[13,24,33]. However the pharmacological modulation of mouse intracardiac neurons have never been investigated. Here, we demonstrated that mouse cardiac neurons showed responsiveness to Ach, ATP and BK, suggesting that they can be modulated by a variety of stimuli. In our experiments, BK induced a small inward current associated to a slight membrane depolarization. This indicates that BK receptors are present in mouse cardiac neurons but their stimulation was not sufficient in our conditions to trigger AP firing as observed in rat[33].

Conclusion

In conclusion, our study is the first detailed report providing phenotypic, electrophysiological and pharmacological characterization of mouse intracardiac neurons. Taken together our results demonstrated that the mouse ICNS shares similar complexity in term of phenotypic, electrophysiological as well as pharmacological properties to that of other species. ICNS complexity deserves to be deciphered since there is growing evidences that ICNS plays an essential role in cardiac modulations and in the initiation and maintenance of cardiac arrhythmias. The emergence of new genetic tools such as DREADDs and optogenetics raise new opportunities to control the activity of specific neurons within a global network. Therefore, these tools represent an excellent approach to understand the role of targeted neurons in global cardiac modulation as described in the recent review of Scalco et

al.[34]. However, these transgenic technologies are almost only available in mouse, and despite the extensive use of mouse models in cardiovascular research, little was known about their ICNS. In this context, our study is paving the way for future investigations using a combination of cre-mice systems and DREADD/optogenetic tools in order to decipher the functional organization of the ICNS as well as its implication in pathological states such as arrhythmias.

Author Contributions

G.L and A.C performed and analyzed the experiments. C.P conceptualized the imageJ pipeline. A.T performed technical imaging acquisition. G.L, C.P, S.P and A.C wrote the manuscript. A.C conceptualized and supervised the study.

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312 **Table 1**. **Neurochemical profile of mouse intracardiac neurons**.

Neurochemical phenotype	Percentage (number of profiles)	Multiple phenotype (% of total)
ChAT	98,7%	
TH	28,8% (251/873)	27,9% are ChAT/TH
NPY	66,5% (581/874)	
nNOS	1,7% (10/577)	
Calb	45,7% (596/1305)	11,8% are Calb/ChAT/TH
CART	60,8% (578/950)	

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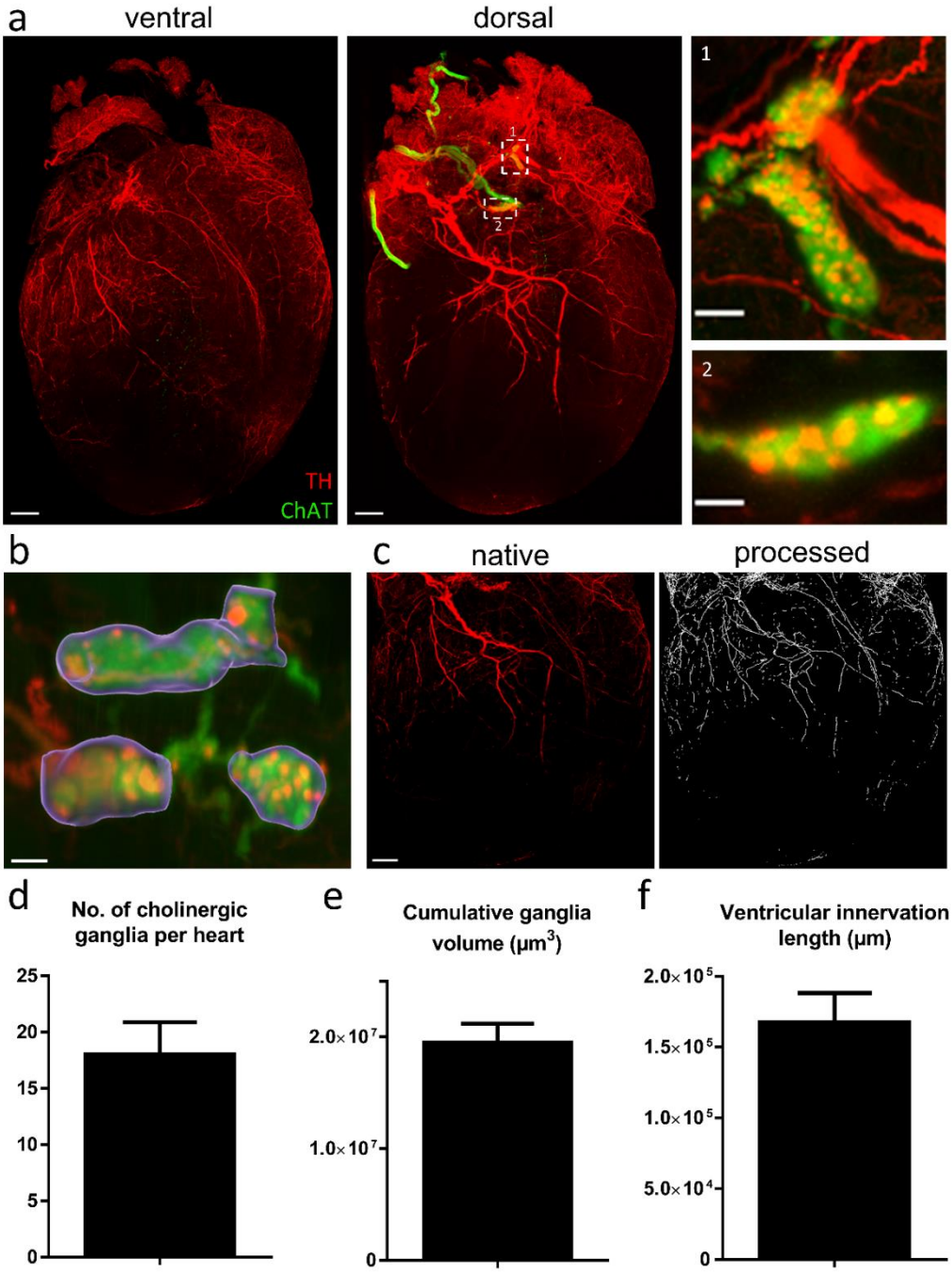
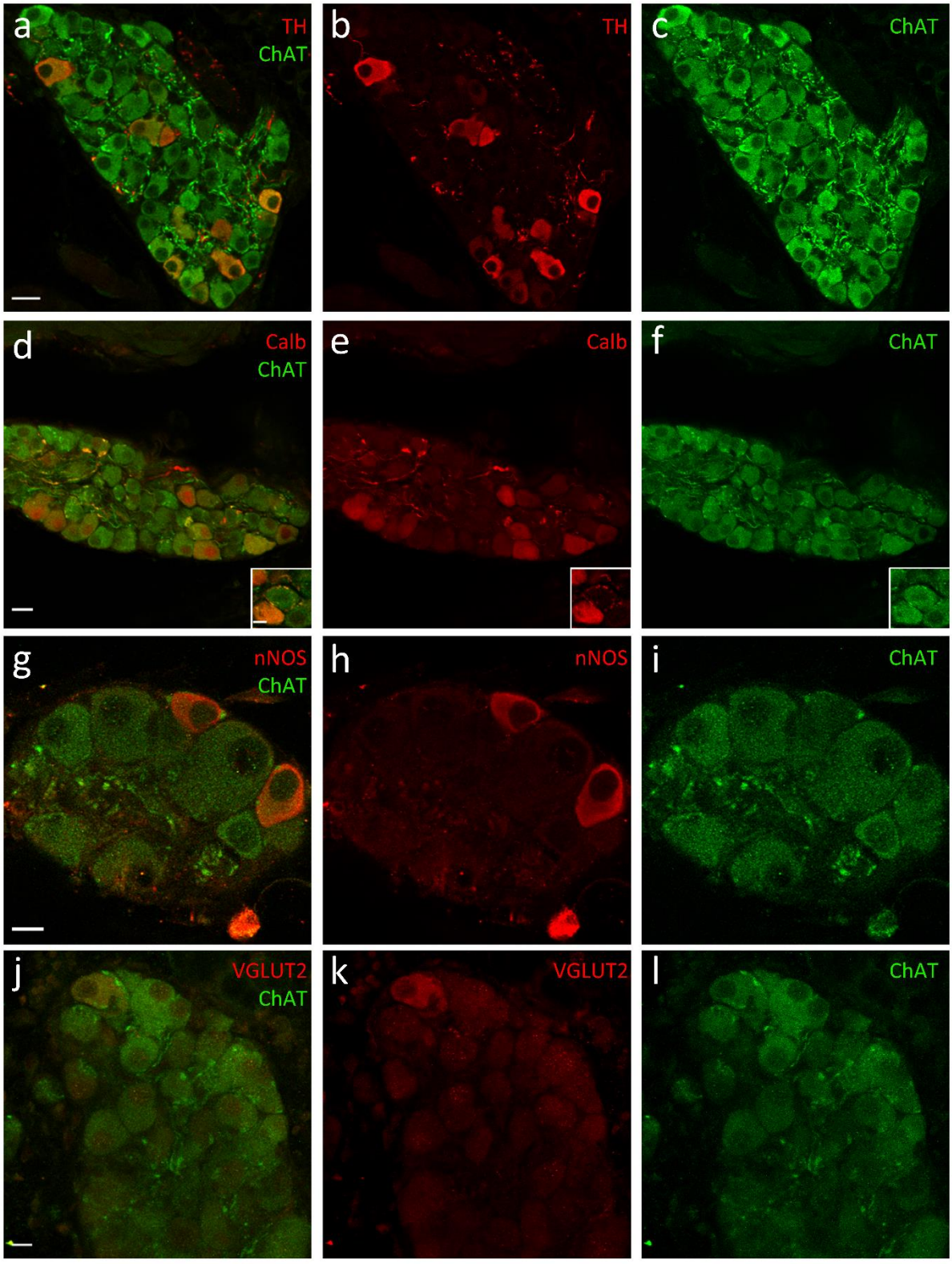
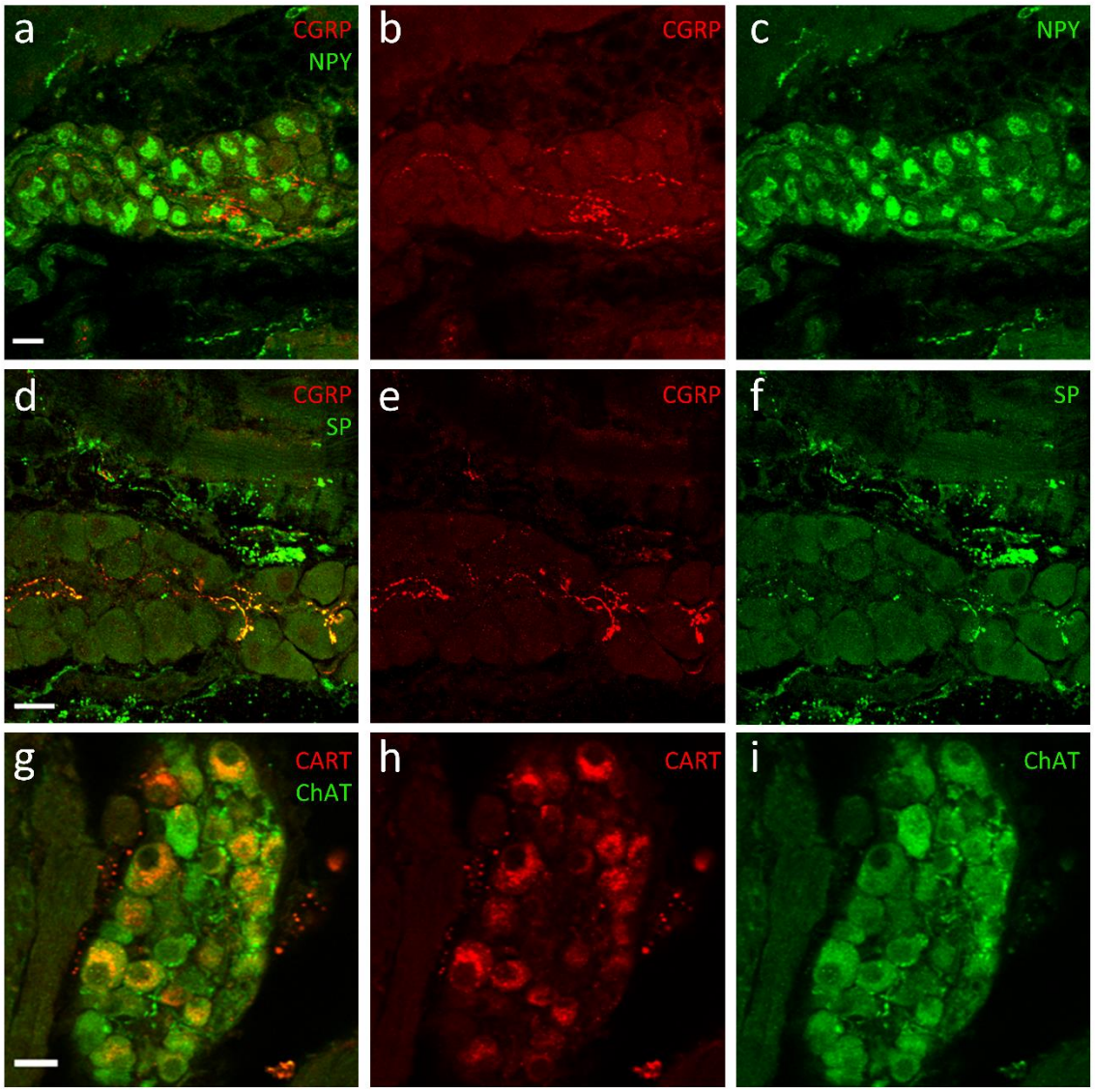


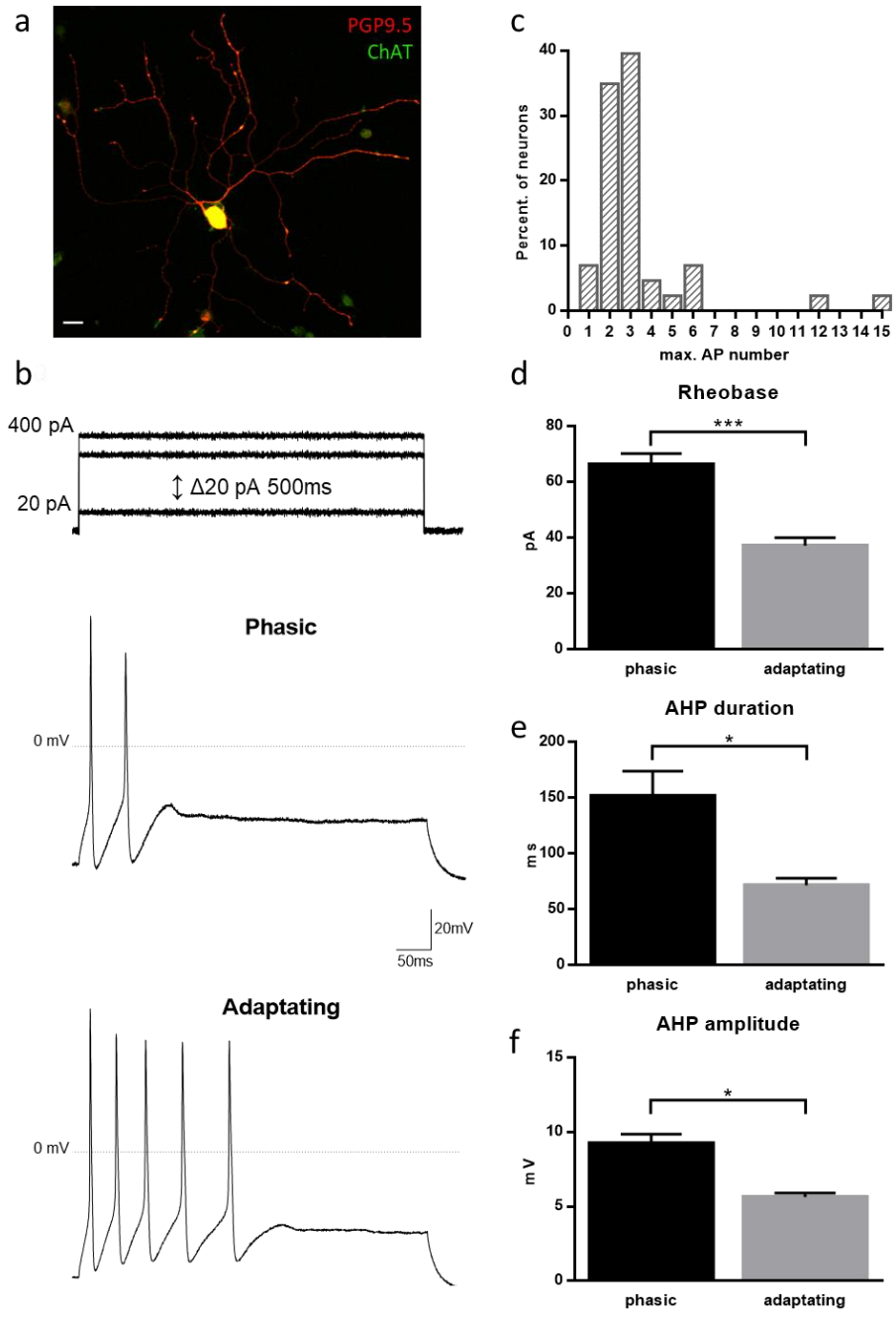
Figure 1. **Cholinergic and catecholaminergic cardiac innervation**. **a)** 3D projections of the ventral (2500µm z-stack) and dorsal side of a cleared heart (2440µm z-stack) with TH (red) and ChAT (green) staining. Magnification of ganglia are presented in inset 1 and 2. (**b-c)** 3D image processing used for the quantification of ganglia volume (purple) and ventricular innervation (white). **(d-f)** Determination of the total number of ganglia per heart **(d),** the cumulative ganglion volume **(e)** and the total lenght of adrenergic fibers innervating both ventricles **(f)**. Scale bars are 500µm (**a**(ventral and dorsal) and **c**) and 100µm (**a**(insets 1 and 2) and **b**).



321 **Figure 2**. **Phenotypic properties of intracardiac neurons**. Confocal images of sections of cardiac
322 ganglia immunostained with TH **(a-c)**, calbindin **(d-f)**, nNOS **(g-i)**, VGLUT2 **(j-l)**, and ChAT. Insets in
323 d-f show typical calbindin-IR pericellular baskets surrounding a neuronal somata. Scale bars are 10µm
324 (inset d, g-i and j-l) and 20µm (all others).



325 **Figure 3**. **Peptide expression in mouse intracardiac neurons**. Confocal images of sections of cardiac
326 ganglia immunostained with NPY and CGRP **(a-c)**, SP and CGRP (d-f) and CART **(d-f)**. Scale bars are
327 20µm.



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329 **Figure 4**. **Action potential discharge profiles in dissociated mouse intracardiac neurons**. **(a)**
330 Isolated cardiac mouse neuron costained with the neuronal marker PGP9.5 and ChAT.**(b)** Maximum
331 discharge of AP observed in two distinct neurons in response to 500ms depolarizing current injection
332 from 20 to 400pA (Δ20pA). Upper trace represents AP obtained in a phasic neuron whereas lower trace
333 was obtained in an adapting one **(c)** Distribution of the maximum number of AP recorded with the
334 stimulation protocol presented in a. **(d)** Determination of the rheobase of phasic (n=35) versus adapting
335 neurons (n=7) ($p<0,0005$) **(e-f)** Determination of the duration and amplitude of AHP in phasic (n=14)
336 versus adaptating (n=4) neurons ($p<0,05$) with the stimulation protocol used in figure5**.**

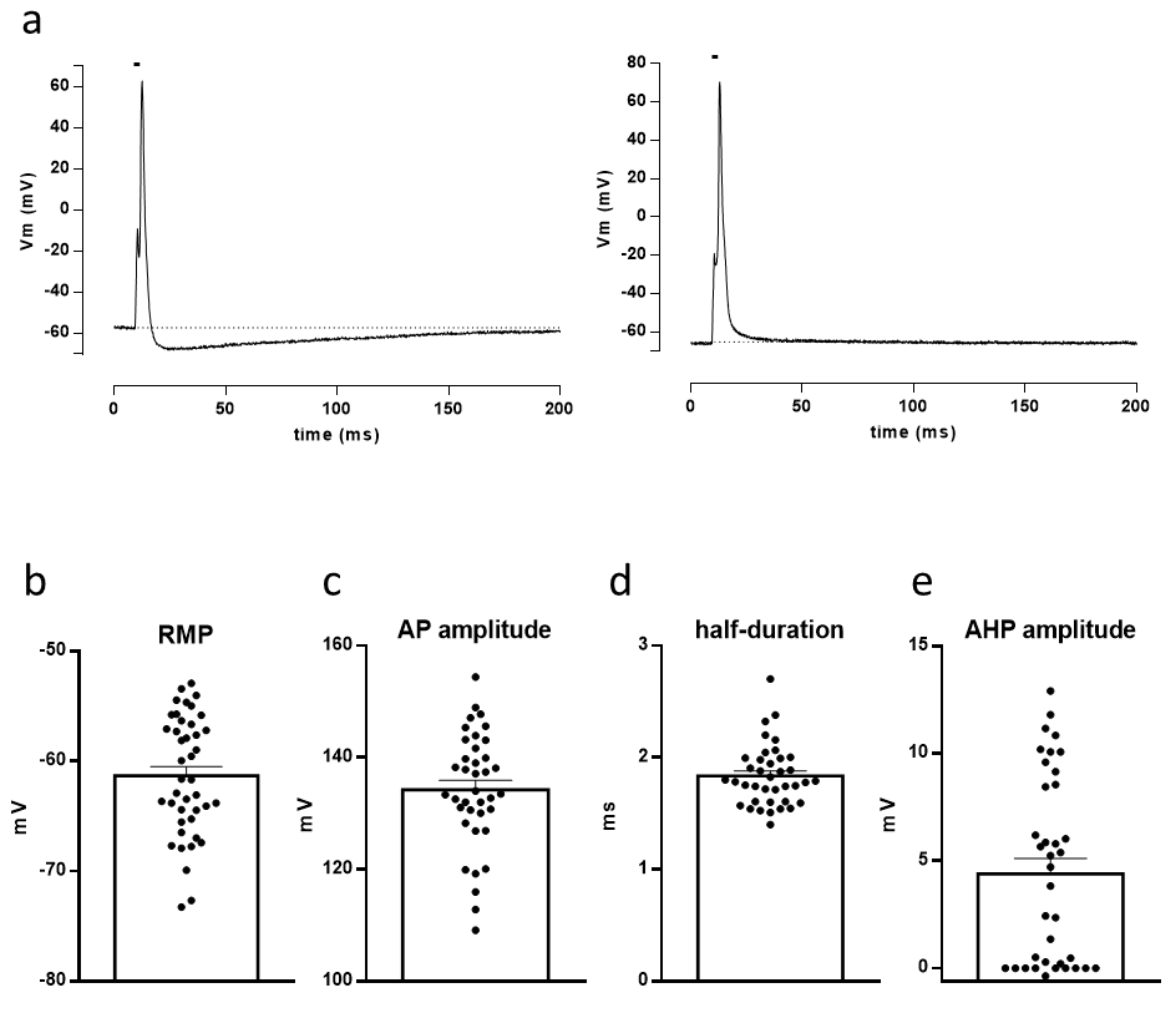

Figure 5. **Action potentials properties in mouse intracardiac neurons.** Typical AP recorded in response to a brief injection (2ms, black line) of subthreshold depolarizing current **(a)**. Determination of the resting membrane potential (RMP) (n=42) **(b)** AP amplitude (n=38) **(c)** AP half duration (n=38) **(d)** and AHP amplitude (n=38) **(e)**.

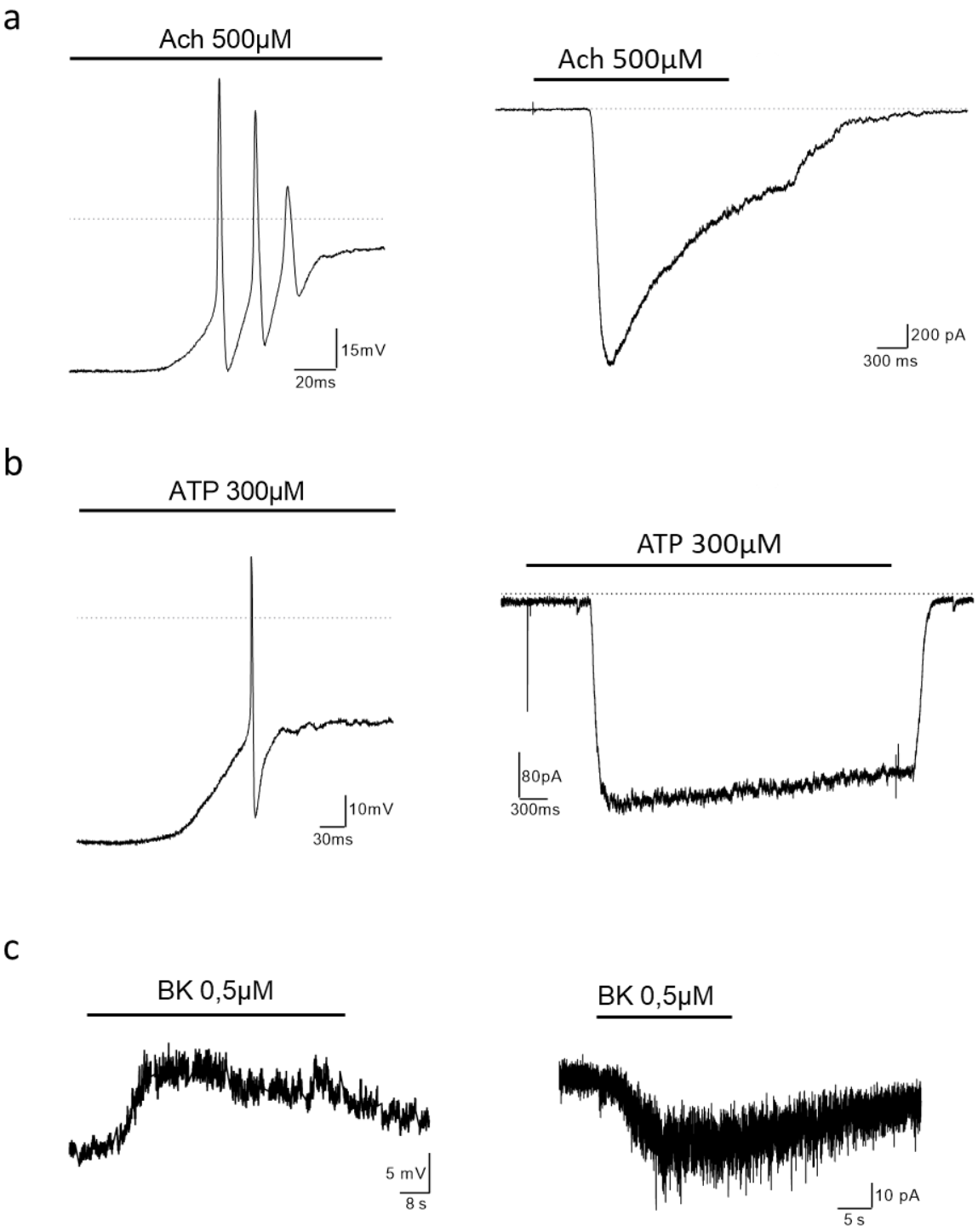

Figure 6. **Representative traces of** p**harmacological responses in mouse intracardiac neurons.** Membrane voltage (left) and current (right) responses to Ach (500µM)**(a)**, ATP (300µM)**(b)** and BK (0,5µM)**(c)**. For voltage clamp experiments HP was -60mV(**a** and **c**) and -50mV(**b**).

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