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# Molecular and functional characterization of the mouse intrinsic cardiac nervous system



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**BACKGROUND** The intrinsic cardiac nervous system (ICNS) refers to clusters of neurons, located within the heart, that participate in the neuronal regulation of cardiac functions and that are involved in the initiation of cardiac arrhythmias. Therefore, deciphering its role in cardiac physiology and pathophysiology is mandatory.

**OBJECTIVE** The aim of this study was to provide a phenotypic, electrophysiological, and pharmacological characterization of the mouse ICNS, which is still poorly characterized.

**METHODS** Global cardiac innervation and phenotypic diversity were investigated using immunohistochemistry on cleared murine hearts and on tissue sections. The patch clamp technique was used for the electrophysiological and pharmacological characterization of isolated mouse intracardiac neurons.

**RESULTS** We have identified the expression of 7 distinct neuronal markers within the mouse ICNS, thus proving the neurochemical diversity of this network. Of note, it was the first time that the

existence of neurons expressing the calcium-binding protein calbindin, neuropeptide Y, and cocaine and amphetamine regulated transcript peptide was described in the mouse. Electrophysiology studies also revealed the existence of 4 different neuronal populations on the basis of their electrical behavior. Finally, we showed that these neurons can be modulated by several neuromodulators.

**CONCLUSION** This study showed that the mouse ICNS presents a molecular and functional complexity similar to other species and is therefore a suitable model to decipher the role of individual neuronal subtypes regarding the modulation of cardiac function and the initiation of cardiac arrhythmias.

**KEYWORDS** Intrinsic cardiac ganglia; Autonomic nervous system; Heart; Cardiac innervation; Peripheral neuron electrophysiology

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## Introduction

The 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 increasing attention. Indeed, whereas intracardiac neurons were initially considered as simple parasympathetic postganglionic neurons, studies conducted over the past 30 years have suggested a more complex organization, including sensory, local regulatory, and motor neurons within intracardiac ganglia, finally leading to the concept of “little brain on the heart.”<sup>1</sup>

Phenotypic studies have been conducted in many species to identify several neuronal subpopulations. Besides cholinergic phenotype, the presence of catecholaminergic, glutama-

tergic, and nitergic phenotypes have been described in intracardiac neurons.<sup>2–10</sup> This neurochemical diversity has been further confirmed by the expression of several neuropeptides, such as neuropeptide Y (NPY), vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, somatostatin, or cocaine and amphetamine regulated transcript (CART) peptide.<sup>3,6,10–13</sup>

From a functional point of view, several studies have also identified various types of cardiac neurons on the basis of their membrane electrical properties.<sup>14–17</sup> In guinea pig, electrophysiological experiments combined with a morphological characterization of intracardiac neurons led to the identification of 3 distinct types of neurons, including a putative sensory one.<sup>16</sup> However, evidence that this neuronal diversity is associated with a functional specialization of neurons is still lacking. Such a characterization would bring essential information to assess the influence of the cardiac neuronal network on the cardiac physiology. This would be even more important since the ICNS has also been implicated in arrhythmias. For example, atrial fibrillation (AF) has been correlated with excessive activity of intracardiac neurons<sup>18</sup> and specific stimulation of cardiac ganglia has been able to trigger AF.<sup>19</sup> Moreover, it has

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been showed that ablation of ganglionated plexus reduces AF and is now one of the strategies used in therapy.<sup>20</sup> Deciphering modulation of cardiac function by the ICNS through the role of individual neuronal subtypes is therefore essential but has been for a long time hindered by technical limitations.

Recent advances in genetic engineering have opened new opportunities to improve our understanding of the ICNS. For example, cre-lox systems combined with fluorescent reporter, optogenetic, or Designer Receptors Exclusively Activated by Designer Drugs (DREADD) approaches represent powerful tools to precisely address the function of 1 particular subtype of intracardiac neurons. However, because these technologies are almost exclusively available in mouse models, a better understanding of the mouse ICNS is required.

To date, the few existing studies focused on the mouse model identified choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), and neuronal nitric oxide synthase (nNOS) within cardiac neurons.<sup>2,7</sup> However, the expression of other neuronal markers has not yet been investigated.

Moreover, little is known about the electrical and pharmacological properties of mouse cardiac neurons. To our knowledge, only 1 study investigated this aspect.<sup>21</sup>

This study was therefore designed 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 strengthened by the characterization of passive and active electrical membrane properties as well as by 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 sex 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.<sup>22</sup> Images were acquired with the ALICE's custom-built mesoSPIM microscope at Wyss Center, Geneva, Switzerland (see the Online [Supplement](#)).

### Immunohistochemistry

Immunohistochemistry was performed on 40–50  $\mu\text{m}$  heart sections and acquired using a confocal laser scanning microscope (FV3000 Olympus, Tokyo, Japan) (see the Online [Supplement](#)).

## Neuron dissociation

Ganglia were enzymatically digested with 2 mL of Hanks' Balanced Salt solution containing 3 mg/mL of collagenase type II (Worthington, Lakewood, NJ), 7.5 mg/mL of dispase II (Sigma-Aldrich, Lyon, France), and 0.25 mg/mL of deoxyribonuclease I (Sigma-Aldrich, Lyon, France; 30 minutes at 37°C). This was followed by incubation in 2 mL of trypsin-ethylenediaminetetraacetic acid 0.25% supplemented with 0.25 mg/mL of deoxyribonuclease I (35 minutes at 37°C). Finally, cells were gently triturated with fire-polished Pasteur pipettes coated with fetal bovine serum and plated on laminin-coated 35 mm Petri dishes.

## Electrophysiology

The electrical membrane properties of isolated cardiac neurons were determined using the whole-cell configuration of the patch clamp technique. Recordings were done at room temperature within 30 hours of isolation. Data acquisition and analysis were performed using pCLAMP software (Molecular Devices, San Jose, CA).

## Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are presented as mean  $\pm$  SEM. The Mann-Whitney test was used for statistical comparison. Statistical significance was accepted when  $P < .05$ .

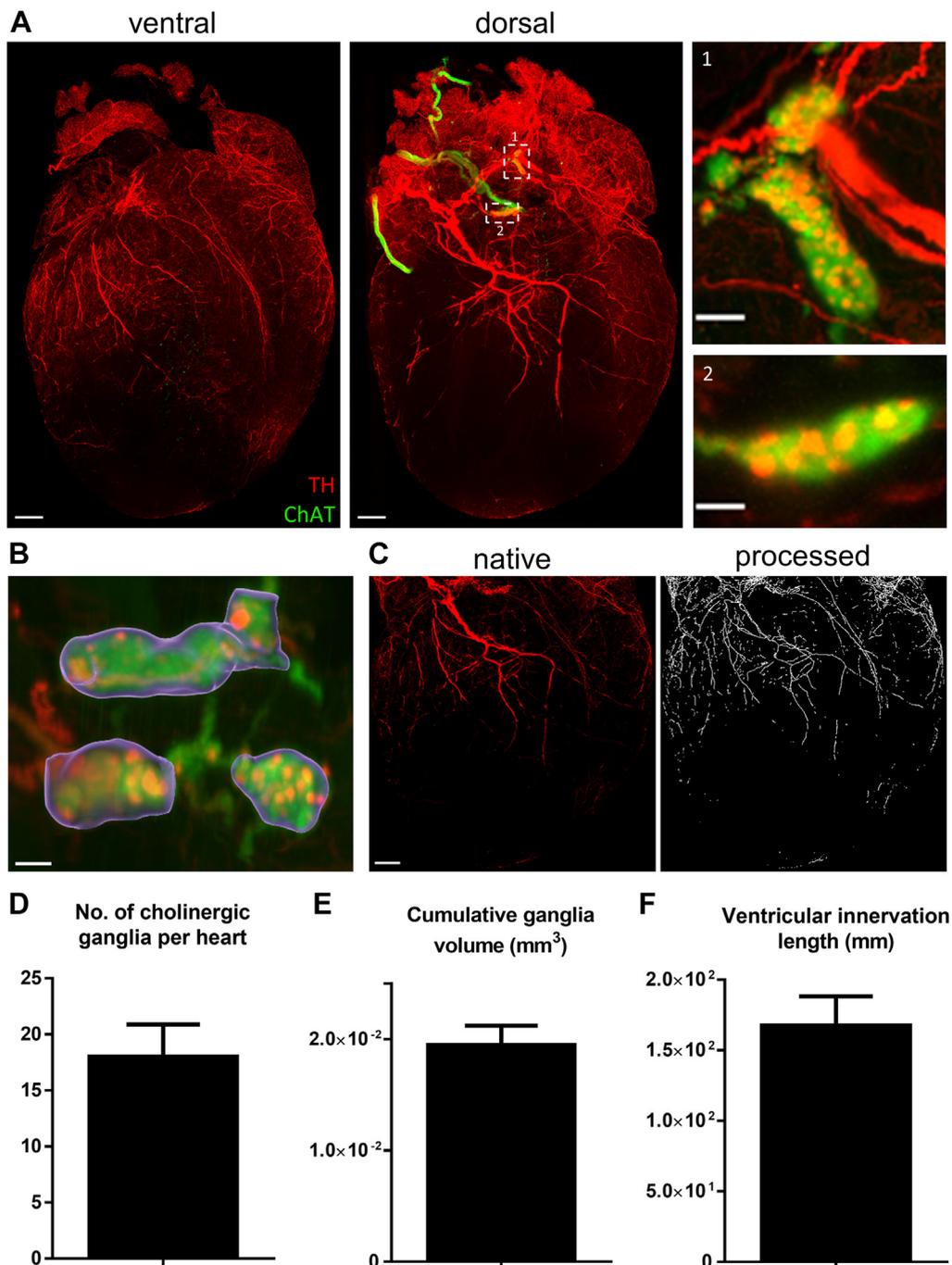
## Results

### Cholinergic and catecholaminergic innervation of the mouse heart

The neural control of the heart involves a combination of peripheral and intrinsic neural structures.<sup>1</sup> In order to investigate global cardiac innervation in the mouse, we performed detection of cholinergic (ChAT-IR) and catecholaminergic immunoreactivity (TH-IR) structures on cleared murine hearts.

This approach allowed us to appreciate the network of TH-IR nerves innervating mouse hearts (Online [Supplemental Movie 1](#)). Large bundles of nerves accessed the heart through the heart hilum, at the base of the heart, and extended toward the dorsal and ventral sides of both ventricles ([Figure 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 (Online [Supplemental Movie 1](#)).

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 was  $18 \pm 3$ , ranging from 13 to 23 ( $n = 3$ ) ([Figure 1D](#)). Most of them were characterized by TH-IR puncta, allowing us to see individual neurons ([Figure 1A](#), insets 1 and 2). Unlike TH, ChAT-IR fibers were almost exclusively located around ganglionated plexus, where they interconnected multiple ganglia.



**Figure 1** Cholinergic and catecholaminergic cardiac innervation. **A**: Three-dimensional (3D) projections of the ventral (2500  $\mu\text{m}$  z-stack) and dorsal (2440  $\mu\text{m}$  z-stack) sides of a cleared heart with tyrosine hydroxylase (TH; red) and cocaine and amphetamine regulated transcript (ChAT; green) staining. Magnification of ganglia is presented in insets 1 and 2. **B** and **C**: 3D image processing used for the quantification of ganglion volume (purple) and ventricular innervation (white). **D–F**: Assessment of the total number of ganglia per heart (panel D), the cumulative ganglion volume (panel E), and the total length of adrenergic fibers innervating both ventricles (panel F). Scale bars are 500  $\mu\text{m}$  (panels A [ventral and dorsal] and C) and 100  $\mu\text{m}$  (panels A [insets 1 and 2] and B).

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  $(1.95 \pm 0.17) \times 10^{-2} \text{ mm}^3$  ( $n = 3$ ) per heart (Figures 1B and 1E). The total length of TH-IR nerve fibers innervating both ventricles was quantified using ImageJ software.

The total length was found to be  $(1.68 \pm 0.21) \times 10^2 \text{ mm}$  ( $n = 3$ ) (Figures 1C and 1F).

### Neurochemical phenotype of mouse intracardiac neurons

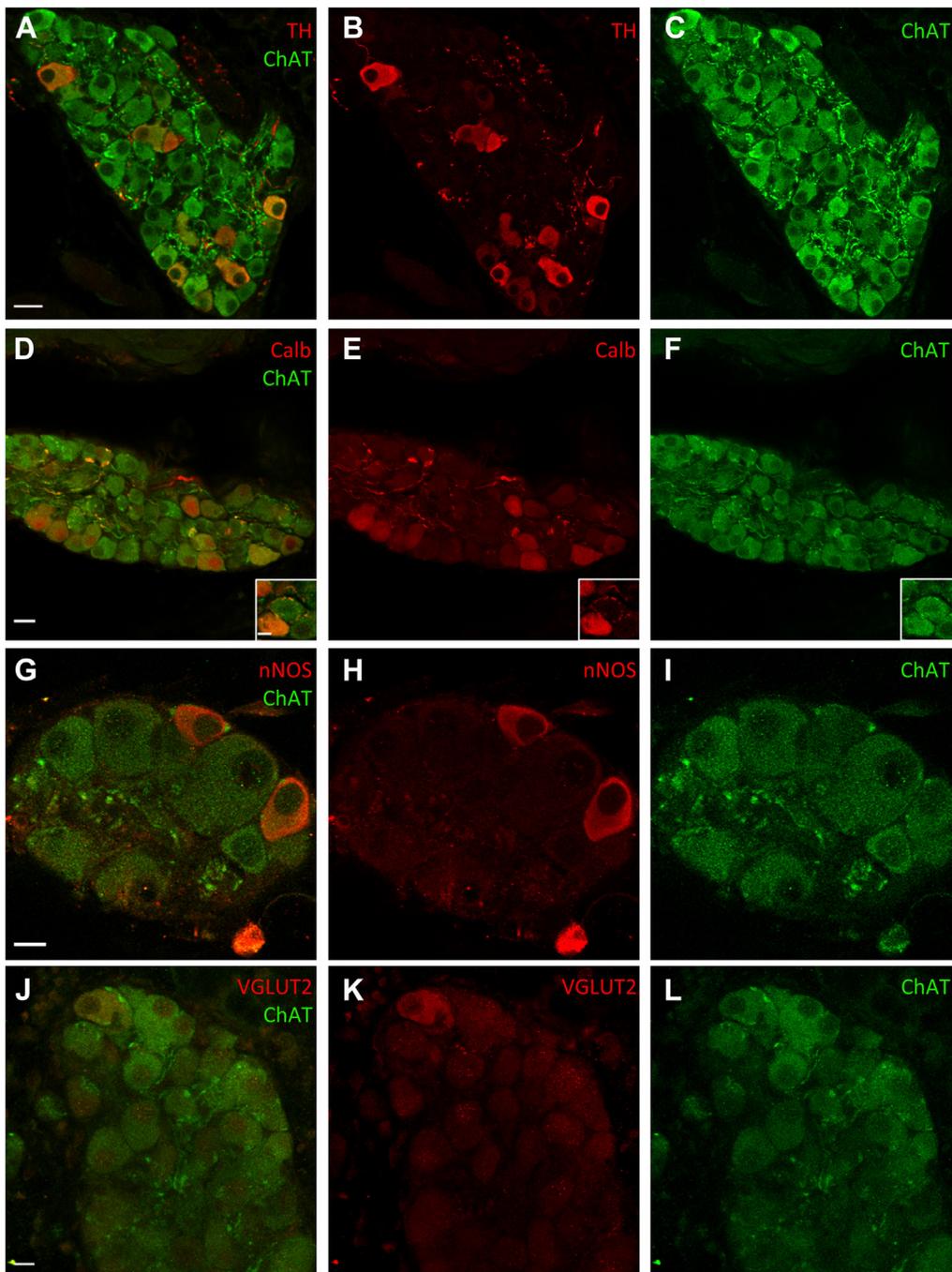
Immunodetection of the cholinergic marker ChAT confirmed the prominent cholinergic phenotype of intrinsic cardiac

neurons (98.7%). This marker also labeled intra- and interganglionic nerve fibers, as well as varicose terminals surrounding neurons. Immunoreactivity for TH was detected in ~30% of cardiac neurons and in numerous nerve fibers (Figures 2A–2C).

Apart from the widely established cholinergic and catecholaminergic markers, we observed that a large proportion of neurons (46%) expressed the calcium-binding protein calbindin D-28k (calbindin). In neuronal somata, calbindin-IR was

always observed in the cytoplasm and often accompanied by a strong nucleus staining (Figures 2D–2F). Calbindin-IR was also present in multiple intra- and interganglionic nerve fibers and occasionally in pericellular baskets surrounding neuronal somata (Figure 2D, inset). Calbindin neurons were always costained by ChAT, and 11.8% of cardiac neurons showed immunoreactivity for calbindin, ChAT, and TH (Table 1).

A small population of neurons stained with nNOS, the neuronal enzyme responsible for the synthesis of NO, were



**Figure 2** Phenotypic properties of intracardiac neurons. Confocal images of sections of cardiac ganglia immunostained with tyrosine hydroxylase (TH) (A–C), calbindin (D–F), neuronal nitric oxide synthase (nNOS) (G–I), vesicular glutamate transporter 2 (VGLUT2) (J–L), and cocaine and amphetamine regulated transcript (ChAT). *Insets* in panels D–F show typical calbindin-immunoreactivity (Calb) pericellular baskets surrounding neuronal somata. Scale bars are 10  $\mu\text{m}$  (inset in panel D and panels G–I and J–L) and 20  $\mu\text{m}$  (all other panels).

also present in cardiac ganglia. These neurons were not present in all ganglia and accounted for only 2% of total neurons (Figures 2G–2I). Even though most nNOS-IR somata coexpressed ChAT, not all of them did.

On rare occasions, we observed immunoreactivity for vesicular glutamate transporter 2 (VGLUT2) (Figures 2J–2L). However, no soma was stained by the other vesicular glutamate transporter VGLUT1. Moreover, despite the use of different markers such as glutamate decarboxylase 67, gamma aminobutyric acid and tryptophan hydroxylase 2, we did not observe any GABAergic or serotonergic phenotype.

Intracardiac neurons have been shown to express several neuropeptides in various species, especially in rodents.<sup>6,11</sup> In mouse hearts, a large proportion of neuronal somata were immunoreactive for NPY (67%) and CART peptide (61%). However, antibody cross-reactions prevented us from quantifying the number of neurons coexpressing both peptides. In somata, both stainings were granular with a perinuclear localization, suggesting a localization in the endoplasmic reticulum and the Golgi apparatus (Figure 3). Numerous intra- and interganglionic nerve fibers were also labeled by these 2 peptides.

Calcitonin gene-related peptide-IR and substance P (SP)-IR, 2 peptides distinctive of sensory neurons, were also observed in nerve fibers, but never in neuronal somata (Figures 3A–3F). Both peptides were frequently colocalized, although SP-IR nerve fibers were far less numerous. IR for somatostatin and vasoactive intestinal peptide was also tested. However, we did not observe 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 extensions emerging from it (Figure 4A). Most neurons were multipolar, even if some uni- and bipolar neurons were also observed.

After 12–30 hours of culture, passive and active electrical membrane properties of isolated mouse cardiac neurons were assessed using the patch clamp technique in the current clamp mode.

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

Neurochemical phenotype	Percentage (number of profiles)	Multiple phenotype (percentage 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)	

Calb = calbindin; CART = cocaine and amphetamine regulated transcript; ChAT = choline acetyltransferase; nNOS = neuronal nitric oxide synthase; NPY = neuropeptide Y; TH = tyrosine hydroxylase.

Under our experimental conditions, no spontaneous firing activity was observed. An investigation of discharge characteristics revealed 2 different profiles: 77.4% of neurons showed limited firing activity (phasic neurons) characterized by a maximum of 1–3 action potentials (APs) at the onset of the stimulus, while 22.6% of neurons exhibited adapting firing behavior (Figures 4B and 4C). Adapting neurons also showed a lower rheobase ( $40.0 \pm 2.3$  pA;  $n = 14$ ) than did phasic neurons ( $64.0 \pm 3.3$  pA;  $n = 48$ ) ( $P < .0001$ ) (Figure 4D). In addition, adapting neurons displayed shorter afterhyperpolarization (AHP;  $67.0 \pm 5.2$  ms [adapting] vs  $132.8 \pm 17.0$  ms [phasic];  $P < .01$ ) (Figure 4E).

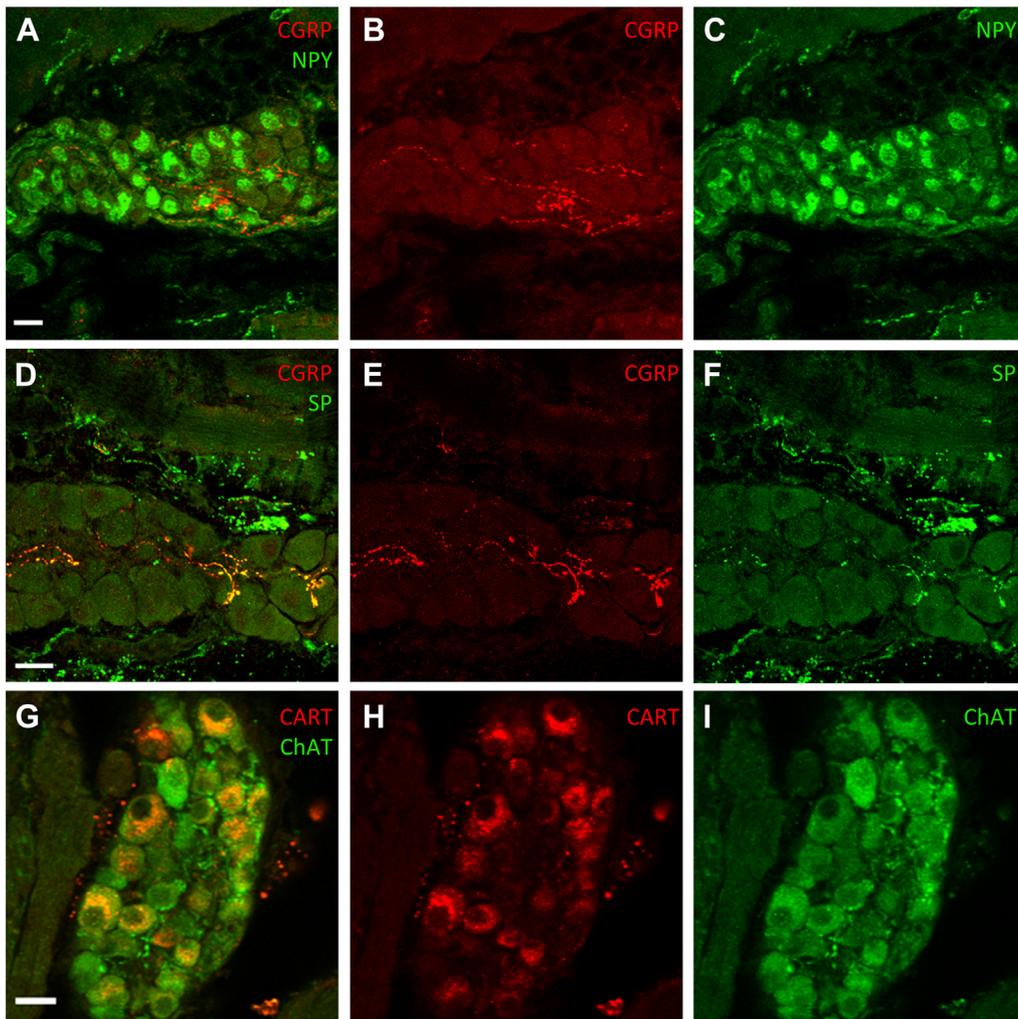
Upon brief injection of suprathreshold depolarizing current, intracardiac neurons were divided into 2 additional groups according to the presence or absence of AHP. Fifty-five percent of APs (30 of 55) were followed by AHP, while other neurons did not exhibit any (Figure 5). Neurons with AHPs were characterized by a more depolarized resting membrane potential ( $-57.4 \pm 0.9$  mV [AHP] vs  $-62.7 \pm 1.1$  mV [no AHP];  $P < .001$ ) (Figure 5B), a smaller AP amplitude ( $120.8 \pm 2.8$  mV [AHP] vs  $137.5 \pm 2.1$  mV [no AHP];  $P < .0001$ ) (Figure 5C), and a smaller maximum rate of depolarization ( $147.9 \pm 7.8$  V/s [AHP] vs  $189.2 \pm 5.6$  V/s [no AHP];  $P < .001$ ) (Figure 5D). It is noteworthy that these 2 newly defined types of neurons were not correlated to phasic or adapting patterns (Fisher exact test,  $P > .05$ ). The electrophysiological properties of mouse intracardiac neurons are summarized in Online Supplemental Table 1.

## Pharmacological response of mouse intracardiac neurons

Since the ICNS depends on several modulators,<sup>23</sup> we investigated the pharmacological response of isolated mouse cardiac neurons to known peripheral neuromodulators.

All tested neurons ( $n = 28$ ) exhibited membrane voltage and current responses to acetylcholine (ACh) perfusion, and the resulting inward current was sufficient to trigger AP (Figure 6A). The superfusion of ACh resulted in the development of a large inward current, whose amplitude decreased before the end of the drug application, probably owing to channel desensitization. The peak current density evoked by ACh was similar in phasic and adapting neurons but was significantly smaller in neurons displaying AHP ( $P < .01$ ) (Figure 6B).

The expression of several purinergic receptors has been described in rat cardiac neurons,<sup>24</sup> and adenosine triphosphate (ATP) and other purinergic compounds have been reported to modulate intracardiac neurons.<sup>23</sup> In our study, the exogenous application of ATP was accompanied by a strong membrane depolarization in all tested neurons ( $n = 17$ ), resulting most of the time in AP firing (Figure 6C). In voltage clamp, ATP induced a rapidly activating and sustained inward current, distinctive of purinergic receptor activation. There was no statistical difference between phasic and adapting neurons, while AHP neurons were characterized by a smaller current amplitude as compared with neurons without AHP ( $P < .05$ ) (Figure 6D).



**Figure 3** Peptide expression in mouse intracardiac neurons. Confocal images of sections of cardiac ganglia immunostained with neuropeptide Y (NPY) and calcitonin gene-related peptide-immunoreactivity (CGRP) (A–C), substance P (SP) and CGRP (D–F), and cocaine and amphetamine regulated transcript (CART) (D–F). Scale bars are 20  $\mu\text{m}$ .

In our study, perfusion of bradykinin (BK) was able to slowly depolarize mouse cardiac neurons without eliciting AP (0.2  $\mu\text{M}$ :  $8.4 \pm 1.1$  mV [ $n = 7$ ]; 0.5  $\mu\text{M}$ :  $13.0 \pm 2.4$  mV [ $n = 8$ ]). This was confirmed by the observation of a slowly activating inward current of small amplitude after BK exposure in voltage clamp experiments (Figure 6F).

## Discussion

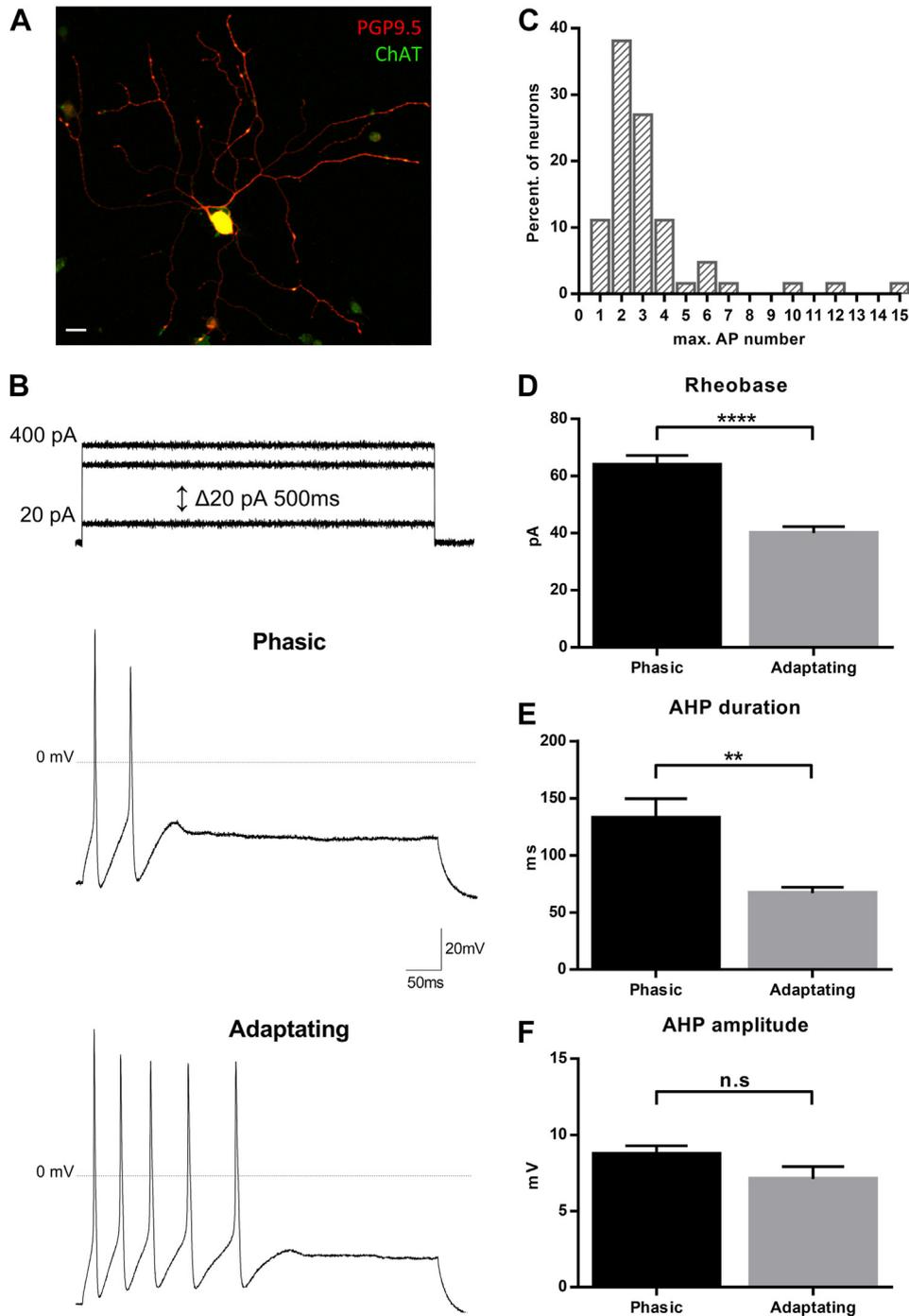
This study presents for the first time a detailed report of anatomic, phenotypic, electrophysiological, and pharmacological properties of the mouse ICNS.

### Cholinergic and catecholaminergic innervation of the 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 essentially report superficial information and suffer

from limitations because of sample damaging. The recent development of optical clearing techniques combined with advances in 3-dimensional imaging of a large-scale specimen helped overcome these limitations, opening new opportunities to study global cardiac innervation.

The iDISCO clearing method allowed us to visualize and quantify in 3 dimensions the sympathetic and parasympathetic innervation of the mouse heart, thus enabling the determination of the exact location and number of cardiac ganglia in the whole nonsectioned murine heart. Our results are in accordance with Rysevaite et al,<sup>25</sup> who reported a mean number of  $19 \pm 3$  ganglia. Since a 3-dimensional analysis of ganglia was missing in the literature, we quantified ganglion size using a volumetric approach and found that the cumulative ganglion volume is  $0.02 \text{ mm}^3$ . 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,<sup>26</sup> who developed a clearing-imaging-analysis pipeline to assess the diameter and orientation of

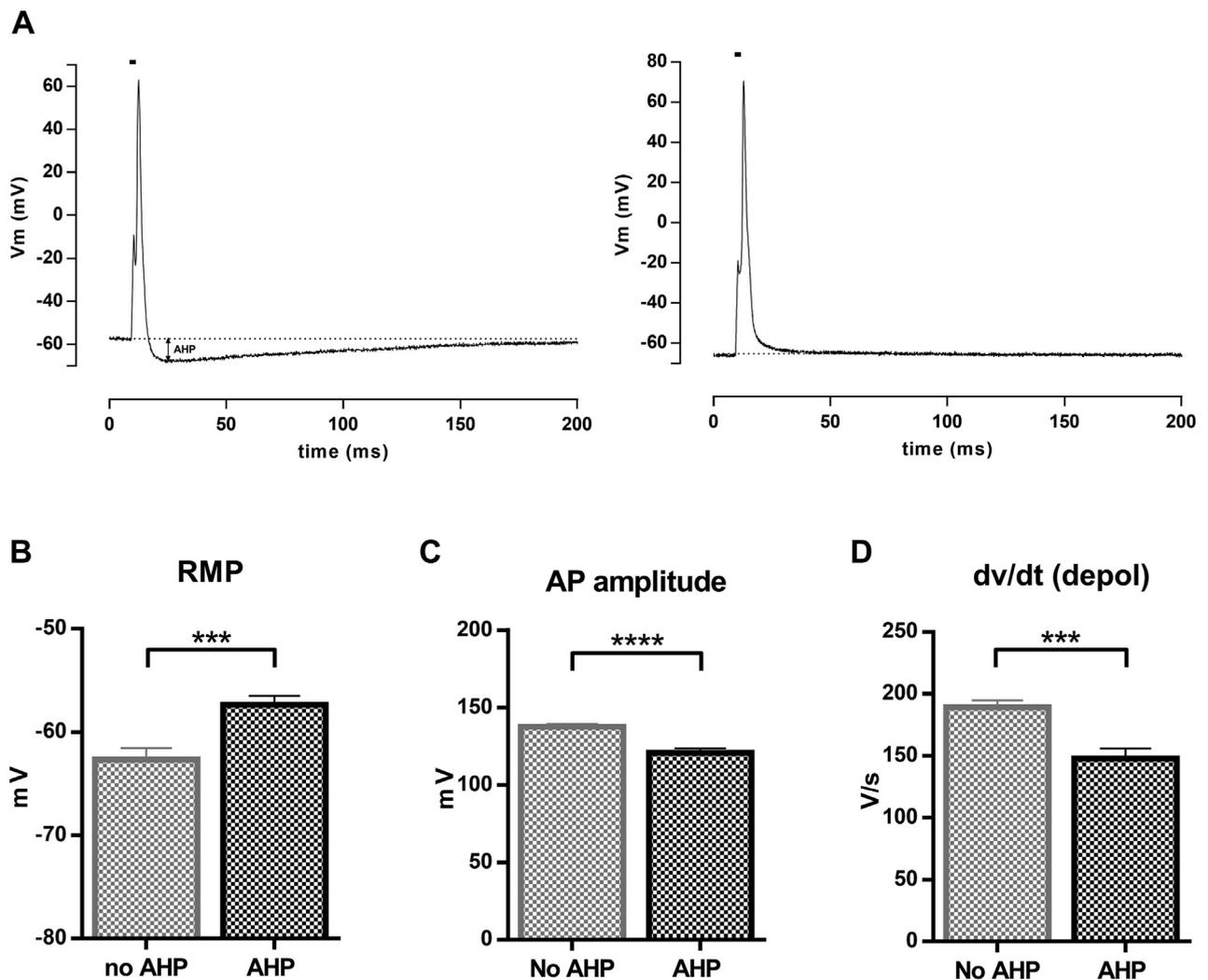


**Figure 4** Action potential (AP) discharge profiles in mouse dissociated intracardiac neurons. **A:** Mouse isolated cardiac neuron costained with the neuronal marker Protein gene product 9.5 and choline acetyltransferase (ChAT). **B:** Maximum discharge of AP observed in 2 distinct neurons in response to 500 ms depolarizing current injection from 20 to 400 pA ( $\Delta 20$  pA). The upper trace presents AP obtained in a phasic neuron, whereas the lower trace was obtained in an adapting one. **C:** Distribution of the maximum numbers of AP recorded with the stimulation protocol presented in panel A. **D:** Assessment of the rheobase of phasic ( $n = 48$ ) vs adapting ( $n = 14$ ) neurons. **E and F:** Assessment of the duration and amplitude of afterhyperpolarization (AHP) in phasic ( $n = 21$ ) vs adapting ( $n = 7$ ) neurons with the stimulation protocol used in Figure 5.  $**P < .01$ ,  $****P < .0001$ . n.s. = not significant ( $P = .14$ ).

nerve fibers innervating the mouse heart. This anatomical description of neuronal cardiac circuits will bring useful information to better understand the autonomic cardiac innervation and to identify abnormal cardiac innervation in pathological states, as already described in myocardial infarction or in cardiac autonomic neuropathy.<sup>27,28</sup>

### Neurochemical phenotype of mouse intracardiac neurons

So far, little was known about the neurochemical diversity of intracardiac neurons in mouse, with only 3 different neuronal markers (ChAT, TH, and nNOS) identified.<sup>2,7,8</sup> Our results confirm that almost all intracardiac neurons express the



**Figure 5** Action potential (AP) properties in intracardiac neurons with or without afterhyperpolarization (AHP). AP with (*left*) or without (*right*) AHP recorded in response to brief injection (2 ms; *black line*) of suprathreshold depolarizing current (A). Assessment of resting membrane potential (RMP) (B), AP amplitude (C), and maximum rate of depolarization ( $dv/dt$  (depol)) (D). \*\*\* $P < .001$ , \*\*\*\* $P < .0001$ ; no AHP:  $n = 25$ ; AHP:  $n = 28$ .

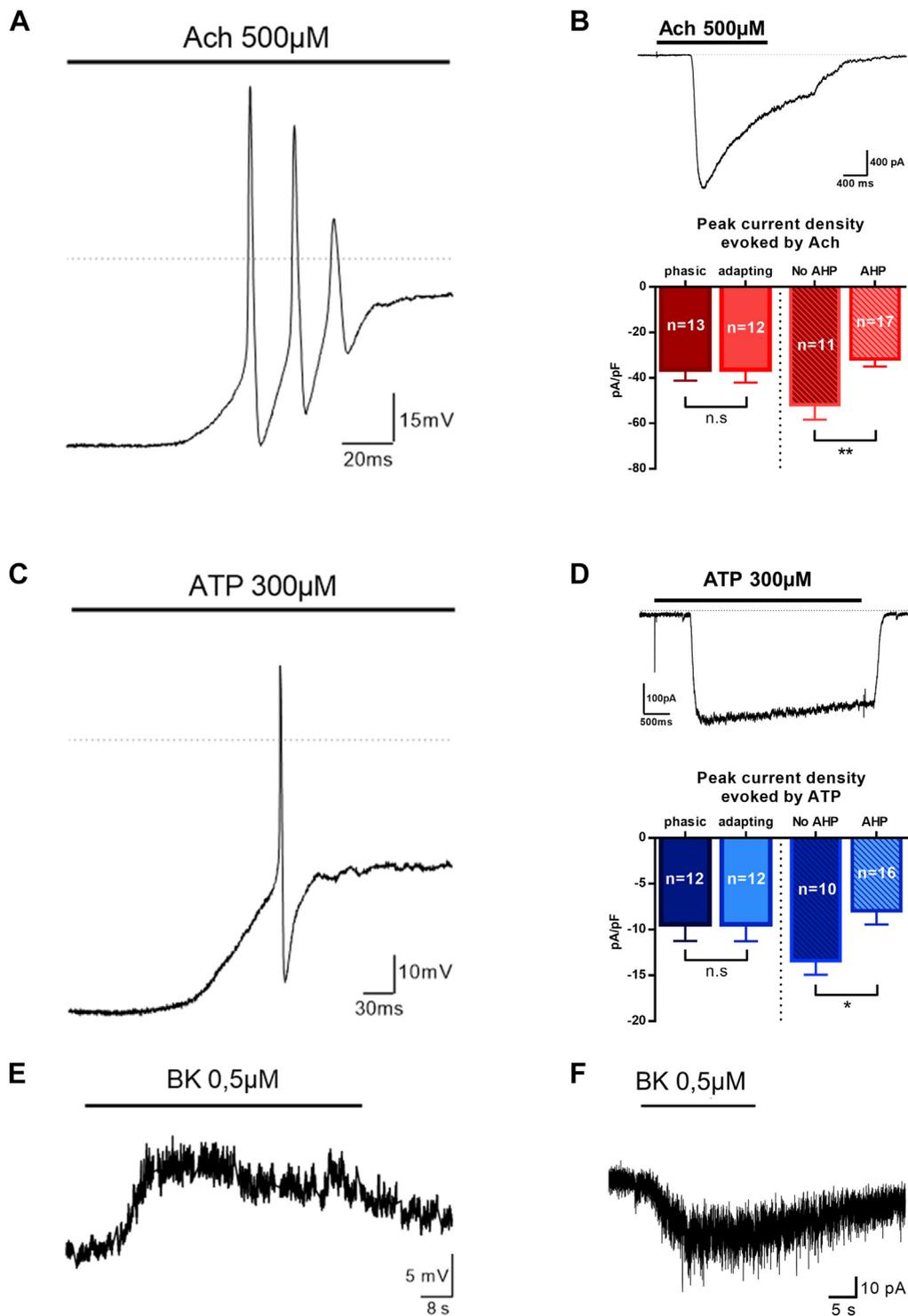
cholinergic marker ChAT and that  $\sim 30\%$  of them coexpress ChAT and TH.<sup>7,8</sup> We also identified a small population of nitrergic neurons, which is consistent with previous investigations that have reported such a phenotype in rat, guinea pig, rabbit, and human.<sup>3–5</sup>

For the first time, we described the expression of 4 additional markers within the somata of mouse intracardiac neurons, thus showing that this species is characterized by a relative phenotypic diversity, as reported in others mammals.<sup>3,5,10</sup> 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 expressed the calcium-binding protein calbindin. To date, only 1 study had identified such neurons in rat hearts, where they represent only 7% of total neurons.<sup>3</sup> The expression of this protein has been reported in central and peripheral neurons and is often used as a marker to discriminate different functional subpopulations of neurons, such as sensory neurons.<sup>29</sup> We also

observed a lot of intra- and interganglionic fibers, as well as terminals surrounding cell bodies that are immunoreactive for calbindin, suggesting that calbindin-expressing neurons may be crucial components of local circuits. Whether the expression of this protein is associated with any specialized function still remains to be determined.

Recently, glutamatergic neurons immunoreactive for VGLUT1, VGLUT2, and glutaminase, the synthetic enzyme for glutamate, have been found within the ICNS of rat.<sup>9</sup> In our study, we occasionally observed neurons immunoreactive for VGLUT2, yet without detecting any VGLUT1 immunoreactivity. Therefore, we suggest that in the mouse, glutamatergic intracardiac neurons may exist but do not represent a significant neuronal population.

The expression of a variety of neuropeptides also accounts for the neurochemical diversity of intracardiac neurons, especially in rodents.<sup>6,11</sup> However, apart from the description of sensory fibers immunoreactive for calcitonin gene-related peptide-IR and SP, the expression of other



**Figure 6** Pharmacological responses of mouse intracardiac neurons. Effects of 500  $\mu$ M acetylcholine (Ach), 300  $\mu$ M adenosine triphosphate (ATP) or 0.5  $\mu$ M bradykinin (BK) on membrane potential (A, C and E respectively) and current (B, D and F respectively). The peak current densities of Ach and ATP evoked currents are compared between the different neuronal populations (respectively in B and D). \* $P < .05$ , \*\* $P < .01$ . n.s. = not significant ( $P = .97$  in B and  $.87$  in D).

neuropeptides had not yet been investigated in the mouse.<sup>7</sup> Here, we highlighted the expression of 2 neuropeptides, NPY and CART, within mouse intracardiac neurons. The expression of NPY within these neurons is not surprising, since it is known to be widely distributed in the autonomic

nervous system and its cardiovascular effects have been well documented.<sup>30</sup>

CART peptide expression concerns only a small number of cardiac neurons in guinea pig while it was observed in 46% of neurons in rat.<sup>11,12</sup> This proportion is even higher in the

mouse, with 61% of neurons showing immunoreactivity for CART. This peptide has been extensively studied in the enteric nervous system, where it is expressed in many neurons, but experimental evidence elucidating its biological function is still lacking.<sup>31</sup> Further studies will be necessary to clarify its function within the cardiac context.

### Electrophysiological and pharmacological properties of mouse intracardiac neurons

The complex organization of the ICNS was further supported by the examination of its electrophysiological properties. Indeed, on the basis of their electrical behavior, different subtypes of neurons have been identified within mammals, demonstrating that intracardiac neurons are forming a heterogeneous population.<sup>14,16,17</sup> Here, we report the first detailed investigation of passive and active electrical properties of mouse intracardiac neurons. By studying their firing activity, we identified 2 distinct neuronal populations as observed previously.<sup>15,17,21</sup> While most neurons were classified as phasic because of limited firing activity, a small proportion of neurons were able to discharge more APs (adapting neurons). Phasic neurons have a significantly higher rheobase as well as a higher AHP duration than do adapting neurons, which confirms the existence of 2 types of neurons displaying different excitability features. Little is known about the different ionic channels expressed by intracardiac neurons, especially in the mouse. Thus, it would be interesting to closely investigate the molecular determinants of these 2 types of electrical behaviors.

It is important to emphasize that our results were not obtained from freshly isolated cardiac neurons. Hence, they may have been affected by cell culture conditions. However, it is worth mentioning that our results are consistent with those previously obtained *in situ*.<sup>32</sup> Another limitation is that our experiments were carried out at room temperature; thus, it would be interesting to assess these electrophysiological properties at a more physiological temperature.

A significant number of studies have revealed the diversity of neuromodulatory sources of intracardiac neurons, which further suggests that they could play the role of integrative centers. In dogs, neuronal activity is known to be regulated by mechanical and chemical stimuli.<sup>27</sup> Similarly, a variety of substances have been reported to modulate the excitability of intracardiac neurons in rat and guinea pig.<sup>13,23,33</sup> However, the pharmacological modulation of mouse intracardiac neurons has never been investigated. Here, we showed that mouse cardiac neurons respond to Ach, ATP, and BK, suggesting that they could be modulated by a variety of stimuli. In our experiments, BK induced a small inward current associated with 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.<sup>33</sup>

### Conclusion

Our study is the first detailed report providing the phenotypic, electrophysiological, and pharmacological characterization of mouse intracardiac neurons. Our results demonstrate that the mouse ICNS shares similar complexity to that of other species. ICNS complexity deserves consideration since there is growing evidence that the ICNS plays an essential role in cardiac modulations and in the initiation and maintenance of cardiac arrhythmias. To this purpose, the use of transgenic mouse models will allow the functional investigation of neuronal phenotypes by expressing a fluorescent reporter in specifically targeted neurons. Furthermore, the emergence of new genetic tools such as DREADDs and optogenetics opened new opportunities to control the activity of specific neurons within a global network. This represents a promising strategy to understand the role of targeted neurons in global cardiac modulation as described in the recent review of Scalco et al.<sup>34</sup> However, these transgenic technologies are essentially available in the mouse, where the ICNS had been poorly characterized yet. Hence, our study is paving the way for future investigations using a combination of cre-lox systems and DREADDs/optogenetic tools in order to decipher the functional organization of the ICNS as well as its implication in pathological states such as arrhythmias.

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### Appendix Supplementary data

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.hrthm.2022.04.012>.

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