SYMPATHETIC INNERVATION DIRECTS LOCALIZATION OF ANKYRIN G, NA_V AND KCNQ1 CHANNELS TO NEURO-CARDIAC JUNCTIONS.

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Abstract

The sympathetic nervous system plays a central role in the cardiovascular response to acute stress by increasing heart rate and contractility. Despite the importance of functional connections between sympathetic nerves and cardiac myocytes, very little is known about structural and functional organization of the neuro-cardiac synapses. Earlier, we have demonstrated that specialized signaling domains are organized in cardiac myocytes at sites of contact with sympathetic neurons [Shcherbakova O.G., 2007]. In the present study, we addressed the question if sympathetic innervation may affect localization of the cardiac ion channels. We have found that scaffold protein ankyrin G is localized at the postsynaptic sites in the innervated cardiac myocytes in co-culture with sympathetic neurons. Consistent with roles of ankyrin G in targeting Na_v channels to excitable domains in neurons and cardiac myocytes, we have observed an increased density of Na_v channels at the neuro-cardiac junctions. We have also found that KCNQ1 channels are enriched at that sites. The increased density of Na_v and KCNQ1 channels at the sites of sympathetic innervation is likely to enable a fine-tuning of particular currents by neuronal input.

Keywords

Sympathetic neurons; neuro-cardiac junction; Ankyrin G; Nav channels; KCNQ1 channels.

Abbreviations

SGN, sympathetic ganglion neurons; APD, action potential duration.

Introduction

Cardiac performance is regulated by neural inputs to the heart from the sympathetic and parasympathetic nervous systems [1]. According to the existing model, sympathetic neurons form *en passant* synapses with cardiac myocytes. We [2] and other authors [3] have demonstrated direct coupling between neurotransmitter releasing sites and cardiomyocytes membranes. We have shown that the myocyte membrane develops into specialized zones that surround contacting axons and contain accumulations of the scaffold proteins SAP97 and AKAP79/150 but are deficient in caveolin-3. The β 1 ARs are enriched within these zones, whereas β 2 ARs are excluded from them after stimulation of neuronal activity [2]. Therefore, it would be of interest to explore the molecular organization of the neuro-cardiac junctions.

Ankyrin polypeptides play critical roles in ion channel and transporter targeting in excitable cells. Ankyrin G is a key organizer of the axon initial segments (AIS) in neurons since it controls localization of membrane-associated proteins such as Kv and Nav channels as well as the cell adhesion molecules NF186 and NrCAM [4,5,6]. In cardiac myocytes ankyrin-G associates with Nav1.5, primary ion channel responsible for the upstroke of cardiac action potential. It has been demonstrated that the mutation in $Na_v1.5$ channel that abolishes binding of $Na_v1.5$ to ankyrin-G and prevents accumulation of $Na_v1.5$ at cell surface sites in ventricular cardiomyocytes, cause Brugada syndrome, which is a severe form of arrhythmia leading to sudden death [7].

Cardiac action potential is formed by the finely balanced activity of multiple ion channels and transporters. In human ventricular myocytes, KCNQ1-KCNE1 channels generate I_{Ks}, a slowly activating K^+ current that is important for timely myocyte repolarization. Mutations in two human I_{ks} channel subunits, hKCNQ1 and

hKCNE1, prolong action potential duration (APD) and cause inherited cardiac arrhythmias known as LQTS (long QT syndrome) [10,11]. The input of sympathetic nervous system, mediated by β-adrenergic receptor activation, increases the slow outward potassium ion current (I_{KS}) to accelerate repolarization, shorten the cardiac APD and increase cardiac contractility [8,9].

In the present communication, we addressed localization of ankyrin G, Nav and KCNQ1 channels at the sites of sympathetic innervation in cardiac myocytes.

Methods

Co-culture of sympathetic ganglion neurons (SGN) and neonatal mouse ventricular myocytes.

SGN were isolated from the cervical ganglia of newborn mouse pups by treating ganglia with collagenase type 1A-S (Sigma-Aldrich) and trypsin T XI (Sigma-Aldrich) followed by trituration. All procedures met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Stanford University. Neurons were plated on cover slips coated with laminin (Sigma-Aldrich) for immunocytometry as described previously [2]. Spontaneously beating neonatal cardiac myocytes were prepared from hearts of newborn mouse pups as described previously [12] and were added to already plated SGNs on the same day. After culturing for 24 h, co-cultures were treated with 1 μM cytosine arabinoside (Sigma-Aldrich) for 24 h to inhibit fibroblasts growth. Co-cultures were maintained in Leibovitz's L-15 medium supplemented with Nu serum (BD Biosciences), NGF (Invitrogen), and ITS liquid media supplement (Sigma-Aldrich). After cytosine arabinoside treatment, media were changed every 3 d as previously described.

Immunofluoresence microscopy.

Co-cultures were fixed by adding PBS (Mediatech, Inc.) containing 8% PFA directly to the culturing media to achieve a final PFA concentration of 4%. Cells were permeabilized with 1% BSA solution in PBS containing 0.2% Triton X-100. Cells were then stained with the desired antibody. The antibodies used were as follows: anti - tyrosine hydroxylase (mouse monoclonal; 1:800; Transduction Laboratories), anti-Ankyrin G and anti-KCNQ1 (1:800, rabbit polyclonal) were described earlier [13](Mohler et al.,2003); anti-pan Na_v (mouse monoclonal; 1:200; NeuroMab). The primary antibodies were detected with AlexaFluor594-conjugated goat anti–mouse IgG (1:1,000; Invitrogen) and AlexaFluor488 goat anti–rabbit IgG (1:1,000; Invitrogen). The slices for imaging were mounted with Vectashield mounting media (Vector Laboratories). The images were acquired at room temperature on an imaging microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) using a plan-Apochromat 63X 1.40 NA oil lens (Carl Zeiss MicroImaging, Inc.), a camera (RTE/CCD-1300-Y/HS; Roper Scientific), and IPLab software (BD Biosciences). Confocal images were acquired using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.) using Argon and He/Ne lasers and a plan-Apo 63X 1.4 NA or plan-Apo 100x 1.1 NA oil lenses, and images were analyzed by Volocity software (Improvision) and ImageJ. Immunostaining experiments using sets of six separate cover slips were repeated at least three times.

Results

Scaffold protein ankyrin G serves to target and stabilize membrane proteins in cardiomyocytes. It localizes at critical domains in cardiac myocytes, such as T-tubule membranes and intercalated disks, which are specialized sites of contact between cardiomyocytes that connect individual cardiac myocytes to work as a single functional organ or syncytium [14]. At the intercalated disc, gap junctions electrically couple adjacent cells, acting as low resistance pathways to propagate action potentials between cardiomyocytes. Based on the localization of ankyrin G to critical sites in cardiac myocytes, we proposed that ankyrin G might be localized at another important compartment – junctions of cardiac myocytes with sympathetic neurons. We have tested this in the co-cultures of cardiac myocytes and SGN by immunostaining with antibodies to ankyrin G and tyrosine hydroxylase. Indeed, we observed that ankyrin G is localized at the sites of contact of cardiac myocytes and SGN (Fig.1).

Ankyrin G is known to interact with Na_v channels and retain them at the excitable domains of membranes in neurons and in cardiac myocytes [4,5,6,15]. Consistent with this, we have observed an increased density of Na_v channels using pan-Na_v channel antibodies (Fig.2).

The excitable domains in neurons are organized by means of accumulation of Na_v channels responsible for initiation and propagation of action potential and K_v channels that shape the downstroke of the action potential [5,6]. In cardiac myocytes, repolarization is primarily achieved by I_{ks}, mediated by KCNQ1-KCNE1 and possibly other KCNQ1-KCNE combinations [10, 11]. It is well established that

input of sympathetic nervous system increases the slow outward potassium ion current (I_{KS}) carried by KCNQ1-KCNE1 channel [8,9]. Thus, we have tested localization of KCNQ1 channel at the neuro-cardiac junction. Indeed, we have observed an increased density of KCNQ1 channels at the sites of sympathetic innervation in cardiac myocytes (Fig.3).

Discussion.

Localization and targeting of the cardiac ion channels are critically important for efficient action potential initialization and propagation through myocardium. We have found ankyrin G as well as an increased density of Na_v channels at the sites of sympathetic innervation in cardiac myocytes. It was demonstrated that the condition ankyrin-G knockout mice display bradycardia and arrhythmia associated with catecholaminergic stress [15]. Taken together with our results, this suggests that local modulation of Na_v channels by sympathetic input is important for the proper transmission of the catecholaminergic signals to the heart.

KCNQ1 is a unique potassium channel that have the capacity to form either channels that are voltage-dependent and require membrane depolarization for activation, or constitutively active channels [16]. KCNQ1 interacts with all five members of the KCNE family of single transmembrane domain ancillary or beta subunits, also called MinK-related peptides, MiRPs [10,11,17,18]. The human KCNQ1-KCNE1 channel heteromer in cardiac myocytes is a part of a macromolecular complex formed by direct interaction with Yotiao, PKA, protein phosphatase1, phosphodiesterase 4D3 (PDE4D3), and adenylyl cyclase 9 [9, 19, 20]. Further studies are warrantied to address the localization and dynamics of other

proteins of this macromolecular complex.

It is worth noting that the localization of KCNQ1 and Na_v channels is not exclusive for the neuro-cardiac junctions: they are enriched at these sites, but they also expressed over the cardiac myocytes plasma membranes. This argues for compartmentalized regulation of these channels in the heart. For instance, it has been shown that there are two populations of K_v4 channels responsible for I_{to} current in cardiac myocytes, one localized in caveolae and the other localized in flat rafts. Interestingly, only the population with K_v4 channels located in caveolae can be regulated by α1-AR stimulation and its responsiveness of the K_v4 channels to catecholaminergic stimulation depends on scaffold protein AKAP100 [21].

Variations in channels density and surface expression are known to contribute to the modulations of particular currents [22]. Moreover, sympathetic neurons can differentially release multiple transmitters – epinephrine, nor-epinephrine, dopamine, NPY, substance P, ATP and UT in stimulus-dependent manner [23,24]. Therefore, increased density of ion channels at the sites of sympathetic innervation may provide means for fine-tuning of particular currents. Functional significance of localization of Na_v and KCNQ1 channels to the neuro-cardiac sympathetic junctions is yet to be determined.

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Figure legends.

Figure 1. Ankyrin G localizes to neuro-cardiac junctions.

A. Co-culture of cardiac myocytes and sympathetic neurons (DIV 5) immunostained with antibodies to Tyrosine Hydroxylase (red) and Ankyrin G (green). Confocal imaging, x100, single slice. B. Enlarged fragment of the image A, 3D reconstruction. C, D. Tyrosine Hydroxylase immunostaining (red channel of the images A and B correspondingly). E, F. Ankyrin G immunostaining (green channel of the images A and B correspondingly).

Figure 2. Na_v channels are enriched at the neuro-cardiac junctions.

A. Co-culture of cardiac myocytes and sympathetic neurons (DIV 5) immunostained with antibodies to Tyrosine Hydroxylase (green) and pan-Na_v (red). Epi-fluorescent image, x63. B. Tyrosine Hydroxylase immunostaining (green channel). C. Immunostaining for pan-Na_v antibodies (red channel).

Figure. 3. KCNQ1 channels are enriched at the neuro-cardiac junctions.

A. Co-culture of cardiac myocytes and sympathetic neurons (DIV 5) immunostained

with antibodies to Tyrosine Hydroxylase (red) and KCNQ1 (green). Confocal imaging, x63, single slice. B. Enlarged fragment of the image A, 3D reconstruction. C,D. Tyrosine Hydroxylase immunostaining (red channel of images A and B correspondingly). E,F. KCNQ1 immunostaining (green channel of images A and B correspondingly).

Fig.1: Ankyrin G localizes to neuro-cardiac junctions.

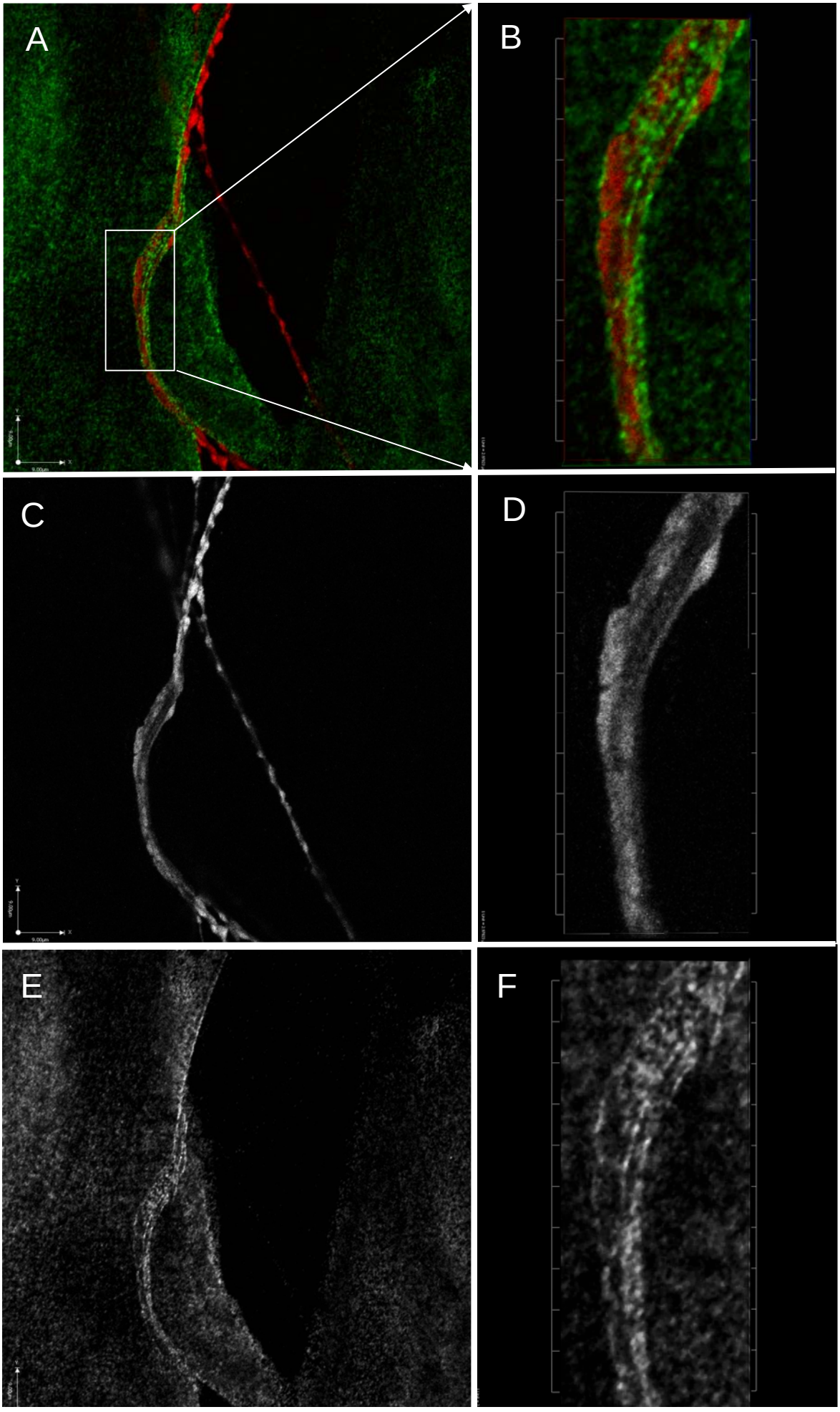
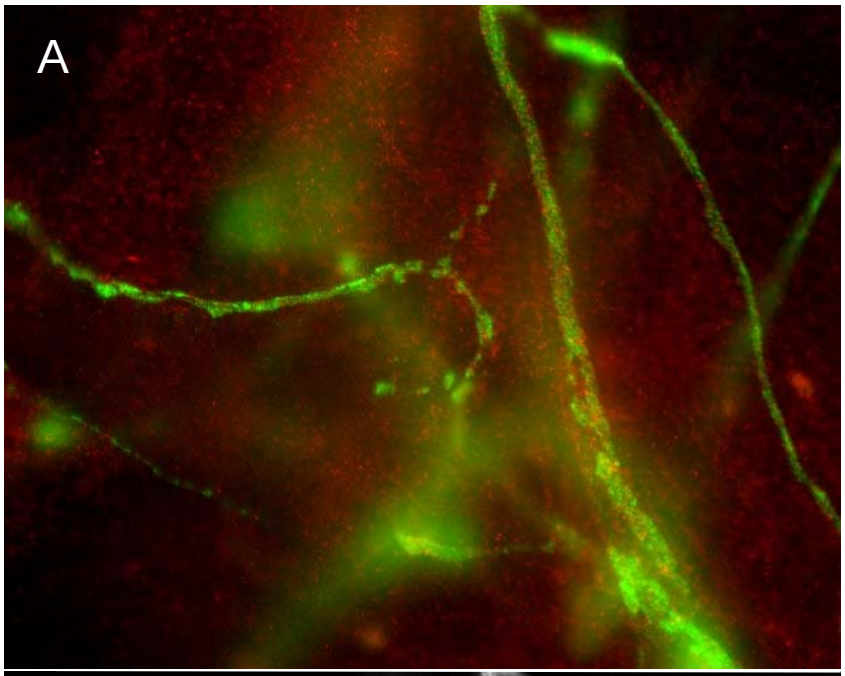
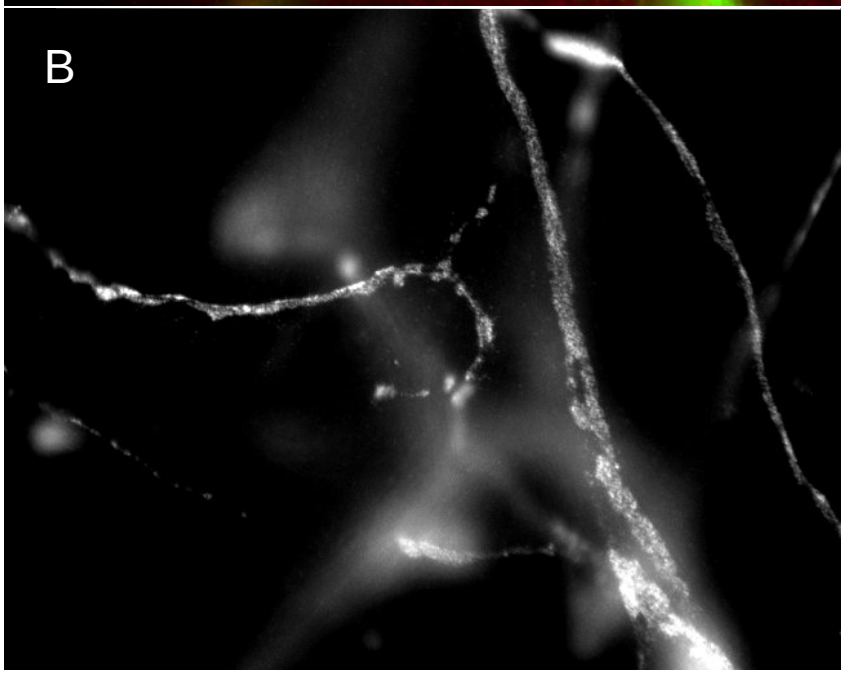
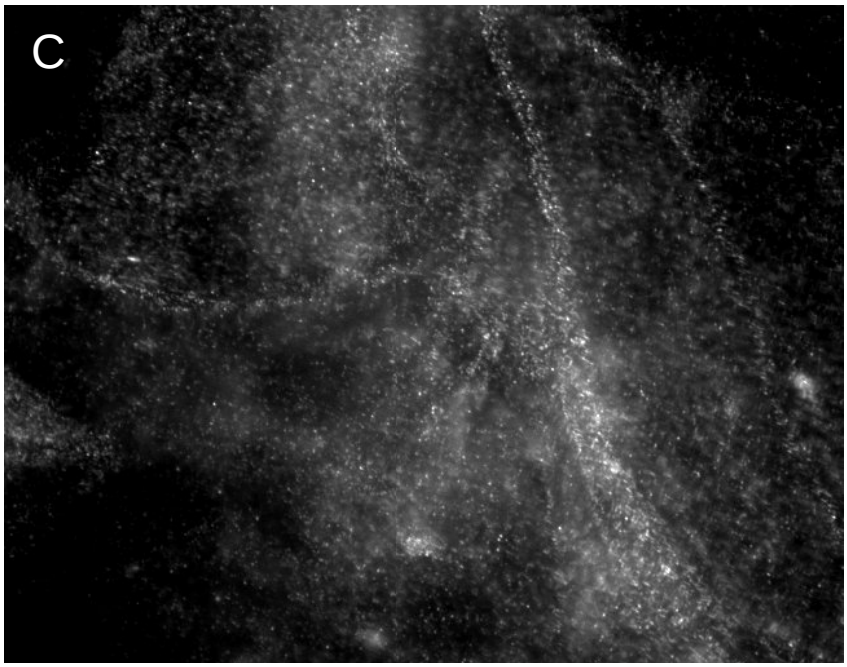


Fig.2: Na_V channels are enriched at the neuro-cardiac junctions.







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