Ulf Henrik Petersson

Immunohistochemical analysis of pig atrioventricular node morphology and its innervation

Final master’s thesis

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1. SUMMARY

Name of the author is Ulf Henrik Petersson and the title of this study was Immunohistochemical analysis of pig atroventricular node [AVN] morphology and its innervation.

The aim of this study was to investigate the pacemaker cells distribution and its innervation of the AVN. To obtain the aim of this study following objects were investigated: 1) area of AVN, 2) innervation of AVN, 3) neurochemical properties of structures within AVN and 4) the morphology of intracardiac neurons.

The AVN dissected from piglets that died during their first day of life, in total 4 piglets hearts were used for this study. The immunohistochemical markers were used to label pacemaker cells, working myocardium and neural cells. Hyperpolarization-activated cyclic nucleotide 4 [HCN4] was used to label pacemaker cells. Connexin [Cx43] was used to label working cardiomyocytes. Choline acetyltransferase [ChAT] was used to label cholinergic neurons. Tyrosine hydroxylase [TH] was used to label dopamine sensitive neurons. Nitric oxidase synthase [nNOS] was used to label nitrergic neurons. The immunohistochemical procedure was used to measure and identify the HCN4 positive cells in the intraatrial septum, innervation of the AVN, the variety of neurons in the AVN, size of the neuronal somata and the shape of the intracardial neurons.

The intracardial ganglia were found in all preparations, 2.5 ganglia per section of AVN, and contained 4.59 neurons per ganglia and those counted for 81.2% of neurons found in this study, the other 18.8% were solitary neurons. The AVN occupied 3.96±0.48 mm² intracardial septum. Two types of preparations were used for analysis of neurons neurochemical type and these were labeled for 1) ChAT+nNOS and 2) ChAT+TH. 6 phenotypic groups were observed 1) ChAT(+)/nNOS(-), 2) ChAT(+)/nNOS(+), 3) ChAT(-)/nNOS(+), 4) ChAT(+)/TH(+), 5) ChAT(+)/TH(-) and 6) ChAT(-)/TH(+). In the ChAT+nNOS group the 1) ChAT(+)/nNOS(-) was the most abundant group counting for 56.94±14.76%, 2) 4) ChAT(+)/nNOS(-) counting for 28.13±14.57% and the ChAT(-)/nNOS(+) counting for 14.93±9.16%. In the ChAT+TH group the most abundant was the 1) ChAT(+)/TH(+) counting for 52.86±4.10%, 2) ChAT(-)/TH(+) counting for 31.95±3.38% and the smallest being the 3) ChAT(+)/TH(-) counting for 15.19±2.65%. The phenotypic group that had the biggest neurons was the 1) ChAT(+)/nNOS(+) group ranging from 42.84±3.059µm, tightly followed by the 2) ChAT(-)/TH(+) with a mean size of 38.23±1.67µm, 3) ChAT(+)/TH(+) group with a mean size of 38.22±1.67µm, 4) ChAT(+)/TH(-) group with a mean size of 24.84±1.54µm, 5) ChAT(-)/nNOS(+) group with a mean size of 23.63±1.62µm, and 6) ChAT(+)/nNOS(-) group with a mean size of 16.71±1.55µm. The shape of the neurons were generally elongated and the nerves innervating the axons of the intracardiac neurons were found in almost all pictures, and had a mean diameter of 4.506±1.78µm.

Conclusively two parts of the AVN was seen 1) the loose part and the 2) compact part. The AVN was composed of nerves, neurons, ganglia and axons. All nerves found were ChAT(+)/TH(+) or
ChAT(+)\text{nNOS}(+) positive. The sizes of intracardiac neurons with different neurochemical phenotype varied greatly in size and in their variability through out the AVN.
2. ACKNOWLEDGEMENTS

I would like to thank Lekt Hermanas Inokaitis for his advice and encouragement throughout this final master thesis.

3. CONFLICTS OF INTERESTS

The author reports no conflicts of interests during this study.

4. CLEARANCE APPROVAL BY COMMITTEE OF BIOETHICS

All the procedures were performed in accordance with local and state guidelines for the care and use of laboratory animals (Permission Nr. LT-61-19-004).
5. ABBREVIATIONS

AChE- Acetylcholinesterase
AVN- Atrioventricular node
ChAT- Choline Acetyltransferase
Cx43- Connexin 43
HCN4- Hyperpolarization-activated cyclic nucleotide 4
nNOS- nitric oxide synthase
PBS- Phosphate buffer saline
SAN- Sinoatrial node
SIF- Small intensively fluorescent cells
TH- Tyroxine hydroxylase
6. INTRODUCTION

The immunohistochemistry techniques used enables us to quantify the expression of different types of intracardiac neurons through a reaction between an antibody and an antigen localized in the AVN. The importance of immunohistochemical protocols from a medical research approach can be applied to what animal cardiac valves (to be used in aortic valve replacements), to suit us humans better [1].

Previous studies of the sinoatrial and atrioventricular cells of mammal’s hearts have shown that there are different intracardiac neurons with different immunohistochemical properties, such as ChAT sensitive neurons, TH sensitive neurons and nNOS sensitive neurons. These different phenotypic groups of intracardiac neurons differ in size, numbers and how many of them are located inside the ganglia of the heart. However this kind of immunohistochemical reactions show us what neurotransmitters are more common to regulate the cardiac functions, such as cholinergic, nitrergic and dopaminergic neurotransmitters are having the most prominent function within the cardiac autonomic nervous system [2].

Immunohistochemical studies of the innervating nerves of the heart have shown that acetylcholinesterase [AChE] positive nerve fibres (parasympathetic nerve fibres) are more concentrated than TH positive nerve fibres (sympathetic) in the atriums and vice versa the TH positive fibres are more concentrated in ventricles comparing to the AChE positive nerve fibres [3-4].

However due to usage of HCN4 as a neurochemical marker in immunochemistry, it’s been detected that the AVN is much wider than anticipated before [5], because of the distribution of HCN4 positive pacemaker cells. Due to this we expect to find a wide variety of different neurochemical phenotypic intracardiac neurons, nerves, axons and being able to measure the size of the AVN and also to see more HCN4 positive cells than Cx43 in the cross-sections.

The overall aim of this study, is to evaluate the pacemaker cells and their innervation of the AVN.
7. AIMS AND OBJECTIVES

7.1 AIMS

The aim of the study was to determine the distribution of pacemaker cells and their innervation in pig AVN.

7.2 OBJECTIVES

1. To determine the area of the AVN.

2. Investigate the innervation of the AVN:
   a) Investigating axons of both solitary and clustered neurons
   b) Investigating the intracardiac nerves of the AVN

3. To investigate the neurochemical properties of structures within the AVN.

4. Determine the morphology of the intracardiac neurons:
   a) Size of neuronal somata
   b) Shape of intracardiac neurons
8. LITERATURE REVIEW

8.1 INNERVATION OF THE HEART

The cardiac function is regulated by the autonomic cardiac nervous system. The cardiac autonomic nervous system is divided into two parts: 1) extrinsic cardiac autonomic nervous system and 2) intrinsic cardiac autonomic nervous system. It’s also believed that those two different parts of the cardiac autonomic nervous system can work either by themself independently or by depending on each other, this statement have been proven by dividing the two different parts of the cardiac autonomic nervous system and the intrinsic cardiac autonomic nervous system was still able to provide for the heart to maintain its most crucial functions to work. However even today the question how the two different parts interacts with each other is not completely understood as the there is a very complicated situation over the which part is the highest in the hierarchy of controlling the function of the heart [6].

However as this very complicated hierarchy of nervous control of the heart is compromised of afferent, efferent and interneurons. A long this road of nervous control, the involved neurons are connected to each other with different so-called interneurons. As this hierarchy of the cardiac autonomic nervous system communicates with itself via the interconnecting neurons it ultimately controls the functions of the different parts of the heart [7].

![Schematics of the autonomous nervous system hierarchy controlling the heart in humans](image)

Fig 1. Schematics of the autonomous nervous system hierarchy controlling the heart in humans [8].
8.1.1 EXTRINSIC INNERVATION OF THE HEART

Researches have shown that the heart innervated by parasympathetic branches from the X cranial nerve, also known as cardiac branches. The cardiac branches are classified according to which structural part of the vagal nerve that they originate from. The cardiac branches are divided into three groups: 1) Superior cardiac branch, 2) Inferior cardiac branch and 3) Thoracic cardiac branch. The sympathetic trunk in the cervical region of new world monkeys are composed of two sympathetic ganglia, superior cervical ganglia and a middle cervical ganglia, however in humans and gibbons there is also a vertebral ganglion. The superior cervical ganglion was found in all humans, the middle cervical ganglion and the vertebral ganglion were not found in all of the cases. It has also been proven that a small portion of the population has an inferior cervical ganglion that does not fuse with the first thoracic ganglion, however most parts of the populations instead presented with stellate ganglion. The superior cervical ganglion is located posteriorly to the bifurcation of the common carotid artery and between the 1st and 3rd cervical vertebrae. The superior cervical ganglion communicate with the first cervical spinal nerve and second cervical spinal nerve in all cases, it doesn’t communicate with the third and fourth cervical spines in all cases. The middle cervical ganglion isn’t present in all population, but could still be regarded, as it is present in a majority of the population. It is located between the 3rd and 7th cervical vertebrae of sympathetic trunk and communicates with the first cervical spinal nerve in all population, it doesn’t however communicates with the rest of cervical spinal nerves in an subpopulation. The vertebral ganglia is located on the anterior surface of ventral artery between the joining of anterior and posterior roots of ansa subclavius, it differs from the other ganglia by the fact that it doesn’t communicate with the cervical spinal nerves on both sides. The stellate ganglion is located between 7th cervical vertebra and the 1st thoracic [3-4].

The sympathetic ganglion of the superior cervical ganglion is made up of two types of cells: 1) Small intensively fluorescent cells [SIF] cells and 2) sympathetic ganglion cells. All SIF cells are extremely sensitive to the TH marker and with further us of immunoreactions the SIF cells can be divided into three subtypes depending on their reactivity to different markers [9].

Cardiac sympathetic nerves are divided into 4 types: 1) superior cardiac nerve, 2) Middle cardiac nerve, 3) inferior cardiac nerve and 4) thoracic cardiac nerve. The superior cardiac nerve originates in most cases from the superior cervical ganglion, or in fewer cases between the sympathetic trunk between the superior and middle ganglia. The middle cardiac nerve originates from middle cervical ganglion in most cases, vertebral ganglion or the sympathetic trunk between the middle and inferior ganglia. The inferior cardiac nerve originates from the inferior cervical ganglion in most of cases or the stellate ganglion. The cardiac nerves (superior, middle and inferior) follow the great arteries (common carotid, subclavian and brachiocephalic artery). The thoracic cardiac nerve
originates from the thoracic ganglion. As it is divided into right and left thoracic, the pathways are
divided into one left pathway, one right pathway. The right thoracic cardiac nerve have two pathways:
1) Oblique course along intercostal vessels and enter into the right venous porta. 2) Oblique course
along the intercostal vessel until the 6th to 9th thoracic vertebra where it turns to form a connection with
the cardiac plexus. The left thoracic cardiac nerve enters into the left venous porta. The vertebral nerve
originates from inferior or stellate ganglia follow the carotid artery and enter the \textit{foramen
transversarium}. The autonomic cardiac nervous system can enter the heart through reflected portions
of the pericardium. Cardiac nerves that follow ascending aorta, pulmonary trunk and superior vena
cava are usually named the atrial group, and usually are thicker than its counterparts in the venous
group. They run along the coronary arteries and are distributed to the atrial part of the heart. The
venous part that runs along the pulmonary veins and inferior vena cava usually are thinner. They are
going on the pericardium and do not form any plexuses until they enter at the pericardial reflection [3-4].

However in humans who have similar structure of the cardiac autonomic nervous system, it’s
also divided into a 1) sympathetic nervous system and 2) parasympathetic nervous system that arises
from different ganglia. The sympathetic originates from the stellate ganglion and as in other mammals
the stellate ganglion give rise to parasympathetic nerves that travels on both the right and the left side
in different numbers. As the stellate ganglion gives rise to the parasympathetic nerves, the
parasympathetic nerves have two different origins 1) recurrent laryngeal nerve and 2) the thoracic part
of the X cranial nerve (\textit{nervus vagus}). It’s also proven that humans as other mammals have the
interconnecting neurons previously mentioned. It’s also mentioned that the human cardiac autonomic
nervous system have the same ganglia as monkeys, which further proves the hypothesis that there
similarities between different mammal species. As there are evidence that there are similarities
between different mammals, as mentioned before with similarities between humans and monkeys.

There are as previously mentioned also similarities between piglets, humans and monkeys as the
piglets also have the similar ganglia as the humans and the monkeys have the similar ganglia such as
1) stellate ganglion and 2) middle cervical ganglion. These statements prove that the cardiac nervous
structures are similar in most mammals. Studies made on human hearts have shown that the density of
ACHE positive intracardiac nerves increases as the human ages and will reach a peak in a certain point
of life and after that gradually decrease as aging continues [10-11; 12-13].

\section*{8.1.2 INTRINSIC INNERVATION OF THE HEART}

The nerves of the heart enter the hearts of pigs at 5 different locations. After the entrances into
the heart the nerves continues their pathways into the different parts of the heart, these pathways that
are composed of intrinsic cardiac nerves are also known as sub plexuses. The different sub plexuses supplies all parts of the heart but with a slight difference in which sub plexus supply which part of the heart [14].

However other researches have proven that there are up to 7 different sub plexuses instead of the previous data of only being 5 sub plexuses. The statement that there is seven sub plexuses instead of five is being further discussed and proven of the rabbit heart as well, and also as mentioned earlier that the number of neurons is affected by aging is confirmed to be in rabbits hearts as well. This can be the case in piglets as well, because the rabbits hearts are similar to most mammals in their anatomic build up [15-16].

Researches have shown that the intrinsic cardiac nerves can be immunoreactive to different types of neuromarkers, such as TH and ChAT. This is important as it tell us that the intracardiac nerves are reacts to different neurotransmitters. It’s also further hinted that the TH immunoreactive nerves are generally having a bigger diameter than the ChAT immunoreactive nerves. However it’s also implied that the number of nerves generally differs a lot in size depending on where they are located [2].

The autonomic cardiac nervous system have a higher concentration of PGP 9,5 which make it easy to differentiate the cardiac conduction system from the surrounding working myocardium that have much less density of PGP 9,5 reactivity. The AVN however is very sensitive to TH and AChE in calves and can be applied to piglets [17].

The statement have been proven when the same research have been done surrounding piglet hearts instead of calves, the research did prove that the AV node in piglets have very high density of TH sensitive fibres [18].

As it’s known that the porcine heart is used as a base when it comes to research of the human heart, however in human cardiac conductive system the most common sensitivity is to cholinergic acetyltransferase and nitric oxide synthase, while as mentioned above the most common in pigs are the TH [19].

There are many different cardiac neurotransmitters that are used in the mammals, but there are few that also are used in the fish that have come to be known as a very important neurotransmitter known as nitric oxide. This neurotransmitter is detectable both in fish and mammals [20].

Researches of the intrinsic cardiac innervation of mouse hearts have shown that the intrinsic cardiac ganglia vary greatly in size and distribution. It’s also proven that intrinsic cardiac ganglia containment of neuronal somata greatly differs from very small numbers to very high numbers [22-23].
8.2 HUMAN HEART COMPARING TO A PIGLETS HEART

The shape of a pig’s heart contra a human heart have a few differences such as that while the human heart have a trapezoid shaping and the pig heart is more valentine shaped on the x-ray. The ventricular tissues of a pig look like a cone that has the “top” part toward the diaphragm while the anterior surfaces is aligned with the sternal surfaces of the chest. Those differences comparing to that of humans are quite obvious and depends solely on the fact that the humans and pigs have different posture, pigs are walking on four legs while humans are standing up on two legs. Just like in humans the atriums of pigs are divides by 1) septum and 2) venous components. The right atrium of both pigs and humans receive blood from the same blood vessels: 1) superior vena cava, 2) inferior vena cava and 3) coronary sinuses. However there is a difference in what manner the two species receives the blood from the mentioned blood vessels: 1) Humans receives the blood from the blood vessels in a line while 2) the pig receives the blood from the vessels an right angle. Additionally the pigs have a very big azygous vein that enters on the left side of the heart and then later drains into the sinuses of the pig hearts [23].

However the conductive system of the piglets are supplied by a posterior septal artery that similar to human will make the heart right sided as in humans. Whilst in pigs instead of the posterior septal artery, the atroventricular nodal artery supplies the human AVN. Due to this there is a future problem to be solved, such as is the blood supply to the AVN the same in humans and pigs or is this difference that have been mentioned above due to there is anatomical difference or is it because limitations of previous researches done on the piglets hearts. It may not have any clinical importance but however it more so signifies the importance of the right coronary artery in both human and piglets cardiac conduction systems [24-25].

While a human have four different orifices for the pulmonary veins to enter the left atrium of the heart a pig only have two orifices as a pig only have two pulmonary veins that will enter the left atrium to provide oxygenated blood. Question arises if this affects the pigs ability contrary to humans to provide their bodies with oxygenated blood in situations requiring a bigger amount of oxygen or if the pig body is compensated by having a bigger intraluminal diameter of the pulmonary vein than humans. As known since long there is a difference in the size of the human atria, such that the right atrium usually are larger than that of the left atria, however in pigs there is usually only a small or none at all difference between the right and the left atria. There are no significant differences of the cardiac ventricles between humans and pigs [24].
8.3 PHYSIOLOGY OF THE HEART

The physiology of the heart is divided into different phases (phase 0, phase 1 and phase 2, phase 3 and phase 4), and is regulated by the influx and out fluxes of Na⁺, K⁺ and Ca²⁺. These mechanisms cause the heart to depolarize and repolarize and cause contractions of the heart [25].

As the physiology of the sinoatrial node [SAN] is well documented, the physiology of the AVN is not as well documented. However it’s widely known that the AVN have two different entry points that are known as the: 1) Fast pathway and the 2) Slow pathway. Whilst the fast pathway is well researched upon and documented there is less information about the second slow pathway. As their names implicates the main difference between the two pathways is that the fast pathway has a very short conduction delay while the slow pathway have a much longer conduction delay. These differences in the conduction delay of the two different pathways are believed to synchronize the ventricular contractions [24].

Pathology of the AVN can lead to a variety of different arrhythmias, most commonly the atrioventricular node re-entry tachycardia. But however it’s indicated that the atrioventricular node re-entry tachycardia is not in all cases caused by changes in the morphology of the atrioventricular node. But it’s proven that by blocking the parasympathetic nervous control and the adrenergic nerves have widely different results as blocking the parasympathetic nervous system cause the atrioventricular node re-entry tachycardia to have reduced inducing ability, while blocking of the adrenergic nerves caused the atrioventricular node re-entry tachycardia to be induced. This further proves that there is no need for morphological changes of the AVN to cause arrhythmias of different individuals [26-27].

8.4 ANATOMY AND HISTOLOGY OF THE HUMAN HEART CONDUCTION SYSTEM

The Atrioventricular bundle and fibres connect the atrial and ventricular musculature. In all mammal animals the AV node originates from atrioventricular septum and goes through the fibrous septum and finally reaches different parts of the ventricular wall. The connecting system is always separated from the musculature by connective tissue and does not connect with muscular tissue. There are different specialized tissues within the heart; they are as follows Sinus node, atrioventricular conduction system and the purkinje network. The atrioventricular junction is made up of three main parts: 1) Transitional cell zone, 2) AVN and the 3) atrioventricular bundle. The transitional cells is the connecting area between the AVN and the working myocardium, thus it’s not being isolated by neither connective nor fatty tissue [28-31].

The AVN is located inferior to the posterior epicardium of right atrium, anterior to the ostium of coronary sinus and above the septal leaflet of the tricuspid valve. At the apex of the triangle of Koch
the AVN becomes the penetrating bundle of His. The atrioventricular node artery that originates from right coronary artery in most individuals supplies the AV node, but in a small portion the artery originates from the right circumflex artery [32].

The AVN is an oval structure that measures 1x3x5 mm. Histologically the AVN is made up of so called star cells that are pale, they are organized in groups and interconnect with each other within a meshwork of collagen and elastic fibres. The cardiac conduction system is composed of 4 different cells: P-cells, Transitional cells, common myocardial cells and Purkinje cells, which can be seen with electron microscope [33-34].

The bundle of His is largely viewed as a continuation of the AV node, and it doesn’t have any histological difference from the AV node that can separate them from one and another. The bundle of His is around 20 mm long and can be up to 4 mm thick. The bundle of His starts when the specialized myocardial cells loose their network and instead form parallel strands. The bifurcation of bundle of His is the lowest part of the bundle of His, it is started when the bundle of His comes out from the central fibrous body. The right bundle branch goes along the right-sided part of ventricular septum. The cells of right branch are generally described as Purkinje cells. Arteries originating from the right anterior descending coronary arteries and left anterior descending coronary arteries supply the right bundle branch. The left bundle branch runs along the left ventricular septum and is supplied by arteries originating from left anterior descending artery and the right posterior descending artery [29].

There are “two” atrioventricular rings, rightward ring and the leftward ring. They both originate from an inferior extension of AV node. They run in two different directions, the right goes around the vestibule of tricuspid valve and the left ring circles the mitral valve. When returning again toward the atrial septum, the right ring crosses the penetrating part of His bundle, and here it reunites with the left part of the rings. When they reunite the form a retroaortic node that is located superiorly. The AV conduction system continues beyond the origin of the right and the left bundle branches and form an aortic ring, this ring however doesn’t make any connections with the retroaortic node. The failure of the aortic node to make contact with retroaortic node is because of the fibrous tissues between aortic-to-mitral valve areas [29].

8.5 DISEASE IMPACT ON INTRACARDIAC INNERVATION

When there is a changing of innervation of organs such as the heart there will be different functional changes of the affected organ. By measuring the nerves of the heart when there are changes of the innervation, two types can be distinguished: 1) hypo-innervation or 2) hyper-innervation. These two different type are distinguished from each other that 1) hypo-innervation will show loss of intracardiac axons while 2) hyper-innervation show that there will be increased intracardiac axons.
This can be used on human patients with the diagnosis of Alzheimer’s disease, as they are shown to have hypo-innervation as they have loss of axons [35].
9. MATERIAL AND METHODS

9.1 STUDY MATERIAL

4 piglets of both genders with a weight of 2-2.5 kg were used in this study. Piglets’ hearts were dissected from the chest of the pigs 2 – 6 hours after death. Hearts were cleaned with 0.01M phosphate buffer saline [PBS], and the coronary vessels were perfused with PBS. To obtain the AVN, the intraarterial septum with koch’s triangle was dissected from the rest of the heart. The part of the AV-node containing atria were then fixated in 4% paraformaldehyde, 4°C, with 0.01M PBS (pH 7.4) solution acting as a buffer, for 40 minutes. Then the preparations were washed and immersed to cryoprotect them in PBS containing 20-25% glucose for 3×10 minutes. After the cryoprotection the preparations were frozen using tissue-medium, after the freezing of preparations they were mounted on the cryomicrotome. The preparations were sliced into 20 μm by using the microtome at a degree of -23°C and put onto microscope slides.

9.2 PRIMARY REACTIONS

Then the slides were washed in PBS solution+0,5% Triton-X-100 (200ml PBS and 10μl Triton-X-100) 3×5 minutes. Then it was incubated in 5% donkey serum with PBS solution (200μl PBS+10μl NDS) for 40 minutes to prevent non-specific binding during the study. After this the preparations were incubated with the correct antisera for 2 hours.

9.3 SECONDARY REACTIONS

After the incubation for 24 h the preparations were incubated in 0,01M PBS for 3-5 minutes and incubated with correct antisera (table 1) for 1 hour. These combinations of anti-sera were used during the study, 1) ChAT+TH, 2) ChAT+nNOS and 3) Cx43+HCN4(table 1). Then the preparations were washed in 0,01M PBS solution for 3×5 minutes. The preparations were then covered and sealed with transparent nail polish.
9.4 STATISTICAL ANALYSIS AND QUANTITATIVE ANALYSIS

Data are presented as mean±standard error of the mean (SE), the program statPlus was used in this study and descriptive analytics was used on all results obtained in this study. All results were measured on digital images using the Fiji imageJ. The size of neuronal somata was expressed as the mean of their long and short axes expressed in µm. The size of intracardiac nerves and axons were expressed as the mean of their diameter, expressed in µm. The variety of different phenotypic groups was expressed as the mean of their percentage. The HCN4 positive cells were expressed as the mean of their percentage. The area of the AVN covering the intrarterial septum was expressed the mean of the mm².

Table 1. Table with all antisera and their properties and their dilution used in the study.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Host</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td><strong>Primary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAT</td>
<td>Goat</td>
<td>1:100</td>
</tr>
<tr>
<td>TH</td>
<td>Mouse</td>
<td>1:2000</td>
</tr>
<tr>
<td>TH</td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>nNOS</td>
<td>Rabbit</td>
<td>1:1000</td>
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<td>HCN4</td>
<td>Mouse</td>
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</tr>
<tr>
<td>Cx43</td>
<td>Goat</td>
<td>1:500</td>
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<tr>
<td>Rabbit</td>
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10. RESULTS

10.1 THE DISTRIBUTION OF HCN4 POSITIVE CELLS

AVN occupied 3.96±0.48mm² area of the cross section of interatrial septum.

HCN4 cells were distributed typically in all observed preparations. These myocytes were distributed diffusely and encapsulated by the connective tissue. In the inferior corner of the node they were distributed more compact, then in rest of the area, thus it was possible relatively to identify two parts of AVN: compact and lose one. In the transitional area were compact and loose parts met each other 2-3 bigger blood vessels were observed. HCN4 positive cells occupied 70%±0,075% of the whole AVN area. In the preparations were double immunohistochemical staining for HCN4 and Cx43 was applied, all HCN4 positive cells were negative for Cx43 (Fig. 2).

![Distribution for HCN4+Cx43 in the AVN.](image)

**Fig. 2 Distribution for HCN4+Cx43 in the AVN. The HCN4 (marked green) indicates the distribution of pacemaker cells in the AVN, while the Cx43 (marked red) indicates the distribution of working cardiomyocytes in the AVN.**

10.2 INNERVATION OF ATRIOVENTRICULAR NODE

The area of the AVN contained a dense meshwork of nerve fibres. Several bigger nerves, solitary neurons and ganglia were observed in the area of the AVN. Nerves were distributed irregularly and were only found as biphenotypic groups of ChAT(+)nNOS(+) and ChAT(+)TH(+) positive nerves. The ChAT(+)nNOS(+) positive nerves had a mean diameter of 182,23±51,91µm. The ChAT(+)TH(+) positive nerves had a mean diameter of 204,06±41,47µm.

Axons were found in many of the solitary neurons and the clustered neurons, with a mean diameter of 4,506±1,78µm.

On average 2,5 ganglia were found in each cross section of AVN. It’s worth to mention that ganglia were observed in all preparations. Ganglia were usually distributed in the loose part of AVN.
Mostly in the superior corner, and middle part of AVN on the top of protuberance of central fibrous body. These ganglia were clustered of 4.59±0.63 neurons on average. The neurons clustered in ganglia constituted 81.2% of all intracardiac neurons found in this study. 18.8% of the intracardiac neurons was found as solitary neurons spread out irregularly throughout the obtained pictures.

**Fig. 3 ChAT(-)/TH(+) sensitive neurons clustered as a ganglion. The arrowheads mark the different neurons.**

**Fig 4. Schematics of a nitrergic and cholinergic nerve of the AVN. Red color marks the ChAT(+) and the green color marks the nNOS(+).**
10.3 THE VARIETY OF NEURONS WITHIN ATRIOVENTRICULAR NODE

Two types of preparations were used in this study to describe neurochemical phenotypes of neural components. These were double labelled for ChAT+nNOS and ChAT+TH. Six neurochemical phenotypes of neural cells were observed within AVN area, the different phenotypical groups found were 1) ChAT(+)nNOS(+), 2) ChAT(+)nNOS(-), 3) ChAT(-)nNOS(+), 4) ChAT(+)TH(+), 5) ChAT(+)TH(-) and 6) ChAT(-)TH(+). Neuronal somata were purely positive for ChAT, nNOS, TH and biphenotypic positive for ChAT+nNOS and TH+ChAT. SIF cells were also observed in this area.

![Figure 5](image.jpg)

*Fig. 5 Picture showing a ChAT(+) solitary neuron. Arrowhead A marks the nucleus and arrowhead B marks the body of the neuron.*

10.3.1 ChAT+TH

In the preparations double labelled for ChAT+TH. ChAT(+)/TH(+) positive neurons took the majority and amounted 52.86±4.10% of all population in this group. The second biggest population was the ChAT(+)/TH(-), these neurons counted for 31.95±3.38% of all observed in this preparations group. The smallest population was the ChAT(-)/TH(+) neurons, as they constituted for 15.19±2.65% of all neurons observed in this group.

10.3.2 ChAT+nNOS

In the preparations double labelled for ChAT+nNOS. ChAT(+)/nNOS(+) neurons were dominant ones averaging 56.94±14.76% of all population in this group. While the second most prominent population that was only ChAT(+)/nNOS(-) neurons, counting for 28.13±14.57% of the
population. The smallest population was the ChAT(-)/nNOS(+) sensitive group counting for 14.93±9.16% of the total population (table 2).

10.4 MORPHOLOGY OF INTRACARDIAC NEURONS

10.4.1 SIZE OF INTRACARDIAC NEURONAL SOMATA

The size of distinct neurochemical phenotype neurons showed significant differences in size (table 2). The biggest group was the ChAT(+)/nNOS(+) positive group, with a mean size of the group was 42.84±3.05 µm. The second biggest group was the ChAT(-)/TH(+) sensitive group that was 38.23±1.67 µm. The third biggest group was the ChAT(+)/TH(+) sensitive group, with a mean size of 38.22±1.67 µm. The fourth biggest group was the ChAT(+)/TH(-) sensitive group with a mean size of 24.84±1.54 µm. The second smallest group was the nNOS(+)/ChAT(-) sensitive group, with a mean size of 23.63±1.62 µm and finally the smallest group was the ChAT(+)/nNOS(-) sensitive group with an mean size of 16.71±1.55 µm.

10.4.2 SHAPE OF THE INTRACARDIAC NEURONS

The observed data of this study proved that the significant differences of between mean long axis and mean short axis of both the solitary and the neurons clustered in ganglia that the ChAT(+)/nNOS(-) group could be differentiated from the rest of the phenotypic groups due to its generally oval shape contrary to the elongated shape of the rest of the groups.
### Table 2. Summary of the different phenotypic groups long axis, short axis, size and their percentage of the variety of the intracardiac neurons.

<table>
<thead>
<tr>
<th>Group</th>
<th>Long axis±SE</th>
<th>Short axis±SE</th>
<th>Size±SE</th>
<th>Percentage±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) ChAT+nNOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAT(+)/nNOS(-)</td>
<td>41,75±2,99 µm</td>
<td>16,68±1,67 µm</td>
<td>42,84±3,05 µm</td>
<td>56,94±14,76%</td>
</tr>
<tr>
<td>ChAT(+)/nNOS(-)</td>
<td>16,29±1,33 µm</td>
<td>12,17±1,70 µm</td>
<td>16,71±1,55 µm</td>
<td>28,13±14,57%</td>
</tr>
<tr>
<td>ChAT(-)/nNOS(+)</td>
<td>22,36±1,78 µm</td>
<td>11,96±0,59 µm</td>
<td>23,63±1,62 µm</td>
<td>14,93±9,16%</td>
</tr>
<tr>
<td>2) ChAT+TH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChAT(+)/TH(+)</td>
<td>38,27±1,65 µm</td>
<td>17,75±0,74 µm</td>
<td>38,22±1,67 µm</td>
<td>52,86±4,10%</td>
</tr>
<tr>
<td>ChAT(+)/TH(-)</td>
<td>24,81±1,51 µm</td>
<td>13,36±0,81 µm</td>
<td>24,84±1,54 µm</td>
<td>15,19±2,65%</td>
</tr>
<tr>
<td>ChAT(-)/TH(+)</td>
<td>27,21±2,33 µm</td>
<td>14,50±1,12 µm</td>
<td>38,23±1,67 µm</td>
<td>31,95±3,38%</td>
</tr>
</tbody>
</table>
11. DISCUSSION OF THE RESULTS

11.1 DISCUSSION OF THE HCN4 POSITIVE CELLS DISTRIBUTION AND AREA OF AVN

The HCN4 positive cells found in the cross-sections identifies the pacemaker cells of the AVN and distinguishes them from the working myocardioocytes of the AVN. These results can be further used to study the extent of pacemaker cells in atrioventricular cells and it’s extension further extent of the AVN in different species. The results of this study can be compared with those of Pauza et al [5] and Inokaitis et al [36], this distribution of HCN4 cells indicates that as in rabbits SAN as in this study the AVN is mainly composed of pacemaker cells explaining their ability to produce impulses.

The area covered by the AVN was 3.96±0.48 mm$^2$. However as shown previously by Jerrold Widran [37], the AVN varies in size in different individuals and that is getting larger with aging. This implies that the size of AVN in piglets would enlarge with age. This knowledge can be used when to operate atrioventricular node re-entry tachycardia or when to dissect AVN for studying purposes of cadavers.

11.2 DISCUSSION ABOUT INNERVATION OF THE AVN

11.2.1 AXONS

In this study the axons were found as unipolar and multipolar and as in amphibious animals such as frogs done by Batulevicius et al [38] the diameter of the axons are similar ranging from 4-8µm. This indicates that in the future when researching axons in the future it’s possible to use frogs, as they are comparable to pigs in this fashion. This indicates that there are similarities of intracardiac neural cells of cold-blooded and warm-blooded animals.

11.2.2 NERVES

As previously done by Neringa Pauziene et al [39] intracardiac nerves can also be distinguished with immunohistochemical reactions as with electron microscope. Both are significant in distinguishing nerves from other intracardiac structures but as with the electron microscope there are such advantages that it can be used to differentiate different nervous structures as mentioned by Pauziene et al [39], but as indicated in this study the immunohistochemical examinations can be used.
to differentiate the different types of nerves and if different phenotypic nerves have different size. This study indicates that biphenotypic nerves of ChAT(+)\text{nNOS}(+) and ChAT(+)\text{TH}(+) are similar in diameter.

11.3 DISCUSSION OF THE VARIETY OF THE NEURONS PRESENT IN AVN

The results from the ChAT+nNOS and the ChAT+TH preparations in this study indicate that there is different prominence of different types of neurons of the cardiac conduction system. The most abundant phenotypic group of the ChAT+nNOS preparations was the 1) ChAT(+)/nNOS(+), 2) ChAT(-)/nNOS(+) and 3) ChAT(+)/nNOS(-). The most abundant phenotypic groups of the ChAT+TH preparations was 1) ChAT(+)/TH(+), 2)ChAT(-)/TH(+) and 3) ChAT(+)/TH(-). These results can be compared to those of Pauza et al \[40\] who was doing a similar research on bats which indicates that bats have mostly ChAT sensitive neurons, while there is fewer TH and nNOS sensitive neurons. This indicates that there is a difference between hibernating mammals (bats) and non-hibernating animals (pigs), in perspective that bats have a much more adapted cardiac structure to withstand hypoxia than pigs. This can be used in studies surrounding heart transplantations as how to prepare the heart for transplantation without causing any damage to the cardiac structure.

According to previous studies done by Kieran Brack et al \[14\] and Crick et al \[16\] have shown that there is difference of the distribution of different phenotypic intracardiac neurons of different mammals, such as rabbits have dominating TH sensitive neurons, these implicates that due to different life styles such as activity can have lead to different variety of neurons to answer the demand of the heart.
11.4 DISCUSSION ABOUT THE MORPHOLOGY OF THE INTRACARDIAC NEURONS

11.4.1 SIZE OF THE NEURONAL SOMATA

The results indicate that the mean length of all long axis of intracardiac neurons obtained in this study have the similar length as in human infants as previously studied by Ruta Jurgatiene et al [41].

The results of this study indicate that there are major differences in the sizes of the different phenotypic groups in the objects used in this study, and this should be examined in post mortem examinations of patients deceased due to cardiac reasons and cross referred to the results if there’s correlation with the differences of the sizes of the different phenotypic groups that could be the cause of death.

11.4.2 SHAPE OF INTRACARDIAC NEURONS

The measurements of this study indicated that all phenotypic groups of intracardiac neurons was elongated except the ChAT(+)/nNOS(-) group. These results indicate that there is usage of this information in today’s clinical usage. By using other methods of obtaining information of the morphological properties of intracardiac neurons, the ChAT(+)/nNOS(-) group could be distinguished from other phenotypic groups of intracardiac neurons due to the difference of their shape.

The result of Batulevicius et al [38] indicates that there is no correlation in the morphology between mammals and amphibious animals and should therefore not be used as a model to compare human and amphibious animals hearts on a morphological level.
12. CONCLUSIONS

1. The area of AVN was 3.96±0.48 mm² and two parts of node could be easily distinguished:
   a) Lose one that was observed on the fibrous body.
   b) Compact part, was observed at the orifice of the tricuspid.
2. AVN contained a great variety of neural structures. The whole AVN area was fulfilled with nerve fibres, nerves and neurons:
   a) Intracardiac neurons clustered in ganglia was dominating those that was solitary
   b) Axons were seen in all pictures with both intracardiac neurons clustered as ganglia and solitary neurons.
   c) All intracardiac nerve found, had mixed neurochemical phenotypic features. (ChAT(+)/nNOS(+) and ChAT(+)/TH(+)).
3. Great variety of distinct neurochemical phenotypic neurons were observed: 1) ChAT(+)/nNOS(+), 2)ChAT(+)/nNOS(-), 3) ChAT(-)/nNOS(+), 4) ChAT(+)/TH(+),5) ChAT(+)/TH(-) and 6) ChAT(-)/TH(+). The phenotypic groups of ChAT(+)/nNOS(+) and ChAT(+)/TH(+) was the most abundant found in this study.
4. The size of intracardiac neurons varied greatly over the different neurochemical phenotypic groups.
   a) The largest neurons was the ChAT(+)/nNOS(+) and ChAT(-)/TH(+).
   b) Most the neurons had an oval shape except ChAT(+)/nNOS(-) cells, that had a round shape.
13. PRACTICAL RECOMMENDATIONS

6 piglets were used to do the immunohistochemical reactions on, but due to factors influencing the results such as the time death of the objects until arrival at the department where the experiments took place varied greatly that lead to causing 2 hearts be decomposed. Thus also affecting on how exact the results were on the 4 objects that were used to perform the immunohistochemical reactions.

To minimize the time frame between the time of death of the piglets and the arrival to the department of anatomy is essential to get more suitable hearts to work with. A good but more expensive way to minimize the time frame between time of death of the objects and the time of dissection of the objects is to euthanize the objects at the site of experimentation.
14. REFERENCES


[8] Fig 1. [http://ajpcell.physiology.org/content/ajpcell/306/2/C121/F1.large.jpg](http://ajpcell.physiology.org/content/ajpcell/306/2/C121/F1.large.jpg)


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