Ion Channel Transcript Expression at the Rabbit Atrioventricular Conduction Axis

Ian D. Greener, PhD; James O. Tellez, PhD; Halina Dobrzynski, PhD; Mitsuru Yamamoto, MD; Gillian M. Graham, PhD; Rudi Billeter, PhD; Mark R. Boyett, PhD

Background—Little is known about the distribution of gap junctions and ion channels in the atrioventricular node, even though the physiology and pathology of the atrioventricular node is ultimately dependent on them.

Methods and Results—The abundance of 30 transcripts for markers, gap junctions, ion channels, and Ca\(^{2+}\)-handling proteins in different regions of the rabbit atrioventricular node (nodal extension and proximal and distal penetrating bundle of His as well as atrial and ventricular muscle) was measured using a novel quantitative polymerase chain reaction technique and in situ hybridization. The expression profile of the nodal extension (slow pathway into penetrating bundle) was similar to that of the sinoatrial node. For example, in the nodal extension, in contrast to the atrial muscle and as expected for a slowly conducting tissue with pacemaker activity, there was no or reduced expression of Cx43, Na\(_1\).5, Ca\(_1\).2, K\(_1\).4, KChIP2, and RYR3 and high expression of Ca\(_1\).3 and HCN4. The expression profile of the penetrating bundle was less specialized. In situ hybridization revealed a transitional zone with reduced expression of Cx43, Na\(_1\).5, and KChIP2 that may form the fast pathway into the penetrating bundle.

Conclusions—At the atrioventricular node, the expression of gap junctions and ion channels in the nodal extension (slow pathway) and a transitional zone (putative fast pathway) as well as the penetrating bundle (output pathway) is specialized and heterogeneous and roughly matches the electrophysiology of the different regions. (Circ Arrhythmia Electrophysiol. 2009;2:305-315.)

Key Words: atrioventricular node ▪ conduction system ▪ ion channels ▪ connexins ▪ qPCR ▪ in situ hybridization

The function of the atrioventricular node (AVN), located in the triangle of Koch (formed by coronary sinus, tendon of Todaro and tricuspid valve; Figure 1A) is to transmit the action potential from the atria to the ventricles after a suitable delay. The AVN is also a subsidiary pacemaker. Both structurally and functionally, the AVN is complex. The penetrating bundle of His, extending from the compact node (small group of compactly organized nodal myocytes) to the left and right bundle-branches, transmits the action potential through the central fibrous body into the ventricles (Figure 1A). The compact node-penetrating bundle has dual inputs from the atrial muscle, the slow and fast pathways (Figure 1A). The slow pathway, extending from the coronary sinus to the compact node, corresponds to an inferior (or posterior) extension of nodal tissue (Figure 1A). The fast pathway, extending from the direction of the interatrial septum to the compact node, is thought to correspond to transitional myocytes in the triangle of Koch. There are large differences in the conduction velocity of the action potential in different parts of the AV conduction axis; whereas the conduction velocity of the atrial muscle is high (80±29 cm/s), the conduction velocity of the slow pathway is low (2 to 10 cm/s). Consistent with this, electric coupling within the center of the AVN is likely to be poor. There are also large differences in the action potential in different parts of the AV conduction axis; although

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Billette has described 6 myocyte types (classified according to action potential configuration), the presence of 3 types (N, AN, and NH) is well established. Nodal (N) myocytes are assumed to be present in the compact node. N myocytes have a characteristic Ca\(^{2+}\)-dependent action potential with a low upstroke velocity (maximum upstroke velocity =18 V/s) and a relatively positive resting potential (~−64 mV). The slow upstroke together with the poor electric coupling can explain the slow conduction through the AVN, essential for the delay

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From the Cardiovascular Research Group (I.D.G., J.O.T., H.D., M.R.B.), Faculty of Medical and Human Sciences, University of Manchester, Core Technology Facility, Manchester, United Kingdom; the Department of Circulation (M.Y.), Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan; Institute of Membrane and Systems Biology (G.M.G.), University of Leeds, Leeds, United Kingdom; and Centre for Integrated Systems Biology (R.B.), University of Nottingham, Nottingham, United Kingdom.

Drs Billeter and Boyett are joint senior authors.

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Correspondence to M.R. Boyett, PhD, University of Manchester, Core Technology Facility, 46 Grafton St, Manchester M13 9NT, UK. E-mail mark. boyett@manchester.ac.uk.

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between atrial and ventricular systole. Atrio-nodal (AN) myocytes are transitional cells making up the fast pathway and nodo-His (NH) myocytes are transitional cells within the penetrating bundle; AN and NH myocytes can have a faster action potential and a more negative resting potential (intermediate between that of N and atrial myocytes\(^6,7,9,10\)). The differences in the action potential in the different regions of the AV conduction axis are likely to be the result of differences in ionic currents.\(^9,11\) Munk et al\(^9\) (see also Ren et al\(^11\)) have shown that the majority of putative N myocytes do not have a Na\(^+\)/H\(^+\) current (\(i_{Na}\)) but do have the hyperpolarization-activated current (\(i_f\)); this explains why N myocytes have a slow action potential and exhibit pacemaking. In contrast, Munk et al\(^9\) (see also Ren et al\(^11\)) have shown that the majority of putative AN myocytes have \(i_{Na}\) but not \(i_f\). The regional differences in electric coupling, the action potential, and ionic currents along the AV conduction axis are likely to be the result of differences in ionic currents.\(^9,11,12\) Munk et al\(^9\) (see also Ren et al\(^11\)) have shown that the majority of putative AN myocytes have \(i_{Na}\) but not \(i_f\). The regional differences in electric coupling, the action potential, and ionic currents along the AV conduction axis are likely to be the result of differences in the expression of gap junctions and ion channels. The aim of this study was to describe the distribution of gap junction and ion channel transcripts in the different regions of the AVN. The work was carried out on the rabbit because of the plethora of functional data collected from rabbit AVN.

**Methods**

Methods are described in brief here (see the online-only Data Supplement for a full description). AVN preparations were dissected from male New Zealand White rabbits (weight, 1.5 to 2.5 kg) for both quantitative polymerase chain reaction (qPCR) and in situ hybridization. For qPCR, total RNA was isolated from (1) atrial muscle, (2) nodal extension, (3) compact node and proximal penetrating bundle, (4) distal penetrating bundle and bundle-branches, and (5) ventricular muscle (Figure 1A) from 8 rabbits. The techniques used ensured that there was little contamination (\(<3%\) to \(<5%\)) of the nodal tissues by working myocardium (Data Supplement). The total RNA was reverse-transcribed to produce cDNA, and qPCR was then performed for 30 transcripts. To compare the abundance of a selected transcript in different tissues, we used a double standardization method. These data are shown in the figures, in which the transcript abundance is expressed as a percentage of the mean value in the atrial muscle. To compare the abundance of different cDNAs, we used a single standardization method; from this, tentative conclusions can be made about the abundance of different mRNAs (see Data Supplement); these data are discussed in the text. In situ hybridization was carried out for 11 selected transcripts using cRNA probes. Mean±SEM values are shown in the figure. Statistical analysis of mRNA abundance in different tissues (as measured by qPCR) was carried out on complete data sets only (for 6 or 7 rabbits, depending on transcript). Significant differences were identified by ANOVA (for normally distributed data) or ANOVA on ranks (for non-normally distributed data), followed by the Student-Newman-Keuls test. A difference was considered significant if \(P<0.05\).
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Marker Proteins, Connexins, and Ca^{2+}-Handling Proteins
To test the efficacy of the tissue sampling for qPCR, the relative abundance of transcripts for marker proteins was measured. In the rabbit, middle (160/165 kDa) neurofilament is known to be expressed by the tissues of the cardiac conduction system only, whereas atrial natriuretic peptide (ANP) is known to be expressed by the atrial muscle only; neurofilament and ANP are, therefore, markers of nodal and atrial tissue, respectively.\(^{13}\) As expected, neurofilament mRNA was found at negligible levels in the atrial and ventricular muscle, but it was abundantly expressed in the nodal tissues (Figure 1B). In contrast, ANP mRNA was abundantly expressed in the atrial muscle but not in the other tissues (Figure 1C). This distribution of neurofilament (data not shown) and ANP (supplemental Figure S1) mRNA was confirmed by in situ hybridization. This confirms that the sampling of tissue for qPCR was accurate (see the Data Supplement for further discussion of this issue).

Connexins are responsible for gap junctions and therefore, electric coupling. Three connexin isoforms were investigated. Connexin43 (Cx43) mRNA, responsible for large conductance (60 to 100 pS) gap junction channels, was abundant in the atrial muscle (Figure 1D). It was least abundant in the nodal extension, more abundant in the proximal penetrating bundle, and highly abundant in the distal penetrating bundle and ventricular muscle (Figure 1D). This pattern was confirmed by in situ hybridization; as an example, Figure 2A,B shows that whereas Cx43 mRNA was abundant in the atrial and ventricular muscle at the AV junction, it was much less abundant in the compact node (red circled area in Figure 2A).

Figure 2. A, Distribution of Cx43 mRNA as detected by in situ hybridization in section cut at level of compact node. Red dashed line outlines compact node. Black dashed line shows border between Cx43 mRNA expressing (above) and nonexpressing (below) tissue. B, High magnification images of atrial muscle, transitional zone, compact node, and ventricular muscle. C, Confocal image of in situ hybridization product (blue; Na,1.5 mRNA; rabbit atrial muscle). D, Confocal image of in situ hybridization product (blue; HCN4 mRNA; rabbit sinoatrial node) and nuclei (red; propidium iodide).
At low magnification, the labeling of Cx43 mRNA in the working myocardium was in the form of dark spots (e.g., at red arrow in Figure 2A). At high magnification, the labeling of Cx43 mRNA in the working myocardium could be seen to be in the form of rings (Figure 2B). This has been the case for all mRNAs coding for membrane resident proteins we have analyzed so far using in situ hybridization. The dark purple stain formed in situ hybridization shows significant fluorescence, and Figure 2C shows a confocal micrograph of 2 rings (blue; in this case, transcript was Nav1.5). As shown in Figure 2D, the rings (blue; in this case, transcript was HCN4) are located around nuclei (red; stained with propidium iodide). These rings most likely correspond to the rough endoplasmic reticulum in which the mRNA is translated. Figure 2 (A and B) also shows that Cx43 mRNA was absent from a tract of tissue above the compact node. In the Discussion, we speculate that this may form the fast pathway.

In contrast to Cx43 mRNA, the mRNAs for connexin40 (Cx40; data not shown) and connexin45 (Cx45; Figure 1E), responsible for large (200 pS) and small (20 to 40 pS) conductance gap junction channels, respectively, were equally abundant in all regions investigated.

AVN myocytes have an intracellular Ca\(^{2+}\) transient, and there is evidence that intracellular Ca\(^{2+}\) may be important in pacemaking in the AVN (as it is in sinus atrial node). Four Ca\(^{2+}\)-handling proteins were investigated: The ryanodine receptor is the sarcoplasmic reticulum Ca\(^{2+}\) release channel. In the PCR, cDNA fragments corresponding to 2 ryanodine receptor isoforms, RYR2 and RYR3, were detected. RYR2 cDNA was more abundant than RYR3 cDNA (in the nodal extension, the ratio of RYR2:RYR3 cDNAs was \( \approx 700:1 \)); this suggests that RYR2 mRNA is much more abundant than RYR3 mRNA. Both RYR2 and RYR3 mRNAs were significantly less abundant in the nodal extension than in the atrial and/or ventricular muscle (Figure 1, F and G). SERCA2A is the sarcoplasmic reticulum Ca\(^{2+}\) pump, and its mRNA was significantly less abundant in the penetrating bundle than in the atrial and ventricular muscle (Figure 1H). mRNA for the Na\(^{+}\)-Ca\(^{2+}\) exchanger, NCX1, was equally abundant in all regions investigated (data not shown).

**Na\(^{+}\), Ca\(^{2+}\), and HCN Channels**

The cardiac Na\(^{+}\) channel, Nav1.5, is responsible for \( I_{Na} \) and therefore the rapid action potential upstroke in the atrial and ventricular muscle. Na\(^{+}\)1.5 mRNA, as inferred from qPCR, was abundant in the atrial muscle, and tended to be less abundant in the nodal extension (Figure 3B). Interestingly, Na\(^{+}\)1.5 mRNA abundance was higher (than in nodal extension) in the penetrating bundle and higher again in the ventricular muscle (Figure 3B). The pattern of expression of Nav1.5 was confirmed by in situ hybridization: The examples in Figure 4 show that whereas Nav1.5 mRNA was abundant in the atrial and ventricular muscle and penetrating bundle, it was absent from the nodal extension. In the tissue above the nodal extension (transitional zone/putative fast pathway), there was also reduced expression of Nav1.5 mRNA (Figure 4A and 4C).

As well as the cardiac Na\(^{+}\) channel isoform (Nav1.5), cardiac myocytes can express neuronal Na\(^{+}\) channel isoforms, for example, Nav1.1. The PCR yielded a ratio of Na\(^{+}\)1.5:Nav1.1 cDNAs of 7:1 in the nodal extension; this suggests that there is a significant amount of Nav1.1 mRNA in the nodal tissues (comparable to the level of Nav1.5 mRNA). Na\(^{+}\)1.1 mRNA was measurable in the atrial and ventricular muscle, but it was significantly more abundant in the nodal extension (Figure 3A). Na\(^{+}\)1.1 protein has been detected in the AVN.

L-type Ca\(^{2+}\) channels are known to be responsible for the upstroke of the action potential in N myocytes at the AVN (because \( I_{Na} \) is absent). Two L-type Ca\(^{2+}\) channel isoforms were investigated, Ca\(^{1+}\)1.2 and Ca\(^{1+}\)1.3. In the atrial and ventricular muscle, Ca\(^{1+}\)1.2 is known to be the dominant cardiac isoform. Figure 5A and 5B show the abundance of Ca\(^{1+}\)1.2 mRNA as determined by in situ hybridization in a section at the level of the distal penetrating bundle. As expected, Ca\(^{1+}\)1.2 mRNA was abundant in the atrial and ventricular muscle. However, Ca\(^{1+}\)1.2 mRNA was largely absent from the myocytes of the distal penetrating bundle (Figure 5A and 5B); Ca\(^{1+}\)1.2 mRNA was also absent from the nodal extension and compact node (supplemental Figure S2). qPCR showed Ca\(^{1+}\)1.3 mRNA to be present in the atrial and ventricular muscle but more abundant in the nodal tissues (Figure 3C). This was confirmed by in situ hybridization: Figure 5C and 5D show that Ca\(^{1+}\)1.3 mRNA was not expressed (or little expressed) in the atrial and ventricular muscle but was abundant in the distal penetrating bundle; it was also abundant in the nodal extension and compact node (supplemental Figure S2). The PCR yielded a ratio of...
Cav1.3:Cav1.2 cDNAs of 4:1 in the nodal extension. It is concluded that from the working myocardium to the nodal tissue of the AV junction there is an isoform switch from Cav1.2 to Cav1.3.

HCN channels are responsible for the hyperpolarization-activated current, $I_h$ (current known to be involved in pacemaking); 2 HCN isoforms, HCN1 and HCN4, were investigated. HCN4 is the principal HCN isoform in the heart. qPCR indicated that, as expected, HCN4 mRNA was poorly abundant in the atrial and ventricular muscle (Figure 3E). Interestingly, HCN4 mRNA was highly abundant in the nodal extension but poorly abundant in the other nodal tissues (Figure 3E); this was confirmed by in situ hybridization (data not shown). This is consistent with functional data (see Discussion). Interestingly, the pattern of expression of HCN1 mRNA was different: As compared with the ventricular muscle, at least, HCN1 mRNA was significantly more abundant in all the nodal tissues (Figure 3D). The PCR yielded a ratio of HCN4:HCN1 cDNAs of 3:1 in the nodal extension; this suggests that their mRNAs probably occur in roughly similar amounts.

**K⁺ Channels**

In the rabbit, 60% of putative N myocytes do not possess the transient outward K⁺ current, $I_{to}$, and although >90% of
putative AN cells do possess $I_{to}$, the current is smaller than in atrial cells.\textsuperscript{9} $K_{1.4}$, $K_{4.2}$, $K_{4.3}$, and KChIP2 are all involved with the transient outward K$^+$ current ($I_{to}$) in the heart: $K_{1.4}$, $K_{4.2}$, and $K_{4.3}$ are pore-forming $\alpha$-subunits responsible for $I_{to}$. KChIP2 is a $\beta$-subunit responsible for trafficking $K_4$ subunits to the membrane and thus increasing the density of $I_{to}$.\textsuperscript{21} As measured by qPCR, $K_{1.4}$ mRNA was significantly less abundant in the nodal extension than in the atrial muscle (Figure 6A). In contrast, $K_{4.2}$ mRNA tended to be more abundant in the nodal tissues ($t$-test showed that it was significantly more abundant in nodal tissues than in working myocardium; Figure 6B). $K_{4.3}$ mRNA was equally abundant in all regions investigated (data not shown). In the nodal extension, the ratio of $K_{1.4}$:$K_{4.2}$:$K_{4.3}$ cDNAs was 3:2:1; this suggests that their mRNAs probably occur in roughly similar amounts. There was a distinct decrease in the abundance of KChIP2 mRNA from the atrial and ventricular muscle to the nodal tissues (Figure 6C). In situ hybridization revealed a more complex pattern of KChIP2 mRNA expression: Figure 7 shows that KChIP2 mRNA was absent from the compact node, consistent with Figure 6C (it was also absent from nodal extension and distal penetrating bundle; data not shown). Figure 7 shows that KChIP2 mRNA was abundant in part of the atrial muscle (again consistent with Figure 6C); however, it shows that KChIP2 mRNA was absent from a tract of tissue above the compact node (transitional zone/fast pathway). Intriguingly, Figure 7 also shows that KChIP2 mRNA was only abundant in part of the ventricular muscle: the ventricular muscle facing the right ventricle (not ventricular muscle facing left ventricle); why this should be the case is not known.

It is known that the rapid delayed rectifier K$^+$ current, $I_{Kr}$ (for which ERG is responsible), plays an important role in repolarization at the rabbit AVN,\textsuperscript{22,23} whereas the slow delayed rectifier K$^+$ current, $I_{Ks}$ (for which $K_{LQT1}$ and minK are responsible), does not.\textsuperscript{23,24} Consistent with this, ERG cDNA was more abundant than $K_{LQT1}$ and minK cDNAs. However, $K_{1.5}$ cDNA (responsible for ultrarapid delayed rectifier K$^+$ current, $I_{Kr}$) was even more abundant. In the nodal extension, the ratio of $K_{1.5}$:ERG:$K_{LQT1}$:minK cDNAs was $\approx$70:20:1:1. The expression of $K_{1.5}$, ERG, $K_{LQT1}$, and minK mRNAs did not vary among the tissues investigated (eg, Figure 6D).
Kir2.1 and Kir2.2 are, in part, responsible for the background inward rectifier $K^+$ current, $I_{K,1}$. Kir2.1 mRNA was abundant in the ventricular muscle but low in all other tissues (Figure 6E); this was confirmed by in situ hybridization (data not shown). Kir2.2 mRNA was equally abundant in all tissues investigated. The PCR yielded a ratio of $K_{ir2.1}:K_{ir2.2}$ cDNAs of $\approx 70:1$; Kir2.1 mRNA is therefore likely to be more abundant than Kir2.2 mRNA. Kir6.2 is the $\alpha$-subunit and SUR2A is the $\beta$-subunit for the ATP-sensitive $K^+$ channel in the heart. Both Kir6.2 and SUR2A mRNAs were significantly less abundant in the nodal extension and proximal penetrating bundle than in the atrial or ventricular muscle (Figure 6F and 6G).

**Summary**

From 9 different regions of the rabbit AVN, Billette\(^6\) has made action potential recordings and classified them into 6 types: N-type, 3 AN-types (AN, ANCO, and ANL), NH-type, and H-type (His-type). In Figure 8A, we have plotted (as color contour maps) the frequency of occurrence of each action potential type in the different regions of the triangle of Koch, and in Figure 8B, as a summary, we superimposed the regions in which the 6 action potential types occur most frequently. As expected, AN-type action potentials (dark gray) were most frequent toward the atrial muscle (top of Figure 8B). Again, ANCO-type action potentials (middle gray) were most frequent toward the atrial muscle (top of Figure 8B), and, interestingly, ANL-type action potentials (light gray) were most frequent in a region in which N-type action potentials were also frequent (perhaps compact node). N-type action potentials (red) were most frequent in the nodal extension and perhaps in the compact node (bottom left of Figure 8B). NH-type action potentials (green) were most frequent in a region perhaps corresponding to the lower nodal tract in the proximal penetrating bundle (bottom middle of Figure 8B). As expected, H-type action potentials (yellow) were most frequent in the distal penetrating bundle (bottom right of Figure 8B). As a summary, for the transitional zone, nodal extension, compact node–proximal penetrating bundle, and distal penetrating bundle–bundle branches, in Figure 8B we have listed the transcripts that are increased or decreased (as compared with atrial muscle) as determined by qPCR or in situ hybridization; as explained in the Discussion, the altered levels of these transcripts can perhaps explain the characteristic action potentials in the different regions.

**Discussion**

In this study, a unique picture of ion channel expression at the AV junction of the rabbit has been obtained. This can tentatively be compared with the well-known electrophysiology of the rabbit AVN.

**Nodal Extension/Slow Pathway**

Whereas AN and NH myocytes are transitional, N myocytes are nodal. N myocytes have a small amplitude action potential with a slow upstroke.\(^6,9,11\) During diastole, the membrane is relatively depolarized and there is a pacemaker potential; N myocytes show pacemaker activity.\(^5,9\) Consistent with this, N myocytes generally lack $I_{Na}$ and $I_{K,1}$ but possess $I_{If}$.\(^9,11,23\) N myocytes are thought to be located in the region of the compact node\(^2\) (not specifically targeted in present study), but we suggest that they are also located in the nodal extension. This is consistent with the high frequency of N-type action potentials between the coronary sinus and tricuspid valve in the work of Billette,\(^6\) as shown in the summary in Figure 8B (red; bottom left). Furthermore, in a later study by Billette,\(^1\) N-type action potentials with an upstroke velocity of only 6 to 12 V/s were specifically recorded from the nodal extension in the rabbit.\(^1\) Finally, the possibility that N myocytes are located in the nodal extension is consistent with the pattern of ion channel expression in the nodal extension as explained below.

The lack of expression of Cx43 mRNA in the nodal extension (Figure 1D) is consistent with the previously reported lack of Cx43 protein expression in the nodal extension.\(^25\) There is little or no expression of $Na_{1.5}$ mRNA in the nodal extension (Figures 3B and 4), and this can explain the lack of $I_{Na}$ and slow upstroke in N myocytes.\(^9,11\) The lack of both Cx43 and $Na_{1.5}$ mRNAs in the nodal extension can explain the slow conduction velocity of the nodal extension (see Introduction).
The lack of Kv1.4 and KChIP2 mRNAs in the nodal extension (Figure 6) explains the absence of $I_{to}$ in the majority of N myocytes in the rabbit. The repriming kinetics of $K_v1.4$ (ie, speed of recovery of $K_v1.4$ from inactivation) are markedly slower than those of $K_v4$ channels. In rabbit atrial muscle, repriming of $I_{to}$ is slow (time constants, 1.4/6.7 s), consistent with a dominant role of $K_v1.4$. In the rabbit sinoatrial node, as compared with the atrial muscle, there is a decline in $K_v1.4$ mRNA (similar to that in nodal extension; Figure 6A), as well as an increase in $K_v4.2$ mRNA (more marked than in nodal extension; Figure 6B) and decline in KChIP2 mRNA (less marked than in nodal extension; Figure 6C), and, in the rabbit sinoatrial node, repriming of $I_{to}$ is fast (time constant, 45 ms), consistent with a dominant role for $K_v4.2$. Perhaps consistent with the similar pattern of changes in the transcripts in the nodal extension, 56% of $I_{to}$ in the rabbit AVN reprimers slowly (time constant, 2.7 s), whereas 44% reprimers rapidly (time constant, 196 ms), consistent with a role for both $K_v1.4$ and $K_v4$ channels in $I_{to}$ in the rabbit AVN.

The lack of $K_v2.1$ mRNA in the nodal extension (Figure 6E) can explain the lack of $I_{Kr}$ and depolarization of the membrane during diastole in N myocytes, and the expression of HCN4 (and HCN1) mRNA (Figure 3D and 3E) explains the presence of $I_f$ in the majority of N myocytes. Together, the lack of $K_v2.1$ and expression of HCNs explains why the N myocytes show pacemaking and the leading pacemaker site at the AV junction in the rabbit is in the nodal extension. We have previously reported HCN4 protein to be present in the nodal extension of the rabbit. We also observed HCN4 protein in the compact node (in present study, expression of HCN4 in compact node was not explicitly investigated) and, therefore, the compact node is also expected to show pacemaking. In the nodal extension, expression of $Ca_{v1.2}$ mRNA was reduced and $Ca_{v1.3}$ mRNA was expressed instead (Figure 3C). In the absence of $Na_{v1.5}$, the action potential upstroke and pacemaker activity is dependent on the L-type $Ca^{2+}$ current, and $Ca_{v1.3}$, because of its more negative activation threshold, is more appropriate for these functions. Knockout of $Ca_{v1.3}$ in mice disturbs AV conduction. In the absence of $I_{Kr}$, action potential repolarization and the development of the maximum diastolic potential is dependent on delayed rectifier $K^{+}$ currents. In rabbit AVN myocytes, $I_{Kr}$ but not $I_{ks}$ is reported to be present. Consistent with this, whereas ERG cDNA was abundant in the nodal extension (as abundant as in other regions studied, including working myocardium; Figure 6D), K$_{LQT1}$ cDNA was 13 times less abundant.

Of the tissues at the AV junction, the ion channel expression profile of the nodal extension (high expression of $Na_{v1.1}$, $Ca_{v1.3}$ and HCN1/4, and low expression of $Cx43$, $Na_{v1.5}$, $Ca_{v1.2}$, $K_v1.4$, KChIP2, $K_v6.2$ and SUR2A mRNAs) is most like that of the sinoatrial node. In the sinoatrial node of the rabbit, mRNA for the $Ca^{2+}$-handling protein, RYR2, is less abundant but RYR3 mRNA is more abundant than in the surrounding atrial muscle. In the nodal extension, both RYR2 and RYR3 mRNAs were less abundant (Figure 1). In the embryo, the AVN and sinoatrial node are formed of primary myocardium, whereas the developing chambers are formed of working myocardium. Tbx3 is a transcriptional repressor...
expressed in the primary but not working myocardium.\textsuperscript{32} Its absence in the working myocardium allows the expression of the working myocardium gene expression program, but its expression in the primary myocardium allows the retention of the primary myocardium gene expression program.\textsuperscript{32,33} Ectopic expression of Tbx3 in atrial muscle converts the atrial muscle into a nodal-like tissue: Consistent with the expression profile of the rabbit AVN, Tbx3 causes a downregulation of ANP, Cx43, RYR2, SERCA2A, Nav1.5, Kir6.2, and SUR2A and an upregulation of HCN1/4.\textsuperscript{32,33}

### Transitional Zone/Fast Pathway

The fast pathway is known to be made up of transitional AN cells. In the present study, in situ hybridization showed that within the right atrium, next to the bundle of nodal tissue (neurofilament-expressing), a region with a different ion channel expression profile (compared with that of ordinary atrial muscle) was observed. We tentatively suggest that this region corresponds to the fast pathway. In the region in which altered ion channel expression was observed, in a previous study we observed transitional myocytes: The myocytes were small and diffusely arranged (and therefore nodal-like) but were neurofilament-negative (and therefore atrial-like).\textsuperscript{34} In the present study, in this region, the tissue did not express neurofilament or HCN4 mRNA (data not shown), but like the nodal tissue, it only weakly expressed Cx43, Na,1.5, and KChIP2 mRNAs (Figures 2, 4A, and 7). This is consistent with the properties of the AN region and AN myocytes.\textsuperscript{9} The absence of HCN4 mRNA suggests that the tissue will not show robust pacemaking and the weak expression of KChIP2 mRNA suggests that the density of $I_{to}$ is low. The weak expression of Cx43 and Na,1.5 mRNAs suggests that electric coupling will be weak and the action potential upstroke slow, and therefore conduction will be paradoxically slow in the fast pathway. However, the transitional zone is in close contact with the atrial muscle (eg, Figure 4A), and the electrotonic influence of the atrial muscle is expected to moderate the changes resulting from the specializations in ion channel expression in this region. Furthermore, it is not known that the conduction velocity of the fast pathway is fast; it is only known that conduction through the fast pathway is quick and this could result from this pathway being short (slow pathway is longer).\textsuperscript{34}

The transitional zone also did not express ANP unlike the ordinary atrial muscle (supplemental Figure S1). The shape of the transitional zone as revealed by the absence of Cx43, Na,1.5, KChIP2, and ANP mRNAs (Figures 2, 4A, 7, and S1) was variable; one possible explanation of this is that different transcripts are distributed in subtly different ways.

### Penetrating Bundle/Output Pathway

The penetrating bundle forms the output pathway at the AV junction. It is presumed to be made up of transitional NH myocytes (proximally) and His myocytes (more distally). In the present study, the expression pattern of the proximal and distal penetrating bundle was transitional: The 2 regions had more Cx43 and Na,1.5 mRNA and less HCN4 mRNA than...
the nodal curvature. Curiously, the penetrating bundle (both proximal and distal parts) had the same level of Ca$_2^+$ and HCN1 mRNAs as the nodal extension, despite that it had more Na$_{\text{slo}}$ mRNA (and presumably, therefore, it does not have characteristic Ca$_2^+$-dependent action potential of N-myoocytes) and less HCN4 mRNA.

Limitations of the Study

Although this study provides clues about the electrophysiological heterogeneity of the AVN, further work is clearly required to match in detail the molecular portrait of the AVN with the electrophysiology. For example, it would be of interest to extend the study to more transcripts and more microdomains (such as compact node or upper and lower nodal myocytes in nodal extension and penetrating bundle). However, the principal technique used, qPCR, measures the average properties of many myocytes in a particular region, and there is evidence that even within 1 region of the AVN there is heterogeneity. For example, 1 of the principal findings of Billette et al. is that although a particular action potential type is primarily encountered in one region, it may also be encountered in other regions, as shown in Figure 8A. Single-cell PCR will be needed to shed light on this level of complexity.

Conclusion

The expression of gap junctions and ion channels at the AV junction is specialized and heterogeneous, but, as expected, it roughly matches the specialized and heterogeneous electrophysiology of the AV junction.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

The atrioventricular node is the only conduction pathway for the action potential from the atria to the ventricles—it is the cause of the delay between atrial and ventricular systole (because it conducts the action potential slowly), it is a subsidiary pacemaker, and it is a source of arrhythmias. Failure of the atrioventricular node results in heart block. The functioning of the atrioventricular node is the consequence of the gap junctions and ion channels expressed in the tissue. For the first time, we show what gap junctions and ion channels are expressed in the atrioventricular node and in what quantities. We show that the expression is specialized (different from that in the working myocardium), heterogeneous (it varies in the different regions of the atrioventricular node), and matches the electrophysiology of the atrioventricular node. For example, slow conduction of the action potential through the atrioventricular node can be attributed to the poor expression of the gap junction channel, Cx43, and the sodium channel, Na\(_{\text{v}1.5}\). The pacemaker activity of the atrioventricular node can be attributed to abundant expression of the pacemaker ion channel, HCN4, the expression of a specialised calcium channel, Ca\(_{\text{v}1.3}\) (rather than Ca\(_{\text{v}1.2}\)), and the poor expression of K\(_{\text{v}2.1}\) (responsible for the stable resting potential and absence of pacemaker activity in the ventricles at least). This understanding could lead to the development of new therapies for the atrioventricular node.
Ion Channel Transcript Expression at the Rabbit Atrioventricular Conduction Axis
Ian D. Greener, James O. Tellez, Halina Dobrzynski, Mitsuru Yamamoto, Gillian M. Graham, Rudi Billeter and Mark R. Boyett

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Tissue preparation

Atrioventricular node (AVN) preparations were dissected from the hearts of male New Zealand White rabbits (1.5-2.5 kg). The rabbits were killed humanely according to the United Kingdom Animals (Scientific Procedures) Act, 1986, and the hearts removed. The bulk of the ventricles were removed close to the junction with the atra. The left atrium was removed to leave the intact right atrium. The right atrium was opened by making an incision in the ventral surface. The right atrial free wall together with the superior and inferior vena cava were removed to leave the posterior wall of the right atrium (including the sinoatrial node and triangle of Koch). Finally, a horizontal incision was made immediately superior to the tendon of Todaro to separate the triangle of Koch from the sinoatrial node. Seven triangle of Koch preparations were used for RNA isolation. The preparations were immersed in freezing medium (OCT, BDH), frozen in isopentane cooled by liquid N₂ (-162°C), and stored at -80°C. For RNA isolation, sets of five 14 µm cryosections followed by three 60 µm cryosections were repeatedly cut. The 14 µm sections were mounted on Superfrost Plus glass slides (VWR) and one was stained with haematoxylin and eosin to show the histology of the tissue. The 60 µm sections were placed in pre-cooled (-25°C) cryotubes and subjected to freeze-drying overnight. The next day, guided by a neighbouring 14 µm section, which was stained with haematoxylin and eosin, specific tissues (atrial muscle; nodal extension; proximal penetrating bundle; distal penetrating bundle; ventricular muscle) were microdissected from the freeze-dried sections under a dissection microscope and placed into cryotubes and stored in a chamber containing dessicant. For RNA isolation, tissue was microdissected from roughly 20-25 60 µm sections per region of tissue per rabbit heart. Various features in the haematoxylin and eosin stained sections were used to help identify different regions. First, we were helped by an understanding of the general anatomy of the region (we have recently published a detailed model of the anatomy of the rabbit AVN). Secondly, we knew the position of any section within the original AVN preparation (and, therefore, we knew roughly where to expect a given region). Thirdly, the nodal extension and proximal penetrating bundle are located on the atrial side of the central fibrous body, whereas the distal penetrating bundle is located on the ventricular side (on the apex of the ventricular septum). Fourthly, the penetrating bundle can be distinguished from the nodal extension, because it is enclosed in connective tissue. Various other features, not discussed here, were also used to help identify the different regions. Finally, recognisable differences in the micro-structures of freeze-dried connective tissue and myocytes allowed the excision of the tissues with good precision. Samples of atrial and ventricular muscle were taken from the working myocardium neighbouring the AVN. Finally, 14 µm cryosections from five of the hearts were stored at -80°C and later used for in situ hybridisation.

RNA isolation and generation of cDNA

Total RNA was isolated from the freeze-dried samples using a modified Qiagen fibrous tissue protocol with a DNase digestion step. The RNA was ethanol precipitated and the pellets dissolved in 20 µl of RNase-free water. RNA concentrations were estimated with the RiboGreen assay (Molecular Probes) and the integrity confirmed by formaldehyde gel electrophoresis and capillary electrophoresis on RNA 6000 Nano total RNA Chips (Agilent Technologies, West Lothian, UK). 150 ng of total RNA from each sample was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) in a 20 µl reaction according to the manufacturer’s instructions using random hexamer priming. Aliquots of the resulting cDNA were diluted 10-fold in water for direct use in quantitative PCR (qPCR). The RNA and cDNA samples from different rabbits were kept separate and not pooled.

qPCR and primers

The relative content of selected cDNA fragments was determined, in triplicate at least, with qPCR on 1 µl aliquots of the diluted cDNA using a LightCycler 1.0 (Roche) and detection with SYBR green in 10 µl reactions (primer sequences and annealing temperatures have been described previously) or with an ABI 7900 HT (Applied Biosystems) and detection with a TaqMan probe (6-FAM/TAMRA) in 25 µl reactions (primer and probe sequences have been described previously). All runs were 40 cycles in duration. Results were analysed using one of two methods described below.

Rough comparison of the abundance of different transcripts – single standardisation method

A single standardisation method was used to compare the abundance of different cDNAs. If it is assumed that the efficiency of the reverse transcription step (and the generation of cDNA) is the same for all transcripts, this method can be used as a measure of the relative abundance of different mRNAs. However, it is only a rough estimate of the relative abundance of different mRNAs, because in our experience the yields
for different cDNA fragments along the same RNA can vary by up to a factor of 10 after random priming of the reverse transcription. Therefore, a >10 fold difference between two cDNAs is likely to be the result of a difference in the corresponding mRNAs, whereas a <10 fold difference may not. This method has been used previously. e.g.4

During the PCR, as the cDNA of interest is amplified, a fluorescent product accumulates. For each cDNA and sample, the CT value was determined: the number of PCR cycles required for the fluorescence to reach a critical threshold (chosen to be within the logarithmic phase of the increase in fluorescence; for the single normalisation method, the fluorescence at which the CT value is measured should be the same for all transcripts). The more abundant the cDNA, the lower the number of cycles required to reach the critical threshold (i.e. the lower the CT value). During each PCR cycle, the number of copies of the cDNA is ideally doubled, i.e. increased by a factor of 2; in practice the value (known as the efficiency, \(E\)) may be close to but less than 2. In the present study, for each cDNA, an average efficiency from at least three runs was used. At the critical threshold, the number of copies of a cDNA (\(N_{CT}\)) is:

\[
N_{CT} = N_{cDNA,s}E^{CT_{cDNA,s}},
\]  

where \(N_{cDNA,s}\) is the number of copies of the selected cDNA in the sample (s) and \(CT_{cDNA,s}\) is the CT value of the selected cDNA in the sample. To correct for variations in sample input, the abundance of a selected cDNA in a sample was expressed as a ratio of the abundance of a housekeeper cDNA (h) in the same sample: \(N_{cDNA,s}/N_{h,s}\). In the case of the housekeeper cDNA,

\[
N_{CT} = N_{h,s}E^{CT_{h,s}}.
\]  

It follows from equations 1 and 2 that:

\[
\frac{N_{cDNA,s}}{N_{h,s}} = \frac{E^{CT_{h,s}}}{E^{CT_{cDNA,s}}},
\]  

The housekeeper cDNA should be of equal abundance in all samples. In the present study, we used the average abundance of three housekeeper cDNAs (cDNAs for 28S, GAPDH and the \(\alpha_1\) isoform of the \(\text{Na}^+-\text{K}^+\) pump), which were roughly constant in different samples. In situ hybridisation confirmed that 28S rRNA and GAPDH mRNA were uniformly distributed throughout the triangle of Koch.

**Accurate measurement of the abundance of a transcript in different tissues – double standardisation method**

To measure accurately the abundance of a selected cDNA in different tissues, a double standardisation method (a modified ‘\(\Delta\Delta CT\) method’) was used. This method cannot be used to compare the abundance of different transcripts. With this method, the efficiency of the reverse transcription step is not relevant, because different transcripts are not being compared. The method has been used previously. e.g.3

The method involved a double standardisation. Each PCR run included a calibrator sample (a cDNA sample containing an equal mixture of all tissue types). First, the abundance of the selected cDNA in the sample was expressed as a ratio of the abundance of the selected cDNA in the calibrator sample, \(c\). The abundance of the housekeeper cDNA was calculated in the same way. This first standardisation allowed for variations between runs. Secondly, the abundance of the selected cDNA (as a ratio of the abundance of the selected cDNA in the calibrator sample) was again expressed as a ratio of the abundance of the housekeeper cDNA (as a ratio of the abundance of the housekeeper cDNA in the calibrator sample):

\[
\frac{N_{cDNA,s}/N_{cDNA,c}}{N_{h,s}/N_{h,c}} = \frac{E^{CT_{cDNA,s}}/E^{CT_{cDNA,c}}}{E^{CT_{h,s}}/E^{CT_{h,c}}} = \frac{E^{CT_{cDNA,s}}}{E^{CT_{h,s}}}\frac{E^{CT_{h,c}}}{E^{CT_{cDNA,c}}},
\]  

For all cDNAs and samples, at least three separate measurements were made. After the removal of any outliers, an average was used. The ratio of ratios (equation 4) is close to one. To make the measurements more meaningful, finally, the measurements were expressed as a percentage of the mean atrial muscle measurement.

**Accuracy of sampling for qPCR**

To test whether our sampling was accurate and if there was contamination from atrial muscle, we used ANP as a marker of atrial muscle (it is assumed that no ANP is expressed in AVN tissue). The maximal contamination of the samples of nodal extension, proximal penetrating bundle and distal penetrating bundle was estimated to be ~2-5 % (Fig. 1C). To confirm that there was little contamination, neurofilament was used as a marker of AVN tissue. There was an approximately 6- to 15-fold enrichment of neurofilament mRNA in the AVN samples as compared to the atrial muscle sample (Fig. 1B). Contamination of the AVN samples from ventricular muscle was unlikely during dissection, because the ventricular muscle was clearly
separated from the AVN tissue by connective tissue. It is concluded that there is little contamination of the AVN samples by atrial and ventricular muscle. Marionneau et al. measured the abundance of various transcripts in the mouse AVN. Unlike in the present study, they did not investigate the different regions of the AVN and they did not microdissect the nodal tissue. Instead, they used the whole of the triangle of Koch region - they reported a maximum 43 % contamination of the AVN sample by atrial muscle and <23 % contamination by ventricular muscle. In the present study, the results of in situ hybridisations confirmed our findings with qPCR. For example, in situ hybridisation showed KChIP2 to be absent in the nodal extension, proximal penetrating bundle and distal penetrating bundle, but present in the atrial and ventricular muscle (Fig. 7) - a similar pattern of expression was seen with qPCR (Fig. 6C). It cannot be ruled out that there was contamination from other sources (e.g. neuronal tissue); however, in situ hybridisation shows unequivocally myocyte (versus non-myocyte) mRNA expression and, for all transcripts for which both qPCR and in situ hybridisation were used, the myocyte expression of the transcript matched the expression pattern revealed by qPCR.

**In situ hybridisation**

For in situ hybridisation, we used digoxigenin (DIG) labelled cRNA probes. These probes have been described previously. In brief, cDNA fragments for ion channels etc. were isolated from rabbit heart or brain cDNA by PCR and linked to SP6 or T7 promoters either by subcloning into pGEM-T Easy (Promega) or by the use of the Lig n’ Scribe system (Ambion). cRNA probes were synthesised by in vitro transcription from SP6 or T7 promoters using the Mega Script kit (Ambion), at a ratio of ~4 unlabelled UTPs (Ambion) to 1 DIG-UTP (Roche).

The protocol for in situ hybridisation was adapted from Braissant et al. The 14 µm sections (see above) were fixed in 4 % paraformaldehyde/phosphate buffer saline (PBS) for 10 min at room temperature and incubated twice for 10 min in acetic anhydride (0.25 %) in 0.1 M triethanolamine (pH 8). The sections were brought to boil in a microwave oven at 800 W in 10 mM sodium citrate (pH 6) and kept boiling for 2.5 min, before letting the solution cool at room temperature to ~70°C. This step was repeated three times. Thereafter, the sections were equilibrated in 5x saline-sodium citrate buffer (SSC) at room temperature for 15 min before a 2 h prehybridisation at 58°C in 50 % formamide, 5x SSC and 40 µg/ml herring sperm DNA. Sections were then hybridised for 60 h at 58°C, with 0.3 ng/µl of DIG-labelled cRNA probes in 50 % formamide, 5x SSC and 40 µl/ml herring sperm DNA. Some of the mRNAs were simultaneously hybridised with up to three different probes, which resulted in increased sensitivity. In this case, each of the probes was used at a concentration of 0.3 ng/µl. Antisense (complementary to the mRNA fragment) and sense (identical to the mRNA fragment) cRNA probes were used. The antisense probe detected the mRNA, whereas the sense probe served as a negative control. In all cases, the sense probes did not produce labelling. After hybridisation, the sections were washed for 30 min in 2x SSC at room temperature, followed by two 1 h washes at 65°C, the first in 2x SSC, the second in 0.1x SSC. This was followed by RNase treatment with 0.5 µg/ml RNase A (Sigma) for 30 min in order to degrade unbound probe. The sections were then washed twice in 2x SSC for 5 min before another stringent wash in 0.5x SSC and 20 % formamide at 60°C for 10 min. The sections were washed again twice in 2x SSC for 5 min. They were then adjusted for 10 min in DIG buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), followed by DIG buffer 2 (same as DIG buffer 1, but with 1 % blocking reagent; Roche) for 1 h, before the application of anti-DIG alkaline phosphatase coupled Fab (Roche), in DIG buffer 2 at a dilution of 1:5000 for 2 h. Unbound antibody was subsequently washed away by two 15 min washes in DIG buffer 1. Before the visualisation of the bound phosphatase, the sections were adjusted for 5 min in DIG buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl2, pH 9.5). They were then incubated for 8-56 h in DIG buffer 3 supplemented with 10 % polyvinyl alcohol (PVA), and 0.4 mM NBT (4-nitro-blue-tetrazolium-chloride, Roche) and 0.4 mM X-phosphate (5-bromo-4-chloro-3-indolyl-phosphate, Roche). The staining reaction was stopped with three 15 min washes in TE buffer (0.1 M Tris, 1 mM EDTA, pH 8.0). The sections were embedded in Kaiser’s gelatine.

The in situ hybridisation product was visualised either by standard light microscopy or by confocal microscopy. In the case of confocal microscopy, the section was illuminated with 565 nm light and emitted light at 823 nm was detected.

**Histology**

14 µm sections were stained with Masson’s trichrome as previously described or haematoxylin and eosin stain.

**COMPARISON WITH PREVIOUS STUDIES**

Using qPCR, Marionneau et al. studied ion channel expression in the mouse AVN, but they dissected and isolated RNA from the whole of the triangle of Koch region and this will include various cell types – they estimated 43% contamination of the AVN tissue by atrial muscle. Bearing in mind that we have
examined three nodal regions, whereas Marionneau et al.\textsuperscript{4} studied one mixed region, their data for Cx43, Cx45, Na\textsubscript{a}.1.1, Ca.1.3, ERG and K\textsubscript{LQT1} are qualitatively similar to the data shown here, whereas their data for Cx40, RYR2, SERCA2, Na\textsubscript{a}.1.5, Ca.1.2, HCN1, HCN4, K\textsubscript{r}.1.4, K\textsubscript{r}.1.5, K\textsubscript{r}.4.2, KChIP2, minK, K\textsubscript{a}.2.1, K\textsubscript{a}.6.2 and SUR2 are not. Although the differences in ion channel expression in the two studies can be explained in part by the nature of the tissues sampled in the two studies, some of the differences may be species differences. For example, in the present study of the rabbit, Cx40 mRNA was present and did not vary between tissues. In contrast, in the mouse, Marionneau et al.\textsuperscript{4} reported Cx40 mRNA to be abundant in atrial muscle, present (but less abundant) in the AVN and negligible in the ventricles in agreement with other studies.\textsuperscript{5} Using similar techniques to those used in the present study, in the human, we have observed a similar expression pattern to that in the mouse: for example, we observed a significantly greater abundance of Cx40 mRNA in the distal penetrating bundle as compared to the ventricles (H. Dobrzynski and I.D. Greener, unpublished data). It is possible, therefore, that Cx40 expression varies in different species. In the AVN, as in the sinoatrial node, Cx45 is a major connexin isoform and yet, surprisingly, in the present study, Cx45, although present, did not vary among the tissues (Fig. 1). However, the same uniform distribution has been observed in the rabbit sinoatrial node,\textsuperscript{3} mouse AVN,\textsuperscript{4} and human sinoatrial node and AVN (H. Dobrzynski, N.J. Chandler and I.D. Greener, unpublished data).

REFERENCES


Figure S1. Distribution of ANP mRNA as detected by *in situ* hybridisation in a section cut at the level of the nodal extension. Red dashed line outlines nodal extension. Black dashed line shows border between ANP mRNA expressing (right) and non-expressing (left) tissue.
Figure S2. Distribution of Ca\textsubscript{v}1.2 (left) and Ca\textsubscript{v}1.3 (right) mRNAs as detected by \textit{in situ} hybridisation in sections cut at the level of the compact node. Red dashed line outlines compact node.