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Developmental Changes in Postnatal Murine Intestinal Interstitial Cell of Cajal Network Structure and Function

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Abstract

The mammalian gastrointestinal (GI) tract undergoes rapid development during early postnatal life in order to transition from a milk to solid diet. Interstitial cells of Cajal (ICC) are the pacemaker cells that coordinate smooth muscle contractility within the GI tract, and hence we hypothesized that ICC networks undergo significant developmental changes during this early postnatal period. Numerical metrics for quantifying ICC network structural properties were applied on confocal ICC network imaging data obtained from the murine small intestine at various postnatal ages spanning birth to weaning. These imaging data were also coupled to a biophysically-based computational model to simulate pacemaker activity in the networks, to quantify how changes in structure may alter function. The results showed a pruning-like mechanism which occurs during postnatal development, and the temporal course of this phenomenon was defined. There was an initial ICC process overgrowth to optimize network efficiency and increase functional output volume. This was followed by a selective retaining and strengthening of processes, while others were discarded to further elevate functional output volume. Subsequently, new ICC processes were formed and the network was adjusted to its adult morphology. These postnatal ICC network developmental events may be critical in facilitating mature digestive function.

Keywords

Confocal microscopy; gastrointestinal; pruning

Introduction

In mammals, nutrition supply via the placenta ceases at birth and the animal becomes reliant upon the GI tract to digest and absorb nutrients from milk¹. Shortly after, the GI tract has to be prepared for the dietary changes that occur during weaning¹⁰. The GI tract must therefore undergo adaptations to cope with this changing workload.

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GI motility is facilitated by specialized cells called ICC⁴. One population of ICC which resides in the myenteric plexus (ICC-MP) between the circular and longitudinal smooth muscle layers is responsible for generating the pacemaker potential that co-ordinates small intestinal motility¹². In mice, ICC or their precursors first appear in the intestinal wall at embryonic day 12 (E12)^{16, 32}. An intact ICC-MP network with functional pacemaker activity is formed by postnatal day 2 (P2)¹⁸, whereas another population of ICC in the small intestine at the level of the deep muscular plexus (ICC-DMP) form shortly after birth, before P5^{31, 32}. Despite the early appearance of ICC, these networks continue to develop postnatally. ICC-MP cell density increases after birth and peaks at approximately two weeks of age before decreasing^{16, 23, 31}, and the network morphology adapts throughout postnatal life²³. Also, the full adult ultrastructural phenotype of ICC-MP is only obtained by P17⁶ and after P21 for ICC-DMP⁵.

It is well known that the central nervous system (CNS) ensures the development of a functional neuronal circuitry through the robust mechanism of initial excessive overgrowth followed by selective pruning of axon branches¹⁹⁻²¹, and a recent study has also identified postnatal development in the electrophysiological and morphological properties of neurons in the enteric nervous system (ENS)⁷. Similar to these concepts, we recently proposed a potential pruning-like mechanism that occurs in postnatal ICC network development by comparing 3-day-and 4-week-old murine intestinal ICC-MP networks⁹. This study therefore aimed to identify, quantify, and characterize structural changes in ICC network imaging data acquired at multiple ages throughout early postnatal life to test the presence of a pruning-like mechanism, and to elucidate the temporal course of this process. In addition, biophysically-based computational simulations of ICC pacemaker activity over the imaged ICC networks^{3,8} were performed to gain quantitative insights into the functional significance of the identified structural changes.

Materials and Methods

ICC Network Imaging Data

ICC network imaging data was collected using methods as described previously^{17,23}. All experiments were performed in accordance with the Health Guide for the Care and Use of Laboratory Animals of the Third Military Medical University (Chongqing, China). In summary, five to ten minutes prior to laparotomy the calcium antagonist papaverine (0.12 mg/g) was injected intraperitoneally into 29 BALB/c mice at postnatal day 0 (P0), P2, P5, P8, P12, P16, and P24 (P0-P8: n=5 for each age; P12-P24: n=3 for each age) in order to abolish contractile activity of the small intestine. The entire small intestine (from the pylorus to the ileocecal junction) was removed and placed into PBS containing papaverine (0.5 mg/ml) and the diameter and length of the small intestine was measured immediately. To obtain whole-mount preparations, the small intestine was inflated back to these original dimensions and fixed for 30 min at 4°C with acetone. The longitudinal smooth muscle layer containing the ICC-MP network was prepared under a dissecting microscope, rinsed in PBS and placed in PBS containing 0.3% Triton X-100 at 4°C for 5-10 min. The specimens were first incubated with a blocking solution (4% BSA/PBS) for 30 min at room temperature to avoid non-specific staining, followed by a rat monoclonal anti-c-*Kit* antibody (ACK2, 1:100;

eBioscience) for 8 hr at 4°C. Immunoreactivity was then detected using a Cy3-conjugated secondary antibody (anti-rat IgG, 1:100; Zymed). All specimens were handled in a consistent manner. Confocal image slices of the *Kit*-positive ICC-MP structures were sequentially taken throughout the transmural depth of the specimen, but as the ICC-MP network was thin in the transmural direction ($\approx 10 \mu\text{m}$) and the majority of processes laid in-plane, the image slices were stacked into a 2D representation of network structure containing all positively labeled cells and processes as performed previously^{3,23}. A total of 61 tissue samples were taken, and the number of samples at each age is shown in Table 1. The physical dimensions of the samples ranged from $0.363 \times 0.379 \text{ mm}$ to $0.379 \times 0.388 \text{ mm}$, whereas the resolutions ranged from $0.379 \mu\text{m/pixel}$ to $0.424 \mu\text{m/pixel}$.

Unbiased thresholding algorithms were used to segment the images and identify the *Kit*-positive structures, thereby minimizing any human influence in the process. This imaging data was then pre-processed to remove artifacts for the application of the ICC network structural metrics⁹. Briefly, small gaps with radius of 2 pixels ($\approx 0.8 \mu\text{m}$) or less in the network structure were joined, and small objects of less than 4 pixels ($\approx 0.65 \mu\text{m}^2$) were removed.

ICC Network Structural Metrics

Four numerical metrics we recently proposed⁹ were used to quantify the structural changes that occur in ICC-MP networks throughout postnatal development:

- 1 *Density*: measures the ICC network volume, representative of the amount of bioelectrical current generated and propagated;
- 2 *Thickness*: measures the width of cellular structures within the ICC network plane, which may impact electrical activity propagation through the network;
- 3 *Hole size*: measures the radius of non-ICC regions within the ICC network plane. Reflects the distribution of ICC throughout the MP (*i.e.*, the ‘tightness’ of the network), and may relate to the uniformity of smooth muscle cell (SMC) activation;
- 4 *Connectivity*: measures the connectivity of the ICC network. Reflects the structural integrity of the ICC network, indicating the cohesion of entrainment pathways³⁴;

Also, to quantify the abundance of ICC processes, an additional fifth metric was developed:

- 5 *Branching index*: computed as *density* divided by *thickness* (*i.e.*, a dense network with thin processes indicates numerous processes).

ICC Pacemaker Activity Simulations

ICC pacemaker activity over the ICC-MP network structures was conducted using a similar approach to previous studies^{3, 8}. Briefly, the ICC networks were discretized into a finite element triangular mesh, with each node point corresponding to a pixel in the ICC network imaging data. The cellular activity of the ICC node points was represented using the biophysically-based Corrias and Buist ICC model² modified to incorporate a finite-state

machine (FSM) approach²⁸, whereas the activity of the non-ICC node points was represented using a passive cell model with a zero active ionic current. The FSM modeled a voltage-dependent entrainment mechanism where the cellular activity of the ICC was divided into the two states of *Active* and *Passive*, and the transition between the states was determined by the membrane potential (V_m), Ca^{2+} dynamics and a non-refractory period which defines the intrinsic frequency of the pacemaker activity. The following rules were defined for the FSM ICC model²⁸:

- 1 If the cellular activity of a node is *Active*, it remains *Active* until the Ca^{2+} dynamics return to a quiescent state. Then it changes to *Passive*.
- 2 If the cellular activity of a node is *Passive*, it remains *Passive* until V_m exceeds an excitation potential threshold while in the non-refractory period, or until the cellular activity of the node has passed the non-refractory period.

The established continuum-based bidomain equations widely used to model cardiac electrical activity¹¹ were employed to model the tissue level activity, and the model was solved using the CHASTE computational framework²⁴. The solution process of the bidomain equations was modified for each ODE time step as described previously²⁸:

- 1 V_m at each node point was evaluated using the bidomain equations.
- 2 Based on the newly obtained V_m , the cellular activity states of the ICC nodes were updated using the FSM rules.
- 3 For *Passive* cellular activity ICC nodes, a zero active ionic current was imposed, which is equivalent to the passive cell model used to represent the non-ICC node points.
- 4 For *Active* cellular activity ICC nodes the ionic current was evaluated using the Corrias and Buist ICC model².
- 5 When transitioning from *Passive* to *Active*, the state variables of the cell model were reinitialized to represent the beginning of a new cycle of Ca^{2+} dynamics.

ICC pacemaker activity was simulated over each of the 61 networks for 1000 ms with an ODE time step of 0.1 ms and a PDE time step of 1 ms. ICC node points within a square corresponding to 1% of the total network area at the corner of the network were set to activate at time $t = 0$ ms as the initial stimulus to the simulations, whereas the remaining ICC node points were activated via the voltage-dependent entrainment mechanism of the ICC model²⁸. The conductivity parameters of the model were selected such that the Ca^{2+} wavefronts propagated through the network at approximately 2 mm/s as observed experimentally²⁶, and were normalized against the resolution of the network imaging data.

Four measures were used to quantitatively assess the simulated ICC pacemaker activity (Fig. 1). One measure was based on the electrical event (average V_m over the network), as this electrophysiological activity contributes to the coordination of small intestinal motility¹², whereas the other three measures were based on the average intracellular calcium concentration over the network ($[Ca^{2+}]_i$):

- 1 *Activation rate*: computed as the network area divided by the activation time (t_a), which was defined as the time taken for the average V_m over the network to reach a threshold of -30 mV. This value was chosen to be slightly lower than the ICC model plateau potential of approximately -25 mV to account for the non-ICC node points;
- 2 *Peak $[Ca^{2+}]_i$ (c_p)*;
- 3 *Time to peak $[Ca^{2+}]_i$ (t_p)*;
- 4 *Half peak $[Ca^{2+}]_i$ time ratio*: computed as the ratio of time to half peak ($t_{0.5p}$) to time to peak $[Ca^{2+}]_i$ (t_p). This measure gauges the dynamics of the $[Ca^{2+}]_i$ upstroke, with a small value (≈ 0) indicating an early initial rise, and a large value (≈ 1) indicating a late initial rise.

More emphasis was placed on the Ca^{2+} dynamics as it plays a key role in the initiation of the electrical event^{27,33}. Specifically, Ca^{2+} release through inositol triphosphate receptors in the endoplasmic reticulum and subsequent Ca^{2+} entry into mitochondria is a prerequisite of ICC electrical pacemaker activity generation³⁶.

Statistical Analysis

The Kruskal-Wallis one-way ANOVA on ranks followed by multiple comparisons (Dunn's test) was applied to identify differences in structure and function between sampling age pairs. Due to the logarithmic nature of the connectivity metric, the logarithm (base 10) of the connectivity metric values were taken before conducting the tests. A p -value of less than 0.05 was considered statistically significant.

Results

Figure 2 shows representative ICC-MP networks from the murine intestine at the various postnatal ages, as objectively defined by having structural metric values near the median of the respective groups.

The simulation time over each network was approximately 2 hr using 48 cores from Intel Xeon X5660, E5-2680, or E7-2870 CPUs. An example of a simulated Ca^{2+} wave propagation sequence from $t = 200$ -1000 ms is shown in Fig. 3. The underlying ICC network was 885×909 pixels at a resolution of $0.42 \mu\text{m}/\text{pixel}$. The Ca^{2+} wave was initiated in the top-right corner and in general, propagated toward the bottom-left corner of the network.

Structural Changes

Significant changes through time were identified in the density, thickness, hole size, and branching index metrics (Kruskal-Wallis test: $p < 0.01$ for all four metrics; Fig. 4), but no change was found in the connectivity metric ($p = 0.07$; data not shown).

The network density from P12 to P24 was higher than that at P5, with the density at P16 being higher than that at P0 as well. The thickness of the ICC processes decreased from P2 to P5, and then increased between P5 and P16. The network hole size decreased by P16 compared to P0, and by P24, the hole size was smaller than at both P0 and P2. The

branching index was higher at P12 and P24 compared to P0 and P2, indicating an increased number of processes between these ages.

Integrating these observations, the temporal course of postnatal ICC network structure development is summarized in Table 2. In the short period immediately after birth (P0 to P2), the ICC network remained in an immature state. Subsequently, an overgrowth in the number of ICC processes had begun by P5 and lasted until P12, as the branching index was higher at P12 than at P0 and P2, but not P5 onwards. This process overgrowth also resulted in an increased density at P12 compared to P5. The decreased thickness at P5 compared to P2 may facilitate the ICC process overgrowth by allowing more processes to be formed per unit cell volume. The lack of a significant difference in branching index at P16 compared to earlier ages indicated that ICC processes had been discarded. However, the retained processes were strengthened due to the increased network density and process thickness at P16 compared to P5, and this strengthening of processes also led to an increased density at P16 compared to P0. Finally, by P24 the higher branching index compared to P0 and P2 again showed formation of new processes while the density remained higher than that at P5. Also, by P16 and P24, the network had smaller holes than at P0 and P2.

Functional Changes

Significant changes through time were identified in the activation rate, peak $[Ca^{2+}]_i$, and time to peak $[Ca^{2+}]_i$ (Kruskal-Wallis test: $p < 0.01$ for all three measures), but no change was found in the half peak $[Ca^{2+}]_i$ time ratio ($p = 0.11$; Fig. 5).

The activation rate at P12 and P16 was higher than at P5. The peak $[Ca^{2+}]_i$ at P12 was higher than at P5, and was higher than that at both P0 and P5 by P16. The time to peak $[Ca^{2+}]_i$ was lower between P12 to P24 compared to P5, with P12 and P16 being lower than P0 as well.

The functional changes are reported in relation to the structural changes below (Table 2). By P12, at the end of the overgrowth of ICC processes, the network had attained higher pacemaker activity transmission efficiency than in the immature state (P0) and before the overgrowth (P5), as seen from the increased activation rate and decreased time to peak $[Ca^{2+}]_i$. The functional output volume quantified by the peak $[Ca^{2+}]_i$ also increased since before the overgrowth (P5). At P16, after the pruning-like phenomenon of ICC processes, the high pacemaker activity transmission efficiency was maintained, and the functional output volume increased since both the immature state (P0) and before the overgrowth (P5). Finally, at P24, after the formation of new ICC processes, the pacemaker activity transmission remained efficient, as the time to peak $[Ca^{2+}]_i$ was still lower than at the start of ICC process overgrowth (P5), but perhaps not as efficient as the optimal levels observed after the overgrowth (P12) and pruning-like phenomenon (P16).

Discussion

This study was motivated by the hypothesis that intestinal ICC-MP networks undergo major structural changes during early mammalian postnatal development, serving to adapt and optimize gut function for acquiring nutrients for growth and survival. In particular, we

wanted to test the presence of a pruning-like mechanism occurring in postnatal ICC network development⁹, similar to that observed in the CNS¹⁹⁻²¹. Understanding this early postnatal developmental process is important because impaired ICC development has been associated with several early postnatal GI disorders, including transient neonatal pseudo-obstruction¹³ and infantile hypertrophic pyloric stenosis³⁵. To achieve this, we applied numerical metrics for quantifying ICC network structural properties on murine intestinal ICC-MP imaging data at various postnatal ages, and successfully evaluated the structure-function relationships in these networks by simulating ICC pacemaker activity over tissue-specific networks using biophysically-based computational simulations.

There was excellent correlation between the timing of statistically significant changes in ICC network structure and function throughout postnatal development, as revealed by the separate approaches of structural metric analysis and biophysically-based modeling. Together, these results elucidated the various events and temporal course of ICC network development, as well as the physiological significance of it. We identified that during postnatal development of ICC networks, there is an initial process overgrowth followed by a pruning-like phenomenon of processes, and subsequently formation of new processes. The concept of axon overgrowth followed by pruning during development is well established in the CNS¹⁹⁻²¹ as a mechanism to ensure the development of a robust neuronal circuitry. Similarly, in ICC network development, an abundance of ICC processes are initially grown to achieve high pacemaker activity transmission efficiency, which may also ensure propagation stability and avoid the development of arrhythmias, as well as to increase the output volume of the pacemaker activity. Subsequently, during the pruning-like phenomenon, the less efficient or redundant processes are discarded without impacting the overall efficiency of the network, and the retained processes are also strengthened to further elevate the pacemaker activity output volume. Mice wean at three weeks of age, and hence the formation of new processes after the overgrowth-pruning-like cycle may offer a chance for the ICC network to adjust to the altered luminal contents and workload. Our simulation results showed that pacemaker activity transmission was still efficient after the formation of new processes, but was not as pronounced as after the overgrowth and pruning-like phenomenon. Therefore, these new processes may develop with objectives other than optimizing pacemaker activity. One possibility would be development in response to myenteric neurons, which adapt morphologically and electrophysiologically throughout postnatal life until adulthood⁷, as ICC are also responsible for integrating neurotransmission with its pacemaker activity¹⁴. The reduction in hole size observed around the time of weaning (P16 and P24) indicates an improved spatial distribution of the generated pacemaker activity, which may aid in propagating this pacemaker activity through the thickened smooth muscle layers²³.

It is also interesting to note the close correspondence between the structural changes identified in this study and other aspects of ICC and intestinal development presented previously. After birth, the ICC network structure remained in an immature state until P2. It has been shown that ICC-MP only establish mature ultrastructural features and electrophysiological activity during this period¹⁸, and hence development in the network structure may not occur before the individual cells have fully developed (*i.e.*, development

during this period focuses on intracellular rather than intercellular aspects). Also, it is known that the *Kit* receptor is essential for ICC development and intestinal pacemaker activity^{12,22}, but *W^{bd}/W^{bd}* mutant mice with a down-modulation of *Kit* expression¹⁵ display a normal network of ICC at P5, and the lack of a functional ICC network and intestinal pacemaker activity in adult *W^{bd}/W^{bd}* mice is due to hampered ICC development after P5¹⁶. Therefore, inadequate signaling through the *Kit* receptor may lead to a failure in the initial overgrowth of ICC processes and consequently disrupts the downstream developmental events.

Structural metric analysis did not show any change in ICC network connectivity from birth through to weaning. This result was as expected since cohesion of the entrainment pathways is crucial for functional pacemaker activity³⁴, which is present throughout normal postnatal development. Also, the lack of any differences in the half peak $[Ca^{2+}]_i$ time ratio functional measure indicates that the $[Ca^{2+}]_i$ upstroke dynamics do not change throughout postnatal life. That is, the $[Ca^{2+}]_i$ upstroke waveform is approximately linearly scaled at different postnatal ages.

The small intestine grows significantly in size throughout postnatal life^{23,29} which should result in continuous stretching of the ICC network, but the identified non-monotonic variation in network structure through time indicates that these observed changes are not simply an epiphenomenon of growth and the accompanying stretch. On the other hand, stretch to the tissue induced during the experimental procedure may influence some of the structural metric and functional measure values reported. Although we have taken precautions to minimize these errors (*i.e.*, meticulous care in tissue handling, multiple samples), it is difficult to quantify the extent of the induced stretch and hence the magnitudes of the introduced errors. However, as all animals and tissues were treated in exactly the same manner, we can anticipate that the degree of stretch is quite consistent across specimens, therefore reducing the contribution of this variable to the data variance. Nonetheless, methods for further addressing this issue need to be investigated. One such strategy is to co-stain and count smooth muscle nuclei, which we used in a previous publication to demonstrate that ICC loss in gastroparesis is not a secondary consequence of gastric distension in these patients²⁵. Although stretch effects would not be eliminated, smooth muscle nuclei counts can quantify the relative levels of stretch between different tissue samples, and hence can be used as a normalization factor for standardized analysis.

Both the structural and functional analysis techniques employed in this paper view the ICC network as a functional syncytium and individual cell boundaries are not considered. We believe this approach is appropriate as ICC-MP networks are highly connected and hence individual cell boundaries (*i.e.*, which processes belong to which cells) cannot be distinguished at the levels of magnification employed in this study³⁰. Therefore, the reported changes in the branching of ICC processes throughout postnatal development are relative to per unit cell volume as opposed to per cell, and as the volume of individual ICC may not remain constant throughout development, the results of this study cannot be directly correlated to changes in ICC numbers.

The intervals between the sampling ages were selected to be sparser for the older ages (*e.g.*, only 2 days from P0 to P2 but 8 days from P16 to P24) because we expected the rate of

development to decay with time. We believe these sparser sampling ages have been able to exhibit a general trend in the postnatal development of ICC networks, but further studies with a more refined temporal resolution may better capture the details of this developmental process. Another potential limitation of this work is that the ICC networks have been sampled from throughout the small intestine (*i.e.*, from the duodenum, jejunum, and ileum), and we have assumed comparable developmental patterns across the organ. Another avenue of future work will be to examine whether any differences in developmental pattern exist between the different small intestinal regions, and if present, identify the interaction effects between spatial region and temporal development.

In conclusion, this study has employed quantitative metrics and computational modeling to assess the structural and functional changes that occur in postnatal development of murine ICC networks. The analysis identified a pruning-like mechanism during postnatal ICC network development which may facilitate mature digestive function, and elucidated the temporal course of this developmental process.

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Abbreviations

CNS	central nervous system
DMP	deep muscular plexus
ENS	enteric nervous system
GI	gastrointestinal
ICC	interstitial cells of Cajal
MP	myenteric plexus
SMC	smooth muscle cell(s)

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Fig. 1.

Example (a) average V_m and (b) average $[Ca^{2+}]_i$ traces of an ICC pacemaker activity simulation. t_a is the activation time, defined as the time when the average V_m over the network reaches the threshold of -30 mV, used to calculate the activation rate; c_p is the peak $[Ca^{2+}]_i$; t_p is the time to peak $[Ca^{2+}]_i$; and $t_{0.5p}$ is the time to half peak $[Ca^{2+}]_i$ used to calculate the half peak $[Ca^{2+}]_i$ time ratio.

Fig. 2.

Example ICC-MP networks from the murine intestine at various postnatal ages as revealed by *Kit* immunoreactivity. The networks in the second and third rows are enlarged views of the bottom-right quarter and sixteenth of the networks in the top row respectively, as indicated by the white squares in the P0 network in the first row. Scale bar (bottom right) = 100 μm , 50 μm and 25 μm for the first, second and third rows respectively.

Fig. 3.

Example simulated Ca^{2+} wave propagation sequence over the P0 network in Fig. 2. The black represents the non-ICC regions with a $[\text{Ca}^{2+}]_i$ of 0 nM, whereas the blue to red represents the varying levels of $[\text{Ca}^{2+}]_i$ in the ICC over time. The first image on the left in the top row shows the Ca^{2+} distribution at time $t = 200$ ms, and each subsequent image, from left to right, then down to the next row, is 100 ms apart. The underlying ICC network was 885×909 pixels at a resolution of $0.42 \mu\text{m}/\text{pixel}$. The Ca^{2+} wave was initiated in the top-right corner and in general, propagated toward the bottom-left corner of the network.

Fig. 4.

Variation in the structural metric values through time. Cross (x) symbols indicate the respective mean metric values at each age. Asterisk (*) symbols indicate a significant difference in metric values compared to the age noted below the asterisk.

Fig. 5.

Variation in the functional measures through time. Cross (×) symbols indicate the respective mean measure values at each age, and plus (+) symbols indicate outliers. Asterisk (*) symbols indicate a significant difference in functional measure values compared to the age noted below the asterisk.

Table 1

Number of ICC network samples taken at each postnatal age.

Age	Number of samples
P0	8
P2	9
P5	5
P8	11
P12	10
P16	10
P24	8
Total	61

Table 2

Summary of the structural and functional changes in ICC network during postnatal development.

Age	P0	P2	P5	P8	P12	P16	P24
Structure	-		Increased abundance of ICC processes.			Selected processes are retained and strengthened, while others are discarded.	New ICC processes are formed, and the network is adjusted to its adult morphology.
Function	-		Pacemaker activity transmission efficiency and output volume increased.			Pacemaker activity transmission efficiency maintained and output volume further elevated.	Pacemaker activity transmission remained efficient.