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BIOLOGICAL PACEMAKERS IN CANINES EXHIBIT POSITIVE CHRONOTROPIC RESPONSE TO EMOTIONAL AROUSAL

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ABSTRACT

Background: Biological pacemakers based on the HCN2 channel isoform respond to beta-adrenergic and muscarinic stimulation, suggesting a capacity to respond to autonomic input.

Objective: The purpose of this study was to investigate autonomic response to emotional arousal in canines implanted with murine HCN2-based biological pacemakers using gene therapy.

Methods: An electronic pacemaker was implanted with its lead in the right ventricular apical endocardium (VVI 35 bpm). An adenoviral HCN2/GFP construct (Ad-HCN2, n=7) or saline (control, n=5) was injected into the left bundle branch on day 2 after radiofrequency ablation of the atrioventricular node to induce complete atrioventricular block. Emotional arousal was achieved by presenting food following an overnight fast. Autonomic control was evaluated with Poincaré plots of R-RN against R-RN+1 intervals to characterize heart rate variability (HRV) and with continuous RR interval assessment via 24-hour ambulatory ECG. The 24-hour ECG and Poincaré plot shape were analyzed.

Results: During day 1 after biological pacemaker implantation, Poincaré HRV parameters and RR intervals were unchanged with food presentation. However, on day 7, food presentation was accompanied by an increase in HRV (SD1, P<0.07, and SD2, P<0.05) and shortening of RR interval (P<0.05) in dogs with Ad-HCN2 but not in controls.

Conclusion: This is the first demonstration that biological pacemakers are capable of responding to natural arousal stimuli to elicit appropriate chronotropic responses, a potential advantage over electronic pacemakers.
INTRODUCTION
A distinct advantage of intrinsic cardiac pacemakers over their electronic replacements is their seamless response to physiologic stimuli, thereby adapting heart rate to the metabolic needs of the body. Although electronic pacing is a lifesaving technology and rate-responsive units now are in use, the nuanced input of the autonomic nervous system is difficult to replicate. Recent experiments in the engineering of biological pacemakers based on the HCN family of genes has demonstrated good responsiveness to infusion of catecholamines and to vagal stimulation. However, such testing does not provide information regarding autonomic control in a conscious animal. Specifically, whether activation of the sympathetic nervous system in response to emotional arousal would be associated with a significant increase in the activity of a genetically engineered biological pacemaker remains unknown.

The purpose of this study was to evaluate whether a stimulus that reproducibly evokes emotional arousal during sinus rhythm does so as well in dogs implanted with a biological pacemaker. A positive outcome would provide evidence of the autonomic responsiveness of these pacemakers. The paradigm is based on a standardized protocol in which we consistently observed significant cardiovascular responses to presentation of food following an overnight fast. To evaluate the responsiveness of the biological pacemakers to emotional arousal, we performed quantitative Poincaré analysis of heart rate variability (HRV), which circumvents the stationarity requirements associated with conventional time- and frequency-domain HRV techniques. RR interval was also continuously assessed.

MATERIALS AND METHODS
Experiments were performed using protocols that were approved by the Columbia University Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Preparation and implantation of the adenoviral HCN2 construct
Adenoviral constructs of murine HCN2 (Ad-HCN2) and enhanced green fluorescent protein (Ad-GFP) driven by the CMV promoter were prepared as previously described. To obtain high-titer stocks suitable for in vivo injections, both constructs were amplified in HEK293 cells, harvested, purified, and concentrated via CsCl banding and subsequently titrated. The final titer for each virus was ≥10^11 fluorescent-forming units per milliliter. The adenoviral construct delivered to all dogs was a mix of Ad-HCN2 and Ad-GFP, 3×10^10 fluorescent-forming units each. This delivered construct is referred to throughout the text as Ad-HCN2. For implantation of Ad-HCN2 or saline, 2- to 4-year-old male mongrel dogs weighing 23 to 27 kg (Chestnut Ridge Kennels, Chippensburg, PA, USA) were anesthetized with sodium thiopental induction (17 mg/kg intravenously) followed by inhalational isoflurane (1.5%-2.5%). A pacemaker lead (Flextend, Guidant Corp., St. Paul, MN, USA) was introduced into the right ventricular apex via a jugular
venous approach, and an electronic pacemaker (Discovery II, Guidant) was placed in a subcutaneous pocket in the neck and set at VVI35. Electronic pacing was discontinued completely during arousal testing. Complete heart block was created by radiofrequency ablation of the atrioventricular node (Atakr, Medtronic, Inc., Minneapolis, MN, USA). A custom-modified bipolar 8Fr steerable catheter with a retractable 29-gauge needle (Guidant Corp.) was used to deliver the adenoviral constructs or saline subendocardially. The catheter was introduced into the left ventricle under fluoroscopic guidance via an 8Fr introducer sheath through the right carotid artery. After a stable left bundle branch potential electrogram had been identified, the needle was advanced 3 mm, and 0.1 to 0.2 mL of 3:1 saline-diluted contrast material (50% Hypaque, Nicomed, Melville, NY, USA) was injected to confirm that the needle tip was in the left ventricular wall. The adenoviral construct or normal saline was injected at three sites identified by left bundle branch electrograms (total volume=0.6 mL in each animal). After each injection, cardiac pacing via the injection catheter was performed for pace mapping. The injection sites were within 4 mm of one another. During surgery, ECGs and electrograms were monitored and stored on a personal computer (EMKA Technologies, Falls Church, VA, USA). The 24-hour recordings of heart rhythms were made with a Holter monitor and analyzed on a personal computer (Rozinn Electronics, Inc., Cleveland, OH, USA). Results were reviewed by two independent readers.

Behavioral paradigm
At the time of behavioral testing, a six-lead ambulatory ECG (Dr. Vetter PC-ECG program, Baden-Baden, Germany) was attached to the animals. After an overnight fast, 30-minute baseline ECG recordings were made while the animals rested on days 1 and 7 after implantation of the biological pacemaker or saline. Food then was presented, and the ambulatory ECG recording of the arousal state was obtained. Poincaré HRV parameters and RR intervals were assessed.

Quantitative analysis of Poincaré HRV
The Poincaré plot is a diagram in which each RR interval of a tachogram is plotted as a function of the previous RR interval for a predetermined segment length (Figure 1), producing a graphic display of the plots for visual interpretation and allowing quantitative analysis of the shape of the scattergrams. The center point of the scattergram is located at \((R_{\text{aver}}^\text{R}, R_{\text{aver}}^\text{R})\), where \(R_{\text{aver}}^\text{R}\) is the average RR-interval length for the tachogram. The length of the transverse axis describes the instantaneous beat-to-beat variability of the data, or standard deviation 1 (SD1). The length of the longitudinal axis describes the continuous, longer-term versus instantaneous variability of the data, SD2.

Statistical analysis
Results are given as mean±SEM. Depending on the protocol, statistical significance was determined by Student’s t-test for unpaired data or analysis of variance for repeated measures. Linear regression analysis was also used. \(P<0.05\) was considered significant. The authors had full access to and take responsibility for the integrity of the data.
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RESULTS

As in previous studies from our laboratory, pacemaker function became evident within 2 to 3 days of implantation, with stabilization as a function of time after implantation.

Baseline resting state

ECGs from a representative control animal (Figure 2) and an animal implanted with Ad-HCN2 (Figure 3) illustrate the basic function of spontaneously arising idioventricular rhythm in the resting and arousal states on day 1. Of note, the points of the associated Poincaré plot for the Ad-HCN2 animal during rest (open squares in Figure 4) are tightly clustered. The axis is short and densely packed with some splay in width.

Response to behavioral arousal

The ECG from a representative control animal on day 7 (Figure 5) exhibits a mild increase in heart rate of 8 bpm during arousal. By comparison, an animal implanted with Ad-HCN2 (Figure 6) on day 7, at which time the biological pacemaker is fully expressed, shows a >3-fold greater acceleration in heart rate of 27 bpm in response to arousal. Poincaré analysis of the ECG on day 7 produced a cluster of points along a longer segment of the transverse axis (Figure 7). There is a 616-ms separation of the centroids for rest or arousal in the representative animal, indicative of a more dynamic heart beat pattern during arousal than on day 1, when the separation between the centroids measured 375 ms (Figure 4).

On day 1 following implantation of the biological pacemaker, the response to emotional arousal was minimal in terms of both RR interval decrease (Figure 8A) and

Figure 1. Quantitative analysis of Poincaré plots from a representative animal implanted with HCN2 during a 5-minute rest period on day 1 following biological pacemaker implant. Centroid at 1,500 ms indicates point of the average RR interval. Standard deviation (SD) of instantaneous RR-interval variability, SD1, is visualized as length of the centroid about the transverse axis (lower left to upper right). Standard deviation of long-term continuous RR-interval variability, SD2, is observed as length of the centroid about the longitudinal axis (upper left to lower right). AVG: average.
Figure 2. ECG of a representative control animal on day 1 after saline injection. Slow idioventricular rhythm is observed at baseline during rest (heart rate=42 bpm) and after presentation of food (heart rate=45 bpm).

Figure 3. ECG of the same representative animal shown in Figure 1 on day 1 after injection of Ad-HCN2 biological pacemaker. Slow idioventricular rhythm is observed at baseline rest (heart rate=44 bpm) and increased heart rate during arousal after presentation of food (heart rate=55 bpm).
Figure 4. Poincaré plot of 5-minute ECG recordings during rest and arousal of same animal with biological pacemaker implant in Figures 1 and 3 on day 1. In this example, distinct centroids about the transverse axis are evident for the two behavioral states, with rest (open squares) compared to food presentation (filled squares). The centroid is shifted somewhat by 375 ms upon food presentation, consistent with a general shortening in RR interval.

Figure 5. ECG of same representative control animal shown in Figure 2 on day 7 after saline injection. Slow idioventricular rhythm is observed at baseline rest (heart rate=41 bpm) and after presentation of food (heart rate=49 bpm).
HRV as assessed by quantitative Poincaré analysis (filled squares in Figure 4; Figures 8B and 8C). In striking contrast, on day 7, presentation of food provoked a decrease in RR interval by 425.7±65.9 ms versus 218.0±58.0 ms (mean±SEM) on day 1 ($P<0.05$, Figure 8A). In the seven Ad-HCN2 animals, food presentation on day 7 also elicited significant increases in SD1 and SD2, from 11.3±21.0 and 18.0±22.2, respectively, on day 1 to 37.5±13.4 ($P<0.07$) and 60.4±10.9 ($P<0.05$) on day 7 (Figures 8B and 8C).

Data for day 1 were similar for dogs with Ad-HCN2 and controls during both rest and food presentation. The observed alterations in RR interval, SD1, and SD2 on day 7 in response to food presentation to dogs with adenoviral vector also were significant in comparison with control animals, which exhibited no changes in any parameter from day 1 to day 7 (Figure 8).

**DISCUSSION**

Among the beneficial theoretical advantages that biological pacemakers possess over electronic devices is their potential capacity to adapt to changing physiologic demands associated with mental and/or physical activity. Promising evidence already available indicates that pacemakers based on the HCN2 channel isoform
respond to beta-adrenergic and muscarinic stimulation, suggesting a capacity to respond to autonomic input. We investigated autonomic response to emotional arousal in dogs implanted with Ad-HCN2-based biological pacemakers or saline control in the left bundle branch. Using this methodology, we now provide the first demonstration that biological pacemakers are capable of responding to natural arousal stimuli to elicit appropriate chronotropic responses, a distinct advantage over electronic pacemakers.

Previous studies
In previous studies of biological pacing, approaches included overexpression of $\beta_2$-adrenergic receptors in pig atrium, use of a dominant negative construct to reduce the repolarizing current $I_{K1}$ in guinea pig heart, administration into pig hearts of a cardiogenic cell line developed from human embryonic stem cells, overexpression of the HCN family of pacemaker genes in canine heart via viral or hMSC platforms and administration of various mutant and chimeric ion channel constructs. Autonomic responsiveness was tested in only some of these studies.

Beta-adrenergic responsiveness was observed, as anticipated, in the setting of $\beta_2$-adrenergic receptor expression. Studies of the HCN2 gene administered as an adenoviral construct into atrium revealed a positive chronotropic response to catecholamine infusion. Vagal responsiveness was observed as cessation of impulse initiation following bilateral vagal stimulation. In addition, the HCN2 construct administered adenovirally or via an hMSC cell platform into the ventricle showed an increased idioventricular rate in response to catecholamine infusion. The same was true of an adenoviral construct of the mutant HCN2 channel E324A. These studies of ventricular function did not test any effect of vagal stimulation, given the paucity of vagal innervation to the ventricle. Hence, it was reasonable to conclude, based on preserved cAMP sensitivity of these HCN constructs, that circulating catecholamines could have a positive chronotropic effect, whereas any vagal effect would depend on the proximity of vagal terminals to the pacemaker implant.

Present findings
Our observations are novel in that they demonstrate the capacity of biological pacemakers to respond to emotional arousal, a far more natural stimulus than the infusion of catecholamines or electrical stimulation of the vagi previously reported. The intensity of the stimulus is comparable to that observed during the course of daily activities. Of note, at 7 days after implantation of biological pacemakers, food presentation yielded a positive chronotropic response, indicated by shortening of RR interval and a shift in the centroid of average RR intervals, providing evidence of autonomic responsiveness, which was not present in the control animals. In addition, the animals with biological pacemakers exhibited a significant change in SD1 and SD2, which are indicators of rapid, dynamic heart rate changes and of the long-term variability dynamics of heart beat pattern, respectively. Control animals did not exhibit evidence of autonomic responsiveness.
The capacity of biological pacemakers to respond to emotional arousal after a 7-day period, at which time full expression of the transgene has occurred, has apparent adaptive advantages. Namely, the chronotropic response associated with excitement can act in concert with catecholamine-mediated increases in contractility and stroke volume to increase cardiac output. The adrenergic nature of this behavioral paradigm is supported in prior studies by the facts that plasma catecholamines are markedly elevated and that β-adrenergic blockade blunts the chronotropic response. Although behavioral arousal was used as a test stimulus in the present study, it is likely, but unproven, that the capacity of biological pacemakers to respond to an adrenergic stimulus would also apply to other physiologic challenges, particularly exercise.

A notable feature of the beat-to-beat pattern observed 7 days following implantation is the greater variance in the RR interval, suggested by the changes in SD1 and SD2 (Figure 8). Based on an extensive HRV literature, the presence of variability in the RR interval is associated with cardiovascular health. For example, a number of disease states, including diabetic neuropathy, myocardial infarction, heart failure, as well as advancing age, are associated with reduced HRV. Cardiovascular health-enhancing activities, such as exercise training, are known to increase HRV as well as baroreceptor reflex sensitivity. Thus, the observation that the HRV markers of long-term variance were increased after 7 days suggests a general beneficial impact on

![Figure 7. Poincaré plot of 5-minute ECG recordings during rest and arousal of same Ad-HCN2-injected animal shown in Figure 1, Figure 3, Figure 4 and Figure 6 on day 7. In this example, the separation of the centroids for rest and arousal increased from baseline (open squares) to 616 ms with food presentation (filled squares). The centroid is shifted nearly twice as much along the transverse axis on day 7 (616 ms) as on day 1 (375 ms in Figure 4) upon food presentation, consistent with greater autonomic response.](image)
cardiovascular regulation associated with the dynamics of the chronotropic response to the emotional arousal stimulus following biological pacemaker implantation.

Although the present study does not permit direct elucidation of the mechanisms whereby behaviorally mediated changes in neural activity affect biological pacemaker function, it is likely that the control points center on the slope of spontaneous diastolic depolarization and the resting membrane potential. Behavioral arousal, by enhancing adrenergic activity, would be expected to affect both control points in the direction of increasing the firing rate of biological pacemakers. This effect is evident in the increase
in centroid shift in dogs treated with adenovirus during food presentation on day 7 compared to day 1. Recent evidence suggests that beta-adrenergic stimulation may also increase the firing rate of pacemakers through an influence on Ca\(^{2+}\) clocks.\(^{20}\) The potential role of the latter mechanisms on biological pacemaker activity, as observed in the present study, remains to be determined.

**Study limitations**

Histologic study of the biological pacemakers was not performed. We previously reported the functional outcome of saline versus Ad-GFP versus Ad-GFP, Ad-HCN2 administration and found no significant difference between saline and Ad-GFP.\(^{11}\)

Moreover, histologic study of saline versus Ad-GFP injection sites has shown inflammatory cell infiltration, likely reflecting a combination of trauma from the injection, saline, and adenovirus (unpublished data). Nonetheless, the chronotropic and HRV changes associated with HCN2 administration are consistent with our previous reports with catecholamine administration\(^{7}\) and are seen only in animals receiving HCN2.

**CONCLUSION**

The present study contributes to progress in the field of biological pacemakers in the evolution from proof of concept to experimental confirmation of appropriate chronotropic response to behavioral arousal, an important step in clinical implementation.

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