Engineering prokaryotic channels for control of mammalian tissue excitability

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The ability to directly enhance electrical excitability of human cells is hampered by the lack of methods to efficiently overexpress large mammalian voltage-gated sodium channels (VGSC). Here we describe the use of small prokaryotic sodium channels (BacNa₅) to create de novo excitable human tissues and augment impaired action potential conduction in vitro. Lentiviral co-expression of specific BacNa₅ orthologues, an inward-rectifying potassium channel, and connexin-43 in primary human fibroblasts from the heart, skin or brain yields actively conducting cells with customizable electrophysiological phenotypes. Engineered fibroblasts (‘E-Fibs’) retain stable functional properties following extensive subculture or differentiation into myofibroblasts and rescue conduction slowing in an in vitro model of cardiac interstitial fibrosis. Co-expression of engineered BacNa₅ with endogenous mammalian VGSCs enhances action potential conduction and prevents conduction failure during depolarization by elevated extracellular K⁺, decoupling or ischaemia. These studies establish the utility of engineered BacNa₅ channels for induction, control and recovery of mammalian tissue excitability.
Voltage-gated sodium channels (VGSCs) exist in all animal organisms and govern processes fundamental to life including neuronal communication, muscle contractions or hormonal release. In electrically excitable tissues, VGSCs enable firing and spread of action potentials (APs) and their loss-of-function mutations cause a variety of neuronal\(^1\)-6, cardiac\(^7\)-8 and skeletal muscle\(^9\)-10 disorders. Similarly, acute tissue injuries resulting in permanent excitability loss (for example, stroke, spinal cord injury and heart attack) are associated with long-term disability and death. In addition to excitable tissues, the VGSCs are expressed in many unexcitable cells including macrophages, microglia and islet \(\beta\)-cells, where they play important roles in phagocytosis, migration, insulin secretion and other cellular processes.\(^1\) The ion-conducting \(\alpha\)-subunits of mammalian VGSCs have four structurally homologous domains, each consisting of \(\sim 300\) amino acids.\(^12\) These four domains are simultaneously transcribed and translated from a single, relatively large (\(\sim 6\, \text{kb}\)) gene. This large gene size precludes stable and efficient expression of mammalian VGSCs in primary cells, a strategy that could potentially lead to new remedies for impaired tissue excitability.

The recent discovery of a large bacterial VGSC family (BacNa\(_\alpha\)) has provided novel insights into the structure and biophysical properties of eukaryotic VGSCs.\(^13\)-17. In contrast to their eukaryotic counterparts, BacNa\(_\alpha\)\(^13\) consist of four identical domains, individually transcribed and translated from single genes of only \(\sim 900\) bp in size. Although the small size of BacNa\(_\alpha\) facilitates their overexpression and purification for crystallography studies, it is plausible that BacNa\(_\alpha\) genes could be efficiently packaged into viral vectors, either alone or with other ion channel genes, and used to stably introduce or modify electrical excitability of mammalian tissues. Furthermore, utilization of BacNa\(_\alpha\) orthologues with diverse biophysical properties\(^13\)-16.19,20, combined with targeted channel mutations, may allow custom engineering of cellular electrophysiological properties tailored to specific tissue types, diseases and individuals.

Here we combined electrophysiological, computational and genetic engineering techniques to establish a versatile BacNa\(_\alpha\)-based platform that enables stable conversion of unexcitable primary human fibroblasts into AP conducting cells that can successfully recover conduction slowing in an in vitro model of cardiac interstitial fibrosis. Moreover, we demonstrate that direct expression of engineered BacNa\(_\alpha\) channels in mammalian excitable cells can enhance their excitability and prevent loss of AP conduction in pathological conditions when endogenous Na\(^+\) channels fail to activate.

**Results**

**Genetic engineering of actively conducting human fibroblasts.** To stably convert unexcitable primary fibroblasts into actively conducting cells (named engineered fibroblasts or \(\text{E-Fibs}\)), we first explored a BacNa\(_\alpha\) variant from \(\text{Roseobacter denitrificans}\), \(\text{Na}_\text{RosD}\) G217A, shown in our preliminary tests to be capable of generating AP propagation when transfected in a monolayer of HEK293 cells stably expressing Kir2.1 and connexin-43 (Cx43). We generated a high-titre bi-cistronic lentivirus pRRL-CMV-\(\text{Na}_\text{RosD}G217A\)-P2A-eGFP-Kir2.1 encoding both \(\text{Na}_\text{RosD}\) G217A and inward-rectifier potassium channel Kir2.1 (gene \(\text{KCNJ2}\)) fused with enhanced green fluorescent protein (eGFP) (eGFP-Kir2.1), separated by the self-cleaving peptide P2A. Adult human dermal fibroblasts (HDFs) transduced with this virus stably expressed inward-rectifier \(\text{K}^+\) current, \(I_{\text{K1}}\) (Fig. 1a,b), and bacterial Na\(^+\) current, \(I_{\text{Na,RosD}G217A}\) (Fig. 1c,d), at much higher levels compared with endogenous currents, and exhibited a significantly hyperpolarized resting membrane potential of \(-78.4 \pm 0.2\, \text{mV}\) (\(n = 6\)) compared with that of wild-type (wt)-HDFs (\(-23.3 \pm 2.9\, \text{mV};\) \(n = 5\)). The resulting voltage dependencies of activation and inactivation of \(\text{Na}_\text{RosD}G217A\) (Fig. 1e) in transfected HDFs were similar to those previously reported in transfected HEK293s (ref. 15). Importantly, transfected HDFs became electrically excitable, whereby injection of \(1\, \text{ms}\) current pulses of increasing amplitude elicited reproducible firing of an ‘all-or-none’ AP (Fig. 1f). These results demonstrated the ability of BacNa\(_\alpha\) to yield creation of de novo electrically excitable cells.

To enable electrical coupling in excitable HDFs, we co-transduced them with a pRRL-CMV-Cx43-P2A-mCherry lentivirus. Transduced cells robustly expressed membrane-bound Cx43 gap junctions (Fig. 1g), which resulted in improved intercellular coupling compared with wt-HDFs as revealed by fluorescence recovery after photobleaching, albeit not to the same level as measured in neonatal rat cardiomyocytes (Fig. 1h). These engineered HDFs (E-HDFs) were then cultured at confluence in 2 cm diameter monolayers in a random or aligned configuration (Supplementary Fig. 1). When electrically stimulated by a bipolar platinum electrode, randomly oriented E-HDFs supported circular AP spread at an average conduction velocity (CV) of \(3.63 \pm 0.23\, \text{cm}\, \text{s}^{-1}\) (Fig. 1i and Supplementary Video 1). As expected, aligned E-HDFs exhibited elliptical (anisotropic) AP spread with higher CVs along (3.86 \(\pm 0.14\, \text{cm}\, \text{s}^{-1};\) \(n = 5\) versus across (2.07 \(\pm 0.19\, \text{cm}\, \text{s}^{-1};\) \(n = 5\)) cell orientation (Fig. 1j and Supplementary Video 1).

Although the above approach enabled AP propagation in initially unexcitable human fibroblasts, CVs were low, which could be at least in part attributed to the relatively slow activation kinetics of \(\text{Na}_\text{RosD}G217A\).

**Improved E-Fib electrical properties with NavSheP mutants.** To augment the excitability and conduction of E-HDFs, we developed a combined computational and experimental strategy to screen and identify BacNa\(_\alpha\) variants with more favourable properties (Fig. 2a). We set to explore the BacNa\(_\alpha\), orthologue from \(\text{Shewanella putrefaciens}\), \(\text{Na}_\text{SheP}\), for its fast current kinetics. As the wt \(\text{Na}_\text{SheP}\) channel has a highly hyperpolarized voltage dependency of steady-state inactivation, it does not yield AP firing when co-expressed with Kir2.1. We thus proceeded to shift the activation and inactivation curves of \(\text{Na}_\text{SheP}\) to more depolarized voltages based on the previous studies of specific residues in NaChBac channels\(^22\) and significant homology between different BacNa\(_\alpha\), orthologues (Supplementary Fig. 2); we created a library of \(\text{Na}_\text{SheP}\) mutants where either the D60 or E43 residue was substituted with other standard amino acids. These 38 \(\text{Na}_\text{SheP}\) mutants were transiently expressed in a monoclonal HEK293 line stably expressing Kir2.1 and the resulting sodium current was recorded by voltage clamp. The E43 mutants exhibited highly depolarized voltage dependence of activation (Supplementary Table 1); therefore, we focused on detailed characterization of D60 mutants. The D60 mutant channels exhibited a wide range of steady-state and kinetic parameters (Supplementary Fig. 3 and Supplementary Table 1) and predominantly exhibited fast kinetics compared with the \(\text{Na}_\text{RosD}\) channel (Supplementary Fig. 3c,d and Supplementary Table 1). Based on the recorded parameters, we applied computational modelling to infer AP upstroke, threshold, duration and CV that would result from the same expression level of different \(\text{Na}_\text{SheP}\) mutants in an electrically coupled, Kir2.1 HEK293 line (Fig. 2b-e and Supplementary Fig. 4) and constructed \(\text{Na}_\text{SheP}\) D60X-Kir2.1 lentiviruses for three D60X mutants (D60A, D60N and D60S) that exhibited the steepest AP conduction.
upstrokes and highest CVs among different BacNa<sub>e</sub> models. All three mutants exhibited significantly faster kinetics compared with Na<sub>α</sub>RosD G217A in transduced cells (Fig. 2f,g), which allowed generation of APs with shorter duration and sharper upstroke velocity (Fig. 2h–j). Co-transduction of these viruses with Cx43 in HDFS yielded a 2.5-fold improvement in CV compared with the use of Na<sub>α</sub>RosD (Fig. 2k,l and Supplementary Video 2). This demonstrated the potential for combining BacNa<sub>e</sub> mutagenesis and computational modelling, to engineer primary human fibroblasts with desired electrophysiological phenotypes.

**Fine-tuning of E-Fib electrical properties via FACS.** The use of fluorescence reporters in multi-cistronic lentiviruses (for example, pRRL-CMV-ShEP D60N-P2A-eGFP-Kir2.1) allowed us to further vary I<sub>K1</sub> and I<sub>K1</sub> levels, and characterize their functional effects on E-HDFS. Using fluorescence-activated cell sorting (FACS), we sorted E-HDFS into three populations with low, medium and high GFP expression levels (Supplementary Fig. 5a), which yielded corresponding low, medium and high levels of I<sub>K1</sub> (Fig. 3a) and I<sub>K1</sub> (Fig. 3b), and distinct changes in AP shape (Fig. 3c). Specifically, a trend towards more hyperpolarized resting membrane potential (Fig. 3d, one-way analysis of variance, P = 0.15) and the significantly higher maximum upstroke velocity (Fig. 3e) could be attributed to higher I<sub>K1</sub> and I<sub>K1</sub> (Fig. 3f) respectively, whereas the increased APD (Fig. 3f) resulted from net depolarizing effect of increased I<sub>K1</sub> and I<sub>K1</sub> on membrane repolarization. Although higher AP upstroke velocities expectedly yielded higher CVs (Fig. 3g,h), this effect was moderate, which we hypothesized was due to simultaneous increase in I<sub>K1</sub> along with I<sub>K1</sub>. We thus modelled concurrent increase in I<sub>K1</sub> and I<sub>K1</sub> to simulate the experiment (model 1; Supplementary Fig. 6) and compared resulting CVs with those caused by an increase in I<sub>K1</sub> alone (model 2; Supplementary Fig. 6). Despite the same AP upstroke velocities in both models (Supplementary Fig. 6a), CV in model 1 increased less (Supplementary Fig. 6b), because more depolarizing current was needed to overcome high I<sub>K1</sub> threshold and elicit AP (Supplementary Fig. 6c). Examining a wide range of channel conductances (G<sub>Na</sub> and G<sub>K1</sub>) further revealed that although threshold current required to elicit AP is primarily sensitive to level of I<sub>K1</sub> (Supplementary Fig. 6d), AP upstroke velocity is mainly dependent on I<sub>K1</sub> (Supplementary Fig. 6e), with these two currents inducing opposite effects on CV (Supplementary...
significance was determined by one-way analysis of variance, followed by Tukey’s post-hoc test to calculate P-values.

Fig. 6f). Furthermore, as E-HDFs were also transduced with pRRL-CMV-Cx43-P2A-mCherry lentivirus, sorting cells based on mCherry fluorescence level (Supplementary Fig. 5c) allowed us to vary Cx43 expression and gap junctional coupling of engineered cells. Optical mapping revealed that E-HDFs with higher mCherry/Cx43 expression supported faster AP propagation (Fig. 3i), whereas to eGFP sorting (Supplementary Fig. 5b), mCherry sorting had no effect on APD80 (Supplementary Fig. 5d). Simulation results further revealed that intercellular coupling strength does not influence the relative effects of varying \( I_{\text{K1}} \) and \( I_{\text{NaSheP}} \) on CV (Supplementary Fig. 6g).

**Robustness of E-Fib electrical phenotype.** To test whether E-HDFs can maintain stable electrophysiological phenotype under various perturbations, we expanded them 500-fold over a 4-week period, either directly or after 2 freeze–thaw cycles. In both cases, expanded E-HDFs retained proliferative potential (Fig. 4a) and were able to undergo additional rounds of expansion (for example, 100-fold, 4 passages; Fig. 4b). In addition, as fibroblasts can differentiate into smooth muscle actin expressing myofibroblasts during wound healing and tissue repair,23 we treated the expanded E-HDFs with 10 ng ml\(^{-1}\) transforming growth factor-β1 for 7 days, to induce their conversion to myofibroblasts (Fig. 4c,d). Subsequent electrophysiological analysis revealed that despite significant cell expansion or myofibroblast differentiation, the \( I_{\text{K1}} \), \( I_{\text{NaSheP}} \) (Fig. 4e,f) and AP characteristics (Fig. 4g–i) of E-HDFs remained unchanged, demonstrating the stability of the acquired functional phenotype. The ability of these cells to undergo repeated freeze–thaw cycles without phenotypic alterations suggested the potential for off-the-shelf delivery applications. Electrophysiological phenotype of E-HDFs remained stable until the cells reached senescence (after additional eight passages and \( \sim 10^6 \)-fold expansion; Supplementary Fig. 7a–d). Furthermore, using a classical heterologous expression system—monoclonal HEK293 line expressing Cx43, K\(_{\text{v}}\)2.1 and Na\(_{\text{v}}\)SheP D60A—stable electrical properties were maintained beyond 20 passages.
Figure 3 | Tuning electrical properties of E-Fibs via FACS. (a,b) Steady-state $I_{Na}\text{-}V$ curves (a, $n = 8–12$) and peak $I_{Na}\text{-}V$ curve (b, $n = 8–12$) in three groups (blue, red and green denoting low, medium and high eGFP-expressing cells, respectively), measured at 25°C. (c) Representative AP traces with inset showing AP upstrokes in the three eGFP-sorted E-HDF groups. (d-f) Resting membrane potential (RMP, d), maximum AP upstroke velocity (e) and APD$_{80}$ (f) in the three eGFP-sorted E-HDF groups ($n = 14–18$). (g,h) Representative isochrone maps of AP conduction in electrically stimulated anisotropic monolayers of E-HDFs (g) showing that cells with higher eGFP expression had faster CV (h, $n = 6–10$). All E-HDFs were first sorted for high Cx43-mCherry level to ensure strong intercellular coupling. Pulse signs indicate location of stimulating electrode. Circles denote 504 recording sites. (i) CV in electrically stimulated monolayers of three mCherry-sorted E-HDF groups (left, middle and right bars denoting low, medium and high mCherry-expressing cells, respectively; $n = 5$). *$P < 0.01$ in f; *$P < 0.001$ versus low eGFP group in e.h or versus low or medium mCherry group in i. All electrophysiological data obtained at 37°C, unless otherwise specified. Error bars indicate s.e.m; statistical significance was determined by one-way analysis of variance, followed by Tukey’s post-hoc test to calculate P-values.

(Appendix Fig. 7e–g), with cells adapting to higher pacing rates by decreasing APD$_{80}$ and CV (Supplementary Fig. 7h,i).

Applicability to other sources of human unexcitable cells. To further demonstrate the versatility of this approach, we used the Kir2.1-NaSheP D60N lentivirus to stably convert human ventricular fibroblasts (HVFs), human astrocytes (HAs) and HEK293s into engineered electrically excitable cells (E-HVF, E-HAs and E-HEK293, respectively). All engineered cell types displayed strong $I_{NaShep}$ and $I_{K1}$, and fired APs on stimulation (Supplementary Fig. 8). Expression of the Kir2.1 channels hyperpolarized the resting membrane potentials in all transduced cells to a similar value (Fig. 4j), whereas similar $I_{NaShep}$ levels resulted in comparable upstroke velocities (Fig. 4k). Significant differences in AP duration among different cell types (Fig. 4l) could be attributed to differences in their endogenous currents (Fig. 4m) and potentially other differences in epigenetic, post-transcriptional and posttranslational regulation of ion channel expression and function. Collectively, these results confirmed the robustness of the BacNa$_{a}$ engineering approach against the starting unexcitable cell phenotype.

E-Fibs improve conduction in model of interstitial fibrosis. To test whether E-Fibs could improve impaired AP conduction in native excitable tissues, we designed a zig-zag micropattern of neonatal rat cardiomyocytes (Fig. 5a and Supplementary Fig. 9a), which mimics the tortuous electrical conduction of cardiac tissue with interstitial fibrosis 24,25. The cardiomyocyte zig-zag micropattern displayed normal AP propagation in the longitudinal direction (Supplementary Fig. 9b), but significantly slowed down transverse CV (3.2 ± 0.4 cm s$^{-1}$; Supplementary Fig. 9c), which was fully rescued (12.0 ± 0.5 cm s$^{-1}$) by the electrical coupling of actively conducting E-HDFs and cardiomyocytes (Fig. 5b,c, Supplementary Fig. 9g and Supplementary video 3). In contrast, unexcitable wt-HDFs or excitable Na$_{SheP}$ D60A + Kir2.1 HDIFs coupled only by endogenous junctions (no Cx43 overexpression) did not alter the transverse conduction (Fig. 5c and Supplementary Fig. 9d,e), whereas unexcitable HDFs with overexpressed Cx43 yielded only a small conduction improvement (to 6.5 ± 0.3 cm s$^{-1}$; Fig. 5c and Supplementary Fig. 9f). All groups had similar AP durations (Fig. 5d); thus, wavelength differences (Fig. 5e) reflected the differences in CV. Importantly, these studies suggested that slow electrical propagation in cardiac tissues with interstitial fibrosis could be significantly improved if fibroblasts were engineered to actively conduct APs by becoming both excitable and robustly coupled.

BacNa$_{a}$ augment and rescue mammalian tissue excitability. Our computational results showed that select BacNa$_{a}$ mutant (Na$_{SheP}$ D60A) displayed comparable AP upstroke and slightly lower CV than mammalian Na$_{a}$,1.5 channel in an excitable cell model (Fig. 2d,e), suggesting that in addition to custom design of de novo excitable cells and tissues, BacNa$_{a}$ channels could be exploited to directly manipulate or augment 26,27 mammalian tissue excitability. Specifically, when lentivirally expressed in cardiomyocyte cultures, Na$_{SheP}$ D60A channels significantly improved CV from 15.9 ± 0.8 to 26.1 ± 0.7 cm s$^{-1}$ without
altering AP duration (Supplementary Fig. 10). Furthermore, expression of BacNa\textsubscript{v} channels could ameliorate reduced cardiac tissue excitability and arrhythmogenic conduction slowing in various pathological conditions (for example, ischaemia, infarction and congenital disease) associated with membrane depolarization and inactivation of Na\textsubscript{v}1.5 channels\textsuperscript{2,28,29} or diminished intercellular coupling\textsuperscript{30,31}. For proof-of-concept studies, we used a Na\textsubscript{v}SheP D60A mutant, which exhibits diminished intercellular coupling\textsuperscript{30,31}. For proof-of-concept studies, we used a Na\textsubscript{v}SheP D60A mutant, which exhibits diminished intercellular coupling\textsuperscript{30,31}.

By one-way analysis of variance, followed by Tukey’s post-hoc test to calculate \( P \)-values.

**Figure 4 | Stability and robustness of E-Fib phenotype.** (a,b) E-HDFs transduced with Na\textsubscript{v}SheP D60N-P2A-eGFP-Kir2.1 and Cx43-P2A-mCherry lentiviruses retain proliferative potential as shown by Ki-67-positive staining (a) after \( \sim \)100-fold expansion (b), 4 weeks post-lentiviral transduction. Growth curve is fit to a power function. (c,d) Conversion of smooth muscle actin (SMA\textsuperscript{a}) to SMA\textsuperscript{b} myofibroblasts after transforming growth factor (TGF)-\( \beta \) treatment (d). (e-i) Steady-state \( I_{\text{Na}} \)-V (e) and peak \( I_{\text{Na}} \)-V (f) curves (recorded at \( 25 \) °C) as well as resting membrane potential (RMP, g) and AP upstroke velocity (i) do not differ among control, expanded or TGF-\( \beta \)-treated E-HDFs (n = 5–9). (j-l) RMP (j), maximum AP upstroke velocity (k) and APD\textsubscript{80} (l) in different E-Fibs (E-HVF, human ventricular fibroblasts; E-HA, human astrocytes) transduced with the Na\textsubscript{v}SheP D60N-P2A-eGFP-Kir2.1 lentivirus (n = 6–8). (m) Steady-state I-V curves in different wt Fibs (n = 4–6). *\( P < 0.05 \), **\( P < 0.01 \) versus HVF in l. All electrophysiological data obtained at \( 37 \) °C, unless otherwise specified. Error bars indicate s.e.m; statistical significance was determined by one-way analysis of variance, followed by Tukey’s post-hoc test to calculate \( P \)-values.
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**Discussion**

Since their discovery 15 years ago, prokaryotic Na$^+$ channels have contributed greatly to understanding structure, function, and pharmacology of the more complex eukaryotic VGSCs. Yet, it has remained unexplored if large diversity and small gene size of BacNa$_v$ could be employed to stably modify and augment the excitability of mammalian tissues, a feat difficult to achieve using large mammalian VGSCs. In this study, we presented a combined experimental and computational strategy for use of BacNa$_v$ channels in primary cells, efficacy of this approach was validated using large mammalian VGSCs. In this study, we combined both experimental and computational approaches to use of BacNa$_v$ channels in primary tissues.

![Figure 5 | Improvement of AP conduction by E-Fibs in a model of cardiac interstitial fibrosis.](image)

(a) Schematic depicting use of E-Fibs to improve impaired conduction in an in vitro model of cardiac interstitial fibrosis. (b) Immunostaining corresponding to dashed-line square area in a showing vimentin$^+$ E-Fibs in between sarcomeric α-actinin (SAA)$^+$ neonatal rat cardiomyocytes (CMs). (c–e) Average CV (c), APD$_{90}$ (d) and impulse wavelength (WL = CVxAPD$_{80}$). (e) Values during longitudinal or transverse conduction in CM-only cultures (fibrosis control), CM+wt HDF co-cultures, CM+wt HDF co-cultures and confluent anisotropic CM cultures (healthy control) (n = 5–8). $^*$ < 0.001 versus transverse CVs in fibrosis control group. Error bars indicate s.e.m; statistical significance was determined by one-way analysis of variance, followed by Tukey’s post-hoc test to calculate P-values.

Importantly, lentivirally expressed ion currents in all engineered fibroblasts were much larger than their endogenous currents (Fig. 1). As such, resulting electrophysiological phenotypes of engineered cells was relatively insensitive to the cell’s epigenetic state and both predictable in silico and for the most part reproducible among different cell types in vivo (Fig. 4j–l and Supplementary Fig. 5). The ability to generate actively conducting HVFs, in particular, suggested a potential therapeutic strategy whereby in situ fibroblast engineering could improve impaired conduction in fibrotic heart tissues. As a proof of concept, we showed that well-coupled and excitable (but not weakly coupled and/or unexcitable) fibroblasts successfully restored normal neonatal rat cardiomyocyte conduction in an in vitro model of interstitial fibrosis (Fig. 5). On the other hand, distinct forms of cardiac fibroses, differing in size, and cellular and non-cellular components can result from various types of heart disease or injury. In situ engineering of actively conducting fibroblasts, while potentially beneficial in improving cardiac conduction in small regions of interstitial or diffuse fibrosis where these cells come in close contacts with cardiomyocytes, may not prove as effective in large cardiac scars with low cellularity. Of note is also that without the prominent Ca$^{2+}$ currents, engineered fibroblasts...
are unlikely to spontaneously fire APs or be a source of triggered activity, which could potentially make them less arrhythmogenic compared with the use of pluripotent stem cell-derived cardiomyocytes.\(^\text{42,43}\)

Furthermore, transduction of mammalian excitable tissue cultures with specific BacNa\(_r\) mutants augmented or rescued conduction in healthy (Supplementary Fig. 10) and pathological (depolarization, decoupling and ischaemia; Fig. 6 and Supplementary Fig. 11) conditions, suggesting that BacNa\(_r\)-based gene therapies could be targeted directly to excitable cells. In addition, previous studies have shown that BacNa\(_r\) could be converted into Ca\(^{2+}\)-selective channel (BacCa\(_r\)) by substituting three amino acid residues of the selectivity filter with Asparagine.\(^\text{19,44,45}\) Hence, stable expression of engineered BacNa\(_r\) channels or their Ca\(^{2+}\)-selective derivatives may enable direct therapeutic effects on excitable and unexcitable cells whose function relies on voltage-gated Na\(^+\) or Ca\(^{2+}\) channels.

Although in this study we focused on altering specific amino acid residues (E43 or D60) in a particular BacNa\(_r\) orthologue, variations in other BacNa\(_r\) orthologues and residues\(^\text{18,46,47}\) could be explored to customize phenotype of engineered or native excitable cells. For example, mutations in the 'glycine hinge' region in some BacNa\(_r\) orthologues could change function relies on voltage-gated Na\(^+\) or Ca\(^{2+}\) channels.

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Basic studies in 'lower' organisms, including prokaryotes, have led to versatile approaches to manipulate and study mammalian biology. Recent examples include optogenetics\(^\text{51}\) and genome-editing techniques\(^\text{52}\) that, in addition to becoming powerful research tools, have been extensively investigated for potential medical applications. In line with these undertakings, our work supports expansion of prokaryotic ion channel research to basic studies of mammalian tissue excitability and development of new human therapies.

**Methods**

**Screening of bacterial sodium channels.** To engineer excitable cells, we first explored use of NaChBac channel, the longest studied BacNa\(_r\) orthologue.\(^\text{13,17,43,53}\) However, its slow recovery from inactivation yielded excitable cells with very low maximum capture rate (<1 Hz). This prompted us to instead use NaRosD G217A and NaSheP channels that were kindly provided by Dr. Katsumasa Irie from Kyoto University.\(^\text{15}\) Libraries of SheP(D60X) and SheP(E43X) mutants were generated using Quikchange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), as

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**Figure 6 | Improvement of mammalian AP conduction by BacNa\(_r\) in modeled pathological conditions.** (a) Schematic depicting direct expression of BacNa\(_r\) in mammalian excitable tissues containing endogenous Na\(_{1.5}\), to enhance conduction and prevent conduction block in simulated pathological conditions. (b) Steady-state inactivation (h\(_\text{N},5\) of Na\(_\text{SheP}\) D60A is more depolarized compared with that of Na\(_{1.5}\) (n = 5). (c, d) Conduction failure due to inactivation of Na\(_{1.5}\) by membrane depolarization with elevated extracellular K\(^+\) (c, n = 6) or blocking of intercellular coupling by PA (d, n = 12-18) in Ex293 monolayers (black squares) is rescued by co-expression of Na\(_\text{SheP}\) D60A (white circles). (e) Schematic depicting exogenous expression of BacNa\(_r\) in neonatal rat CMs to increase resistance to conduction block in ischaemic conditions. Black dashed circle denotes position of glass coverslip used to induce regional ischaemia in CM monolayer. (f) Progressive conduction slowing until block with time of ischaemia in control (CM) and Na\(_\text{SheP}\) D60A transduced (CM + D60A) monolayers (n = 6). (g, h) Representative isochrones maps of a CM monolayer showing conduction slowing after 6 min (g) and complete block after 11 min of ischaemia (h). Pulse signs indicate location of stimulating electrode. Circles denote 504 recording sites. (i) Under ischaemic condition, CMs transduced with Na\(_\text{SheP}\) D60A lentivirus (white) resisted conduction block longer than control CMs (black) (n = 6, *P < 0.01). Error bars indicate s.e.m; statistical significance was determined by an unpaired Student’s t-test to calculate P-value.
**Lentivirus production.** Lentiviral plasmonics were constructed from the pRRL-CMV vector (a gift from Dr Inder Verma, Salk Institute). BacNAc (Life Technologies). GFP fluorescence was usually detected after 12 h and cells were patch clamp 24 h after transfection, to derive AP parameters. (Life Technologies). GFP fluorescence was usually detected after 12 h and cells were patch-clamped 24 h after transfection, to derive AP parameters.

**vector pCMV5(CuO)-IRES-GFP (Qbiogene) and transfected into HEK293 per the manufacturer’s instructions.** All the primers used are listed in the main text. BacNAc, Kir2.1 and CuX43 lentiviruses refer to pRRL-CMV-BacNAc, pRRL-CMV-Kir2.1 and pRRL-CMV-CuX43-Kir2.1, respectively. Other lentiviral constructs used in the study were pRRL-CMV-BacNAc, -P2A-Kir2.1, -T2A-eGFP and pRRL-CMV-BacNAc, -T2A-eGFP. High titre lentiviruses were produced using second generation lentiviral packaging system. Briefly, 293FT cells (Life Technologies, R700-07) were co-transfected with lentiviral plasmids, packaging plasmid pSPAX2 and envelope plasmid pMD2.G (2:1 mass ratios) using Lipofectamine 2000 (Life Technologies). Supernatant containing lentiviral particles was collected 72 h after transfection, centrifuged (500 g, 10 min) and filtered through a 0.1 μm acetate filter. (Corning) to remove cell debris before being transfected with Lenti-X Concentrator (Promega) at 2 μg/mL cell concentration. For transduction of HEK293 cells, 1 μg/mL of Lenti-X Concentrator (Promega) was used and 500 g, 10 min, cells were resuspended for 30 min in DMEM/F-12 medium (Gibco, C-013-5C) and resuspended in 1/10 to 1/100 of the original volume in DMEM medium. Plasmids pSPAX2 and pMD2.G were obtained from Didier Trono (Addgene plasmids #12260 and #12259).

**Generation of engineered human fibroblasts.** Primary adult HDFs (Cambrex, C-013-5C) were co-transduced with BacNAc-Kir2.1 and CuX43 lentiviruses at a density of 105 cells per cm2 in fibroblast basal medium (ATCC, PCS-201-030). Five days after lentiviral transduction, GFP +/−mCherry + cells were sorted by FACS analysis, replated in fibroblast growth medium (basal medium supplemented with fibroblast low-serum growth kit, ATCC, PCS-201-041) and assessed with patch-clamp recordings and optical mapping at 1 week and 4 weeks after sorting. Conversion of HDFs into myofibroblasts was induced by adding transforming growth factor-β (10 ng mL−1) to culture media for 5 days. Similar lentiviral transduction protocol was repeated in HFVs (Lonza, CC-2904), HAs (Lonza, CC-2565) and HEK293 cells (ATCC, CRL-1573).

**Isotrophic and anisotropic monolayers of HDFs.** E-HDFs were seeded onto 22 mm-diameter Aclar (Electron Microscopy Sciences) coverslips coated with fibronectin ( Sigma, 15 μg mL−1, 30 min) at a density of 5 × 104 cells per cm2 to form isotrophic monolayers. Anisotropic E-HDF monolayers were formed on Aclar coverslips microcontact printed with a pattern of parallel fibronectin lines (10 μm width, 10 μm spacing) as previously described.35 Cells were cultured for 24 h to become confluent and form intercellular junctions followed by optical mapping of AP propagation and immunostaining.

**Micropatterned zig-zag cultures.** All animals were treated in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee. Neonatal rat cardiomyocytes were enzymatically dissociated from ventricles of 2-day-old Sprague–Dawley rats and enriched using differential pre-plating.35 Specifically, ventricles were excised, minced and incubated with 0.1% trypsin overnight and dissociated in four sequential steps using 0.1% collagenase. Isolated cells were centrifuged (500 g, 200 g) and further enriched by two 45 min pre-plating steps to select for cardiomocyte-enriched cells that remained unattached after the second pre-plating. Zig-zag cultures were generated as previously described.35 Briefly, Aclar coverslips (22 mm diameter) were coated with 5 μg mL−1 collagen (1% in PBS). P815 Mast cells and cervical cancer cell line were cultured as culture media. 2 μg mL−1 is also known as cell suspension media. The following primary antibodies (1 h incubation) were used: anti-sarcomeric α-actinin (Sigma, A5781, 1:200), anti-Cx43 (Life Technologies, 71-0700, 1:100), anti-vimentin (Sigma, V6630, 1:500), anti-smooth muscle actin (Sigma, A2547, 1:200), anti-GFAP (BD Biosciences, 561483, 1:100) and anti-Ki67 (Abcam, ab15580, 1:200). Secondary antibodies were Alexa 488 (Life Technologies, A-21200 or A-21411, 1:200), Alexa594 (Life Technologies, A-21201 or A-21412, 1:200), Alexa647 (Life Technologies, A-21463, 1:200), Alexa488-conjugated phallolidin (Life Technologies, A-12379, 1:300) and 4.6-diamidino-2-phenylindole (Sigma, J300). All immunostaining steps were performed at room temperature.

**Fluorescence recovery after photobleaching.** Functional coupling in wt and Cx43-expressing HDFs was assessed using fluorescence recovery after photobleaching.5,27 as previously described.35 Briefly, confluent HDF monolayers were stained with Calcein AM dye (Molecular Probes, 0.5 μM in DMEM) for 20 min and fixed with 4% paraformaldehyde (PFA) and rinsed with PBS at room temperature. The cells were photobleached with a 488 nm Argon laser and subsequently imaged every 15 s for 6 min using an upright confocal microscope (Zeiss, LSM 510). The level of fluorescence in the target cell was normalized to the fluorescence level in remote unbleached cells and plot as a function of time post photobleaching.

**Whole-cell patch-clamp recordings.** Cells were briefly trypsinized, plated onto poly-lysine-coated glass coverslips and left to attach for 5 h. Cells were then transferred to a patch-clamp chamber perfused with Tyrode’s solutions containing (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 0.3 Na2HPO4, 5 HEPES and 5 glucose. Patch pipettes were fabricated with tip resistances of 1–2 MΩ when filled with pipette solution consisting of (in mM): 140 KCl, 1 NaCl, 2 MgCl2, 10 EGTA, 10 HEPES and 3 MgATP. Whole-cell voltage-clamp and current-clamp recordings were acquired at room temperature (25 ± 3 °C) and 37 °C, respectively, using the Multiclamp 700B amplifier (Axon Instruments), filtered with a 10-KHz Bessel filter, digitized at 40 kHz and analysed using WinWCP software (John Dempster, University of Strathclyde). To measure BacNAc, activation properties, membrane voltage was stepped from a holding potential of −100 mV to −50 to −60 mV, inducing 500 ms test potentials (−50 to −60 mV, increasing 10 mV/step). Inactivation of BacNAc was derived from peak currents measured at 0 mV after varying 3 s prepulse potentials (−160 to −30 mV, increments of 10 mV). For the channels with more hyperpolarized activation and inactivation (NaSheP WT and D60E), holding voltage for steady-state activation protocol was set at −160 mV, whereas for steady-state inactivation protocol was set at −50 mV and recorded for 500 ms test potentials (−180 to −30 mV). Steady-state K1/2− Curve was constructed from the current responses to varying 1 s test potentials (−130 to 30 mV, increments of 10 mV) from a holding potential of −40 mV. APs were elicited by injecting a 1 ms current pulse at 1.1 ± 100 mV.

**Optical mapping of AP propagation.** Cell monolayers or zig-zag cultures were optically mapped with a 20 mm diameter hexagonal array of 504 optical fibres (Redshirt Imaging), as previously described.32,56,57 Specifically, cultures were stained with a voltage-sensitive dye (Di-4-ANEPPS, 10 μM) for 5 min at room temperature, transferred to a recording chamber filled with Tyrode’s solution at 37 °C and illuminated by a solid-state excitation light source (Lumencor, SOLA SM) filtered through a 530 ± 30 nm bandpass filter. Emitted red fluorescence signals (λ>590 nm) were collected by the optical fibre array, converted to voltage signals by photodiodes and recorded at a 2.44 kHz sampling rate with a 750 μm spatial resolution. AP propagation was triggered by a 10 ms, 1.2 ± 100 mV, 1 Hz stimulus from either a bipolar point electrode (in isotropic or anisotropic monolayers of E-HDFs) or a line electrode (in the zig-zag cultures). Generation of isochrone maps and calculation of CV, AP duration at 80% repolarization (APD80) and impulse wavelength were performed using custom MATLAB software, as previously described.59,60

**Depolarization and decoupling studies.** Similar to previous studies,61 membrane depolarization in Ex293 monolayers was induced by applying increasing doses of KCl (1 mM increments starting from 5 mM). After 5 min equilibration at each KCl, monolayers were paced at 1 Hz and AP conduction optically mapped. For gap junction decoupling studies, PA (Sigma, 76168) was dissolved in dimethyl sulfoxide and sonicated (10 min, 37 °C) to yield a stock concentration of 50 mM. Ex293 monolayers were exposed to increasing doses of PA (0, 20, 40 and 60 μM) and 15 min equilibration at each dose, cells were paced at 1 Hz and AP conduction optically mapped.

**Regional ischaemia studies.** Regional ischaemia in neonatal rat cardiomyocyte monolayers was induced as described previously.31,61 Briefly, 12 mm round glass coverslip was placed onto the central area of a 22 mm cardiac monolayer to induce regional ischaemia. Cells were continuously paced at 1 Hz and AP conduction optically mapped every 1 min during ischaemia, until complete conduction failure in the ischaemic region. CV, APD80 and optical AP amplitude measured at the beginning of the ischaemic region were corresponding baseline values before ischemia. Recording site was defined as inactive (not conducting) if its AP amplitude fell below 10% of baseline value.
Computational modelling. Experimentally derived modelling parameters are listed in Supplementary Table 3. The AP generation in HEK293 cells co-expressing K_\text{r2.1} and BacNa channels was simulated using the Hodgkin–Huxley formalism and the following differential equation:

\[ \frac{dv}{dt} = - \frac{1}{C_m} (I_{\text{Na}}(v, t) + I_{\text{k}}(v) + I_{\text{l}}(v) - I_{\text{endo}}(v, t)) \]  

where \( v \) is the cell transmembrane potential, \( C_m \) is the total membrane capacitance, \( I_{\text{Na}} \) is the BacNa channel current, \( I_{\text{k}} \) is the inward-rectifying potassium current, \( I_{\text{l}} \) is the leakage background current and \( I_{\text{endo}} \) is the stimulus current. Based on the similarities between BacNa and mammalian Ca_\text{v} channels, the BacNa conductance was modelled as a simple product of activation and inactivation gating variables and \( I_{\text{endo}} \) was expressed using the following equation:

\[ I_{\text{endo}}(v, t) = G_{\text{endo}}(m(t)) h(v, t) (v - E_{\text{Na}}) \]

where \( G_{\text{endo}} \) is the maximum conductance of the BacNa channel, \( m \) and \( h \) are the first-order activation and inactivation gating variables, respectively, and \( E_{\text{Na}} \) is the sodium Nernst potential given in Supplementary Table 3. Mathematical model of human Na_\text{v}1.5 was adopted from a previous model of human ventricular tissue with a slightly modified inactivation time constant to better fit voltage clamp recordings in Na_\text{v}1.5-expressing HEK293 cells.

From the voltage-clamp activation and inactivation protocols, the steady-state values of the gating variables as a function of \( V \) were plotted and fit to the Boltzmann equation, to derive the midpoint voltage and slope factor listed in Supplementary Table 1:

\[ m_{\text{ss}} = \frac{1}{1 + \exp \left( \frac{V - V_{1/2}}{\text{Slope}} \right)} \text{ and } h_{\text{ss}} = \frac{1}{1 + \exp \left( \frac{V - V_{1/2}}{\text{Slope}} \right)} \]

The initial and late phases of \( I_{\text{Na}} \) traces for each clamp potential were fit as single exponentials to derive time constants of activation (\( \tau_{\text{a}} \)) and inactivation (\( \tau_{\text{i}} \)) as functions of \( V \). The \( \tau_{\text{a}} \) and \( \tau_{\text{i}} \) at \( V = +20 \text{ mV} \) are listed in Supplementary Table 1.

Voltage-clamp recordings of inward-rectifying potassium current were used to fit steady-state \( I_{\text{k}} \) values to a time-independent Nygren et al.\textsuperscript{62} model as follows:

\[ I_{\text{k}} = G_{\text{k}} \frac{V - E_{\text{k}}}{1 + \exp \left( \frac{V - V_{\text{k}}}{\text{Slope}} \right)} \]

where \( G_{\text{k}} \) is the maximum conductance of the K_\text{r2.1} channel and \( E_{\text{k}} \) is the potassium Nernst potential given in Supplementary Table 3. Finally, the endogenous background current set in HEK293 or HDF cells, \( I_{\text{endo}} \), was fit by a time-independent linear equation:

\[ I_{\text{endo}} = 2.6V + 12.3 \text{ (for HEK293)} \text{ or } I_{\text{endo}} = 0.53V + 17.5 \text{ (for HDF)} \]

Data availability. All data supporting the findings of this study are available within the article and its Supplementary Information files or can be requested from the authors.

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Author contributions

H.X.N. and N.B. designed the project. H.X.N. and R.D.K. performed experiments. H.X.N. and N.B. analysed data and co-wrote the manuscript.

Additional information

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