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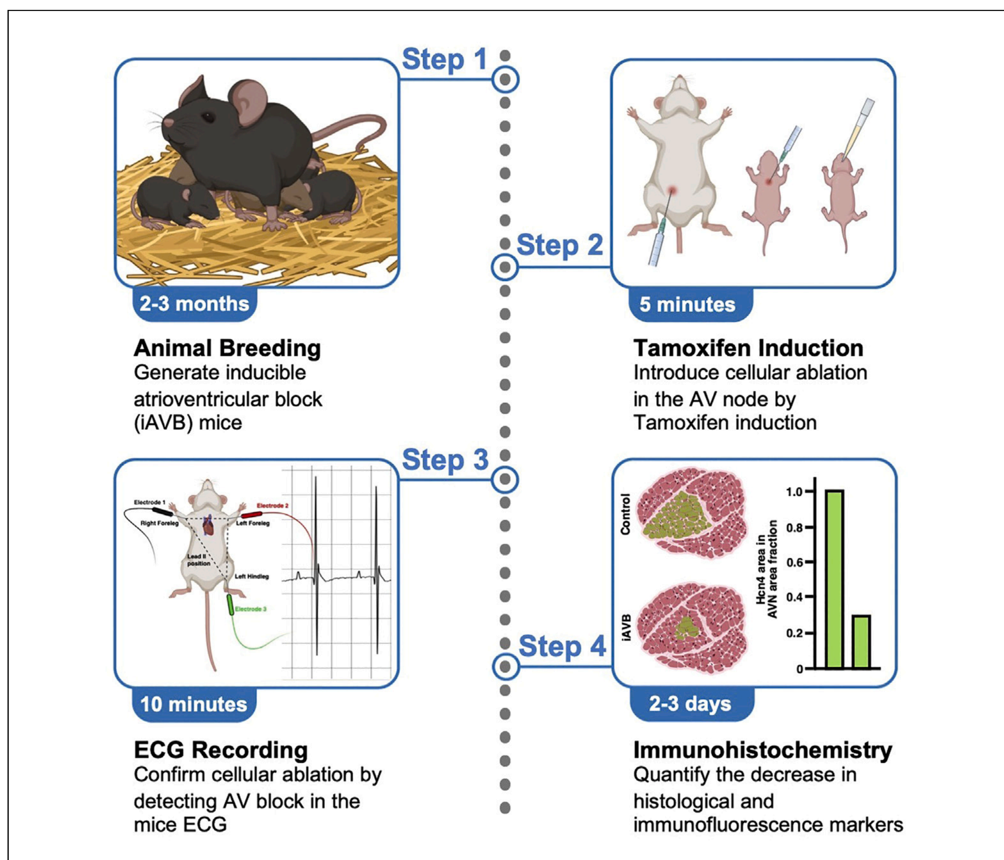
Protocol for inducing cellular ablation in the mouse atrioventricular conduction system



Damage to the atrioventricular conduction system (AVCS), the main electrical connection between the atrial and ventricular chambers, can result in a variety of cardiac conduction disorders. Here, we provide a protocol for selective damage of the mouse AVCS to study its response during injury. We describe tamoxifen-induced cellular ablation, detection of AV block through electrocardiography, and quantification of histological and immunofluorescence markers to analyze the AVCS. This protocol can be used to study mechanisms associated with AVCS injury repair and regeneration.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Reproducible ablation injury model of the mouse atrioventricular conduction system

Different routes of tamoxifen induction among neonatal and adult mice

Simplified instructions on neonatal and adult mice electrocardiography

Detailed walkthrough of immunohistochemical techniques for AVCS tissue

Wang et al., STAR Protocols 4, 102145
March 17, 2023 © 2023 The Author(s).
https://doi.org/10.1016/j.xpro.2023.102145

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Protocol for inducing cellular ablation in the mouse atrioventricular conduction system

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https://doi.org/10.1016/j.xpro.2023.102145

SUMMARY

Damage to the atrioventricular conduction system (AVCS), the main electrical connection between the atrial and ventricular chambers, can result in a variety of cardiac conduction disorders. Here, we provide a protocol for selective damage of the mouse AVCS to study its response during injury. We describe tamoxifen-induced cellular ablation, detection of AV block through electrocardiography, and quantification of histological and immunofluorescence markers to analyze the AVCS. This protocol can be used to study mechanisms associated with AVCS injury repair and regeneration.
For complete details on the use and execution of this protocol, please refer to Wang et al. (2021).[1]

BEFORE YOU BEGIN

Institutional permissions

All animal experiments were performed in an American Association for the Accreditation of Laboratory Animal Care-accredited and specific pathogen-free animal facility at the University of Texas Southwestern Medical Center. All mice were bred, maintained, and housed in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals under a study proposal (protocol no. 2017-102162) approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center.

Animal breeding

Timing: 2–3 months

1. Prepare mutant mice carrying the Connexin 30.2 (Cx30.2)-MerCreMer transgene ($Tg^{Cx30.2\text{-}MerCreMer/+}$) and breed with Diphtheria Toxin A (DTA) reporter mice ($Rosa26^{DTA/DTA}$) to generate $Tg^{Cx30.2\text{-}MerCreMer/+}$; $Rosa26^{DTA/+}$ mice (Figure 1A).

   Note: Male $Tg^{Cx30.2\text{-}MerCreMer/+}$ mice and female reporter mice (i.e., $Rosa26^{DTA/DTA}$ & $Rosa26^{LacZ/LacZ}$) should be used in the breeding crosses.

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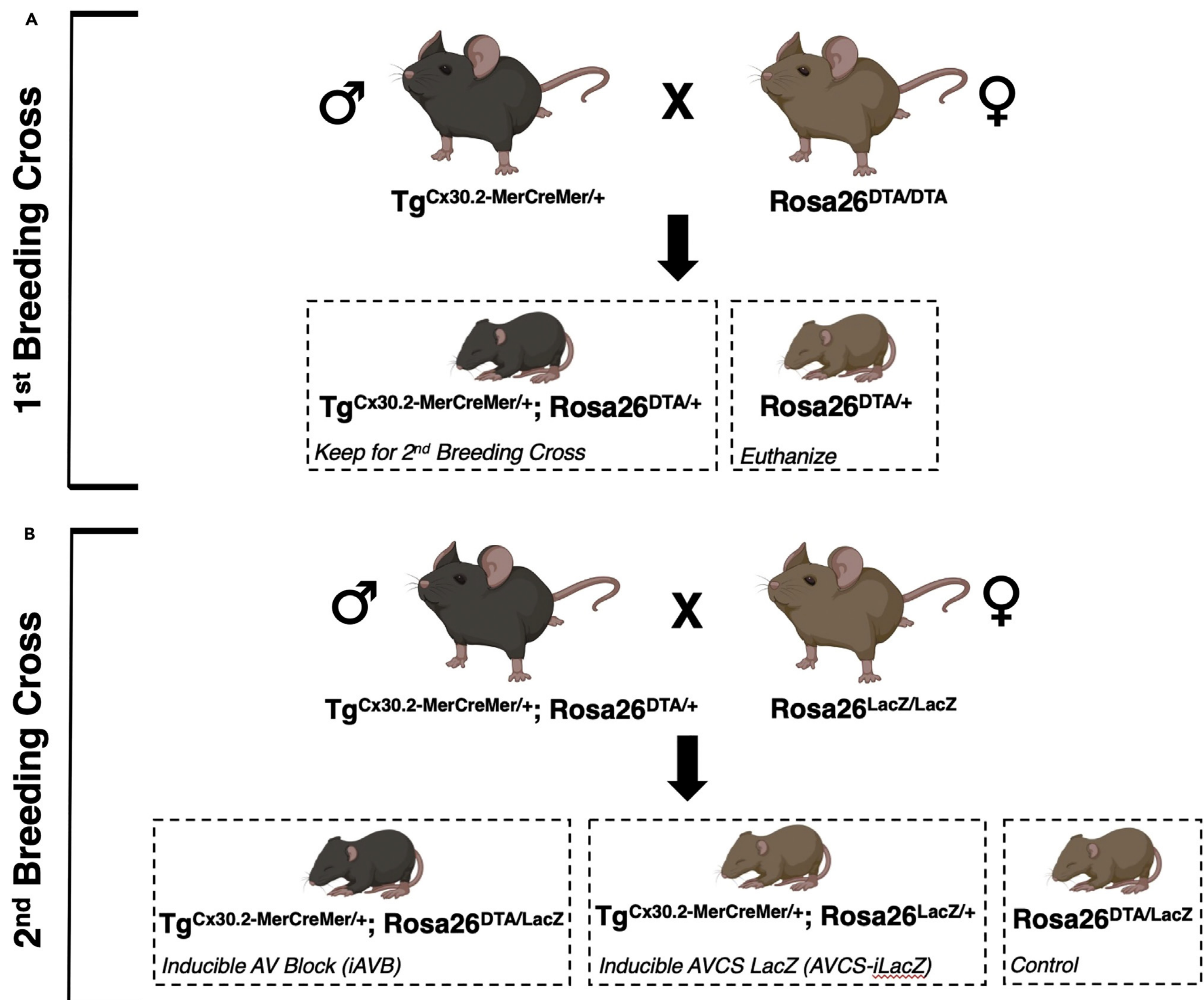

Figure 1. Animal breeding schematic to generate the inducible AV block (iAVB) mouse model and appropriate mouse controls
(A) First breeding cross.
(B) Second breeding cross.

2. Breed $Tg^{Cx30.2\text{-}MerCreMer/+}$; $Rosa26^{DTA/+}$ mice with LacZ reporter mice ($Rosa26^{LacZ/LacZ}$) to generate $Tg^{Cx30.2\text{-}MerCreMer/+}$; $Rosa26^{DTA/LacZ}$ (i.e., inducible AV block or iAVB), $Tg^{Cx30.2\text{-}MerCreMer/+}$; $Rosa26^{LacZ/+}$ (i.e., inducible AVCS LacZ or AVCS-iLacZ), and $Rosa26^{DTA/LacZ}$ (i.e., control) mice (Figure 1B).

   Note: 6- to 8-week-old mice should be used in the breeding crosses.

3. Check for the presence of LacZ, DTA, and Cre recombinase genes by genotyping – use the polymerase chain reaction (PCR) protocol provided in (Tables 1, 2, 3) and the primers listed in the key resources table.

Set up electrocardiography (ECG) and anesthesia machine

Timing: 5 min

Table 1. PCR cycling conditions for Cx30.2-MerCreMer

Steps	Temperature	Time	Cycles
Initial Denaturation	93°C	1 min	1
Denaturation	93°C	20 s	29 cycles
Annealing	68°C	3 min	
Hold	4°C	∞	

4. Turn on Powerlab 16/30 (ADInstruments, CO, USA) connected to a Bio Amp (Figure 2).
5. Respectively connect the anesthesia induction chamber and rodent facemask with F/AIR Canister (Bickford, NY, USA).

   Note: Weigh the F/AIR before and after experiment.

6. Open oxygen tank.
7. Open Sigma Delta Vaporizer (Penlon, UK) by turning the knob for oxygen flow to 1.5–2 L per minute (lpm) and isoflurane to 2%. Direct the oxygen and isoflurane to the induction chamber.

   Note: Make sure there is enough volume of isoflurane (i.e., between the minimum and maximum mark) in the vaporizer.

8. Open laptop and LabChart 8 (ADInstruments, CO, USA).
9. Go to Channel Settings (under Setup) and change the following: Range = 10 mV, Input Amplifier = Bio Amp, Units = mV, and Calculation = Smoothing.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Polyclonal anti-HCN4 guinea pig antibody (1:200 dilution)	Alomone Labs	Cat#AGP-004
Polyclonal anti-Nppa rabbit antibody (1:200 dilution)	Abgent	Cat#AP8534a
Alexa Fluor 488 polyclonal goat anti-guinea pig IgG (H+L) (1:400 dilution)	Invitrogen	Cat#A11073
Alexa Fluor 555 polyclonal goat anti-rabbit IgG (H+L) (1:400 dilution)	Invitrogen	Cat#A21422
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Sigma-Aldrich	Cat#T5648
Ethanol	Pharmco	Cat#111000200
Sesame oil	Thermo Fisher Scientific	Cat#241002500
Atropine	Sigma-Aldrich	Cat#A0132
Isoproterenol	Sigma-Aldrich	Cat#I5752
(Para-)Formaldehyde solution 4%	Thermo Fisher Scientific	Cat#J19943.K2
Glutaraldehyde 25%	Thermo Fisher Scientific	Cat#A17876.0F
X-gal	Thermo Fisher Scientific	Cat#B1690
Paraffin	Thermo Fisher Scientific	Cat#171400025
Tissue Freezing Medium	Electron Microscopy Sciences	Cat#72592
Universal blocking buffer 10×	Biogenex Laboratories	Cat#HK0855K
Phosphate-buffered saline (PBS) 1×	Thermo Fisher Scientific	Cat#20012027
Deoxycholic acid	MilliporeSigma	Cat#D2510
NP-40 detergent solution	Thermo Fisher Scientific	Cat#85124
Potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$)	MilliporeSigma	Cat#60299
Potassium hexacyanoferrate(II) ($K_4Fe(CN)_6$)	MilliporeSigma	Cat#60279
Acetylthiocholine iodide	MilliporeSigma	Cat#A5751
Sodium acetate	MilliporeSigma	Cat#241245
Acetic acid	MilliporeSigma	Cat#W200611

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium citrate	MilliporeSigma	Cat#PHR1416
Cupric sulfate ($CuSO_4$)	MilliporeSigma	Cat#931071
Tetraisopropyl pyrophosphoramide (iso-OMPA)	Santa Cruz Biotechnology	Cat#sc-215956
Cedarwood oil	Spectrum	Cat#CE107
Eosin B	MilliporeSigma	Cat#45260
Xylene	MilliporeSigma	Cat#214736
Nuclear fast red	MilliporeSigma	Cat#N3020
Harris hematoxylin	MilliporeSigma	Cat#HHS32
Triton X-100	MilliporeSigma	Cat#X100
VECTASHIELD® Antifade Mounting Medium w/ DAPI	Vector Laboratories	Cat#H-1200-10
Isoflurane	Thermo Fisher Scientific	Cat#AAAL17315-14
Apex Taq Master Mix 2X	Genesee Scientific, Identifier	Cat#42-138
Critical commercial assays		
Trichrome One-Step Blue & Red Stain Kit (includes: Bouin's Fluid, Modified Mayer's Hematoxylin, One-Step Trichrome Stain Blue/Red)	StatLab	Cat#KTTRBPT
Experimental models: Organisms/strains		
Mouse: Tg(Cx30.2-MerCreMer); 6–8-week-old; male	Wang et al.[1]	N/A
Mouse: B6;129S4-Gt(ROSA)26Sor^{tm1Sor}/J (LacZ reporter mice); 6–8-week-old; female	The Jackson Laboratory	Stock no. 003309; RRID: IMSR_JAX:003309
Mouse: B6.129P2-Gt(ROSA)26Sor$^{tm1(DTA)Lky}$/J (DTA reporter mice); 6–8-week-old; female	The Jackson Laboratory	Stock no. 009669; RRID: IMSR_JAX:009669
Oligonucleotides		
Primers: Tg(Cx30.2-MerCreMer) Forward:	Wang et al.[1]	5′-GCATTACCGGTCGATGCAACGAGTGATGAG-3′
Primers: Tg(Cx30.2-MerCreMer) Reverse:	Wang et al.[1]	5′- GAGTGAACGAACCTGGTCGAAATCAGTGCG -3′
Primers: Rosa26-LacZ Forward:	Integrated DNA Technologies (IDT)	5′-AAAGTCGCTCTGAGTTGTTAT-3′
Primers: Rosa26-LacZ Reverse:	Integrated DNA Technologies (IDT)	5′-GCGAAGAGTTTGTCCTCAACC-3′
Primers: Rosa26-DTA Forward:	Integrated DNA Technologies (IDT)	5′-CGACCTGCAGGTCCTCG-3′
Primers: Rosa26-DTA Reverse:	Integrated DNA Technologies (IDT)	5-CTCGAGTTTGTCCAATTATGTCAC-3′
Software and algorithms		
LabChart8	ADInstruments	https://www.adinstruments.com/support/downloads/windows/labchart
Others		
PowerLab 16/30	ADInstruments	https://www.adinstruments.com/products/powerlab-daq-hardware
Bio Amp	ADInstruments	https://www.adinstruments.com/research/animal/autonomic/ecg
Anesthesia induction chamber	Harvard Apparatus	https://www.harvardapparatus.com/induction-chambers.html
F/AIR Canister	Vetamac, Inc.	Cat#80120
Sigma Delta vaporizer	Penlon Limited	https://www.penlon.com/Product-Groups/Vaporizer/Sigma-Delta
Confocal laser-scanning microscope	Nikon Instruments, Inc.	https://www.microscope.healthcare.nikon.com/products/confocal-microscopes/a1hd25-a1rhd25
Mouse pad	E-Z Systems	https://www.ezsystemsinc.com/product/hb-103-bed-breather/
mouse pad alternative	Styrofoam board wrapped in surgical drape	N/A
BD Insulin Syringe	ADW Diabetes	Cat#SY8290328291
CM1850 Cryostat	Leica Biosystems	https://www2.leicabiosystems.com
ECG needle electrodes	ADInstruments	https://www.adinstruments.com/products/needle-electrodes
Alcohol pad	Med Lab Supply	https://www.medical-and-lab-supplies.com/syringes-and-needles/alcohol-prep-pads.html
Rodent facemask	E-Z Systems	https://www.ezsystemsinc.com/product/hb-103-bed-breather/
Rodent facemask alternative	Nitrile glove cut-out	N/A
Oxygen tank	Parkland Scientific, Inc.	Cat#PX-8703-1T

Table 2. PCR cycling conditions for DTA and LacZ

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	94°C	30 s	35 cycles
Annealing	65°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	2 min	1
Hold	4°C	∞	

MATERIALS AND EQUIPMENT

Tamoxifen Solution (100 μg/μL)

Reagent	Final concentration	Amount
Tamoxifen	100 μg/μL	100 mg
Ethanol, 100%	9:1 sesame oil-ethanol	100 μL
Sesame Oil		900 μL
Total	–	**1,000 μL**

Store at –20°C for 6 months.

> *Note:* If Tamoxifen is not dissolving in 37°C water bath, the tube can be placed in a 42°C or 67°C water bath.

Atropine solution (1 mg/mL)

- Dissolve 50 mg of Atropine in 1,000 μL of 1× PBS to make 50 mg/mL Atropine stock solution. In a 15-mL tube, mix 100 μL of 50 mg/mL Atropine and 4,900 μL of 1× PBS.

50 mg/mL Atropine can be stored at –20°C for 1 year.

Isoproterenol solution (10 μg/mL)

- Dissolve 10 mg of Isoproterenol in 1,000 μL of 1× PBS to make 10 mg/mL Isoproterenol stock solution. In a 15-mL tube, mix 5 μL of 10 mg/mL Isoproterenol and 4,995 μL of 1× PBS.

10 mg/mL Isoproterenol can be stored as stock solution at –20°C for 1 year.

Washing buffer for X-Gal staining

Reagent	Final concentration	Amount
Deoxycholic acid, 10%	0.01%	50 μL
NP-40, 10%	0.02%	100 μL
1× PBS	–	49.85 mL
Total	–	**50 mL**

Store at 4°C for 6 months.

X-Gal staining solution

Reagent	Final concentration	Amount
$MgCl_2$, 100 mM	2.0 mM	1.0 mL
$K_3Fe(CN)_6$, 100 mM	5.0 mM	2.5 mL
$K_4Fe(CN)_6$, 100 mM	5.0 mM	2.5 mL
Deoxycholic acid, 10%	0.01%	50 μL
NP-40, 10%	0.02%	100 μL

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Continued		
Reagent	Final concentration	Amount
X-Gal, 40 mg/mL	1 mg/mL	1.25 mL
1× PBS	–	42.6 mL
Total	–	**50 mL**

Note: Always use freshly prepared X-gal staining solution. Wrap in foil to avoid light exposure.

Acetylthiocholine staining buffer		
Reagent	Final concentration	Amount
Acetylthiocholine iodide	1.7 mM	0.58 mg
Sodium acetate, 0.06 N	0.2 mM	633 μL
Acetic acid, 0.1 N	0.2 mM	20 μL
Sodium citrate, 0.1 M	4.8 mM	48 μL
Cupric sulfate, 30 mM	3.0 mM	100 μL
iso-OMPA, 4 mM	0.08 mM	20 μL
$K_3Fe(CN)_6$, 0.5 M	0.5 mM	1 μL
Total	–	**1,000 μL**

Note: Always use freshly prepared acetylthiocholine staining buffer.

Preparation of primary antibody		
Reagent	Final concentration	Amount
Anti-HCN4 guinea pig antibody (1:200)	1:200	2 μL
Anti-Nppa rabbit antibody (1:200)	1:200	2 μL
1× Universal blocking buffer	–	396 μL
Total	–	**400 μL**

⚠ CRITICAL: If a sample requires staining with multiple antibodies targeting different proteins, it is essential to use antibodies raised in different hosts.

Preparation of secondary antibody		
Reagent	Final concentration	Amount
Alexa Fluor 488 goat anti-guinea pig IgG (H+L) antibody (1:400)	1:400	1 μL
Alexa Fluor 555 goat anti-rabbit IgG (H+L) antibody (1:400)	1:400	1 μL
1× Universal blocking buffer	–	398 μL
Total	–	**400 μL**

Note: Multiple secondary antibodies from different hosts could be added. In that case, the amount of universal blocking buffer is adjusted according to the amount of secondary antibody added.

STEP-BY-STEP METHOD DETAILS

Induction of AVCS cellular ablation by Tamoxifen

Timing: 1 min/adult mouse, 5 min/neonatal mouse

Table 3. PCR reaction master mix

Reagent	Amount (10 μL)
DNA template	1.0 μL
Apex Taq Master Mix 2X	5.0 μL
Primer 1	0.4 μL
Primer 2	0.4 μL
ddH_2O	3.2 μL

In this step, we describe the intraperitoneal (i.p.), per orem (p.o.), and subcutaneous (s.c.) administration of Tamoxifen on adult (P42) and neonatal (P0) mice. Unlike adult mice given Tamoxifen for 7 days, we decided to do a single-day administration of Tamoxifen on neonatal mice to minimize maternal cannibalization.

1. Intraperitoneal (i.p.) administration of Tamoxifen on adult mice (Figure 3A).
   a. On day 0, place adult (P42) mouse on metal grid and grab loose skin at the back of the neck between your thumb and forefinger.
   b. Hold tail with little and ring finger.
   c. Perform injection of a single dose of 1 mg Tamoxifen.
      i. Aim for the lower right abdomen with the needle directed towards the thorax in a 30°–40° angle to minimize risk of puncturing intestinal organs.
      ii. Perforate skin and slowly aspirate.
      iii. If there is no aspirated liquid (i.e., blood or green liquid), administer Tamoxifen solution.

Note: Insulin syringe is preferred (e.g., BD Insulin Syringe, NK, USA), as these diameters are less likely to perforate intestinal organs and hurt the mouse, compared to larger needles.

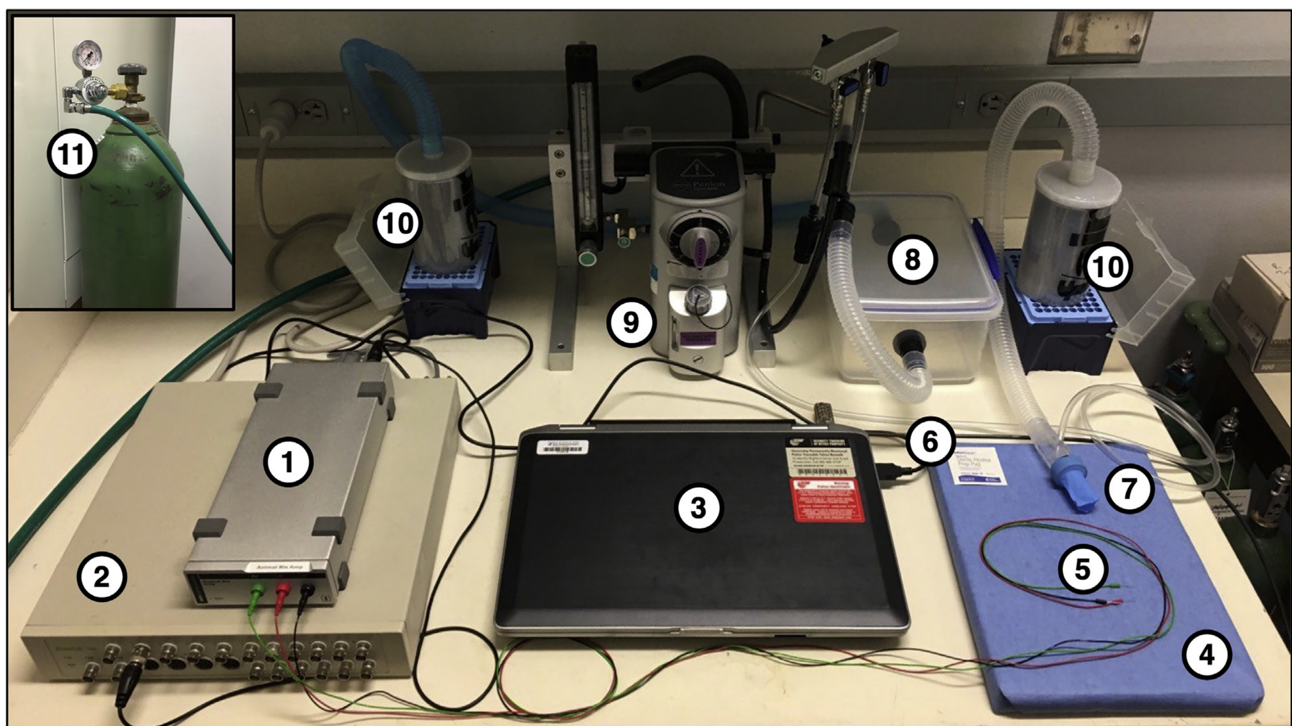

Figure 2. Components of ECG and anesthesia equipment
(1) Bio Amp, (2) PowerLab 16/30, (3) Laptop w/ LabChart8, (4) Mouse pad, (5) ECG needle electrodes, (6) Alcohol pad, (7) Rodent facemask, (8) Anesthesia induction chamber, (9) Sigma Delta Vaporizer, (10) F/AIR Canister, (11) Oxygen tank.

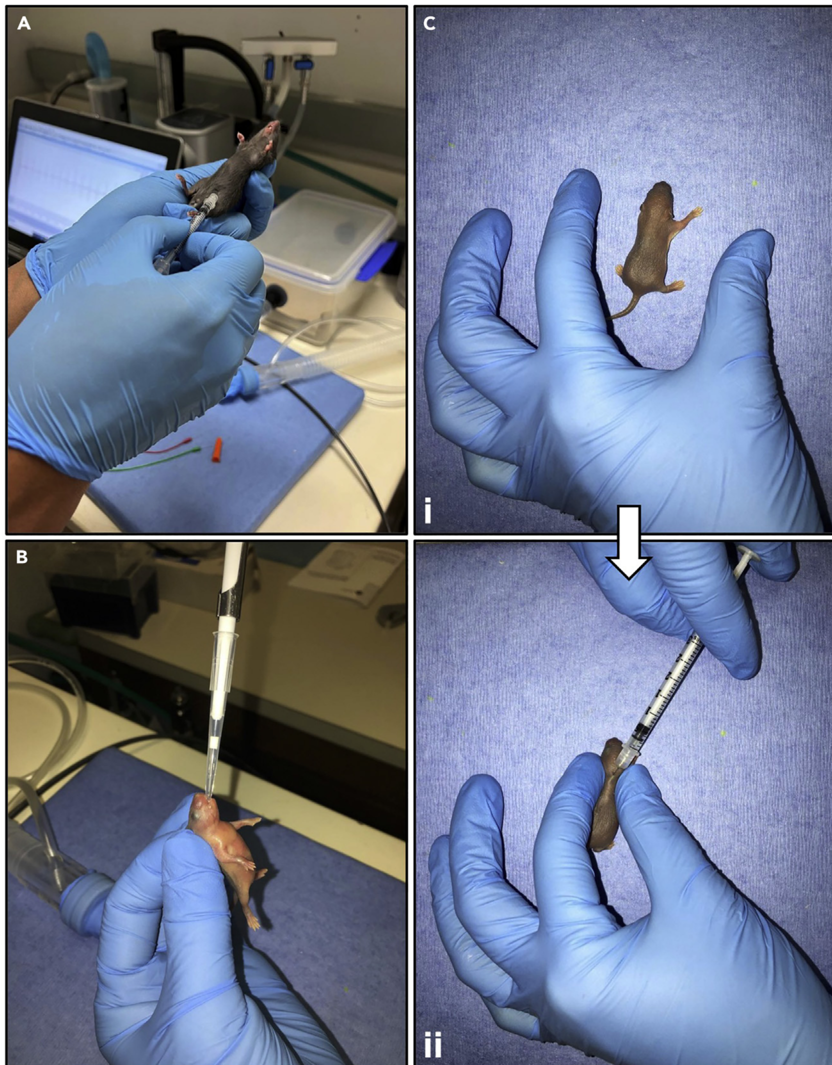

Figure 3. Administration of Tamoxifen solution to adult and neonatal mice
(A) Intraperitoneal (i.p.) administration on adult mice.
(B) Per orem (p.o.) administration on neonatal mice.
(C) Subcutaneous (s.c.) administration on neonatal mice.

   d. Repeat steps a.-b. for day 1–7 of Tamoxifen induction on adult mice.
2. Per orem (p.o.) administration of Tamoxifen on neonatal mice (Figure 3B).
   a. On day 0, collect entire litter of neonatal (P0) mice and place in a separate cage.
   b. Gently grab a neonatal mouse at the back of the neck and visualize the animal's mouth.
   c. Using a pipettor, get 8–10 μL of Tamoxifen equivalent to a single 0.8–1 mg dose.
   d. Position the pipette tip in the animal's mouth and slowly administer the Tamoxifen solution.

Note: Give the neonatal mouse time to swallow the solution and hold the animal upright to ensure ingestion of the Tamoxifen.

   e. Put the neonatal mouse in an extra cage to allow recovery until other neonatal mice are given Tamoxifen.
   f. Relocate all neonatal mice (i.e., entire litter) to their original cage after induction.

3. Subcutaneous (s.c.) administration of Tamoxifen on neonatal mice (Figure 3C).
   a. On day 0, collect entire litter of neonatal (P0) mice and place in a separate cage.
   b. Gently put your thumb and forefinger to the middle of the thoracic spine to immobilize the animal.
   c. Perform injection of a single dose of 1 mg Tamoxifen.
      i. Aim for the skin at the back of the neck with the needle directed towards the tail in a 20°–30° angle.
      ii. Perforate skin and slowly administer Tamoxifen solution.
   d. Put the neonatal mouse in an extra cage to allow recovery until other neonatal mice are given Tamoxifen.
   e. Relocate all mice to their original cage after induction.

ECG recording of adult and neonatal mice

Timing: 10 min/mouse

In this step, we show how to perform electrocardiography (ECG) on adult and neonatal mice to check for the effect of AVCS cellular ablation after Tamoxifen induction.

4. Anesthetize mice for ECG with 2% isoflurane in 2.0 L/min O_2.
   a. Adult anesthesia:
      i. Place adult mouse in anesthesia chamber.
      ii. Once anesthetized, place the animal at the mouse pad and hook to rodent facemask.
   b. Neonatal anesthesia:
      i. Place neonatal mouse in the mouse pad.
      ii. Hook animal directly to rodent facemask.

Note: Assess depth of anesthesia by checking loss of righting reflex or response to pain stimulation (e.g., toe pinch).

5. Place subcutaneous ECG electrodes in the conventional lead II position (Figure 4).
   a. Place positive (red) electrode at the left forelimb.
   b. Place negative (black) electrode at the right forelimb.
   c. Place reference (green) electrode at the left hindlimb.

Note: Clean mouse limbs and ECG electrodes with alcohol pads before inserting them into the mouse.

6. Once ECG electrodes are in place, click start at the LabChart 8 program to record the animal's ECG.
7. Record ECG for at least one to one-and-a-half minutes.
8. Remove ECG electrodes once recording is finished and return the animal to its cage.
9. Repeat on subsequent days as necessary.

Note: Minimize the influence of isoflurane on the heart rate by doing the following: (1) Minimize isoflurane induction time (neonatal mice induction time is longer compared to adult mice); (2) Calculate ECG measurements (i.e., PR interval, QRS interval, etc.) on the first 30 s of an ECG tracing.

Optional: Since mice PR interval shortens as they grow, perform ECG on normal mice of different ages (e.g., P7, P14, p21, p28) to determine normal PR interval values for each age group. Normal PR interval range was set as Mean $\pm$ 2 Standard Deviation in Wang et al.[1] (Table 4).

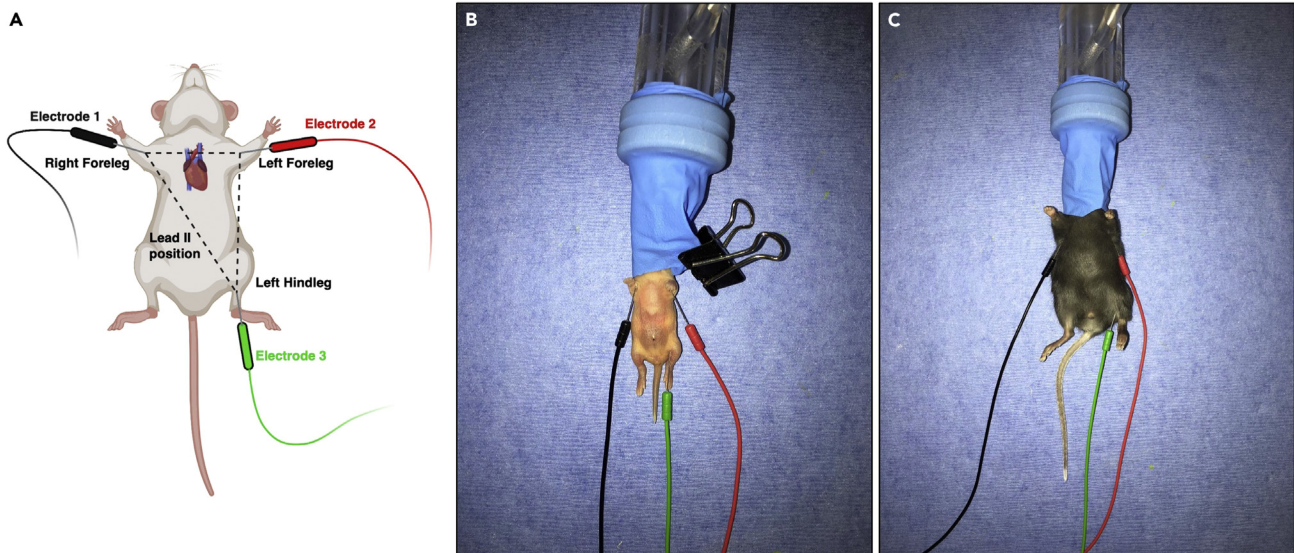

Figure 4. Electrocardiography (ECG) conventional lead II placement in neonatal and adult mice
(A) Schematic of conventional lead II placement of ECG electrodes.
(B) Actual conventional lead II placement of ECG electrodes in neonatal mouse.
(C) Actual conventional lead II placement of ECG electrodes in adult mouse.

Pharmacological challenge post-Tamoxifen induction

Timing: 10 min/mouse

In this step, we describe how to perform pharmacological studies using Atropine and Isoproterenol injection to further check the effect of AVCS cellular ablation after Tamoxifen induction.

10. Freshly prepare pharmacological agents, i.e., Atropine and Isoproterenol.
11. After recording ECG for 1 min, perform IP injection of pharmacological agents at a dose of 2 μg/g or 50 ng/g of Atropine or Isoproterenol, respectively.
12. Continue recording the animal's ECG for 5 min following drug administration.

Mice preparation for heart removal

Timing: 30 min

In the following steps, we show how to collect and process mice hearts for immunohistological staining and analysis.

13. Place mice in an empty cage and slowly fill with CO_2 at 3L/min.
14. Wait until euthanasia is complete (i.e., no more breathing can be observed).
    a. Perform cervical dislocation as secondary method of euthanasia.
15. Place euthanized mice in a clean dissection pad.

Table 4. Normal range of PR intervals across different mice ages with 2% isoflurane/200 mL/min O_2

	P7 (n = 27)	P14 (n = 17)	P21 (n = 16)	P28 (n = 35)
Average PR interval (ms)	51	39	38	37
Mean ± 2SD (ms)	38–63	30–48	28–47	39–44
Number of mice	27	17	22	35

16. Cut the chest open and expose the chest cavity.
17. Cut the major blood vessels connecting the heart to the cardiac base.
18. Place harvested heart in cold 1× PBS.
19. Gently pump the harvested heart with forceps to remove excess blood.

Preparation and X-Gal staining of mouse heart for paraffin block and sliced sections

Timing: 2–3 days

20. Fix the harvested heart in 0.2% glutaraldehyde for one (1) hour at 4°C.
21. Wash with washing buffer for 10 min twice.
22. Incubate and shake the harvested heart in X-gal staining buffer at room temperature (20°C–22°C) overnight (12–16 h), covered with foil.
23. Post-fix the harvested heart in neutral formalin overnight (12–16 h) for paraffin embedding.

> CRITICAL: (Para-)Formaldehyde solution is a harmful material for humans. Handling (para-)formaldehyde solution should be done inside a chemical fume hood and wearing protective gear such as glasses, lab coat, and gloves.

24. Place the harvested heart in a mold in the appropriate orientation.
25. Embed harvested heart in paraffin.

Pause point: Paraffin blocks can be stored at room temperature (20°C–22°C) for years.

26. Section the mouse heart paraffin block in a posterior to anterior (PA) direction.
27. Collect sections where mitral valve and tricuspid valve are present.

Preparation of mouse heart for frozen block and sliced sections

Timing: 1–2 days

28. Fix the harvested heart in 4% paraformaldehyde (PFA) for one (1) hour at 4°C.
29. Sequentially incubate the harvested heart in 10% and 20% sucrose/PBS at 4°C for 12–24 h.
30. Fill a cryomold with Tissue Freezing Medium (TFM).
31. Place the harvested heart in the TFM-filled cryomold.
32. Orient the harvested heart into the four-chamber orientation.
33. Snap-freeze the harvested heart with TFM using dry ice or liquid nitrogen.
34. Store mouse heart frozen block at –80°C prior to sectioning.
35. Section the mouse heart frozen block into 8-micron slices in a posterior to anterior (PA) direction, using a Leica CM3050S cryostat.
36. Collect sections where septal leaflet of the tricuspid valve is present.

Pause point: Frozen sections can be stored at –80°C for years.

Histological staining protocol

Timing: 24–48 h

37. For paraffin sections:
    a. Wash paraffin sections with 100% Cedarwood Oil (CWO) for 5 min three times to deparaffinize sections.
    b. Wash sections with 1:1 CWO and 100% ethanol for 2 min.
    c. Rinse sections three times with 100% ethanol for 5 min.

d. Rinse sections three times with running water for 5 min.

38. For cryosections:
    a. Air-dry tissue sections for 15–20 min at room temperature (20°C–22°C).
    b. Wash thoroughly with PBS for 2 min twice.

Note: Be careful of washing/rinsing cryosections as they come off the slide easier.

39. Hematoxylin and eosin (H&E) staining.
    a. Incubate sections in Harris hematoxylin for 5 s.
    b. Wash thoroughly in running water for 5 min.
    c. Incubate sections in 100% alcohol for 10 s.
    d. Incubate sections in eosin for 5 s.
    e. Wash sections in alcohol for 5 s three times, with one final wash in 100% alcohol for 10 s.
    f. Wash sections in xylene for 10 s, and another two washes in xylene for 5 s each.
    g. Seal sections with cover slips and mounting medium.
40. Masson's Trichrome (MTC) staining.
    a. Immerse sections in Bouin's Fluid (preheated to 56°C) for 60 min or overnight (12–16 h) at room temperature (20°C–22°C).

    Note: All reagents are obtained from Trichrome One-Step Blue & Red Stain kit (#KTTRBPT, American MasterTech Scientific) unless otherwise noted.

    b. Rinse sections with running water for 3 min or until sections are colorless.
    c. Immerse sections in Modified Mayer's Hematoxylin for 7 min.
    d. Rinse sections with running water for 3 min.
    e. Immerse sections in One-Step Trichrome Stain (Blue & Red) for 3–8 min.
    f. Rinse sections with running water for 5 s.
    g. Rinse sections three times with absolute alcohol for 1 min.
    h. Rinse sections three times with Xylene for 1 min.
    i. Seal sections with cover slips and mounting medium.
41. Nuclear-fast red (NFR) staining.
    a. Incubate sections in Nuclear Fast Red solution for 5 min.
    b. Rinse sections with running water for 5 min.
    c. Wash sections three times with 100% ethanol for 2 min.
    d. Rinse sections three times with Xylene for 1 min.
    e. Seal sections with cover slips and mounting medium.
42. Acetylcholinesterase activity staining.
    a. Incubate sections in Acetylthiocholine staining buffer at 37°C overnight (12–16 h).
    b. Rinse section slides with distilled water.
    c. Counterstain section slides with eosin (steps 39d–g).
    d. Seal sections with cover slips and mounting medium.

Immunostaining protocol for cryosections

Timing: 2–3 days

43. Air-dry fixed tissue sections for 15–20 min at room temperature (20°C–22°C).
44. Wash sections three times with PBS for 5 min.
45. Add 0.3% Triton-X100 in PBS to the fixed sections and allow permeabilization for 20 min at room temperature (20°C–22°C).
46. Wash sections three times with PBS for 5 min.
47. Block fixed sections with Universal Blocking buffer for 10 min at room temperature (20°C–22°C).

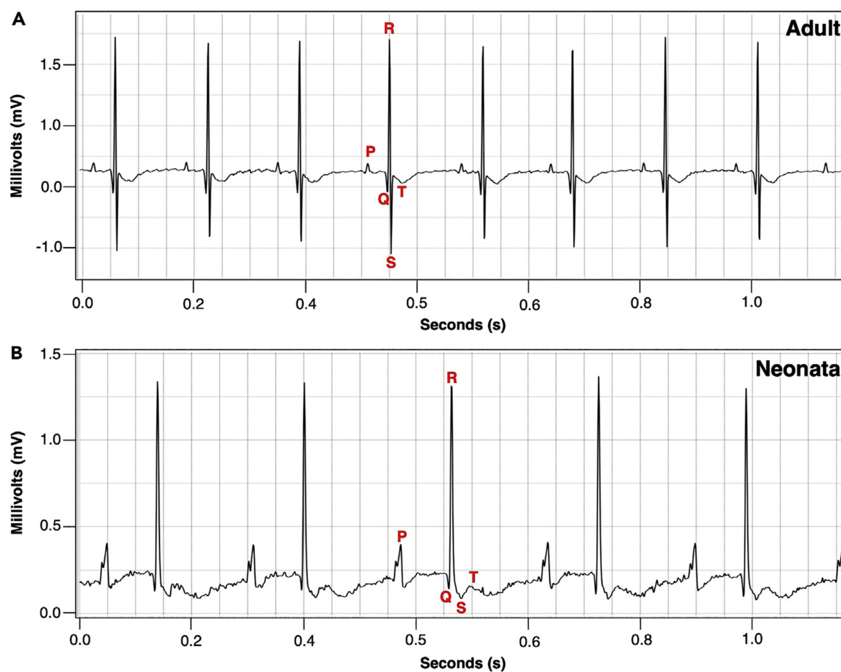

Figure 5. Examples of ECG tracing
(A) ECG tracing of an adult mouse.
(B) ECG tracing of a neonatal mouse.

48. Dilute primary antibodies in the appropriate amount and apply 30 μL to each section.
49. Incubate overnight (12–16 h) at 4°C.
50. Wash sections three times with PBS for 5 min.
51. Dilute secondary antibodies in the appropriate amount and apply 30 μL to each section.
52. Incubate for 1 h at room temperature (20°C–22°C).
53. Wash sections with PBS.
54. Mount tissue sections with Vectashield Mounting Medium with DAPI.

EXPECTED OUTCOMES

In this protocol, we showed how the previously characterized AVCS-specific Cx30.2 enhancer[2] can be utilized to introduce cellular ablation in the mouse AVCS. After crossing $Tg^{Cx30.2\text{-}MerCreMer/+}$ mouse with DTA and LacZ reporter mice ($Rosa26^{DTA/LacZ}$) and induction with Tamoxifen, both adult and neonatal iAVB mice ($Tg^{Cx30.2\text{-}MerCreMer/+}$; $Rosa26^{DTA/LacZ}$) exhibit atrioventricular (AV) block, as detected by electrocardiography (ECG) (Figure 5). Wang et al.[1] presented the classification and descriptions of different degrees of atrioventricular (AV) blocks observed among iAVB mice (Table 5; Figure 6). Pharmacological challenge with Atropine or Isoproterenol can further show the presence of AV block among adult and neonatal iAVB mice (Figure 7).

The AV node of adult and neonatal AVCS-iLacZ mice ($Tg^{Cx30.2\text{-}MerCreMer/+}$; $Rosa26^{LacZ/+}$) highly stains for X-gal, acetylcholine esterase, and HCN4 (Figure 8). Masson's trichrome staining of the AV node of iAVB mice should show an increase in the area of fibrosis compared to control mice AV node (Figure 9). The AV node of iAVB mice should also show decreased HCN4 positive cells compared to control mice AV node, pointing to AVCS cellular ablation (Figure 9).

QUANTIFICATION AND STATISTICAL ANALYSIS

The fibrotic area in the AV node was quantified as the relative area of positive Masson's trichrome-stained area (blue fibrosis) normalized to the total section area using ImageJ/Fiji. HCN4 positive cell

Table 5. Classification and description of atrioventricular block in mice

Atrioventricular (AV) block classification		Description
1° Degree AV Block		PR prolongation without dissociation from QRS complex
2° Degree AV Block	Mobitz Type I	Partial dissociation between P wave and QRS complex as evidenced by intermittent absence of QRS complex following a P wave with gradual PR interval prolongation
	Mobitz Type II	Partial dissociation between P wave and QRS complex as evidenced by intermittent absence of QRS complex following a P wave with consistent PR interval
3° Degree AV Block		Complete dissociation between P wave and QRS complex most evidenced by distinct PP and RR intervals

drop out was calculated as the ratio of HCN4 positive area to the area of atrioventricular node. All data are shown as mean ± standard error of mean (s.e.m). P values are determined using the Fisher's Exact Test or Student's two-tailed t-test. A p value less than 0.05 is considered statistically significant.

LIMITATIONS

The protocol only includes surface ECG since we only needed to detect AV block phenotypes for this protocol. Further characterization of the cardiac dysrhythmia phenotype is outside the scope of the current protocol, but can be pursued with other techniques (e.g., optical mapping and intracardiac ECG).

Masson's trichrome staining was the only method used to evaluate fibrosis coverage in this protocol. Other methods should be considered.

TROUBLESHOOTING

Problem 1

Maternal cannibalization of neonatal mice post-Tamoxifen induction (steps 2 & 3).

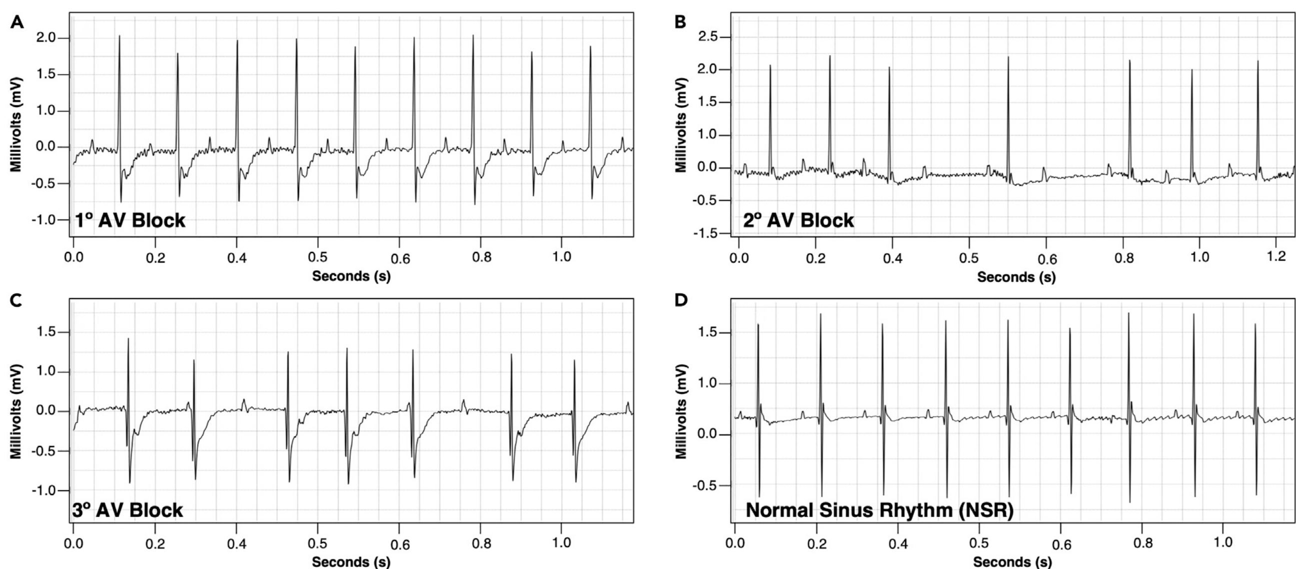

Figure 6. ECG tracings of different degrees of AV block in the murine heart

(A) ECG tracing of 1° AV block.

(B) ECG tracing of 2° AV block.

(C) ECG tracing of 3° AV block.

(D) ECG tracing of a normal sinus rhythm.

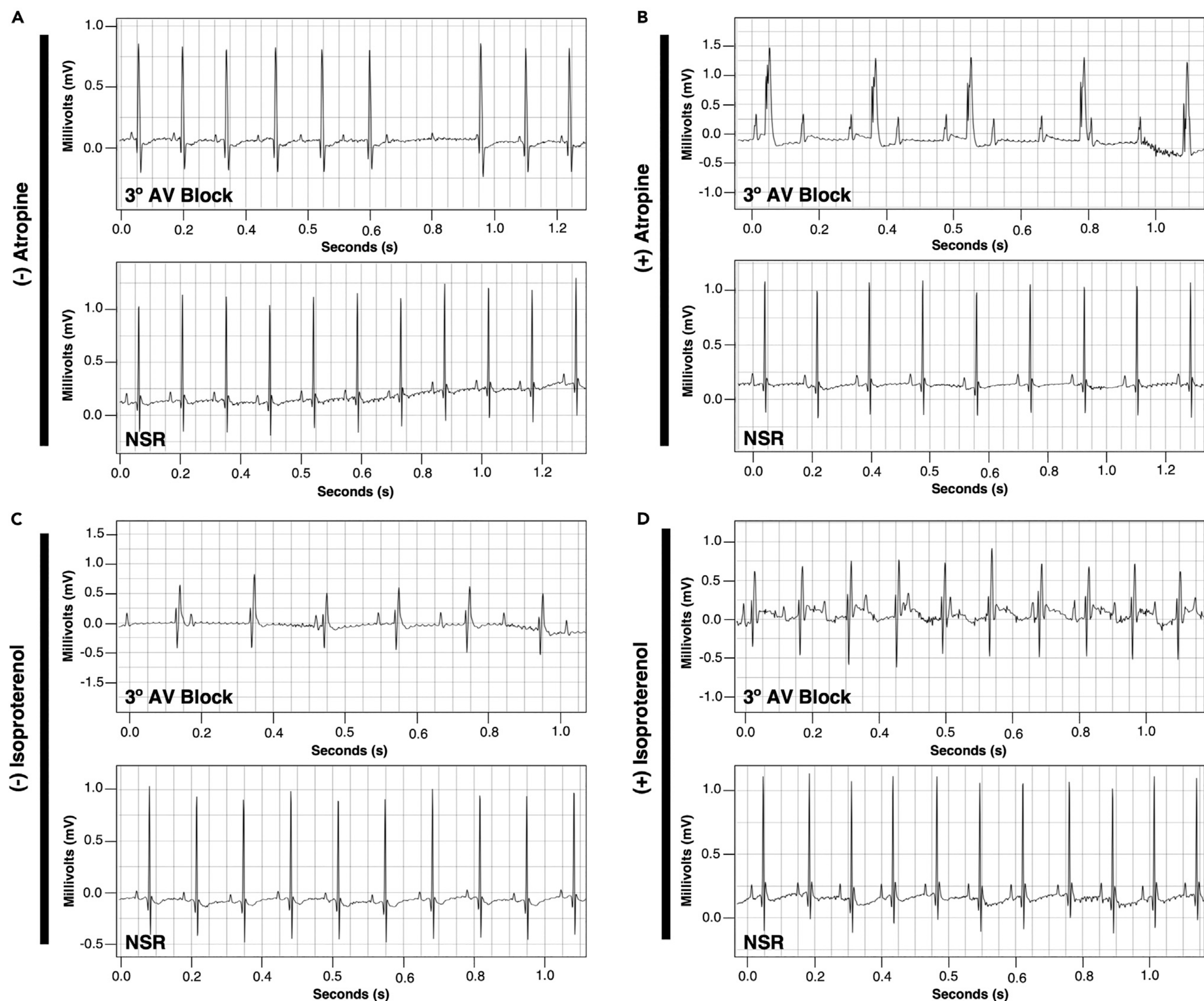

Figure 7. Pharmacological challenge with Atropine and Isoproterenol

(A and B) ECG tracings of iAVB and control mice challenged with Atropine.

(C and D) ECG tracings of iAVB and control mice challenged with Isoproterenol.

Potential solution

The following can decrease the chance of maternal cannibalization of neonatal mice post-Tamoxifen induction: (1) rub hands with cage beddings before handling the neonatal mice; (2) always remove and return entire litter; (3) administer Tamoxifen to neonatal mice at almost P1 of age (<24 h from birth; (4) make sure milk spots are present in neonatal mice before induction with Tamoxifen.

Problem 2

High amount of noise in the ECG recordings (step 5).

Potential solution

In order to decrease the amount of noise in the ECG recordings, avoid inserting the ECG electrodes deeply into the muscle layer and ensure subcutaneous placement. From our experience, needle electrodes are better than micro-hook electrodes. Avoid unnecessary movement of the ECG electrodes while recording (e.g., placing the wirings of the ECG electrodes over the animal's chest and abdomen; moving hands above the mouse while recording). Take particular care and avoid

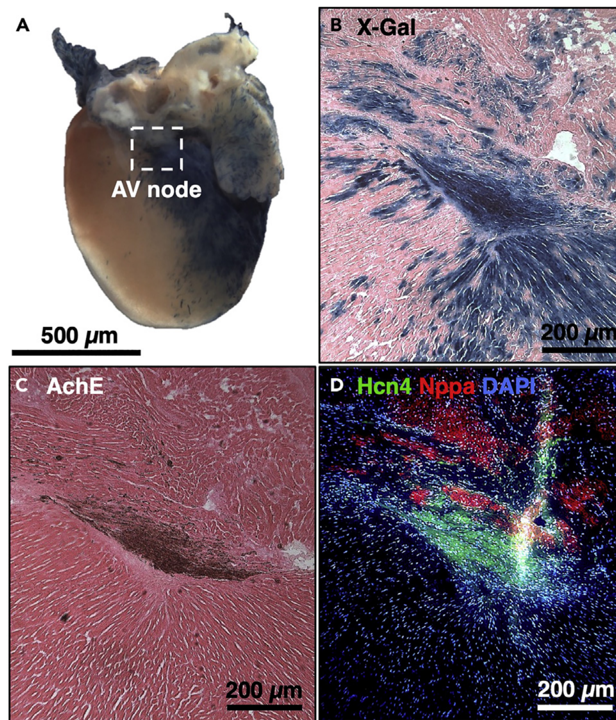

Figure 8. Immunohistochemical staining of the AV node

(A) X-Gal staining of AVCS-iLacZ ($Tg^{Cx30.2-MerCreMer/+}$; $Rosa26^{LacZ/+}$) mouse heart, induced at P0 and harvested at P28, scale bar: 500 μm.

(B) X-Gal staining of the AV node of the AVCS-iLacZ mouse, scale bar: 200 μm.

(C) Acetylcholine esterase staining of the AV node of the AVCS-iLacZ, scale bar: 200 μm.

(D) Immunofluorescence staining of the AV node of the AVCS-iLacZ mouse using HCN4 antibody, scale bar: 200 μm.

damaging the insulators in the electrode wirings. Damaged wiring insulators can also introduce noise in the ECG recordings. We highly recommend using a Bio Amp to enhance the ECG signal and reduce noise. If noise persists, ensure that electrode wirings are well connected to the Bio Amp or replace faulty ECG electrodes if necessary.

Problem 3

No AV block phenotype after Tamoxifen induction (steps 1–3).

Potential solution

Check if the Tamoxifen-induced animals have the correct genotype. Remove from the experiment any mice that have the wrong genotype or any mice that did not exhibit AV block after Tamoxifen induction. If entire litter did not exhibit AV block, genotype Tamoxifen-induced mice and their parents. If both parents and offspring have the correct genotype, prepare a new batch of Tamoxifen solution for another round of induction. If AV block is too severe with low survivability of mice, Tamoxifen dosage can be decreased up to 0.5 mg.

Problem 4

Inadequate cryopreservation and poor sectioning of tissue samples (steps 25 & 26).

Potential solution

Make sure no air bubbles are formed when adding TFM during mounting of heart samples into the cryomold. The presence of bubbles indicates that TFM is not in contact with the heart sample which leads to inadequate cryopreservation. Air bubbles can lead to suboptimal cryosections due to poor transferring of heart sections to microscope slides (e.g., rolling/curling of sections in the slides). Air bubbles can be

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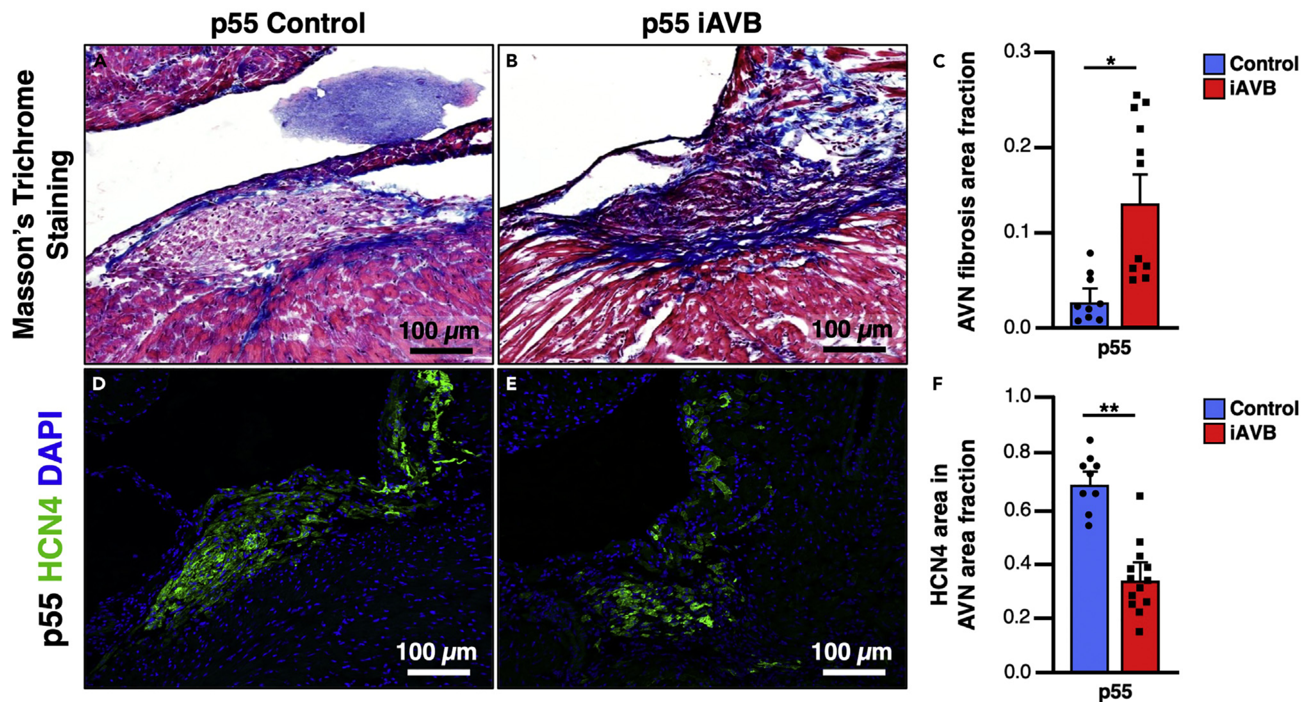

Figure 9. Cellular ablation of the AV node of iAVB (Tg$^{Cx30.2\text{-}MerCreMer/+}$; Rosa26$^{DTA/LacZ}$) mouse heart after Tamoxifen induction

(A and B) Masson's trichrome staining of the AV node of iAVB mouse compared to control, scale bar: 100 μm.

(C) Quantification of the AVN fibrosis of iAVB mouse compared to control, *p-value <0.05. Error bars represent SEM.

(D and E) HCN4 immunofluorescence staining of the AV node of iAVB mouse compared to control, scale bar: 100 μm.

(F) Quantification of HCN4 positive cell dropout in the AVN of iAVB mouse compared to control, **p-value <0.01. Error bars represent SEM.

removed using forceps. Excess sucrose solution around the heart sample ("watery heart") can also lead to suboptimal cryosections due to poor transferring as well. This can be prevented by bathing the heart sample in TFM placed in a foil prior to mounting in the cryomold. Maintaining the cutting chamber temperature at –23°C and ensuring a sharp cutting knife can give optimal heart sections.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nikhil V. Munshi (Email: Nikhil.Munshi@UTSouthwestern.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

This work was supported by the NIH (HL136604, HL133642, and HL135217, to N.V.M.), the Burroughs Wellcome Fund (1009838, to N.V.M.), and the March of Dimes Foundation (5-FY13-203, to N.V.M.). We thank John Shelton and the Histopathology Core Facility for sharing critical reagents and advice.

AUTHOR CONTRIBUTIONS

L.W. developed the study protocol. J.G.L.D.R. wrote the study protocol. N.V.M. obtained funding and supervised the study project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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