Supplemental Material

Materials and Methods

Animals

Wnt1-Cre mice (The Jackson Laboratory, Stock No. 003829) express Cre recombinase in all neural crest-derived cells. tdTomato reporter mice (The Jackson Laboratory, Stock No. 007914) express the red fluorescent protein variant (tdTomato) in response to Cre-mediated recombination. Male Cre/$^{+}$ mice were mated with homozygous female tdTomato mice to produce a litter including Cre/$^{+}$;tdTomato/$^{+}$ mice. The colony was maintained by mating these Cre/$^{+}$;tdTomato/$^{+}$ animals together. Progeny were screened directly using a portable dual fluorescent flashlight and filter unit, NIGHTSEA™ (Electron Microscopy Sciences, DFP1): Cre/$^{+}$;tdTomato/$^{+}$ animals could be identified directly by their red fluorescent heads, due to the fact that neural crest cells contribute to craniofacial structures. Cre/$^{+}$;tdTomato/$^{+}$ animals were used in experiments where direct observation of nerves was required. BALB/cJ animals (The Jackson Laboratory, Stock No. 000651) were used in apical resection experiments due to the relatively large litter size and enhanced maternal care following injury. Studies were performed on 2-day-old neonates and hearts were collected at either day 14 or day 21 post-injury.

Apical Resection

The surgical method was modified from that previously described[1]. Briefly, 2-day-old pups were anesthetized by hypothermia by placing on ice, separated by a latex glove, until akinetic. The animals were secured in a supine position by applying surgical tape to the limbs. The thorax was sterilized with 10% iodine solution and a lateral incision was made into the skin at the level of the 4th intercostal. A perforation was introduced into the chest cavity with needle-nose tweezers and the ribs were spread with blunt-nose

tweezers to avoid damage to the lungs. In a divergence from previous reports, we did not retract the heart by fixing it with tweezers. Instead, we applied gentle pressure to the abdomen, which presented the apex, then "hooked" the apex with a modified 30-gauge needle and retracted it though the surgical site (Supplemental Figure I). A minimal portion of the apex could then be resected in a reproducible manner by cutting proximal to the needle. This method significantly reduced trauma to the heart, compared to that previously caused by fixation[2, 3]. Following resection the heart was allowed to return to the chest cavity and a single suture (Prolene 6-0) was used to close the ribs. The skin was closed using GLUture topical tissue adhesive (Fisher Scientific, NC9855218) and the mice were allowed to recover on their bedding under a heat lamp. Once motile the pups were returned, en masse, to the mother as previously described[1]. The entire procedure was carried out in approximately 7 minutes per animal. Survival rate was approximately 80%.

Denervation

6-Hydroxydopamine hydrobromide (6-OHDA) (Santa Cruz Biotechnology, SC-256988), was dissolved in PBS containing 1% Sodium metabisulfite (Sigma, S-1516) antioxidant at a concentration of 25μg/μL and injected intraperitoneally into neonatal mice at a concentration of 250 μg/g. The 6-OHDA was administered three times at two-day intervals, beginning 48 hours after surgery.

Epifluorescence and Immunofluorescence imaging

Epifluorescent analysis of the neuroanatomical structure of the heart was performed on whole organs. Hearts were isolated from 14 day or 21-day-old mice, washed in cold PBS and fixed in 4% paraformaldehyde at room temperature for 15 minutes. The hearts were washed, submerged in PBS in a 60 mm plastic petri dish and immediately imaged at

592nm using a Zeiss Apo Stemi SV11 fluorescent stereomicroscope with a Spot Flex camera. Immunofluorescent analysis was performed on individually isolated ventricles. The tissue was prepared as described above. After fixation the hearts were incubated in blocking solution consisting of 20% Normal Donkey Serum (Millipore, S30-100KC), 5% Triton X-100 (Fluka analytical, #93443) in PBS for 30 minutes at room temperature. The hearts were transferred to primary antibodies diluted in 1% BSA (Sigma-Aldrich, #A2153)/PBS plus 0.1% Triton X-100 and incubated overnight at 4°C. Primary antibodies used were Rabbit anti-synapsin1 (Millipore, AB1543), Goat anti-ChAT (Millipore, AB144P), Rabbit anti-Tyrosine Hydroxylase (Millipore, AB152), Rabbit anti-Tuj-1 (β-tubulin III) (Covance, mrb-435p-100). Tissues were washed in PBS + 0.2% Tween (3 x 10 minutes) prior to incubation with secondary antibodies diluted in 1% BSA/PBS plus 0.1% Triton X-100 and incubated for 1 hour 15 minutes at room temperature. Secondary antibodies used were Donkey anti-Rabbit Alexa Fluor 488 (Life Technologies, #A21206), Donkey anti-Goat IgG Cy3 (Millipore, #AP180C). Immunofluorescent images were collected using an Olympus IX81 fluorescent microscope and Q Imaging Retiga EX camera. Relative nerve density was calculated using NIH Image J software.

Immunohistochemistry

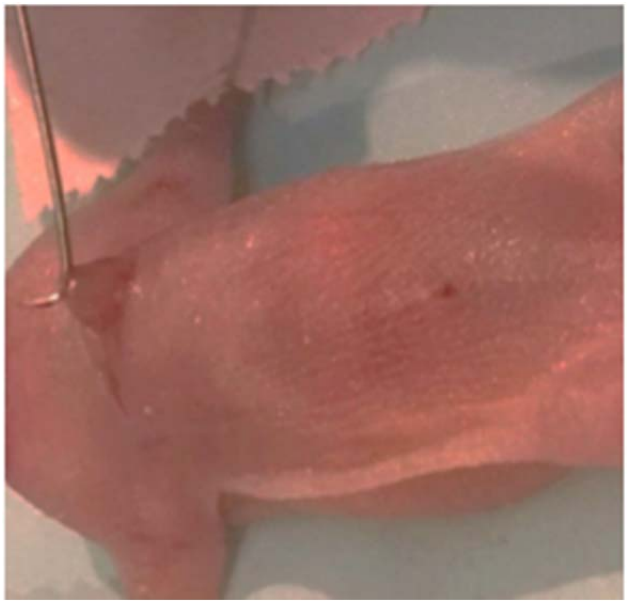
Hearts were harvested from 21-day-old mice, with some requiring mechanical detachment from the wall of the chest cavity. The hearts were washed in cold PBS and fixed o/n at 4°C in 4% paraformaldehyde. After washing twice in cold PBS (5 minutes each) they were transferred to 30% sucrose/PBS solution at 4°C until equilibrium was reached. Hearts were then blotted and embedded in OCT (VWR, #95057-838) and frozen by floating on 100% EtOH cooled with dry ice. 10 µm thick tissue sections were cut using a cryostat (Leica, CM1850) onto Colorfrost Plus microscope slides (Fisher,

#12-550-17). After being allowed to dry, sections were stained with Masson's Trichrome, and imaged using a Pathscan Enabler IV slide scanner. The scarred area was calculated using NIH Image J software and reported as a proportion of the area of the left ventricular myocardium.

Statistical method

Data are presented as mean ± SEM. Comparisons were conducted via unpaired, parametric *t* test. Significant differences between groups are defined by $p<0.01$.

1. Mahmoud AI, Porrello ER, Kimura W, Olson EN, Sadek HA. Surgical models for cardiac regeneration in neonatal mice. *Nature protocols*. 2014;9:305-311
2. Bryant DM, O'Meara CC, Ho NN, Gannon J, Cai L, Lee RT. A systematic analysis of neonatal mouse heart regeneration after apical resection. *Journal of molecular and cellular cardiology*. 2015;79:315-318
3. Bryant DM, O'Meara CC, Ho NN, Gannon J, Cai L, Lee RT. Response to "comment to the article 'a systematic analysis of neonatal mouse heart regeneration after apical resection'". *Journal of molecular and cellular cardiology*. 2015;82:184-185



Supplemental Figure I. Modification of neonatal cardiac resection model. By hooking the apex of the heart with a modified 30-guage needle we reduced confounding trauma associated with fixation and retraction and permitted small, reproducible apical resections.