**Title**

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**Authors**

Bouhidel, Jalaleddinne Omar
Wang, Ping
Siu, Kin Lung
et al.

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# Netrin-1 Improves post-Injury Cardiac Function *in vivo* via DCC/NO-dependent Preservation of Mitochondrial Integrity, While Attenuating Autophagy

**Jalaleddinne Omar Bouhidel, PhD**[*], **Ping Wang, MD, PhD**[*], **Kin Lung Siu, PhD**, **Hong Li, MS**, **Ji Youn Youn, PhD**, and **Hua Cai, MD, PhD.**
Divisions of Molecular Medicine and Cardiology, Departments of Anesthesiology and Medicine, Cardiovascular Research Laboratories, David Geffen School of Medicine at University of California Los Angeles, 650 Charles E. Young Drive, Los Angeles, CA 90095, USA

## Abstract

Reperfusion injury of the heart is a severe complication of angioplasty treatment of acute myocardial ischemia, for which no therapeutics are currently available. The present study aimed to identify whether and how a novel protein netrin-1 induces cardioprotection *in vivo* during ischemia/reperfusion (I/R) injury. Wild type (WT) C57BL6/J mice were subjected to a 30 min coronary occlusion followed by a 24 hr reperfusion with vehicle (normal saline), netrin-1, UO126 (MEK1/2 inhibitor), PTIO (nitric oxide/NO scavenger), netrin-1/UO126 or netrin-1/PTIO intraventricularly. Some were injected of netrin-1 via tail vein. Netrin-1 at 5 μg/kg induced a substantial reduction in infarct size (19.7±5.0% from 41.3±1.8% in the controls), and markedly improved cardiac function as measured by ejection fraction and fractional shortening from echocardiography. Experiments with mice deficient in netrin-1 receptor DCC (deleted in colorectal cancer, DCC+/−), or reperfusion with netrin-1/UO126 or netrin-1/PTIO, attenuated the protective effects of netrin-1, implicating intermediate roles of DCC, ERK1/2 and NO. Netrin-1 induced phosphorylation of ERK1/2 and eNOS was abolished in DCC+/− mice. Electron spin resonance (ESR) determination of NO production from isolated left ventricles demonstrated that netrin-1 improves NO bioavailbility, which was attenuated by UO126 or in DCC+/− mice, suggesting upstream roles of DCC and ERK1/2 in NO production. Netrin-1 further reduced mitochondrial swelling and mitochondrial superoxide production, which was absent when co-treated with PTIO or UO126, or in DCC+/− mice, indicating critical roles of DCC, ERK1/2 and NO in preserving mitochondrial integrity. In a permanent coronary ligation model of myocardial infarction (MI) to assess post-MI remodeling, netrin-1 abolished the marked increase in



Corresponding to: Hua Linda Cai, Divisions of Molecular Medicine and Cardiology, Departments of Anesthesiology and Medicine, Cardiovascular Research Laboratories, David Geffen School of Medicine at University of California Los Angeles, 650 Charles E. Young Drive, Los Angeles, CA 90095, USA, Tel: 310 267 2303, Fax: 310 825 0132, hcai@mednet.ucla.edu.
[*]The authors contributed equally to this study

**DISCLOSURES**
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autophagy. In summary, our data demonstrate robust cardioprotective effect of netrin-1 *in vivo*, as shown by reduced infarct size and improved cardiac function. Mechanistically, this protection is mediated by netrin-1 receptor DCC, and NO dependent preservation of mitochondria. This work clearly establishes a therapeutic potential of netrin-1 for acute treatment of MI, perhaps also for chronic post-MI remodeling.

**Keywords**

netrin-1; cardioprotection; nitric oxide (NO); ischemia reperfusion (I/R) injury; deleted in colorectal cancer (DCC); ERK1/2; endothelial NO synthase (eNOS); NADPH oxidase isoforms 4 (NOX4); myocardial infarction (MI); autophagy

## INTRODUCTION

Acute myocardial infarction (MI) is one of the leading causes of morbidity and mortality in the adult population that strikes 7.6 million American adults, or about one person every 44 seconds in the United States[1]. The mainstay of current therapy for acute MI is the restoration of blood flow (reperfusion) to the affected area through angioplasty, sometimes in conjunction with thrombolytic therapy to remove/prevent thrombi formation. However, the reperfusion of ischemic tissue can itself lead to severe myocardial damage[2]. Intensive research efforts have been focused on the amelioration of various pathophysiological components of ischemia/reperfusion (I/R) injury to limit the extent of cell death[3]. However, data accumulated in the past twenty years have shown that there is no clinically effective therapy for the prevention of I/R injury. Currently, while there are some potential agents under trials for this condition, such as cyclosporine A, there are no clinical approved treatments for the prevention of cardiac I/R injury.

Netrin-1 has been originally identified as a midline-derived chemoattractant that guides axons to the midline during development[4–6]. Its functions are mainly mediated by the receptors of deleted in colorectal cancer (DCC) and the uncoordinated-5 (UNC5) families[7–10]. More recently, netrin-1 has been shown to mediate angiogenesis[11–13]. In our laboratory, we have shown that netrin-1 can induce angiogenesis through an increase in endothelial nitric oxide (NO) synthase (eNOS) activation and NO production[13]. Further, we have shown that netrin-1 induces cardioprotection against I/R via a DCC/ERK1/2/eNOS/NO/DCC feed-forward pathway, using an *ex-vivo* model of Langendorff perfused heart[14].

In the present study we aimed to examine whether and how netrin-1, potentially via production of NO, is cardioprotective *in vivo*. We examined whether netrin-1 improves cardiac function by measuring ejection fraction and fraction shortening using echocardiography at day 1 and day 3 after reperfusion, using animals treated with netrin-1 both intraventricularly and intravenously via tail vein. Treatment with netrin-1 dose-dependently reduced infarct size and improved cardiac function measured by echocardiography. Experiments using pharmacological inhibitors (UO126 as ERK1/2 inhibitor, PTIO as NO scavenger), animals deficient in netrin-1 receptor DCC, and experiments measuring p-eNOS, p-ERK1/2, NADPH oxidase isoform 4 (NOX4) expression and activity, levels of NO and superoxide by electron spin resonance (ESR), as well as mitochondrial damage via mitochondrial swelling, showed that mechanistically netrin-1's cardioprotective effect is mediated by DCC and NO dependent suppression of NOX4-derived oxidative stress, as well as protection of mitochondria. Using an additional permanent myocardial infarction (MI) model we have also observed robust effects of netrin-1 in attenuating autophagy. This work clearly shows the efficacy of netrin-1 as a potent cardioprotective agent against I/R damage *in vivo*, hence the potential as a novel therapeutic for acute treatment of MI, or perhaps also for chronic post-MI remodeling.

## MATERIALS AND METHODS

### Materials

Purified recombinant mouse netrin-1 (R&D Systems, Minneapolis, MN, USA) was administered at doses ranging from 1 to 50 μg/kg, dissolved in normal saline, via injection into the LV lumen at the onset of reperfusion. Some mice were subjected to tail vein injection with netrin-1 (5 μg/kg) also immediately at the onset of reperfusion. UO126, a MEK1/2 inhibitor (200 μg/kg, dissolved in DMSO<0.01%), and PTIO (1 mg/kg, dissolved in normal saline), a specific NO chelator, were purchased from Sigma-Aldrich in highest purity (St. Louis, MO, USA), and administered via injection into the LV lumen at the onset of reperfusion.

Polyclonal antibodies specific for phosphorylated and total forms of ERK1/2, eNOS, were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). NOX4 antibody was purchase from Abcam (Abcam, Cambridge, MA, USA). Anti-actin, antibody, and other chemicals in highest purity, were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

### Animals

Male C57BL/6J mice (6–8 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, ME). The DCC+/− breeding colony was kindly provided by Dr. Marc Tessier-Lavigne from The Rockefeller University. Mice were housed under pathogen-free conditions. The use of animals and experimental procedures were approved by the Institutional Animal Care and Usage Committee at the University of California Los Angeles (UCLA).

### *In vivo* murine model of myocardial ischemia-reperfusion injury

Briefly, mice were pre-medicated with heparin (1,000 IU/kg, i.p.) and anesthetized 5 min later with sodium pentobarbital (60 mg/kg, i.p.). An additional dose of pentobarbital (50 μl; 20 mg/kg, i.p.) was given as needed to maintain anesthesia. After an adequate depth of anesthesia is attained the mouse is fixed in a supine position with tape. Mice were then orally intubated and ventilated mechanically with a Harvard Apparatus Rodent Ventilator (model 845). A mix of oxygen and carbon dioxide (95:5%) was supplied, and body temperature was monitored using a rectal probe thermometer and controlled with a heating pad. Left thoracotomy was performed in order to reveal the left coronary artery (LCA). Myocardial ischemia was achieved by tying a 7-0 Prolene thread around the LCA, which was then subsequently confirmed by the occurrence of regional cyanosis. The LCA was completely occluded for 30 min, and reperfusion was initiated by removal of the 7-0 suture. Reperfusion was confirmed by visualisation of a hyperaemic response. The chest wound was then reapproximated, and mice were extubated and allowed to recover with supplemental oxygen until mobile. All mice received buprenorphine (0.1 mg/kg) subcutaneously to minimize pain. The I/R protocol and the experimental procedures involving treatments with netrin-1 and pharmacological inhibitors are summarized in suppl. Figure 1.

## Infarct size analysis

After 24 hr reperfusion, mice were given heparin (1,000 IU/kg, i.p.), after which they were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The coronary artery was re-occluded at the original site of occlusion, while a solution of Evan blue dye (5% solution) was injected through the apex. The auricle and the right ventricle were removed and the left ventricle (LV) was excised into 6 slices using a mouse heart matrix (Harvard Apparatus, Holliston, MA, USA). Before 2,3,5-triphenyltetrazolium chloride (TTC) incubation, each slice is photographed in order to determine the area at risk (AAR) identified by the absence of Evans blue staining. The infarct area (IA) was identified by incubating each slice with TTC (1% solution, 5 min at 37°C). The AAR identified by the absence of Evans blue staining was expressed as a percentage of the left ventricle weight and the IA was expressed as a percentage of the AAR. Each transverse slice was fixed in 10% neutral buffered formaldehyde and 1 hour later, weighed and photographed. The different areas (AAR, IA, LV circumference, LV cavity circumference) were measured by computerized videoplanimetry (NIH Image J-1.37), and from these measurements infarct size was calculated as a percentage of the AAR.

## Echocardiography

Cardiac morphology and function were assessed using anesthetized (0.6–0.8% isoflurane in 95% oxygen, heart rate: 420–450 beats/min) mice by transthoracic echocardiography (Vevo2100 echocardiograph with MS-400 probe, Visualsonics, Toronto, Ontario, Canada). Two-dimensional images and M-mode tracing were recorded from the parasternal short axis view at the midpapillary level to determine the left ventricular internal diastole diameter (LVID:D) and left ventricular internal systolic diameter (LVID:S). Fractional shortening and ejection fraction were calculated directly from the short axis view of heart contraction.

## Western Blot analysis

At the end of the 10 min or 30 min of reperfusion, the heart was removed and the LV was immediately frozen in liquid nitrogen. Subsequently, the tissue was powderized and homogenized in lysis buffer (Tris 50 mmol/L, EDTA 0.1 mmol/L, EGTA 0.1 mmol/L, protease inhibitor cocktail, phosphatase inhibitors cocktail 2 and 3, pH 7.4). The homogenates were vortexed and centrifuged at 17,600×g for 30 min at 4°C. The supernatants were used for Western blot analysis. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bio-Rad protein assay kit, Bio-rad, Hercules, CA, USA).

Extracted protein samples were denatured at 100°C in loading buffer containing β-mercaptoethanol for 5 min. Proteins (30 μg/lane) were separated by a 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Hybond™, GE Health Care, Piscataway, NJ, USA) and probed overnight with primary antibodies for phospho-ERK1/2 (Thr 202/Tyr204), phospho-eNOS (Ser1177), NOX4, total ERK1/2, total eNOS, and actin. Blots were washed and then incubated with secondary antibody conjugated to horseradish peroxidase (Bio-Rad laboratories, Hercules, CA, USA) prior to incubation with ECL reagent (Pierce, Rockford, IL, USA) and exposed to an autoradiography film (Hyblot CL, Denville scientific, Metuchen, NJ, USA). Bands of interest were scanned and quantified in a blinded manner using gel analysis software NIH Image J-1.37.

## Electron spin resonance detection of nitric oxide radical

Bioavailable nitric oxide radical (NO) from LV tissues was detected using electron spin resonance (ESR) as described [13–16]. In brief, the LV heart homogenates were incubated with equal volume of freshly prepared NO-specific spin trap $Fe^{2+}(DETC)_2$ colloid (0.5 mmol/L) for 60 min, in the presence of calcium ionophore A23187 (10 μmol/L). Gently collected homogenates suspensions were snap-frozen in liquid nitrogen and loaded into a finger Dewar for analysis with an eScan electron spin resonance (ESR) spectrophotometer (Bruker) at the following settings: center field, 3410; field sweep, 100 G; microwave frequency, 9.73 GHz; microwave power, 13.26 mW; modulation amplitude, 9.82 G; 512 points resolution and receiver gain, 356.

## Electron spin resonance measurement of mitochondrial superoxide production

For isolation of heart mitochondria, fresh left ventricular tissues were placed in buffer containing 220 mmol/L mannitol, 70 mmol/L sucrose, 10 mmol/L HEPES, 1 mmol/L EGTA (Sigma-Aldrich, St. Louis, MO, USA), pH 7.4 at 4°C. The tissues were minced with scissors and homogenized on ice using a Teflon Potter homogenizer and were centrifuged at 1,000 g for 5 min. Supernatants were centrifuged at 10,000 g for 10 min. The final pellets were resuspended in homogenization buffer including 0.01 mmol/L EGTA. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bio-Rad protein assay kit, Bio-rad, Hercules, CA, USA). The specific superoxide spin trap methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH, 500 μmol/L, Alexis) solution was prepared freshly in nitrogen gas bubbled Krebs/HEPEs buffer containing diethyldithiocarbamic acid (DETC, 5 μmol/L Sigma) and deferoxamine (25 μmol/L, Sigma). Three μL of the isolated mitochondria was then mixed with spin trap solution and loaded into glass capillary (total volume 100 μL, Fisher Scientific) for analysis of superoxide signal ($CM^{\bullet}$ formed after trapping $O_2^{\bullet-}$) using eScan ESR spectrometer (Bruker) at the following settings: center field, 3410; field sweep, 100 G; microwave frequency, 9.73 GHz; microwave power, 13.26 mW; modulation amplitude, 9.82 G; 512 points resolution and receiver gain, 356. The final superoxide value was normalized by the amount of mitochondrial protein loaded.

## Mitochondrial swelling assay

The activation of the mitochondrial permeability transition pore was determined by $Ca^{2+}$-induced swelling of isolated cardiac mitochondria. Opening of the pore causes mitochondrial swelling, which was measured spectrophotometrically as a decrease in absorbance at 540 nm. Isolated cardiac mitochondria (30 μg) were incubated for 1 min in the swelling buffer, which contained 250 mmol/L sucrose, 10 mmol/L Tris·HCl (pH 7.4) and energized with 2 mmol/L of succinate, to a final protein concentration of 0.3 mg/L. Pore opening was induced by 250 μmol/L of $CaCl_2$ and the decrease in absorbance was measured at 540 nm every minute for 20 min using a 96-well plate.

### NOX4 activity assay

NOX4 activity was determined using isolated membrane fraction. For the isolation of the membrane fraction, hearts were isolated from animals after 10 min of reperfusion, then homogenized on ice in a glass homogenizer in lysis buffer as described above The homogenate was serially centrifuged at 1,200 g for 5 min, 22,000 g for 20 min, and 13,7000 g for 90 min at 4ºC. The final pellet was resuspended in lysis buffer (100 μL per heart). Superoxide production was measured as described above, with 10 μg protein loaded as measured using the Bradford method. Kinetic NADPH-driven (100 μmol/L final concentration) NOX4 activity was measured in the presence or absence of Fulvene-5, a specific NOX4 inhibitor (kindly provided by Dr. Jack L. Arbiser from Emory University)[17].

### Animal Model of MI and Assessment of Autophagy

Male C57BL/6 mice (8–10 weeks old) were used to induce myocardial infarction (MI) by permanent left anterior descending (LAD) coronary artery ligation. Two days before the surgery, the mice were infused with netrin-1 (15 ng/kg per day) or vehicle using subcutaneously implanted osmotic pumps (14 days, Durect Corp). Then the mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and fixed in the supine position with positive pressure respiration. The left thorax was opened and the LAD coronary artery was located and ligated with a 8-0 silk suture 2–3 mm from origin. The ligation was deemed successful when the anterior wall of the LV turned pale. After the ligation, the chest was closed in layers, and the mice were removed from the ventilator when awake. Sham-operated animals were subjected to similar surgery, except that no ligature was placed.

Two weeks later, isolated hearts were subjected to protein isolation and western blotting analysis as described earlier, using specific antibodies to LC3 (1:3000, Sigma) and β-actin. LC3 expression levels were analyzed using Image J program and normalized by β-actin.

### Statistical analysis

All data are presented as Mean±SEM. Comparisons between two groups were made using student’s *t*-test, while multiple comparisons with more than two groups were made using ANOVA follow by Newman-Keuls’s post-hoc test. Statistical significance was defined as $p<0.05$.

## RESULTS

### Netrin-1 attenuates I/R induced myocardial infarction *in vivo*

To examine whether netrin-1 administration is effective in inducing cardioprotection against I/R damage *in vivo*, wild type C57BL6J mice were subjected to a 30 min of ischemia by coronary occlusion, followed by a 24 hr of reperfusion. Netrin-1 or vehicle (normal saline) was injected into the LV lumen at the onset of reperfusion at doses ranging from 1 to 50 μg/kg. Mice receiving vehicle injection displayed a 41.3±1.8% infarct size per area at risk (Inf/AAR), and a 17.1±1.5% infarct size per LV size (Inf/LV). Treatment with netrin-1 at 1 μg/kg, 5 μg/kg and 10 μg/kg all significantly decreased infarct size (37.6±2.5%, 19.7±5.0% and 29.9±1.5% respectively for Inf/AAR; and 15.7±2.0%, 8.1±2.0% and 11.9±0.8% respectively for Inf/LV, Figure 1). Interestingly, netrin-1 at 5 μg/kg resulted in the greatest reduction in Inf/AAR and Inf/LV compared with the other doses (Figure 2). Furthermore, at high doses (50 μg/kg), the cardioprotective effect of netrin-1 disappeared (47.0±1.0% for Inf/AAR, and 20.1±0.6% for Inf/LV). The percent AAR per LV (AAR/LV) was similar among all study groups, indicating similar severities of myocardial ischemia.

### Netrin-1 improves cardiac function after ischemia-reperfusion *in vivo*

To further validate netrin-1's cardioprotective properties *in vivo*, we measured LV dilatation (LVID:d and LVID:s) and cardiac function via echocardiography on animals that underwent I/R injury. The results, shown in Figure 2A and 2C, illustrated that both fractional shortening and ejection fraction were significantly decreased in vehicle treated hearts at both 1 and 3 day after I/R. Treatment with netrin-1 through the LV resulted in significantly increased ejection fraction and fractional shortening, suggesting an improvement in cardiac function. Figure 2A and 2B indicated that LV structures were significantly improved in netrin-1 treated hearts at both 1 and 3 day after I/R. This data shows that netrin-1 can functionally rescue I/R injured heart *in vivo*.

Additionally, separate experiments were performed where the treatment of vehicle or netrin-1 was injected through the tail vein. The results, shown in Figure 2D and 2F, show a similar trend in the recovery of cardiac function as compared to LV injected hearts. Figure 2D and 2E, shows a similar trend in the improvement of LV structures as compared to LV injected hearts. This shows that netrin-1 is effectively protective against I/R injury when given through a more easily accessible route.

### The cardioprotective effect of netrin-1 *in vivo* is dependent on DCC

To examine whether netrin-1's cardioprotective effect is dependent upon its receptor deleted in colorectal cancer (DCC), DCC+/+ and DCC+/− mice were subjected to a 30 min of myocardial ischemia followed by a 24 hr of reperfusion. Mice received either 5 μg/kg netrin-1 or vehicle at the onset of reperfusion. In DCC+/− mice, netrin-1 failed to reduce infarct size (44.4±1.4% vs. 24.7±3.2% in DCC+/+ mice for Inf/AAR, and 18.3±0.6% vs. 11.0±1.2% in DCC+/+ mice for Inf/LV) (Figure 3). AAR/LV was similar for both groups. These data clearly implicate an intermediate role of DCC in mediating netrin-1 induced cardioprotection *in vivo*.

### Role of NO and ERK1/2 in netrin-1 induced cardioprotection *in vivo*

We have previously identified a DCC-ERK1/2-eNOS-NO pathway in mediating netrin-1's potent cardioprotective effect against I/R injury *ex-vivo*[14]. Here, we examined whether similar mechanisms underlie netrin-1 induced cardioprotection *in vivo*. Mice were subjected to a 30 min of myocardial ischemia, followed by a 24 hr of reperfusion, and injected with vehicle (normal saline); netrin-1; UO126 (MEK1/2 inhibitor); PTIO (NO scavenger); netrin-1/UO126, or netrin-1/PTIO (at same doses described earlier) at the onset of reperfusion. As shown in Figure 4, UO126 or PTIO alone had no significant effect on infarct size. Co-treatment of UO126 or PTIO with netrin-1 completely prevented netrin-1's cardioprotective effect, as shown by the infarct size measurements (Figure 4). These data indicate that ERK1/2 and NO are required for the netrin-1 induced cardioprotection *in vivo*. AAR/LV was similar for both groups (Figure 4), showing that I/R injury was comparable among the different groups.

### Netrin-1 activation of ERK1/2 and NO *in vivo* is DCC dependent

As shown above, ERK1/2 and NO are involved in netrin-1 induced cardioprotection against I/R damage *in vivo*. Here, we examined whether mechanistically they lie downstream of DCC. Western blotting of p-ERK1/2 and p-eNOS was performed from DCC+/+ and DCC+/− mice subjected to a 30 min ischemia followed by a 24 hr of reperfusion, and treated at the onset of reperfusion with vehicle (normal saline) or netrin-1. In DCC+/+ mice, treatment with netrin-1 significantly activated ERK and eNOS in cardiac tissue under I/R. This activation was abolished in DCC+/− mice, where netrin-1 resulted in no significant change in ERK and eNOS activation compared with untreated controls (Figure 5A, 5B). These results demonstrate that netrin-1 activation of ERK1/2 and eNOS activation *in vivo* are dependent on DCC. We also examined iNOS expression after reperfusion of netrin-1 (Figure 5C), which showed no significant changes between I/R or I/R/netrin-1 group with sham condition. This further support that observation that eNOS is the major NOS isoform that is responsible for cardioprotective effect of netrin-1.

### Netrin-1 induces NO production *in vivo* in a DCC and ERK1/2 dependent manner

Data described above suggest that both ERK1/2 and eNOS activation lie downstream of DCC. We next measured NO directly via ESR to elucidate the exact relationship between the three components. Wild type C57BL6 mice subjected to I/R injury was treated with vehicle, netrin-1, or netrin-1/UO126 at the onset of reperfusion. The heart was harvested for NO measurements from the LV. The data, shown on Figure 6A, demonstrate that vehicle treated hearts had significantly reduced NO bioavailability, while treatment with netrin-1 markedly corrected this deficiency. Co-treatment of netrin-1 and UO126 abolished the beneficial effects of netrin-1 on NO production, suggesting that activation of ERK1/2 proceeds eNOS/NO activation.

Furthermore, we measured LV NO production using I/R injured DCC+/+ and DCC+/− mice. The results, shown on Figure 6B, indicate that netrin-1 mediated NO increase shown in DCC+/+ animals were completely abolished in DCC+/− animals. Taken together, these data confirm that netrin-1 stimulates NO production in I/R injured hearts through a DCC/ERK1/2/eNOS/NO pathway *in vivo*.

### Netrin-1 attenuates mitochondrial superoxide production *in vivo* via DCC and ERK1/2

Previous studies have shown that the large burst of oxidants in the mitochondria observed during early reperfusion contributes strongly to I/R damage[18]. Mice were subjected to a 30 min of myocardial ischemia, injected with vehicle (normal saline); netrin-1 or U0126 (at same doses described earlier) into the LV at the onset of reperfusion. The heart was then harvested for mitochondrial isolation and subsequent ESR detection of superoxide production from mitochondria. The results, shown in Figure 7A, illustrate that mitochondria from vehicle treated hearts produced ~4 times more superoxide after I/R injury when compared to mitochondria isolated from sham surgery controls. Treatment with netrin-1 significantly reduced this increase to ~2.5 times, while co-treatment of netrin-1 with UO126 abolished this reduction, suggesting the role of ERK1/2 in netrin-1 mediated reduction in I/R induced mitochondrial superoxide production.

To examine the role of the DCC receptor in this phenomenon, identical I/R protocol was performed using DCC+/+ and DCC+/− animals. The results, shown in Figure 7B, illustrate that netrin-1's effect on mitochondrial superoxide production was completely abolished in DCC+/−, implicating a dependency on the DCC receptor.

### Netrin-1 preservation of mitochondrial Integrity *in vivo*: dependence on DCC and ERK 1/2

One of the major events in I/R induced cardiac injury is mitochondrial damage and increased superoxide production from mitochondria. We further examined impact on mitochondrial damage *in vivo* of netrin-1 by employing a calcium dependent swelling assay. As shown in Figure 8A, netrin-1 perfusion resulted in a marked attenuation of the swelling activity, which was reversed by co-treatment with the ERK1/2 inhibitor UO126. Similar experiments were performed using DCC+/− and DCC+/+ animals. The results, shown in Figure 8B, indicates that while netrin-1's treatment is protective against mitochondrial damage in DCC+/+ animals, DCC+/− animals had a complete loss of the protection. Taken together, these data indicate DCC and ERK1/2 are required for mitochondrial preservation induced by netrin-1 during I/R injury *in vivo*.

### Netrin-1 down-regulates NOX4 expression and activity in a NO and ERK1/2 dependent manner

In a previous study using an *ex vivo* model of Langendorff perfused heart, we observed an increase in NOX4 expression and activity from I/R injured hearts, which was abolished by treatment with netrin-1[19]. Here, we examined if this also holds true in the *in vivo* condition and the signaling pathway involved. Western blots for NOX4 and actin were performed on hearts treated with vehicle, netrin-1, netrin-1/PTIO, or netrin-1+U0126 and harvested 30 min after reperfusion. Figure 9A and 9B showed that treatment with netrin-1 significantly reduced NOX4 expression in LV, which was reversed by co-treatment with PTIO and U0126. These results show that netrin-1 reduction of NOX4 is dependent on ERK1/2 and NO. Given that ERK1/2 activation occurs prior to NO production in response to netrin-1, these data suggest a DCC:ERK1/2:eNOS:NO:NOX4 pathway.

To further examine changes in NOX4 activity, we measured NOX4 specific activity using ESR in the presence of Fulvene-5, an inhibitor for NOX4[17]. The results, shown on Figure 9C, indicate that I/R significantly increased NOX4 activity, while netrin-1 perfusion largely abrogated this effect. Further, treatment with PTIO, a specific NO scavenger, completely abolished netrin-1's effect on NOX4 activation, again implicating an upstream role of NO in silencing NOX4 pathway of oxidative activiation.

### Netrin-1 attenuates autophagy in post-MI remodeled heart

In additional experiments we performed permanent LAD ligation to induce myocardial infarction (MI). Chronically, autophagy was markedly increased in post-MI remodeled heart, which was completely attenuated by netrin-1 perfusion (Figure 10). Microtubule-associated proteins 1A/1B light chain 3, LC3, a mammalian homolog of yeast Atg8, is a major mediator of autophagy. During autophagy, LC3-I, a cytosolic form of LC3, is converted into LC3-II which is a specific marker of autophagosomes, and the ratio of LC3-II/LC3-I is an established indicator of autophagy. In this study, we found that LC3-II was up-regulated in the post-MI heart and the myocardial LC3-II/LC3-I ratio was significantly increased in MI animals. However, in mice infused with netrin-1 for 14 days using subcutaneously embedded osmotic minipumps, the myocardial LC3-II/LC3-I ratio decreased to the level similar to the sham group.

## DISCUSSION

The most significant findings of the present study are: 1) establishment of a potent cardioprotective effect of netrin-*1 in vivo*; 2) establishment that netrin-1 not only effectively reduces infarct size after I/R injury, but also markedly improves cardiac function; 3) establishment that netrin-1 exerts cardioprotection *in vivo* via preservation of mitochondrial integrity that is dependent on a DCC/ERK1/2/eNOS/NO pathway; 4) establishments that netrin-1 attenuates oxidative stress in I/R injured heart via ERK1/2/NO-mediated downregulation of NOX4 expression and activity; 5) establishment that in addition to its potent, acute cardioprotective effects during I/R injury, netrin-1 may also be beneficial in treating post-MI remodeling of the heart by attenuating autophagy. These findings mechanistically highlight a therapeutic potential of netrin-1 in treating reperfusion dependent or independent myocardial infarction when delivered to animals *in vivo*.

Netrin-1 has been shown in this work to dose dependently reduce infarct size *in vivo*, when given at lower doses (Figure 1). Further, this protective effect was shown to improve the physiological function of the damaged heart as measured by ejection fraction and fractional shortening via echocardiography (Figure 2). While netrin-1 has been shown by our laboratory in the past to confer cardioprotection in an *ex-vivo* model[14], the use of netrin-1 in an *in vivo*, physiological setting in the present study further confirm its potency and consistency in cardioprotection. The improvement in cardiac function after netrin-1 treatment again supports the use of netrin-1 in a physiological setting. Taken together, this data firmly establishes netrin-1 as a possible therapeutic agent against I/R injury.

The molecular pathway through which netrin-1 exerts cardioprotection *in vivo* is thoroughly characterized in this study. The use of DCC+/− animals (Figure 3) and inhibitors for ERK1/2 and NO (Figure 4) all abolished the cardioprotective effect of netrin-1, which clearly shows involvement of these signaling mediators. To elucidate the precise pathway for this protection, we first examined the activation of ERK1/2 and eNOS in DCC+/+ and DCC+/− animals (Figure 5), which shows that the activation of both ERK1/2 and eNOS by netrin-1 in DCC+/+ animals are abolished in DCC+/−, suggesting that DCC lies upstream of both. Furthermore, by the use of ESR to directly measure NO (Figure 6), we observed that inhibition of ERK1/2 with UO126 abolished netrin-1's effect in augmenting NO production in the I/R injured heart, implicating a dependency of this process on ERK1/2 activation. Taken together, these data clearly shows that netrin-1's cardioprotective mechanism is mediated via a DCC:ERK1/2:eNOS:NO pathway.

Nitric oxide has been shown in the past to be a powerful cardioprotective agent against I/R injury [3,20–23], mediating a number of protective mechanisms such as being anti-oxidative [24,25], anti-inflammatory [26,27], anti-apoptotic [28,29], and anti-detrimental signaling [30]. A number of agents that have shown cardioprotective effects against I/R injury takes advantage of NO, such as estrogen [31], sildenafil [32], and statins [33]. However, these treatments have their own side effects, such as the hormonal problems associated with men taking estrogen, sildenafil being a treatment for erectile dysfunction, and statin's effect on cholesterol. The larger problem is that while at low dose NO may be anti-apoptotic, high doses of NO has been shown to be pro-apoptotic [28,29] and may even contribute to hypotension and sudden cardiac death [34]. In this study, while the dose of netrin-1 used for optimal I/R protection enhanced NO level in the heart (Figure 6A), it is important to note that this enhanced level was still below that of sham surgery controls, suggesting that this dose is safe for the animals. However, the use of high dose of netrin-1 abolished this protective effect (Figure 1), mechanisms of which need to further elucidate.

Cardiac mitochondria are important not only for maintaining sufficient ATP generation, but also involved in cell death and loss of cardiomyocytes [35,36]. Opening of the mitochondrial permeability transition pore (mPTP) leads to cell death, and has been implicated in ischemic injury and the development and progression of heart failure [37–40]. A previous study revealed that NO prevents permeability transition in isolated mitochondria, and reperfusion-induced ROS generation in the reperfused heart [41]. These studies agree well with the results of our current study, where netrin-1, which was shown here to increase NO production, reduces mitochondrial swelling and mitochondrial superoxide production (Figures 7, 8). Co-treatment with UO126, or experiments using DCC+/− animals, shows that this protective effect of netrin-1 against mitochondrial damage lies downstream of DCC and ERK1/2 activation, likely due to DCC/ERK1/2-dependent augmentation in NO production. In a related report from our laboratory, we have shown that netrin-1 preserves mitochondrial Integrity via NO dependent attenuation of NADPH oxidase isoform 4 (NOX4) activation and eNOS uncoupling *ex vivo* [42]. In the present study we found that netrin-1 similarly abolished I/R induced upregulation in NOX4 expression and activity *in vivo*. This would represent an important pathway by which netrin-1 treatment attenuates oxidative stress during I/R injury.

In additional experiments we employed a permanent LAD ligation model of MI to examine chronic effects of netrin-1 on autophagy in post-MI remodeled heart. Autophagy is a fundamental cellular mechanism by which eukaryotic cells degrade and recycle macromolecules and organelles. In the heart, autophagy performs housekeeping functions such as maintaining cardiomyocyte structure and function. However, cardiac autophagy is increased in response to oxidative stress, ATP depletion and mPTP opening [43,44]. Of note, such metabolic changes are involved in both I/R and post-MI remodeling, and previous studies have shown that significantly increased cardiac autophagy is observed during I/R [43,45], and post-MI remodeling [46,47]. In this study, by employing a permanent LAD ligation model of MI, we consistently found that cardiac autophagy, assessed by the ratio of LC3-II to LC3-I, was dramatically upregulated in the post-MI remodeled heart, and this response was completely attenuated by netrin-1 infusion. These data indicate that netrin-1 might be highly protective chronically as well, via modulation of autophagy-dependent post-MI remodeling. Of note, the limitation of the study here is the employment of a permanent MI model rather than a more acute I/R model. However our hypothesis was that autophagy might be a much more importantly modulated by netrin-1 during the chronic process to limit excessive remodeling of heart and enable better repair.

In summary, this study clearly illustrates that netrin-1 effectively protects the heart from I/R injury *in vivo*, when delivered intraventricularly or intravenously via tail vein. This is mediated by DCC dependent ERK1/2/eNOS activation and NO production, and subsequent preservation of mitochondrial integrity that is essential for the survival of cardiomyocytes and the improved cardiac function. These results here establish netrin-1 as a potent therapeutic agent against I/R injury and elucidated several novel mechanisms to this function. In addition to this therapeutic application to acute MI, netrin-1 may also be beneficial in treating post-MI remodeling of the heart chronically, together making netrin-1 a particularly valuable therapeutic option for MI management.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

1. Netrin-1 induces potent cardioprotection *in vivo*, delivered intraventricularly or intravenously
2. Netrin-1 protects the heart *in vivo* via a DCC/ERK1/2/NO-dependent mechanism
3. Netrin-1 attenuates oxidative stress in ischemic heart via NO-dependent inhibition of NOX4
4. Netrin-1 preserves mitochondrial integrity in ischemia heart DCC/ERK1/2-dependently
5. Netrin-1 abrogates autophagy in post-myocardial infarction remodeled heart

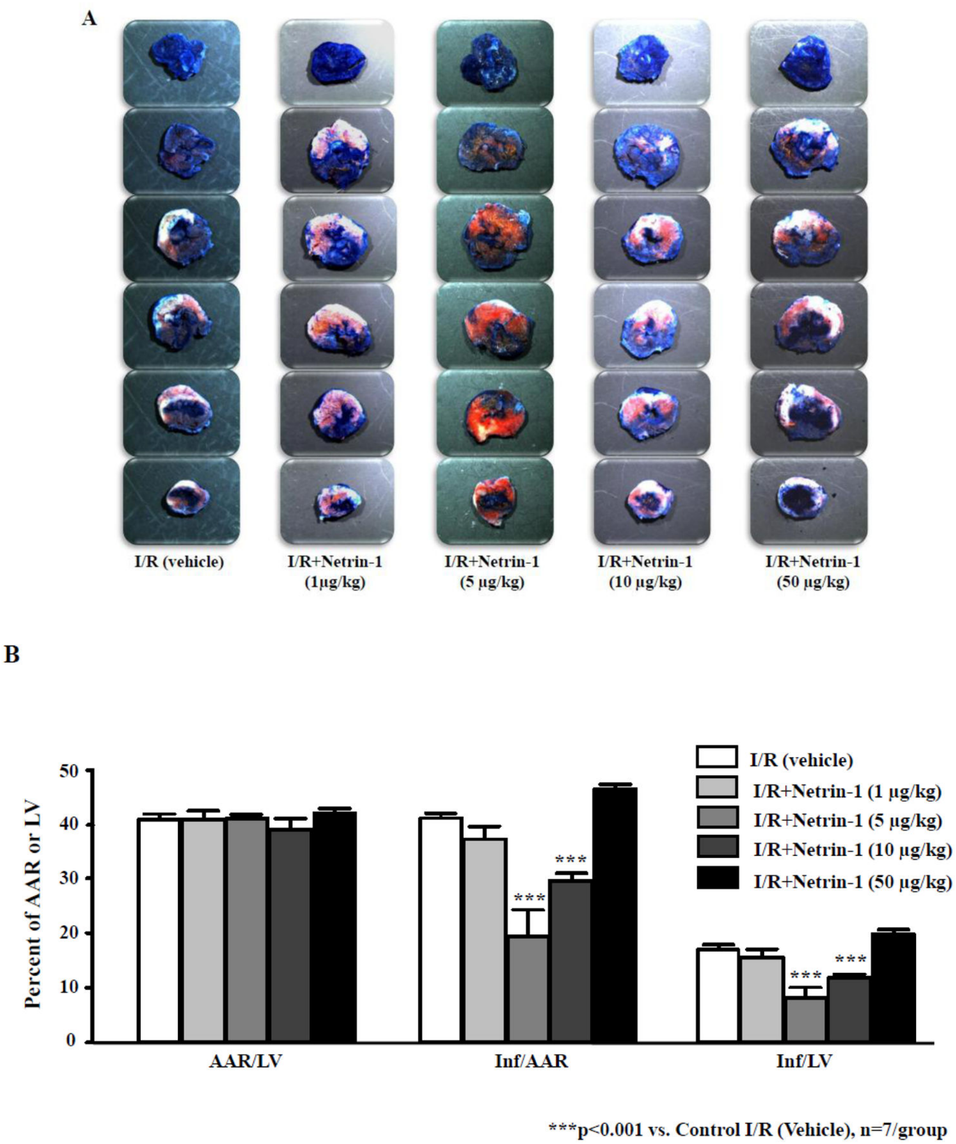
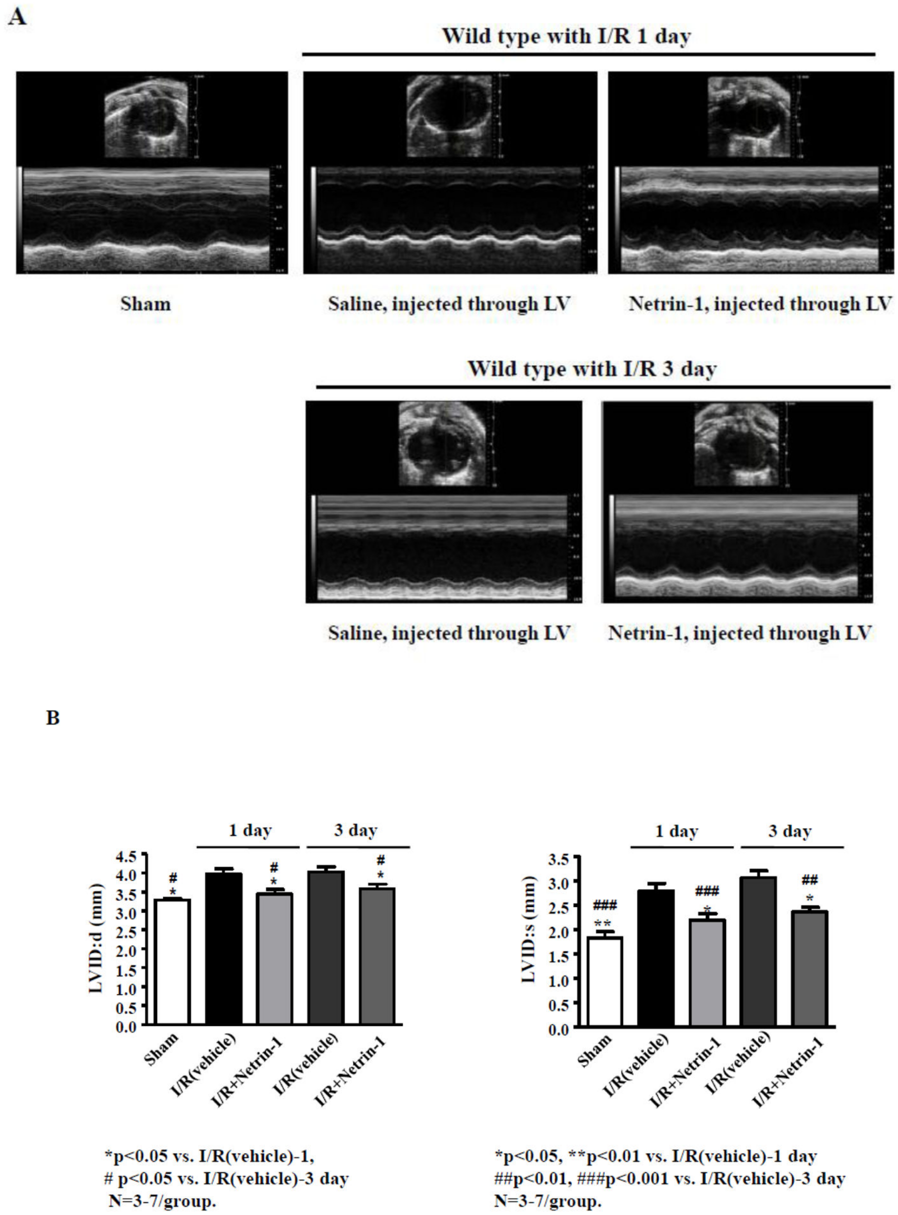
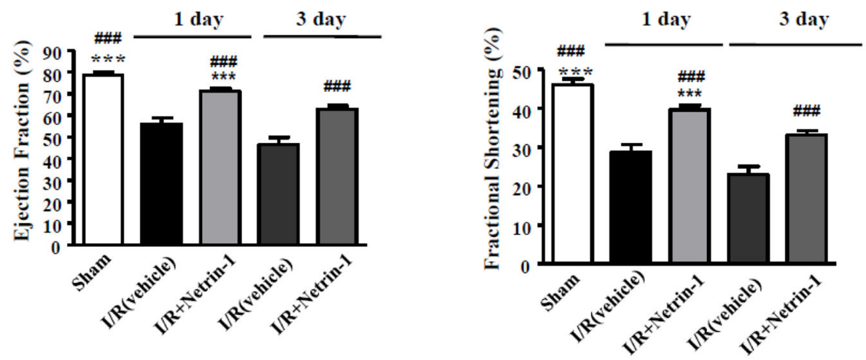



**Figure 1. Netrin-1 attenuates I/R induced myocardial infarct *in vivo***

Myocardial infarction was induced by a 30 min of left coronary artery (LCA) ligation followed by a 24 hr of reperfusion in wild type C57BL6 mice *in vivo*. The mice were treated at the onset of reperfusion with either netrin-1 (1–50 μg/kg) or vehicle (normal saline). Evans blue was used to visualize the non-ischemic area. **A)** Representative TTC-stained heart slices from I/R (vehicle) group, and I/R+Netrin-1 (1–50 μg/kg) groups. The white area indicates infarct zone, while the blue area indicates non infarcted area. The red and white areas represent area at risk. **B)** Infarct size analyzed by: percentage of area at risk divided by left ventricular (AAR/LV), infarct size divided by area at risk (Inf/AAR), and infarct size divided by left ventricular (Inf/LV). The results are presented as Mean±SEM. The number of animal per group was seven. ***p<0.001 vs. Control I/R (vehicle).

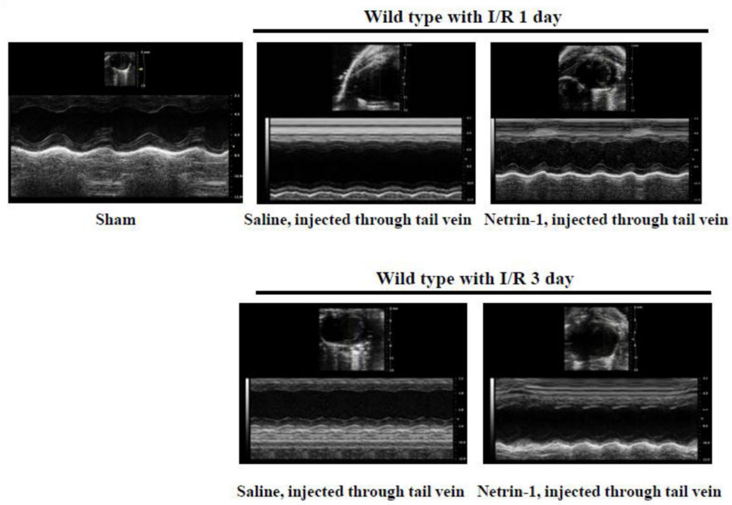

A
Wild type with I/R 1 day
Sham
Saline, injected through LV
Netrin-1, injected through LV
Wild type with I/R 3 day
Saline, injected through LV
Netrin-1, injected through LV
B
1 day
3 day
LVID:d (mm)
Sham
I/R(vehicle)
I/R+Netrin-1
I/R(vehicle)
I/R+Netrin-1
*p<0.05 vs. I/R(vehicle)-1,
# p<0.05 vs. I/R(vehicle)-3 day
N=3-7/group.
1 day
3 day
LVID:s (mm)
Sham
I/R(vehicle)
I/R+Netrin-1
I/R(vehicle)
I/R+Netrin-1
*p<0.05, **p<0.01 vs. I/R(vehicle)-1 day
##p<0.01, ###p<0.001 vs. I/R(vehicle)-3 day
N=3-7/group.

C




*** p<0.001 vs. I/R (vehicle)-1 day
###p<0.001 vs. I/R (vehicle)-3 day
n=3-7/ group

D







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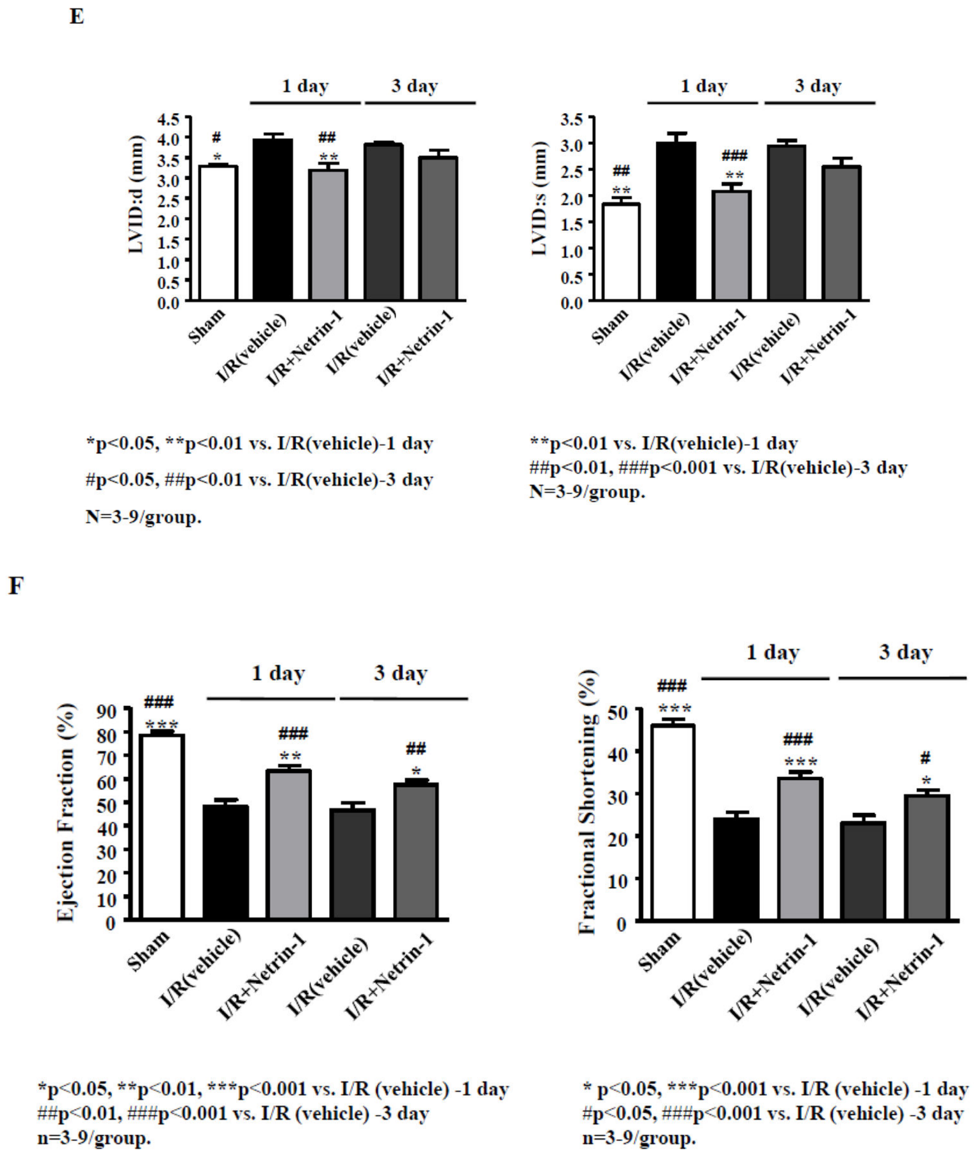


**Figure 2. Intraventricular and Intravenous Delivery of Netrin-1 Improves Cardiac Function after I/R Injury *in vivo***

Echocardiography was performed on wild type C57BL6 mice reperfused for 24 hrs or 72 hrs after a 30 min left coronary artery (LCA) ligation. Left ventricular internal diastole diameter (LVID:D), left ventricular internal systolic diameter (LVID:S), ejection fraction and fractional shortening were measured. **A)** Representative echocardiography data from mice with netrin-1 (5 μg/kg) injected through the LV at the onset of reperfusion. **B)** Grouped data for left ventricular internal dimension (LVID) during systole (*p<0.05, **p<0.01 vs. I/R(vehicle)-1 day, ##p<0.01, ###p<0.001 vs. I/R(vehicle)-3 day) and diastole (*p<0.05 vs. I/R(vehicle)-1, # p<0.05 vs. I/R(vehicle)-3 day) for mice with netrin-1 injected through LV, n=3–7/group. **C)** Grouped ejection fraction and fractional shortening data for mice with netrin-1 injected through LV. *** p<0.001 vs. I/R (vehicle)-1 day, ###p<0.001 vs. I/R (vehicle)-3 day, n=3–7/group. **D)** Representative echocardiography data from mice with netrin-1 (5 μg/kg) injected through tail vein. **E)** Grouped LVID during systole (**p<0.01 vs.




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I/R(vehicle)-1 day, ##p<0.01, ###p<0.001 vs. I/R(vehicle)-3 day) and diastole **(***p<0.05, **p<0.01 vs. I/R(vehicle)-1 day, #p<0.05, ##p<0.01 vs. I/R(vehicle)-3 day) from mice with netrin-1 (5 μg/kg) injected through tail vein, n=3–9/group. **F)** Grouped ejection fraction (*p<0.05, **p<0.01, ***p<0.001 vs. I/R (vehicle) -1 day, ##p<0.01, ###p<0.001 vs. I/R (vehicle) -3 day) and fractional shortening (***** p<0.05, ***p<0.001 vs. I/R (vehicle) -1 day, #p<0.05, ###p<0.001 vs. I/R (vehicle) -3 day) from mice with netrin-1 (5 μg/kg) injected through tail vein, n=3–9/group. Data are presented as Mean ± SEM.

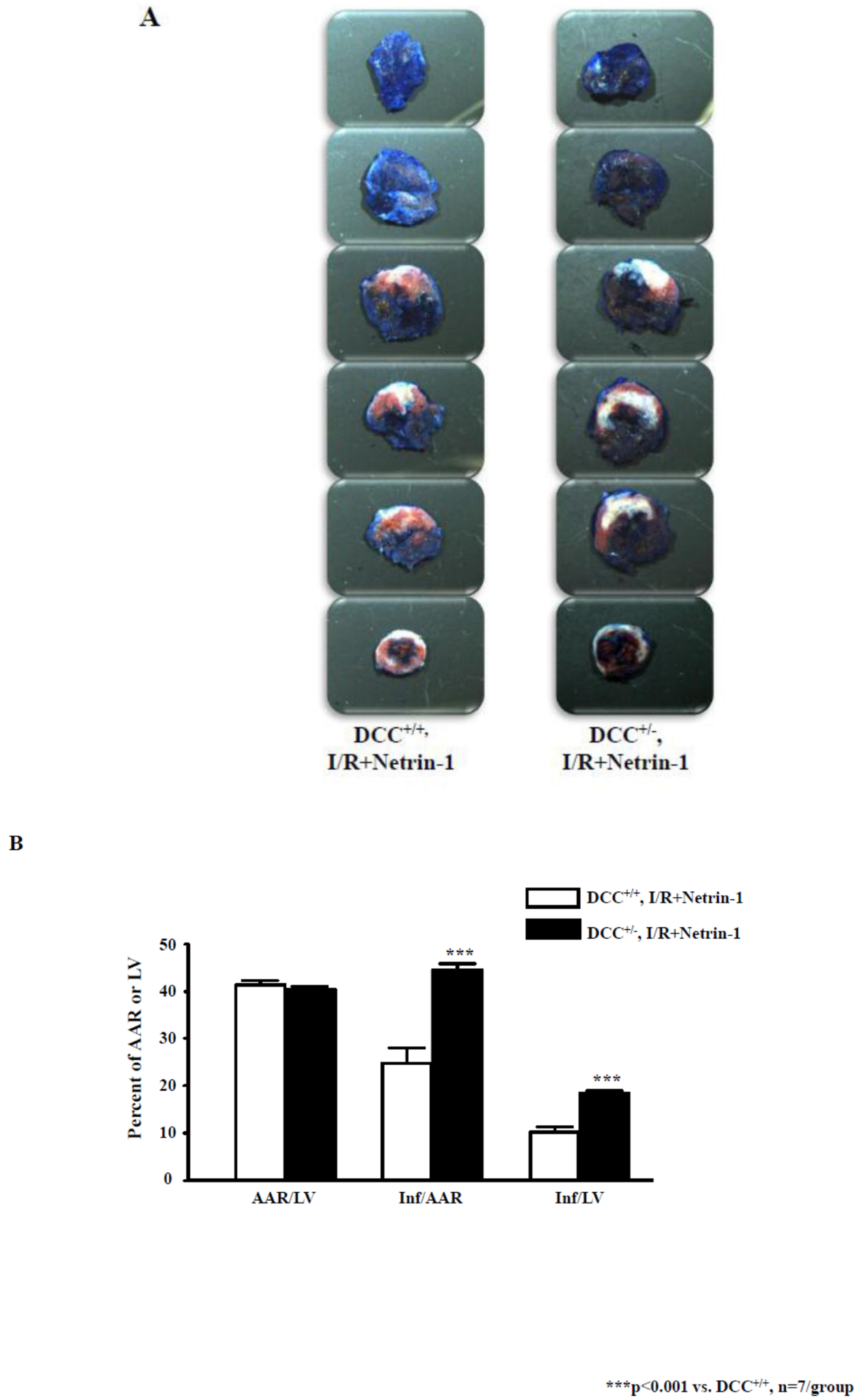


**Figure 3. Netrin-1-mediated cardioprotection *in vivo* is DCC dependent**

Myocardial infarction was induced by a 30 min left coronary artery (LCA) ligation followed by a 24 hr of reperfusion in $DCC_{+/+}$ mice and $DCC_{+/-}$ mice. The mice were injected of 5 μg/kg netrin-1 at the onset of reperfusion. Netrin-1 yielded significant protection in $DCC_{+/+}$




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and not in $DCC_{+/-}$ animals. **A)** Representative TTC-stained heart slices from $DCC_{+/+}$, I/R +Netrin-1 group, and $DCC_{+/-}$, I/R+Netrin-1 group**. B)** Infarct size analyzed by percentage of area at risk divided by left ventricle (AAR/LV), infarct size divided by area at risk (Inf/AAR), and infarct size divided by left ventricle (Inf/LV). The results are represented as Mean±SEM. ***p<0.001 vs. $DCC_{+/+}$, I/R+netrin-1, n=7/group. **I/R+UO126 I/R+Netrin-1**

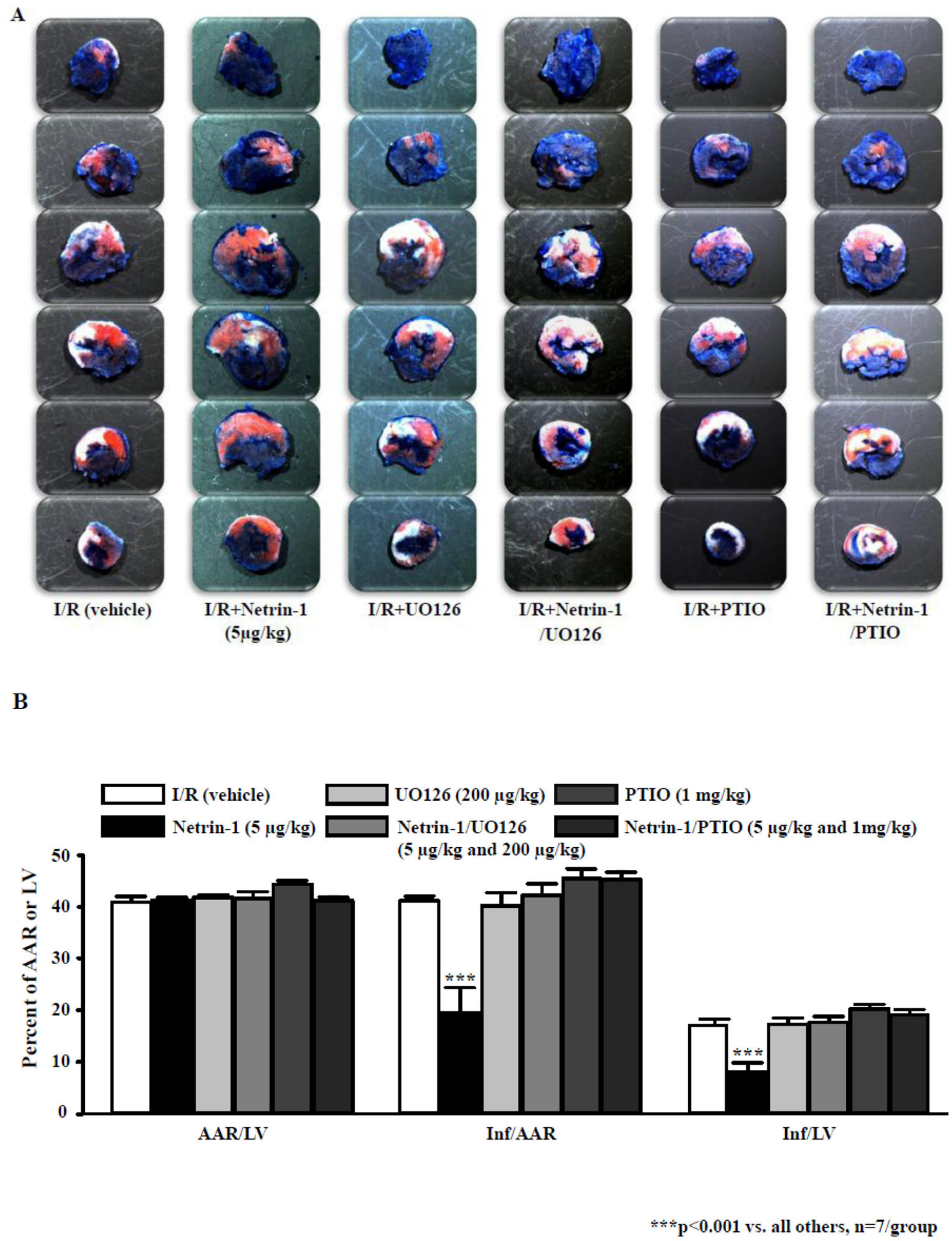


**Figure 4. Netrin-1 induced cardioprotection *in vivo* is ERK 1/2 and NO dependent**

Myocardial infarction was induced by a 30 min LCA ligation followed by a 24 hr of reperfusion. Mice were injected with either 5 µg/kg netrin-1; 200 µg/kg UO126 alone; 1 mg/kg PTIO alone; 5 µg/kg netrin-1/200 µg/k UO126; or 5 µg/kg netrin-1/1 mg/kg PTIO at the onset of reperfusion. UO126 was used to inhibit MERK1/2/ERK1/2 activity and PTIO was used to chelate NO specifically. Cardioprotection elicited by netrin-1 was suppressed in presence of UO126 or PTIO. Infarct size analyses as in Figure 2. The results were represented as Mean±SEM. ***p<0.001 vs. all others, n=7/group.

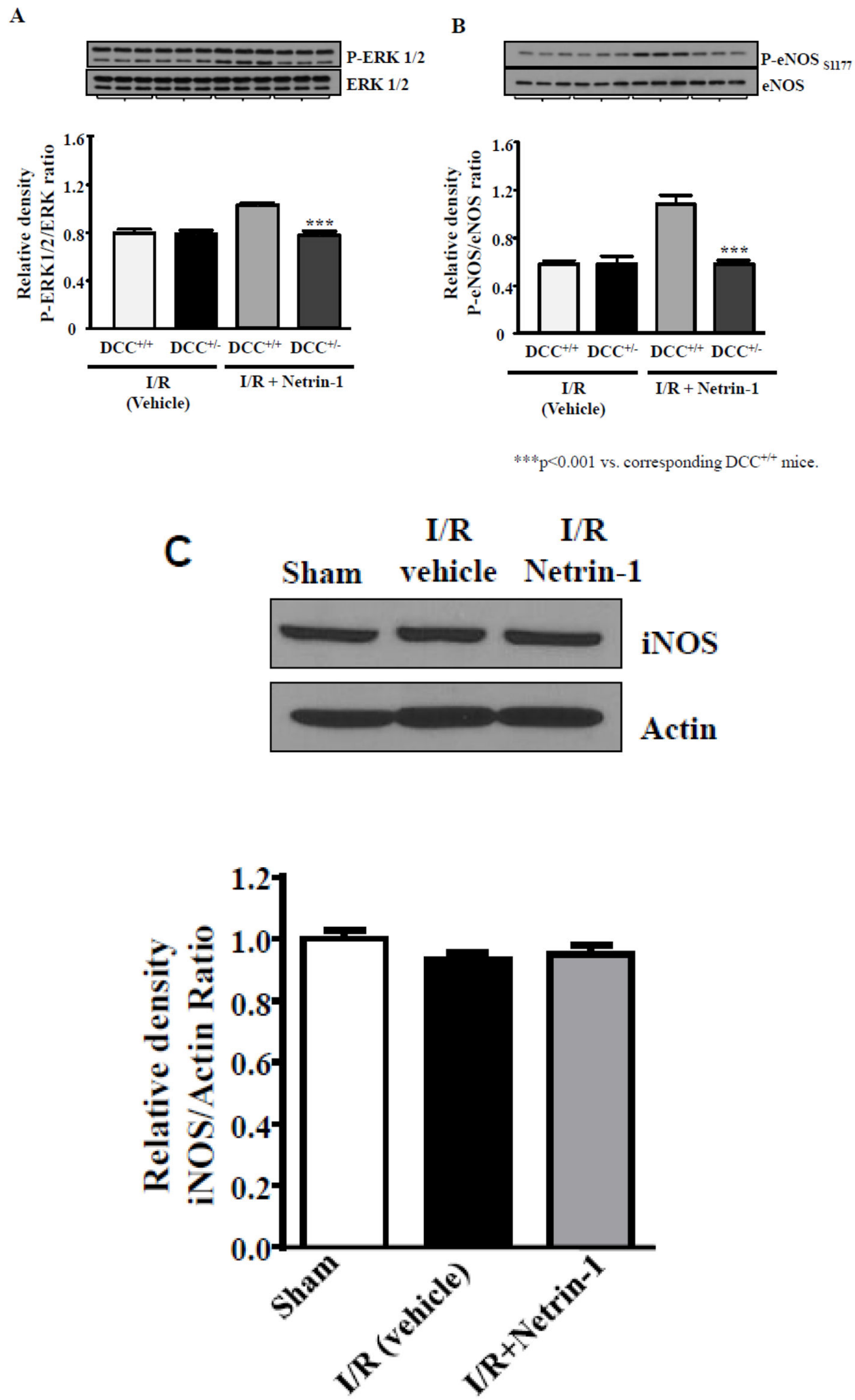


**Figure 5. Netrin-1 activates ERK1/2 and eNOS/NO *in vivo* in a DCC dependent manner, while iNOS expression is unaffected**

Shown are representative western blots and grouped densitometric data from $DCC_{+/+}$ and $DCC_{+/-}$ mice treated at the onset of reperfusion with vehicle (normal saline), or 5 μg/kg of netrin-1. **A)** Grouped densitometric data of ERK1/2 phosphorylation that is normalized by ERK1/2. ***$p<0.001$ vs. corresponding $DCC_{+/+}$ mice, n=3/group. **B)** Grouped densitometric data of $eNOS_{s1177}$ phosphorylation that is normalized by eNOS. ***$p<0.001$ vs. $DCC_{+/+}$ mice, n=3/group. **C)** Representative and grouped data of iNOS expression, n=3/group.

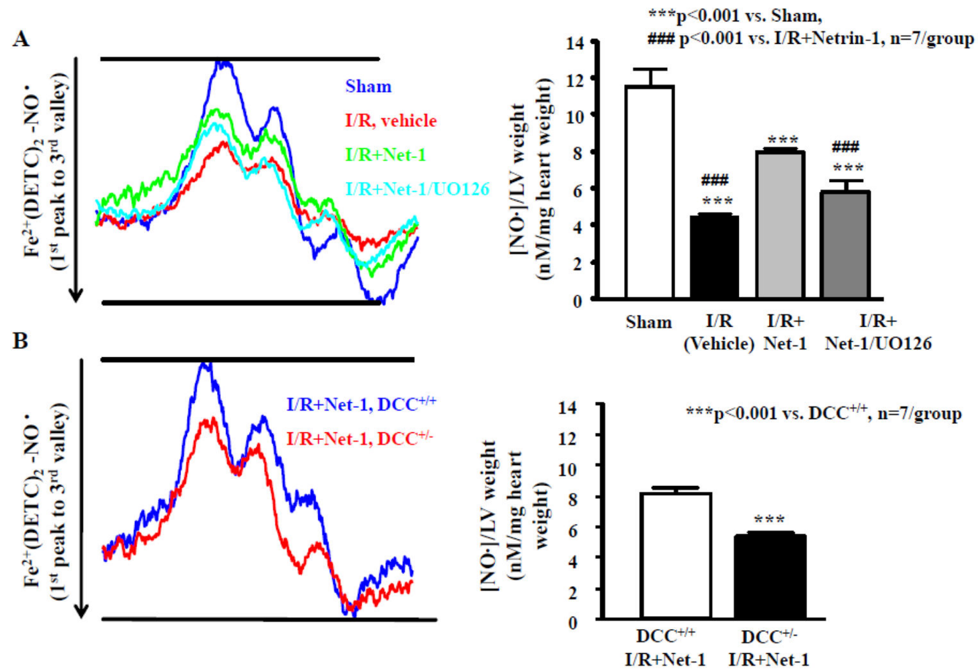


**Figure 6. Netrin-1 induction of NO production *in vivo* is DCC and ERK 1/2 dependent**

Bioavailable NO levels in the left ventricle were determined by electron spin resonance (ESR) from mice treated with either vehicle (normal saline), netrin-1 or netrin-1/UO126 at the onset of reperfusion (at same doses described earlier). Left panels show representative ESR spectra of NO production, while the grouped data are presented on the right. **A)** Netrin-1 markedly restores NO production which was reduced by I/R injury, and this response was inhibited by cotreatment with UO126. ***p<0.001 vs. Sham, ### p<0.001 vs. I/R+Netrin-1, n=5/group. **B)** Netrin-1's effects on restoring NO production was abolished in $DCC_{+/-}$ animals. ***p<0.001 vs. $DCC_{+/+}$, n=5/group.

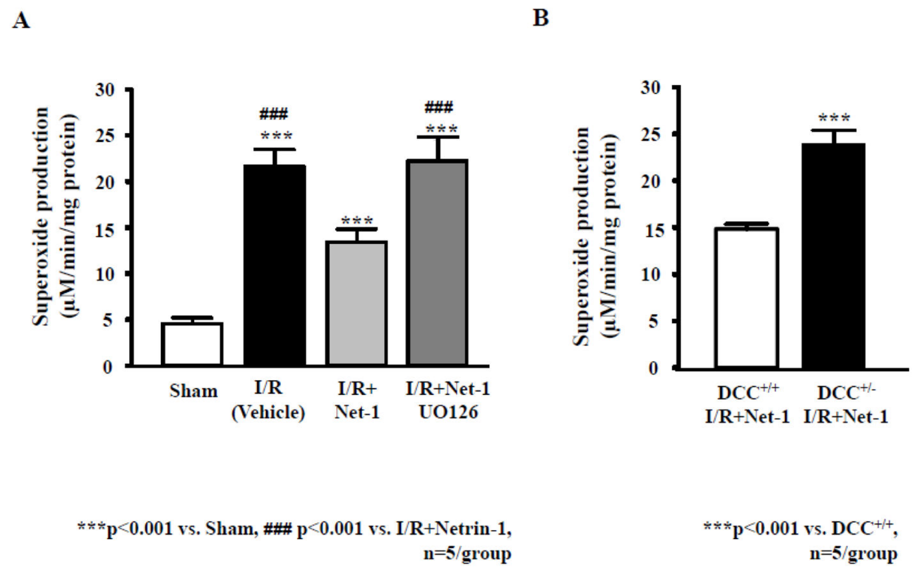


**Figure 7. Netrin-1 attenuation of mitochondrial superoxide production *in vivo* is DCC and ERK 1/2 dependent**

Mitochondrial superoxide production was measured using electron spin resonance (ESR) using freshly isolated mitochondria from left ventricle after I/R surgery. **A)** I/R induced increase in mitochondrial superoxide production was attenuate by netrin-1 infusion, and this response was reversed by co-treatment of ERK1/2 inhibitor U0126. ***p<0.001 vs. Sham, ### p<0.001 vs. I/R+Netrin-1, n=5/group. **B)** Netrin-1's effects on attenuating mitochondrial superoxide production was absent in $DCC_{+/-}$ animals. ***p<0.001 vs. DCC+/+, n=5/group




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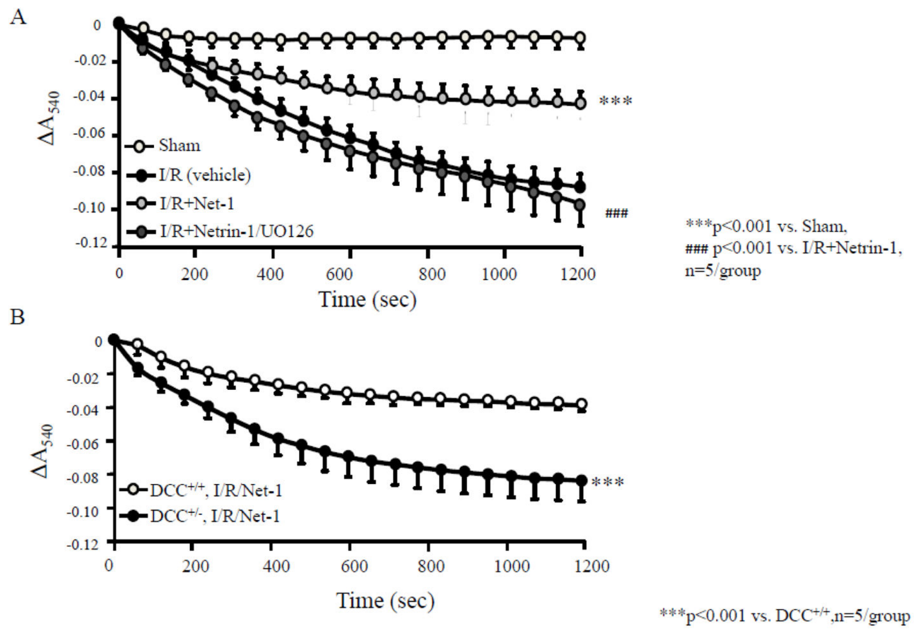


**Figure 8. Netrin-1 preservation of mitochondrial integrity during I/R *in vivo* is DCC and ERK 1/2 dependent**

Calcium induced mitochondrial swelling was measured from freshly isolated mitochondria after I/R injury. **A)** Hearts were injected with vehicle, netrin-1 (5 μg/kg) or netrin-1+UO126 (200 μg/kg), at the onset of reperfusion. The data show that netrin-1 markedly reduced mitochondrial swelling, while co-treatment with UO126 abolished this protective effect. ***p<0.001 vs. Sham, ### p<0.001 vs. I/R+Netrin-1, n=5/group. **B)** Same experiments performed on $DCC_{+/+}$ and $DCC_{+/-}$ animals. Data show that netrin-1's protective effect against mitochondrial swelling was absent in $DCC_{+/-}$ animals. ***p<0.001 vs. $DCC_{+/+}$, n=5/group.

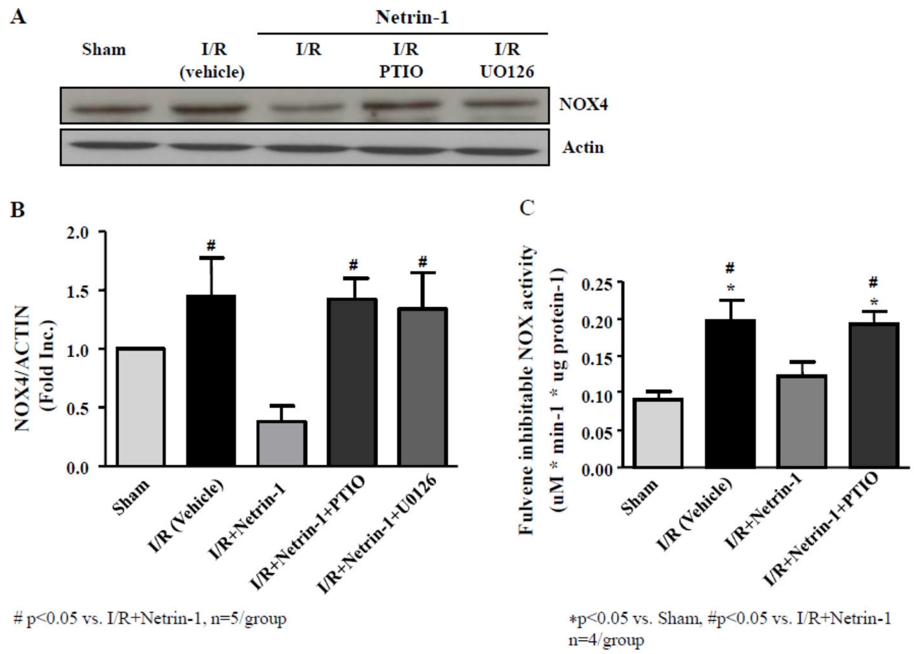


**Figure 9. Netrin-1 downregulates NOX4 expression and activity *in vivo* during I/R**

Shown are representative western blots and grouped densitometric data from mice subjected to sham surgery, permanent LAD ligation for myocardial infarction (MI), or MI surgery with netrin-1 perfusion in osmotic minipumps. A) Western blots showing NOX4 expression. B) Grouped densitometric data of NOX4 protein expression that is normalized by Actin. Results are means ± S.E.M. # $p<0.05$ vs. I/R+Netrin-1, n=5/group. C) Grouped data shows that Fulvene-5 (a specific inhibitor of NOX4) sensitive NOX activity was increased with I/R, and netrin-1 perfusion eliminated this increase. Co-treatment with PTIO, a NO scavenger, abolished this effect of netrin-1. *$p<0.05$ vs. Sham, #$p<0.05$ vs. I/R+Netrin-1, n=4/group.

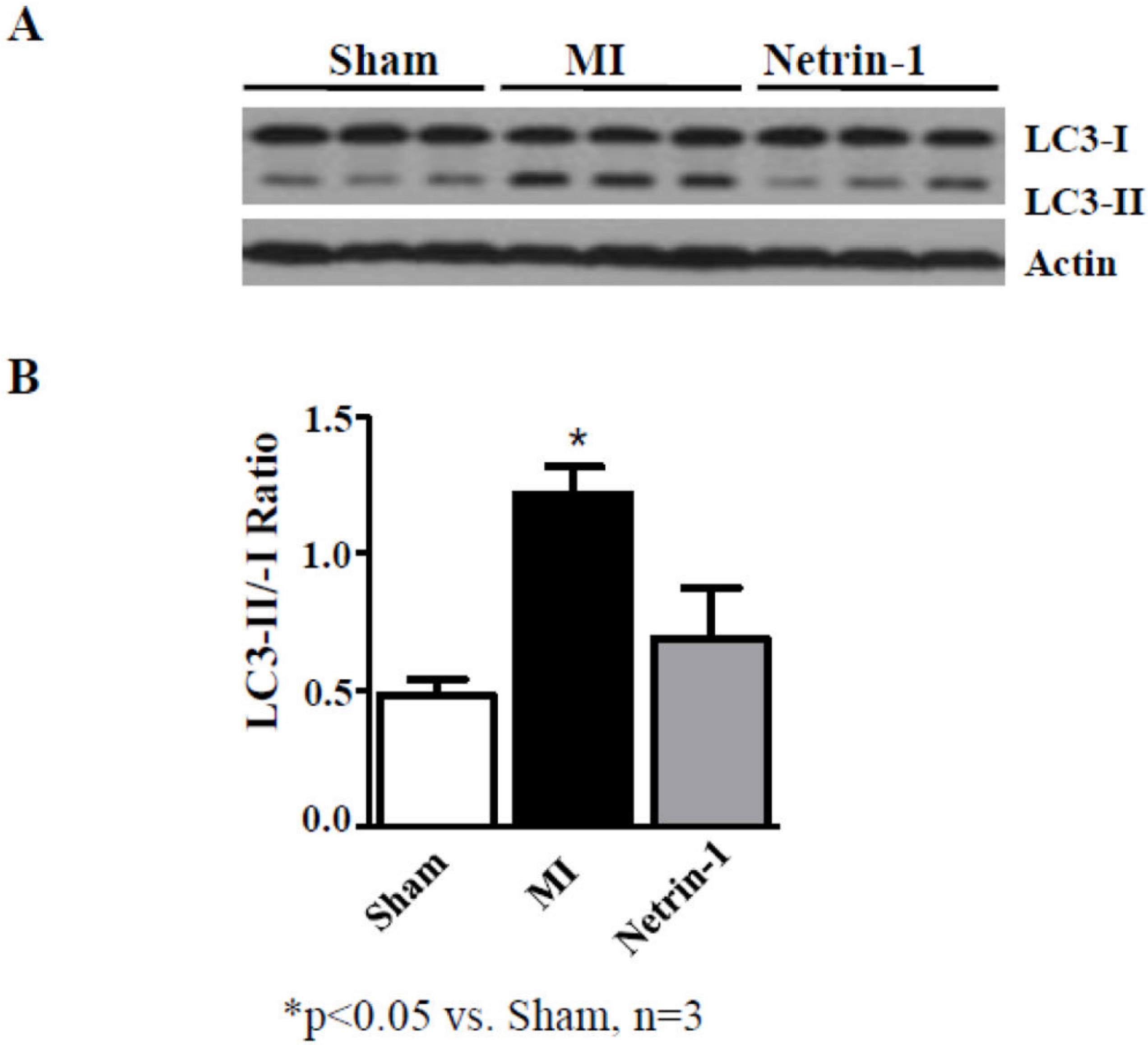


**Figure 10.**

Netrin-1 attenuates autophagy in post-MI remodeled heart. Representative western blots and grouped densitometric data in mice subjected to sham surgery, permanent LAD ligation to induce myocardial infarction (MI), or MI surgery with netrin-1 infused using osmotic minipumps. A) Representative western blots of LCI/LCII and β-actin expression. B) Grouped densitometric data of LCII/LCI ratio after normalized by β-actin. Results are presented as Means±SEM. *p<0.05 vs. Sham, n=3/group.