Identification of an essential nonneuronal function of neurotrophin 3 in mammalian cardiac development

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Neurotrophin 3 (*Nt3*) is one of five neurotrophin growth factors which shape the development of the nervous system by regulating neuronal survival and differentiation. Peripheral neuronal subpopulations expressing the *TrkC* receptor tyrosine kinase respond to *Nt3* with enhanced survival, mitogenesis or cell migration[1–3] and these neurons are lost in homozygous *Nt3* null (–/–) mutant mice[4–7]. The unexplained perinatal lethality in the $Nt3^{-/-}$ mice, however, suggests a wider function for this neurotrophin. Here we report that *Nt3* is essential for the normal development of atria, ventricles, and cardiac outflow tracts. Histological and echocardiographic image analysis of $Nt3^{-/-}$ animals reveal severe cardiovascular abnormalities including atrial and ventricular septal defects, and tetralogy of Fallot, resembling some of the most common congenital malformations in humans. The observed defects are consistent with abnormalities in the survival and/or migration of cardiac neural crest early in embryogenesis[8] and establish an essential role for neurotrophin 3 in regulating the development of the mammalian heart.

Examination of the $Nt3^{-/-}$ mouse has confirmed the essential developmental role of this neurotrophin in promoting the survival of subpopulations of peripheral *TrkC* expressing neurons including proprioceptive sensory neurons[4–7] and sympathetic neurons[4,5]. *Nt3* is not required for the survival of other *TrkC* expressing neuronal populations, including motor, enteric, and hippocampal neurons, as $Nt3^{-/-}$ newborn mice have normal enteric and motor neurons and no obvious anatomical defects in the central nervous system. However, the unexplained death of the $Nt3^{-/-}$ animals within the first days of life does not allow an adequate evaluation of whether this neurotrophin can regulate neuronal function postnatally.

Although the *TrkC* receptor is expressed by numerous non-neuronal cells during development[9–12], the role of *Nt3* outside of the nervous system is unclear[13]. In the mouse, expression of *TrkC* mRNA has been detected both within the heart, the aorta and neural crest cells[9–12], and *Nt3* mRNA has been identified within the heart, aorta, pulmonary artery and neural crest[12,14–16]. The rapid death of the $Nt3^{-/-}$ animals[4–6], along with the observed reduction in birthweight and dusky perinatal appearance suggested a potential defect within the cardiovascular system.

Our examination of $Nt3^{-/-}$ animals revealed numerous cardiac malformations. Upon gross examination of the thorax of newborn homozygous null mutant animals, the hearts appeared markedly enlarged and globular, with dilated atria (Fig. 1). The lungs appeared haemorrhagic although the anatomical relationships were unremarkable.

Microscopic examination of 15 $Nt3^{-/-}$ animals was notable for abnormalities involving chamber septation and size. All $Nt3^{-/-}$ animals had right ventricular dilatation (Fig. 2*a, b*), although the left ventricular size was variable. In three homozygous animals we detected a ventricular septal defect in the region of the membranous septum (Fig. 2*a–c*). In $Nt3^{-/-}$ animals both atria were markedly dilated (Fig. 2*e*, data not shown) with decreased trabeculations, myocyte thinning of the atrial wall and large secundum atrial septal defects (Fig. 2*e*).

We detected developmental defects in structures associated with the truncus arteriosus and aortic arches in the –/– animals. Premature closure of the ductus arteriosus secondary to medial hypertrophy was identified *in utero* or immediately after birth in all of the –/– animals, compared to partial or complete patency of the ductus in 2/4 heterozygotes and 5/5 wild-type littermates (data not shown). In addition, we identified defects in the sinus venosus, and there was aneurysmal dilatation and marked attenuation of the smooth muscle cell layer of the pulmonary veins in 50% of the –/– animals. One $Nt3^{-/-}$ mouse exhibited incomplete aortico-pulmonary septation resulting in persistent truncus arteriosis, and two animals with large ventricular septal defects also had abnormalities in the cardiac outflow tracts, with dilation of the pulmonary artery or an overriding aorta (Fig. 2*b, c*). One mutant animal showed all the defects associated with tetralogy of Fallot, including an atrial septal defect, a ventricular septal defect, pulmonic stenosis and overriding aorta.

All $Nt3^{-/-}$ mutant mice exhibited abnormalities of valvular architecture although specific defects were variable from animal to animal. Pulmonic stenosis, with abnormally thickened semilunar valves (Fig. 2*d*) was detected in five animals. Additional abnormalities in the right ventricular outflow tract, with subpulmonic stenosis, were detected in three of the $Nt3^{-/-}$ mice (data not shown). We also noted thickened leaflets of the aortic valve (Fig. 2*e*) or mitral valves in 50% of the $Nt3^{-/-}$ animals. The most consistent defect was dilatation of the atrioventricular annuli. Examination of the lungs revealed appropriate maturation and normal vascular architecture, but all $Nt3^{-/-}$ mutant mice demonstrated features of pulmonary oedema, with congestion and

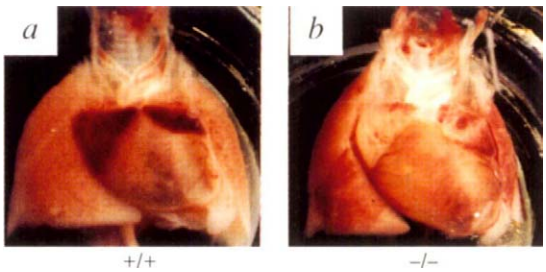

Fig. 1 The homozygous *Nt3* null mutant animal displays cardiomegaly and pulmonary haemorrhage. Dissection of the cardiopulmonary systems was performed *en bloc* from paraformaldehyde (3%) fixed newborn normal (+/+) littermates (*a*) or *Nt3* (–/–) mutant animals (*b*). The genotype of each pup was determined by analysis of tail DNA prior to fixation, using Southern blot analysis as described[6]. Magnification: ×16.

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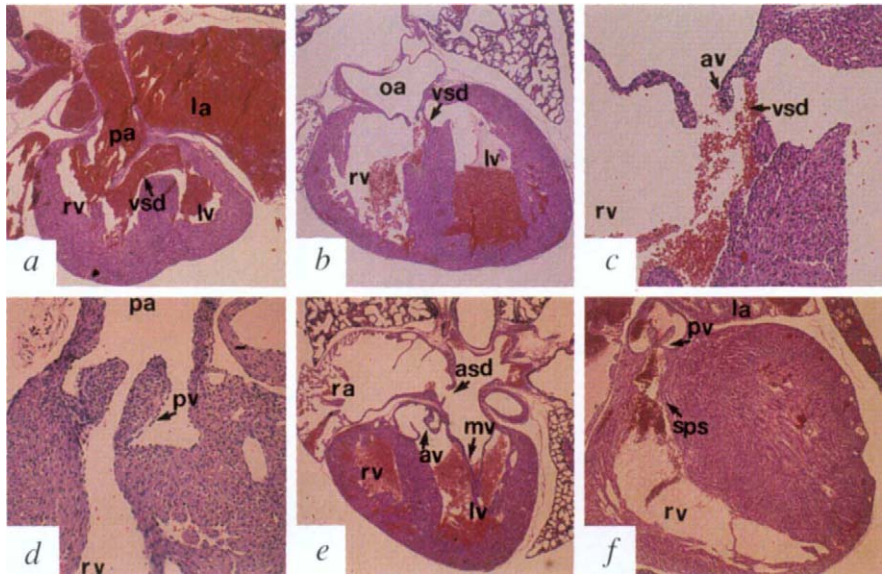

Fig. 2 Malformations in cardiogenesis of *Nt3* $^{-/-}$ and $^{+/-}$ newborns mice. Following fixation and dissection of the cardiopulmonary system, organs were embedded in paraffin, and serially sectioned at 5 microns. Sections were stained with haematoxylin and eosin. The genotype of each animal was determined by Southern analysis[6]. Panels *a–e*, newborn $^{-/-}$ and, panel *f*, $^{+/-}$ mice. *a–b*, Sections of $^{-/-}$ heart demonstrating a ventricular septal defect (vsd), with right and left ventricles (rv or lv), pulmonary artery (pa), left atrium (la) and overriding aorta (oa) indicated. Magnification ×25. *c*, Higher magnification of (*b*), with ventricular septal defect (vsd) and aortic valve (av) indicated. Magnification ×75. *d*, Section of heart demonstrating thickening of pumonic valve leaflets (pv); pulmonary artery (pa) and right ventricle (rv) are indicated. Magnification ×75. *e*, Atrial septal defect (asd) and aortic valve thickening (av) in a *Nt3*$^{-/-}$ mouse heart. Aortic and mitral valves (av, mv), right and left ventricles (rv, lv) and right atrium (ra) are indicated. Magnification ×25. *f*, Section of +/- heart notable for subpulmonic stenosis (sps) and thickening of the pumonic valve leaflets (pv). Right ventricle (rv), left atrium (la). Magnification ×25.

intra-alveolar haemmorhage (Fig. 2*b*, *e*).

To establish the onset of these cardiovascular abnormalities, we examined embryos at 9.5 days gestation. *Nt3*$^{-/-}$ embryos (4/4) exhibited developmental anomalies of the great vessels, including developmental delay in the primitive myofibril organisation of the truncus arteriosis when compared to wild-type littermate embryos (Fig. 3*a*, *b*). We also detected hypoplasia of the sinus venosus in one *Nt3*$^{-/-}$ embryo. In addition, atrial enlargement was found in all mutant embryos. To assess whether the lack of *Nt3* resulted in reduced populations of *TrkC* expressing cells within the developing cardiovascular system, we performed immunohistochemistry using antisera which selectively detects all kinase active isoforms of *TrkC* (see Methods). In wild-type embryos, myocytes within the ventricles, atria, and cardiac outflow tracts express *TrkC* (Fig. 3*b*). In the *Nt3*$^{-/-}$ embryos, however, the level of expression of *TrkC* is decreased, particularly in the ventricles (Fig. 3*a*). Reduced expression of *TrkC* persisted during development, with scant ventricular and atrial immunoreactivity seen in the newborn *Nt3*$^{-/-}$ animals, when compared to *Nt3*$^{+/+}$ littermates (data not shown). Immunohistochemical examination of the developing neural tube in E 9.5$^{+/+}$ embryos identified *TrkC* expressing cells adjacent to the ventral neural tube, consistent in location with the migrating neural crest (Fig. 3*c*).

To assess the functional impairment of the *Nt3* mutant heart and gross anatomic development *in vivo*, we performed real time echocardiography on littermates within 8 hours of birth. Due to the rapid heart rate (between 350 and 400 beats/min) and the size of the hearts in newborn animals (less than 4 mm in diameter), we used a 30 mHz intravascular ultrasound catheter for transthoracic images. *Nt3*$^{-/-}$ animals (3/3) that were imaged displayed biventricular enlargement, relative to normal and heterozygous littermates (Fig. 4*c–f*). In addition, the *Nt3*$^{-/-}$ pups had resting heart rates of approximately 250 beats/min, as compared to wild-type and heterozygous animals with rates of approximately 350 beats/min. Furthermore, the –/– animals displayed a marked sensitivity to transthoracic pressure during imaging, with significant bradycardia which recovered immediately on removal of the transthoracic imaging catheter. This may reflect abnormal autonomic tone in these animals, and is consistent with the 50% reduction in the number of sympathetic neurons in the mutant animals, as compared to normal littermates[4,5].

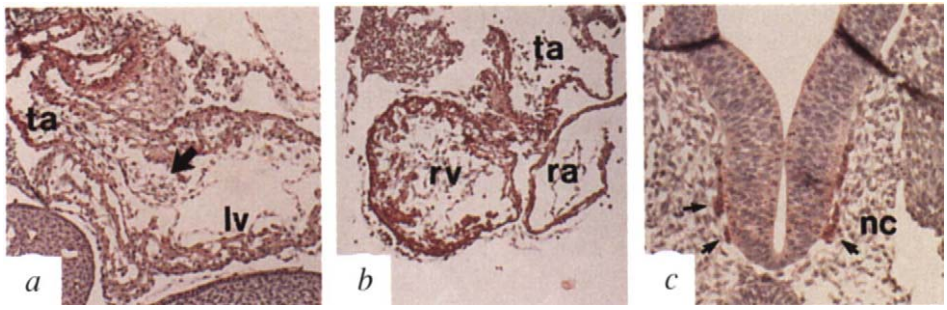

Fig. 3 Immunolocalization of *TrkC* expression in E9.5 *Nt3*$^{+/+}$ and $^{-/-}$ embryos. Sections obtained from E9.5 mutant (*a*) and wild-type (*b,c*) embryos were sectioned and subjected to immunohistochemical analysis with a rabbit polyclonal antisera specific for kinase active *TrkC* isoforms. Immunoreactive products were visualized with Super Fast Red as the chromogenic substrate as described[12]. *a,b*, Sections of the developing heart; right atria (ra), right or left ventricle (rv or lv), truncus arteriosis (ta). Arrow in panel *a* indicates region of reduced circumferential myofibrillar orientation in the truncus. Magnification ×200. *c*, section of the developing spinal cord, with immunoreactive neural crest (nc) indicated by the arrows. Magnification ×200.

Echocardiographic imaging of five *Nt3* heterozygous (+/–) littermates revealed moderate right ventricular enlargement in three of the heterozygous animals. Our histological evaluation of serial sections of the hearts of these +/– littermates, and an additional 12 *Nt3*$^{+/-}$ animals was notable for a range of defects including: pulmonic and subpulmonic stenosis (Fig. 2*f*), small atrial septal secundum defects, and anomalous pulmonary venous return (data not shown). In contrast to the multiple defects detected in each –/– animal,

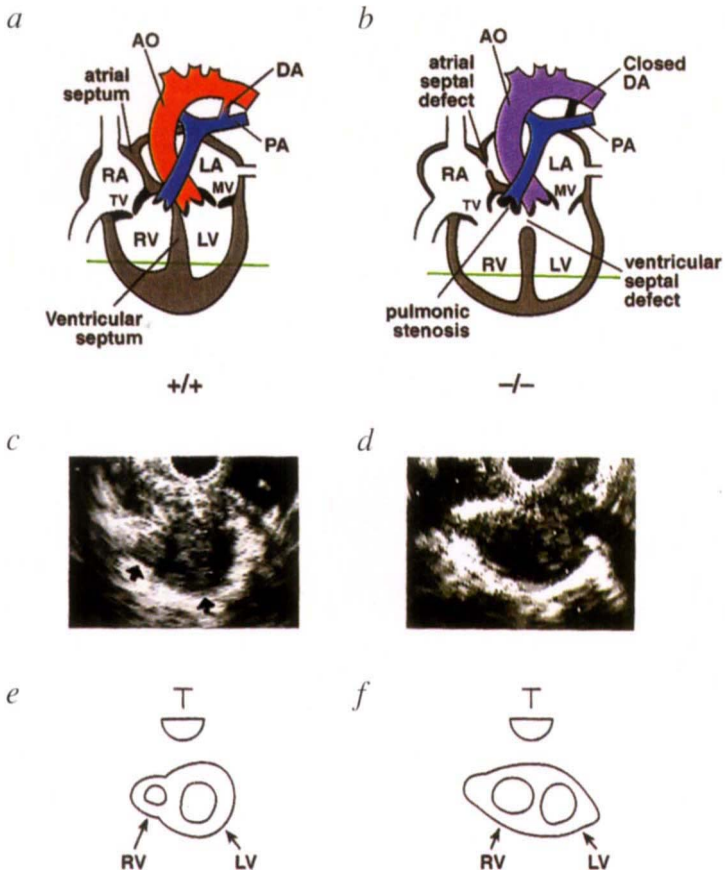

Fig. 4 Schematic representation of cardiac abnormalities in *Nt3* –/– animals and sonographic evaluation of ventricular function. Schematic representation of *a*, normal cardiac anatomy, *b*, the *Nt3* mutant heart; the aorta (AO), ductus arteriosus (DA), right and left atrium (RA, LA), right and left ventricle (RV, LV), tricuspid (TV) and mitral (MV) valve are indicated. The green line indicates the plane of section for the sonographic evaluation. *c,d*, Sonographic image of the cardiac short axis of a *Nt3* $^{+/+}$(*c*) or $^{-/-}$ (*d*) mouse. Newborn mice from heterozygous *Nt3* +/- matings were blindly imaged with a Hewlett-Packard coronary probe using a Sonos 1000 with real time image analysis. Following the imaging, animals were killed and each mouse was genotyped by Southern analysis in a blinded fashion. *e, f*, Schematic drawing of the sonographic images displaying the location of the transducer (T), right (RV) and left (LV) ventricle

only one heterozygous animal exhibited more than one defect in the heart or outflow tracts. We detected no abnormalities in any of the five wild-type littermates examined by either echocardiography or histology.

The cause of death of the homozygous *Nt3* null mutant mice is most likely attributable to the multiple cardiovascular anomalies and resultant cardiac dysfunction. We base this conclusion on the severity of morphologic defects observed, and confirmed it by functional assessment using echocardiography. The timing of death in these animals, in the early postnatal period, is consistent with the increased oxygenation demands after birth, and the severe defects in forward flow resulting from valvular incompetence and stenosis, large septal defects and conotruncal abnormalities.

The development of the vertebrate heart is dependent upon the highly ordered migration and differentiation of cells from three embryologically distinctive populations: the mesoderm of the lateral somites giving rise to cardiac myoctes, cardiac neural crest progenitors migrating to the fourth and sixth aortic arches to contribute to the development of the outflow tracts, endocardial cushions and valves, and the endocardium which develops into the endothelial cells lining the heart chambers[17,18]. Although the majority of studies defining the contributions of cardiac neural crest cells to normal cardiac development have been performed in the avian model because of ease of manipulation of early embryos, the abnormalities of the homozygous *Nt3* null mutant mice follow these avian predictions[19]. From chicken:quail chimaera studies (reviewed in ref. 8), it has been established that the neural crest contributes to the (*i*) ectomesenchyme of the truncus arteriosus, resulting in septation of the pulmonary artery and the aorta, (*ii*) formation of the pulmonary and aortic valves, (*iii*) mesenchyme of the ductus arteriosus and (*iv*) formation of the membranous portion of the interventricular septum. Although each homozygous mutant animal displayed abnormalities in at least two of these structures, a range of defects was seen (Fig. 4*a, b*); this heterogeneity of phenotype is consistent with that seen in avian neural crest ablation studies[8]. The marked dilatation and abnormal septation of the atria, and the dilated, globular ventricles could reflect chronic volume overload secondary to valvular and septal abnormalities.

Congenital cardiac malformations represent the largest group of congenital defects in humans, with 1/200 live births affected[20]. The defects we observed in the *Nt3*$^{-/-}$ mice, with atrial and/or ventricular septal defects, and tetralogy of Fallot, reflect the most common congenital malformations of human infants. Furthermore, the demonstration of both functional and histological abnormalities in hearts and outflow tracts of the heterozygous animals suggests that haplo-insufficiency in *Nt3* expression has developmental consequences. Our findings, together with those of Ernfors *et al.*[4] documenting a reduction in the muscle spindle formation in *Nt3* heterozygotes, provides direct evidence that *Nt3* is present in limiting concentrations in the embryo.

Little is known about the specific growth factors which regulate the survival and migration of the cardiac neural crest. In the avian system, leukaemia inhibitory factor (*LIF*) has been shown to promote the survival of cardiac neural crest *in vitro*[19], and extracellular matrix proteins such as fibronectin and laminin induce directed cell migration of neural crest precursors (reviewed in ref. 21). Abnormalities in the cardiac structures arising from the neural crest are detected in homozygous null mutant mice for neurofibromin[22,23], suggesting that the cardiac neural crest requires the integration of signals from multiple growth factor and extracellular matrix/adhesion receptors.

The reduction in wall thickness of both the atria and ventricles of the null mutant animal suggests that neurotrophin 3 could directly exert survival and/or differentiation effects on cardiac myocytes. Such a role has been amply demonstrated in the *Nt3* $^{-/-}$ mice by the loss of numerous classes of neurons expressing a kinase active isoform of *TrkC*[4–7] . However, the persistence of cardiac mycocytes which display *TrkC* immunoreactivity in the *Nt3*$^{-/-}$ animals suggests that additional fac-

tors are capable of promoting the survival and differentiation of these cells. Further evaluation of the effects of *Nt3* on myocyte function will require the development of strategies to target the ablation or augmentation of *TrkC* signalling in the neural crest or mesenchyme *in utero*.

Although *Nt3* has been described as a mitogen for chick neural crest cells[24], absence of *Nt3* is unlikely to result in a complete lack of neural crest progenitor cell survival, or of neural crest migration to the heart, as the mutant animals have division of the truncus arteriosis into pulmonary arteries and ascending aorta, and valvular development. However, the phenotype of the homozygous mutant animals, with outflow tract, valvular and ventricular septal defects, recapitulates the phenotype described with ablation of the neural crest cells from the first to third somites of chick embryos, indicating that *Nt3* is critical for the function or migration of the neural crest in cardiac development.

Methods

***Nt3* mutant mice and histologic techniques.** Heterozygote (+/–) *Nt3* mice[6] were intercrossed by brother/sister matings for embryo analysis. The morning of the detection of a vaginal plug was considered day 0.5, and gestational age was assigned. At the time of embryo biopsy, morphologic criteria[25] were used in assigning developmental age. Key criteria included somite number, limb bud, eye and ear development and crown-rump length. The genotype of each embryo or newborn mouse was determined by analysis of yolk sac or head-derived DNA as described[6]. Mice were killed and the bodies were immediately fixed in 3% paraformaldehyde in phosphate buffered saline for 18 h, and the contents of the thoracic cavity was dissected *en bloc* prior to embedding in paraffin. Sections of 5 microns were stained using haematoxylin and eosin as described[12] or processed for immunohistochemistry. Rabbit polyclonal antisera (1415), generated using peptide LVDGQPRQAKGELGL, corresponding to aa 639–653 in the cytoplasmic domain of the rat *TrkC* sequence[10], is immunoreactive to *TrkC* kinase active isoforms, but not to *TrkB* or *TrkA*. Immunoreactivity was detected using a biotin streptavidin linked to Super Fast Red as a chromogenic substrate[12].

Echocardiographic imaging. Within 4 h of birth, all animals in a litter were subjected to sonographic imaging in a blinded fashion. Animals were imaged by placement of a Hewlett-Packard coronary probe in warmed gel on the anterior chest wall, using a Sonos 1000 with real time image analysis. Following imaging of the heart for at least 5 min/animal, the cardiac rate, chamber dimensions, and response of these parameters to modest sternal pressure were determined on each animal by analysis of recorded images. The ambient temperature of the newborn animals was maintained at approximately 36 °C and the animals were then killed for genotyping and histologic analysis within 4 h of imaging.

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