Published in final edited form as:
Dev Biol. 2014 April 15; 388(2): 149–158. doi:10.1016/j.ydbio.2014.02.010.

Loss of *Hand2* in a population of *Periostin* lineage cells results in pronounced bradycardia and neonatal death

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Abstract

The *Periostin Cre* (*Postn-Cre*) lineage includes endocardial and neural crest derived mesenchymal cells of the cardiac cushions, neural crest-derived components of the sympathetic and enteric nervous systems, and cardiac fibroblasts. In this study, we use the *Postn-Cre* transgenic allele to conditionally ablate *Hand2* (*H2CKO*). We find that *Postn-Cre H2CKOs* die shortly after birth despite a lack of obvious cardiac structural defects. To ascertain the cause of death, we performed a detailed comparison of the *Postn-Cre* lineage and *Hand2* expression at mid and late stages of embryonic development. Gene expression analyses demonstrate that *Postn-Cre* ablates *Hand2* from the adrenal medulla as well as the sphenopalatine ganglia of the head. In both cases, *Hand2* loss-of-function dramatically reduces expression of *Dopamine Beta Hydroxylase* (*Dbh*), a gene encoding a crucial catecholaminergic biosynthetic enzyme. Expression of the genes *Tyrosine Hydroxylase* (*Th*) and *Phenylethanolamine N-methyltransferase* (*Pnmt*), which also encode essential catecholaminergic enzymes, were severely reduced in postnatal adrenal glands. Electrocardiograms demonstrate that 3-day postnatal *Postn-Cre H2CKO* pups exhibit sinus bradycardia. In conjunction with the aforementioned gene expression analyses, these results strongly suggest that the observed postnatal lethality occurs due to a catecholamine deficiency and subsequent heart failure.

Keywords

Hand2; basic Helix-loop-Helix (bHLH) transcription factor; heart development; sympathetic neurogenesis; bradycardia; heart failure

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Introduction

Hand2 is a member of the Twist family of bHLH transcription factors, exhibiting essential roles in both cardiogenesis and sympathetic neuronal development (Hendershot et al., 2008; Howard, 2005; Vincentz et al., 2011). Loss-of-function experiments in mice demonstrate the necessity of *Hand2* for proper cardiac neural crest cell migration, ventricular chamber expansion, epicardial differentiation, cell type specific gene expression within both sympathetic and enteric neurons, craniofacial development, and digit formation (Barnes et al., 2011; Firulli et al., 2005; Galli et al., 2010; Hendershot et al., 2008; Holler et al., 2010; Lei and Howard, 2011; McFadden et al., 2005; Morikawa et al., 2007; Srivastava et al., 1997; Tsuchihashi et al., 2011). Although these previous studies have done much to improve our understanding of the role of Hand2 in these tissues, the dynamic spatiotemporal expression profile of *Hand2* suggests the existence of additional functions, which remain unexplored.

Hand2 is strongly expressed within both the endocardium of the heart tube (Barnes et al., 2011), and the cardiac neural crest (Holler et al., 2010). The early heart tube consists of an inner endocardial layer and an outer myocardial layer separated by extracellular matrix referred to as cardiac jelly (Abu-Issa and Kirby, 2007). As development proceeds the heart tube loops, expands, and septates to form the distinct ventricular and atrial chambers. A subset of endocardial cells simultaneously undergo an epithelial to mesenchymal transition (EMT) and migrate into the atrioventricular (AV) cushions, which will subsequently remodel into the tricuspid and mitral valves of the heart (VanDusen and Firulli, 2012). Concurrently, cardiac neural crest ectomesenchyme invades the outflow tract (OFT) cushions, which, along with some endodermally-derived cells, will remodel into the aortic and pulmonary valves (Keyte and Hutson, 2012). Thus, *Hand2* is strongly expressed in all of the cellular progenitors of all cardiac valves.

Hand2 is also strongly expressed within the neurons of the sympathetic chain, where it is required to both induce and maintain expression of genes encoding the biosynthetic enzymes that produce nor-epinephrine (Hendershot et al., 2008; Howard et al., 1999). Additionally, Hand2 expression within the catecholaminergic cells of the adrenal medulla has been reported (Wildner et al., 2008), but the role of Hand2 in this tissue remains unknown.

To determine the role, if any, that *Hand2* plays during later stages of embryonic development, we employed the *Postn-Cre* transgenic allele (Lindsley et al., 2007; Takeda et al., 2010) to conditionally delete *Hand2*. *Postn-Cre* lineage overlaps with *Hand2* expression within populations of post-migratory cardiac neural crest and some neural crest-derived components of the autonomic nervous system, as well as the endocardial derived mesenchymal cells of the endocardial cushions (Lindsley et al., 2007; Takeda et al., 2010; VanDusen and Firulli, 2012). Interestingly, these *H2CKOs* do not exhibit detectable cardiac phenotypes within the OFT, and exhibit normally formed tricuspid and mitral valves. Despite the lack of cardiac phenotypes, *H2CKOs* die within 10 days of birth. A low incidence of *Postn-Cre*–independent cleft palate in $Hand2^{fx/fx}$ offspring contributes to some of the observed *H2CKO* lethality, but this hypomorphic phenotype does not account for the complete penetrance of neonatal lethality observed. To better understand the mechanisms

which underlie the major causes of *H2CKO* lethality, we performed detailed *Postn-Cre* lineage-trace analyses and directly compared these findings with *Hand2* expression during mid-(E12.5) and late-stage (E16.5) embryonic development. These studies reveal that *H2CKOs* retain *Hand2* and *Dopamine-β Hydroxylase (Dbh)* expression within the ganglia of the sympathetic chain; however, *Postn-Cre* efficiently ablates *Hand2* expression within the sphenopalatine ganglia and the catecholaminergic cells of the adrenal medulla, an organ that, via its catecholamine production, regulates cardiac homeostatic functions such as blood pressure, metabolism, and heart rate (Axelrod and Reisine, 1984; Fung et al., 2008). Loss of Hand2 function in the adrenal medulla and sphenopalatine ganglia results in a corresponding large decrease in *Dbh* levels, as well as a drop in levels of *Th* and *Pnmt* expression within adrenal glands. We show that this downregulation of genes encoding enzymes crucial for catecholamine synthesis has a functional effect on the heart rates of 3-day postnatal (P3) *H2CKO* pups. In addition, we demonstrate that *H2CKOs* suffer from impaired gastrointestinal motility, which may also contribute to neonatal lethality.

Materials and methods

Mice

Postn-Cre(+) mice (Lindsley et al., 2007) were crossed with *Hand2*$^{fx/fx}$ (Morikawa et al., 2007) mice to generate *Postn-Cre(+);Hand2*$^{fx/+}$ males. These males were then crossed with *Hand2*$^{fx/fx}$*;ROSA26R lacZ* (*R26R*$^{z/z}$) reporter mice to generate conditionally null *Hand2* embryos. Genotyping of *ROSA26R* and *Hand2* conditional alleles was carried out as previously described (Barnes et al., 2011). Similarly, *Nestin-Cre(+);Hand2*$^{+/-}$ males were crossed with *Hand2*$^{fx/fx}$*;ROSA26R YFP* reporter females to generate conditionally null *Hand2* embryos. *Nestin-Cre* mice were genotyped as previously described (Tronche et al., 1999). *Postn-Cre* mice were genotyped by southern blot with a probe corresponding to an EcoRI fragment of the *pTurbo-Cre* cDNA.

Section RNA *In Situ* Hybridization and quantitative RT-PCR

Antisense digoxygenin labeled riboprobes were transcribed with T7, SP6, or T3 (Roche). Section *in situ* hybridization was performed as previously described (Vincentz et al., 2008). Analysis was performed on a minimum of 3 somite-matched embryos for each probe and genotype. For quantitative RT-PCR (qRT-PCR) total RNA was isolated from flash-frozen adrenal glands using the High Pure RNA Isolation Kit (Roche). This RNA served as a template to generate cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). cDNA was amplified using Taqman Probe-Based Gene Expression Assays (Applied Biosystems). Relative gene expression was determined after normalization to GAPDH. Three samples were collected per genotype. The Student's t-test was used to detect significant differences between sample groups, with P-values ≤ 0.05 considered significant.

Histological Preparations

Histology and X-gal staining were conducted essentially as previously described for paraffin embedded embryos (Vincentz et al., 2008). For Alcian Blue staining of endocardial cushions, xylene cleared paraffin embedded embryos were sectioned at 10μm and stained using 0.15mg/ml Alcian Blue in 5% glacial acetic acid. Results reflect an n of 3 or more.

Electrocardiograms

P3 pups were anesthetized with 4% isoflurane before electrode placement. Four percent isoflurane was continuously supplied via facemask and environmental temperature was controlled via heat lamp. ECGs were collected and processed as described previously (Shen et al., 2008). Results reflect n = 4; as previous studies have established that catecholamine insufficiency would only be expected to result in a decreased heart rate, p-value was calculated using a student's one tailed t-test.

Results

H2CKOs exhibit normal cardiac valve development

Timed matings between *Postn-Cre;Hand2*$^{fx/+}$ males and *Hand2*$^{fx/fx}$*;R26R*$^{z/z}$ females were conducted and the embryos collected at various time points from mid-gestation to birth. As *Postn-Cre* activity is evident by E11.5 (Lindsley et al., 2007), hearts from E12.5 embryos were analyzed for β-galactosidase activity to compare Cre lineage in relation to *Hand2* expression. The enhancer controlling *Postn-Cre* activity is reportedly induced at approximately E10.5 in mesenchymal cells of the cardiac cushions (Lindsley et al., 2007). As expected, β-galactosidase staining within the E12.5 heart robustly marks the AV cushion (black arrow Fig. 1A) and OFT cushion mesenchyme (black arrow Fig. 1D), as well as a small population of cells within the epicardium from which a population of cardiac myofibroblasts are derived (Fig. 1A, D). *Hand2 in situ* hybridizations (ISH) similarly confirm that *Hand2* expression overlaps with *Postn-Cre* lineage in each of these cardiac cell populations, but *Postn-Cre* lineage does not overlap with *Hand2* expression within the endocardium (Fig. 1A–C). *Hand2* ISH of *H2CKO* embryos shows significant *Postn-Cre*-mediated deletion of *Hand2* within both the cardiac OFT and AV cushions (asterisks in Fig. 1C, F), in contrast to unaffected *Hand2* expression within the endocardium and epicardium.

H2CKOs are viable at birth, but die within the first 10 days of life (Table 1). A small portion of *Hand2*$^{fx/fx}$ pups die from cleft palate (Sup. Fig. 1, Table 2); however, the occurrence of the palate defect is independent of the presence of the *Cre* allele, and results from hypomorphic expression, which has been previously reported with this *Hand2* conditional allele (Morikawa et al., 2007). To determine whether *H2CKOs* exhibit aortic or AV valve phenotypes, E18.5 hearts were sectioned and analyzed by Alcian Blue staining, which marks proteoglycans within the heart valves (Fig. 2A–D). No significant differences in size or shape between control and *H2CKO* cardiac valves are observed. Alcian Blue staining reveals comparable proteoglycan levels and distribution in control (Fig. 2A, C) and *H2CKO* valves (Fig. 2B, D). E18.5 *Hand2* ISH reveals that *Hand2* expression is restricted to the endocardium overlying both OFT and AV canal valves, and is not detectable within the valve mesenchyme of either control (Fig. 2E, G) or *H2CKO* (Fig. 2F, H) leaflets. *Postn* ISH shows no significant difference in *Postn* expression within the mesenchymal cells of the cushions or within the cardiac fibroblasts between control (Fig. 2I, K) and *H2CKO* (Fig. 2J, L) embryos. These data indicate that Hand2, within the *Postn-Cre* lineage, has no obvious cell-autonomous role in OFT or AV cushion remodeling.

Postn-Cre ablates Hand2 from the enteric nervous system

It has been demonstrated that Hand2 function is required for many aspects of proper enteric nervous system development, including neurogenesis, cell type specification, proliferation of enteric precursor cells, and gangliogenesis (D'Autreaux et al., 2011; Hendershot et al., 2007; Lei and Howard, 2011). As these functions may be impaired in *H2CKOs*, we next looked for defects within the developing gut. Lineage tracing at E12.5 and E16.5 shows that *Postn-Cre* indeed marks the myenteric plexus in the stomachs and intestines of control (Fig. 3A, C) and *H2CKO* embryos (Fig. 3B, D). Similarly, ISH at E16.5 confirms previous reports (Hendershot et al., 2008; Lei and Howard, 2011; Wu and Howard, 2002) that *Hand2* is also expressed within these enteric neurons (Fig. 3E). Furthermore, this expression is ablated in *H2CKOs* (Fig. 3F). To determine if this loss of *Hand2* affects viability, we dissected out and visually assessed the gastrointestinal tracts of P3 pups (Fig. 3G). While deletion of *Hand2* in enteric neural precursor cells results in gut obstruction and severe bowel distention by P20 (Lei and Howard, 2011), P3 *Postn-Cre H2CKOs* displayed a lack of fecal matter posterior to the cecal appendages (arrow Fig. 3E). This is indicative of impaired bowel motility and may contribute to the observed early neonatal death in *H2CKOs*.

H2CKOs display a loss of Dbh expression within sphenopalatine ganglia

We next carefully compared *Postn-Cre* lineage with *Hand2* expression in the sympathetic chain. It has been previously demonstrated that *Hand2* is required for early development of sympathetic neurons. Neural crest-specific *H2CKO* embryos die at approximately E11.0 from low levels of catecholamines, and can be pharmacologically rescued by administration of catecholamine intermediates (Hendershot, Liu et al. 2008). Wholemount analysis at E12.5 reveals robust *Cre* activity in components of the peripheral nervous system, as well as in and around the developing nasal cavity and maxilla (data not shown). As the *Postn-Cre* lineage is reported to include Schwann cells surrounding the sympathetic ganglia (Lindsley et al., 2007), we examined the sympathetic ganglia of *H2CKOs* at E12.5, when *Wnt1-Cre* generated *H2CKOs* die from lack of catecholamines (Hendershot, Liu et al. 2008). *Postn*-lineage analysis in transverse sections at the level of the heart confirms activation of the *ROSA* reporter within the cells surrounding the sympathetic ganglia (Fig. 4B, C). Although the cells enveloping the trunk sympathetic ganglia were clearly marked, ISH demonstrates that expression of *Hand2* (Fig. 4D, E) as well as *Dbh* (Fig. 4F, G), a gene known to be directly regulated by Hand2 (Rychlik et al., 2003; Xu et al., 2003), is unaffected when control and *H2CKO* embryos are compared. Further immunohistochemical analyses using the *Nestin-Cre* (Tronche et al., 1999) and a YFP reporter allele at E14.5 indicate that the *Postn-Cre(+)* population of cells surrounding the trunk ganglia are also selectively marked by the *Nestin-Cre* lineage (Sup. Fig. 2A–I). ISH shows that ablation of *Hand2* within the *Nestin-Cre* lineage does not result in loss of *Hand2* within trunk ganglia cells (Sup. Fig. 2J, M, P), while expression of the pan-neuronal marker Hu is also not affected (Sup. Fig. 2K, N, Q).

Interestingly, more detailed analyses of E12.5 heads reveal that the *Postn-Cre* lineage is not restricted to cells on the outer surface, but also marks the neurons within parasympathetic sphenopalatine ganglia (arrow; Fig. 4H). Despite being commonly considered

parasympathetic and having a parasympathetic root, the sphenopalatine ganglia have an additional sympathetic root derived from the cervical sympathetic ganglia (Coppola et al., 2010). The sphenopalatine ganglia are known to express *Dbh*, with levels peaking at E12.5 and gradually being downregulated (Hirsch et al., 1998). In the rat, at least a subpopulation of sphenopalatine cells express TH and produce catecholamines, while most of the cells produce low levels of TH but do not produce catecholamines (Leblanc and Landis, 1989). Projections from the sphenopalatine ganglia are known to innervate the lacrimal gland, and regulate blood flow to the nasal mucosa, while additional evidence suggests a role in regulating cerebral blood flow (Suzuki et al., 1990; Ter Laan et al., 2013). ISH of frontal sections reveals that *Hand2* is robustly expressed within the sphenopalatine ganglia of control embryos (Fig. 4I), and its expression is ablated in *H2CKOs* (Fig. 4J). This loss of *Hand2* is accompanied by a loss of *Dbh*, which is likely dependent on Hand2 function (Fig. 4K, L). This data suggested that, despite normal *Hand2* expression within the mid-gestation sympathetic trunk, neonatal *H2CKOs* may exhibit a reduction in catecholamine biosynthesis outside the sympathetic chain, or possibly within trunk ganglia due to a later stage deletion. We thus sought to examine additional tissues at later embryonic time points, to better define all of the sources of norepinephrine and epinephrine that might be compromised in *H2CKOs*.

Late-stage Cre activity does not affect the sympathetic chain, but mediates the deletion of Hand2 from the adrenal medulla

To determine whether the *Postn-Cre* lineage, which is initially restricted to cells surrounding the trunk ganglia, later expands expression to include the ganglia neurons, *ROSA* reporter staining was conducted at E16.5. β-galactosidase staining indicates that the *Postn-Cre* lineage includes only a small subpopulation of neurons within sympathetic trunk ganglia (Fig. 5A, B). Importantly, *Hand2* (data not shown) and *Dbh* (Fig. 5C, D) expression within the sympathetic trunk remains robust in *H2CKOs*. In the neonate and the adult, the adrenal medulla is the primary site for synthesis of circulating catecholamines (Malmejac, 1964). *ROSA* reporter staining at E16.5 indicates that *Postn-Cre* lineage includes cells of control (Fig. 5E) and *H2CKO* (Fig. 5F) adrenal medulla. Control and *H2CKO* adrenal glands were then collected from P3 pups, and analyzed by ISH. *Hand2* (Fig. 5G) and *Dbh* (Fig. 5I) are both robustly expressed within control adrenal medulla at P3, whereas *Postn-Cre* efficiently ablates *Hand2* (Fig. 5H), resulting in downregulation of *Dbh*, which would be expected to reduce catecholamine biosynthesis (Fig. 5J) in *H2CKOs*. Furthermore, we confirmed these results by collecting additional P3 adrenal glands, isolating RNA, and conducting qRT-PCR. In *H2CKOs Hand2* expression was reduced to approximately 7% of control levels (p-value = 0.004), while *Dbh* levels were reduced to approximately 11% (p-value = 0.023). Expression of *Th*, which encodes the enzyme responsible for conversion of L-tyrosine to a dopamine precursor, and *Pnmt*, which encodes the enzyme responsible for converting norepinephrine to epinephrine, was reduced to approximately 23% (p-value = 0.001) and 9% (p-value = 0.016) respectively (Fig. 5K)

Postn-Cre H2CKO Pups exhibit bradycardia

When observing *H2CKO* pups, it is clear that the animals are significantly smaller by P3 and exhibit a failure to thrive (Fig. 6A). Given the lack of cardiac structural defects, and the

identification of multiple catecholaminergic *Hand2*-expressing cell populations that overlap with the *Postn-Cre* lineage, a catecholamine deficiency in conjunction with gastrointestinal dysfunction is the most likely cause of the lethal failure to thrive phenotype. As catecholaminergic stimulation is essential for proper cardiac function, we tested this hypothesis by conducting electrocardiograms (ECGs) on P3 pups, and thereby assessing heart rates. After assessing four mutants, and an equal number of littermate controls (Fig. 6B and C), we determined that *Postn-Cre H2CKOs* had significantly slower sinus node rates (P-value = 0.019, Fig. 6D), a condition commonly referred to as bradycardia. Control pups averaged 466 beats per minute (bpm), while *Postn-Cre H2CKOs* averaged only 337 bpm. In addition to heart rate, the ECG traces were used to calculate the average amount of time between the start of atrial activation and the start of ventricular activation (PR interval), but no significant difference was noted (Fig. 6E). Similarly, the duration of atrial depolarization (P-wave duration), and duration of ventricular depolarization (QRS complex duration) were measured, with no significant difference found between controls and *H2CKOs* (Fig. 6E). To determine if *H2CKOs* were experiencing heart failure we examined expression of *Nppa* (*atrial natriuretic factor; Anf*), which becomes upregulated in ventricular muscle when undergoing stress (Edwards et al., 1988). Compared to the highly restricted atrial expression of *Anf* in control hearts (Fig. 6F) a clear marked increase in ventricular *Anf* expression is observed in *H2CKO* hearts (Fig. 6G; see Supplemental Fig. 3 for additional sections), supporting heart failure as a cause of *H2CKO* neonatal lethality.

Discussion

This study demonstrates that, in addition to the established early embryonic roles Hand2 plays in cardiac morphogenesis and the development and function of the sympathetic ganglia, Hand2 plays important homeostatic post-embryonic functions in the production of catecholamines. Deletion of *Hand2* from mesenchymal cells of the cardiac cushions at E11.5, well after initiation of *Hand2* expression in endocardial precursors, does not result in any cardiac valve phenotypic abnormalities. This data supports a model in which essential Hand2 function likely lies within the ventricular endocardium. *Hand2* expression within the early stage cushion mesenchyme is gradually downregulated, and by E18.5, *Hand2* expression within the valves is restricted to only the *Postn-Cre*-negative endothelium covering the valve surface. We conclude that *Hand2* does not play a critical role in endocardial- or neural crest-derived cushion cells post EMT. It is interesting that *Hand2* expression is robustly sustained in overlying valve endocardium, particularly on the backside of valves (ventricular side in AV canal, non-ventricular side in OFT; Fig. 2E–H). These data do not rule out a possible non cell-autonomous role in cushion remodeling, through endothelial to mesenchymal signaling.

Hand2 expression and *Postn-Cre* lineage also overlap within components of the enteric nervous system. Previous studies of enteric function show that ablation of *Hand2* within enteric neural precursors via the *Nestin-Cre*, which is initiated by E11.5, results in a severe bowel distention by P20 (Lei and Howard, 2011). The data in the current study show that *Postn-Cre* is initiated by E12.5, making it possible that *Postn-Cre H2CKOs* share the same gastrointestinal dysfunction as *Nestin-Cre H2CKOs*. Indeed, a lack of fecal matter posterior to cecal appendages of *Postn-Cre H2CKOs* indicates that decreased gastrointestinal motility

could be contributing to neonatal lethality. Interestingly, this lack of motility resembles a human congenital motility disorder called Hirschsprung's disease, which is pathologically characterized by a lack of enteric ganglia in a variable stretch of the distal bowel wall. While *Postn-Cre H2CKOs* do not appear to suffer from a complete loss of enteric ganglia (Fig. 3D), it is possible that a reduction in gangliogenesis within specific regions of the gut has evaded our detection. Indeed, as *Nestin-Cre H2CKOs* suffer from a functional aganglionosis (Lei and Howard, 2011), and the *Postn-Cre* enteric lineage closely resembles that of the *Nestin-Cre*, this would not be surprising; however, as the *Nestin-Cre H2CKOs* survive until P20, while *H2CKOs* generated by the *Postn-Cre* do not, the involvement of additional non-enteric phenotypes is likely.

It is well established that *Hand2* expression within the sympathetic chain is required for neurons to acquire and maintain a catecholaminergic phenotype (Hendershot et al., 2008; Schmidt et al., 2009). Loss of *Hand2* within the sympathetic chain results in downregulation of the crucial biosynthetic enzymes Tyrosine Hydroxylase and Dbh (Hendershot et al., 2008). For a more complete description of Hand2's role within the sympathetic nervous system, as established by various manipulations of *Hand2* expression within multiple genetic systems, see Table 3. Our results confirm previous data showing that *Postn-Cre* expression is restricted to the cells surrounding sympathetic chain ganglia at E12.5 (Lindsley et al., 2007). As expected, *Hand2* expression within the trunk sympathetic ganglia is unaffected in *H2CKOs*, at both E12.5 and E16.5. *Dbh* expression is similarly unaffected, indicating that trunk sympathetic ganglia in *Postn-Cre H2CKOs* produce sufficient concentrations of norepinephrine and epinephrine for embryonic survival to birth. However, we observed that *Hand2* expression and the *Postn-Cre* lineage overlap within the sphenopalatine ganglia at E12.5, and ablation of *Hand2* results in a dramatic decrease in *Dbh* expression in these ganglia. Given that the sphenopalatine ganglia is reported to have a sympathetic root derived from the superior cervical sympathetic ganglion, in addition to parasympathetic and sensory roots, the observation of *Dbh* downregulation is not surprising, and suggests that dysfunction within these ganglia could be contributing to the early neonatal death in *H2CKOs*.

Interestingly, *Hand2* expression and the *Postn-Cre* lineage overlap within the catecholaminergic cells of the adrenal medulla. Similar to what is observed in sphenopalatine ganglia, ablation of *Hand2* within the adrenal medulla also results in a dramatic reduction of *Dbh*. ISH of *H2CKO* adrenal glands indicates that a small population of cells maintains *Dbh* expression, while the vast majority of expression within the adrenal medulla is lost. This is consistent with *Hand2* loss of function studies within the sympathetic chain, where *Dbh* is known to be a direct Hand2 target (Rychlik et al., 2003; Vincentz et al., 2013; Xu et al., 2003). Furthermore, qRT-PCR shows that *H2CKO* adrenal glands exhibit significant decreases in *Th* and *Pnmt*, which like *Dbh*, encode enzymes that catalyze essential steps in the synthesis of the catecholamine adrenalin. In a study of $Dbh^{-/-}$ embryos, it was stated that while most die in utero, approximately 12% survive until birth. The 12% survival was attributed to a flow of catecholamines across the placenta, thus also explaining why of those that survive to birth, 40% die by P2 (Thomas et al., 1995). These data make it tempting to consider that *Postn-Cre H2CKOs* may have enough

catecholaminergic biosynthetic capability to survive to birth as a result of unaffected sympathetic chain ganglia and supplementation from the mother, but at birth this supplementation ceases, leading to lethal catecholamine deficiency. It is well established that the adrenal medulla is a particularly important source of catecholamines that regulate heart rate, blood pressure, blood vessel constriction, and other critical aspects of cardiovascular function (Axelrod and Reisine, 1984; Fung et al., 2008). These data suggest that catecholamine deficiency, in conjunction with an enteric phenotype, causes *H2CKO* postnatal lethality. Heart rates in *H2CKOs* are significantly slower than control littermates. This phenotype is consistent with previous publications on genetic models of catecholamine deficiency, such as *Epas1* null mice, which are reported to have low catecholamine levels and a pronounced bradycardia (Tian et al., 1998), as well as *Th* null embryos, which are reported to have a 28% reduction in heart rate (Ream et al., 2008). In contrast, no difference in PR interval was observed between controls and *H2CKOs*, and while P-wave duration and QRS complex duration both trended toward an increase, no significant differences were observed, indicating that conditional ablation of *Hand2* does not alter dromotropic properties of the P3 heart. The reason for the differential effects of *Hand2* deficiency on chronotropy versus dromotropy is unclear. It may involve differential expression of beta-adrenergic receptors and/or their downstream targets in sinoatrial nodal cells versus cells of the cardiac specific conduction system. The decreased heart rates observed in P3 *H2CKOs* support the conclusion that a catecholaminergic insufficiency, in addition to enteric dysfunction, results in the neonatal *H2CKO* failure to thrive. In further support of this conclusion, upregulation of ventricular *Anf* expression in *H2CKO* hearts indicates pathological changes within these cardiomyocytes that ultimately lead to cardiac failure.

Conclusion

This study establishes that in post-migratory/post-EMT cushion mesenchyme Hand2 function is not required for proper valve development. *Postn-Cre H2CKOs* survive till birth, but fail to thrive and die soon after. While our analyses reveal an absence of cardiac or sympathetic chain phenotypes, *Hand2* is clearly ablated from the adrenal medulla and sphenopalatine ganglia where, similar to the sympathetic trunk, *Hand2* plays an important role in regulating *Dbh* expression. The reduction of *Dbh*, *Th*, and *Pnmt* in *H2CKOs* indicates that pups likely have a decreased ability to synthesize catecholamines, resulting in the observed postnatal lethality. Both ECG data showing that P3 *H2CKOs* have slower heart rates than control littermates and upregulation of *Anf* within the ventricles of *H2CKOs* supports this conclusion, as catecholaminergic sympathetic stimulation is essential for proper cardiac function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Danny Carney and Nichole Northrop for technical assistance and support, and Simon J. Conway (IUPUI) for supplying *Postn-Cre* mice. We also thank the Riley Heart Research Center Group for discussion and helpful feedback. Infrastructural support at the Herman B Wells Center is partially supported by the Riley Children's

Foundation and Division of Pediatric Cardiology. Grant support for this work was provided by: NIH R01 AR061392-03, R01 HL122123, and R01 HL120920 (ABF) and R01NS040644 (MJH)

Literature Cited

Abu-Issa R, Kirby ML. Heart field: from mesoderm to heart tube. Annu Rev Cell Dev Biol. 2007; 23:45–68. [PubMed: 17456019]

Axelrod J, Reisine TD. Stress hormones: their interaction and regulation. Science. 1984; 224:452–459. [PubMed: 6143403]

Barnes RM, Firulli BA, VanDusen NJ, Morikawa Y, Conway SJ, Cserjesi P, Vincentz JW, Firulli AB. Hand2 Loss-of-Function in Hand1-Expressing Cells Reveals Distinct Roles in Epicardial and Coronary Vessel Development. Circulation Research. 2011; 108:940–949. [PubMed: 21350214]

Coppola E, Rallu M, Richard J, Dufour S, Riethmacher D, Guillemot F, Goridis C, Brunet JF. Epibranchial ganglia orchestrate the development of the cranial neurogenic crest. Proc Natl Acad Sci U S A. 2010; 107:2066–2071. [PubMed: 20133851]

D'Autreaux F, Margolis KG, Roberts J, Stevanovic K, Mawe G, Li Z, Karamooz N, Ahuja A, Morikawa Y, Cserjesi P, Setlick W, Gershon MD. Expression level of Hand2 affects specification of enteric neurons and gastrointestinal function in mice. Gastroenterology. 2011; 141:576–587. 587 e571–576. [PubMed: 21669203]

Edwards BS, Ackermann DM, Lee ME, Reeder GS, Wold LE, Burnett JC Jr. Identification of atrial natriuretic factor within ventricular tissue in hamsters and humans with congestive heart failure. J Clin Invest. 1988; 81:82–86. [PubMed: 2961791]

Firulli BA, Krawchuk D, Centonze VE, Vargesson N, Virshup DM, Conway SJ, Cserjesi P, Laufer E, Firulli AB. Altered Twist1 and Hand2 dimerization is associated with Saethre-Chotzen syndrome and limb abnormalities. Nat Genet. 2005; 37:373–381. [PubMed: 15735646]

Fung MM, Viveros OH, O'Connor DT. Diseases of the adrenal medulla. Acta Physiol (Oxf). 2008; 192:325–335. [PubMed: 18021328]

Galli A, Robay D, Osterwalder M, Bao X, Benazet JD, Tariq M, Paro R, Mackem S, Zeller R. Distinct roles of Hand2 in initiating polarity and posterior Shh expression during the onset of mouse limb bud development. PLoS Genet. 2010; 6:e1000901. [PubMed: 20386744]

Hendershot TJ, Liu H, Clouthier DE, Shepherd IT, Coppola E, Studer M, Firulli AB, Pittman DL, Howard MJ. Conditional deletion of Hand2 reveals critical functions in neurogenesis and cell type-specific gene expression for development of neural crest-derived noradrenergic sympathetic ganglion neurons. Dev Biol. 2008; 319:179–191. [PubMed: 18501887]

Hendershot TJ, Liu H, Sarkar AA, Giovannucci DR, Clouthier DE, Abe M, Howard MJ. Expression of Hand2 is sufficient for neurogenesis and cell type-specific gene expression in the enteric nervous system. Dev Dyn. 2007; 236:93–105. [PubMed: 17075884]

Hirsch MR, Tiveron MC, Guillemot F, Brunet JF, Goridis C. Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development. 1998; 125:599–608. [PubMed: 9435281]

Holler KL, Hendershot TJ, Troy SE, Vincentz JW, Firulli AB, Howard MJ. Targeted deletion of Hand2 in cardiac neural crest-derived cells influences cardiac gene expression and outflow tract development. Dev Biol. 2010; 341:291–304. [PubMed: 20144608]

Howard M, Foster DN, Cserjesi P. Expression of HAND gene products may be sufficient for the differentiation of avian neural crest-derived cells into catecholaminergic neurons in culture. Dev Biol. 1999; 215:62–77. [PubMed: 10525350]

Howard MJ. Mechanisms and perspectives on differentiation of autonomic neurons. Dev Biol. 2005; 277:271–286. [PubMed: 15617674]

Keyte A, Hutson MR. The neural crest in cardiac congenital anomalies. Differentiation. 2012; 84:25–40. [PubMed: 22595346]

Leblanc GG, Landis SC. Differentiation of noradrenergic traits in the principal neurons and small intensely fluorescent cells of the parasympathetic sphenopalatine ganglion of the rat. Dev Biol. 1989; 131:44–59. [PubMed: 2462519]

Lei J, Howard MJ. Targeted deletion of Hand2 in enteric neural precursor cells affects its functions in neurogenesis, neurotransmitter specification and gangliogenesis, causing functional aganglionosis. Development. 2011; 138:4789–4800. [PubMed: 21989918]

Lindsley A, Snider P, Zhou H, Rogers R, Wang J, Olaopa M, Kruzynska-Frejtag A, Koushik SV, Lilly B, Burch JB, Firulli AB, Conway SJ. Identification and characterization of a novel Schwann and outflow tract endocardial cushion lineage-restricted periostin enhancer. Dev Biol. 2007; 307:340–355. [PubMed: 17540359]

Lucas ME, Muller F, Rudiger R, Henion PD, Rohrer H. The bHLH transcription factor hand2 is essential for noradrenergic differentiation of sympathetic neurons. Development. 2006; 133:4015–4024. [PubMed: 17008447]

Malmejac J. Activity of the Adrenal Medulla and its Regulation. Physiol Rev. 1964; 44:186–218. [PubMed: 14152905]

McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN. The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development. 2005; 132:189–201. [PubMed: 15576406]

Morikawa Y, Cserjesi P. Cardiac neural crest expression of Hand2 regulates outflow and second heart field development. Circ Res. 2008; 103:1422–1429. [PubMed: 19008477]

Morikawa Y, D'Autreaux F, Gershon MD, Cserjesi P. Hand2 determines the noradrenergic phenotype in the mouse sympathetic nervous system. Dev Biol. 2007; 307:114–126. [PubMed: 17531968]

Morikawa Y, Dai YS, Hao J, Bonin C, Hwang S, Cserjesi P. The basic helix-loop-helix factor Hand 2 regulates autonomic nervous system development. Dev Dyn. 2005; 234:613–621. [PubMed: 16145670]

Ream MA, Chandra R, Peavey M, Ray AM, Roffler-Tarlov S, Kim HG, Wetsel WC, Rockman HA, Chikaraishi DM. High oxygen prevents fetal lethality due to lack of catecholamines. Am J Physiol Regul Integr Comp Physiol. 2008; 295:R942–953. [PubMed: 18635452]

Rychlik JL, Gerbasi V, Lewis EJ. The interaction between dHAND and Arix at the dopamine beta-hydroxylase promoter region is independent of direct dHAND binding to DNA. J Biol Chem. 2003; 278:49652–49660. [PubMed: 14506227]

Schmidt M, Lin S, Pape M, Ernsberger U, Stanke M, Kobayashi K, Howard MJ, Rohrer H. The bHLH transcription factor Hand2 is essential for the maintenance of noradrenergic properties in differentiated sympathetic neurons. Dev Biol. 2009; 329:191–200. [PubMed: 19254708]

Shen WH, Chen Z, Shi S, Chen H, Zhu W, Penner A, Bu G, Li W, Boyle DW, Rubart M, Field LJ, Abraham R, Liechty EA, Shou W. Cardiac restricted overexpression of kinase-dead mammalian target of rapamycin (mTOR) mutant impairs the mTOR-mediated signaling and cardiac function. J Biol Chem. 2008; 283:13842–13849. [PubMed: 18326485]

Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet. 1997; 16:154–160. [PubMed: 9171826]

Suzuki N, Hardebo JE, Kahrstrom J, Owman C. Selective electrical stimulation of postganglionic cerebrovascular parasympathetic nerve fibers originating from the sphenopalatine ganglion enhances cortical blood flow in the rat. J Cereb Blood Flow Metab. 1990; 10:383–391. [PubMed: 2329125]

Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, Shindo T, Sano M, Otsu K, Snider P, Conway SJ, Nagai R. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. J Clin Invest. 2010; 120:254–265. [PubMed: 20038803]

Ter Laan M, van Dijk JM, Elting JW, Staal MJ, Absalom AR. Sympathetic regulation of cerebral blood flow in humans: a review. Br J Anaesth. 2013

Thomas SA, Matsumoto AM, Palmiter RD. Noradrenaline is essential for mouse fetal development. Nature. 1995; 374:643–646. [PubMed: 7715704]

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev. 1998; 12:3320–3324. [PubMed: 9808618]

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet. 1999; 23:99–103. [PubMed: 10471508]

Tsuchihashi T, Maeda J, Shin CH, Ivey KN, Black BL, Olson EN, Yamagishi H, Srivastava D. Hand2 function in second heart field progenitors is essential for cardiogenesis. Dev Biol. 2011; 351:62–69. [PubMed: 21185281]

VanDusen NJ, Firulli AB. Twist factor regulation of non-cardiomyocyte cell lineages in the developing heart. Differentiation. 2012; 84:79–88. [PubMed: 22516205]

Vincentz JW, Barnes RM, Firulli AB. Hand factors as regulators of cardiac morphogenesis and implications for congenital heart defects. Birth Defects Res A Clin Mol Teratol. 2011; 91:485–494. [PubMed: 21462297]

Vincentz JW, Barnes RM, Rodgers R, Firulli BA, Conway SJ, Firulli AB. An absence of Twist1 results in aberrant cardiac neural crest morphogenesis. Dev Biol. 2008; 320:131–139. [PubMed: 18539270]

Vincentz JW, Firulli BA, Lin A, Spicer DB, Howard MJ, Firulli AB. Twist1 controls a cell-specification switch governing cell fate decisions within the cardiac neural crest. PLoS Genet. 2013; 9:e1003405. [PubMed: 23555309]

Wildner H, Gierl MS, Strehle M, Pla P, Birchmeier C. Insm1 (IA-1) is a crucial component of the transcriptional network that controls differentiation of the sympatho-adrenal lineage. Development. 2008; 135:473–481. [PubMed: 18094025]

Wu X, Howard MJ. Transcripts encoding HAND genes are differentially expressed and regulated by BMP4 and GDNF in developing avian gut. Gene Expr. 2002; 10:279–293. [PubMed: 12450220]

Xu H, Firulli AB, Zhang X, Howard MJ. HAND2 synergistically enhances transcription of dopamine-beta-hydroxylase in the presence of Phox2a. Dev Biol. 2003; 262:183–193. [PubMed: 14512028]

Highlights

- *Postn-Cre(+);Hand2$^{fx/fx}$* mice die neonatally
- Mesenchymal Hand2 function is dispensable in the formation of the cardiac valves
- *Postn-Cre* ablates *Hand2* from sphenopalatine ganglia and the adrenal medulla
- *Hand2* ablation results in a loss of *Dopamine Beta Hydroxylase* expression
- *Postn-Cre(+);Hand2$^{fx/fx}$* mice exhibit bradycardia and fail to thrive, resulting in death.

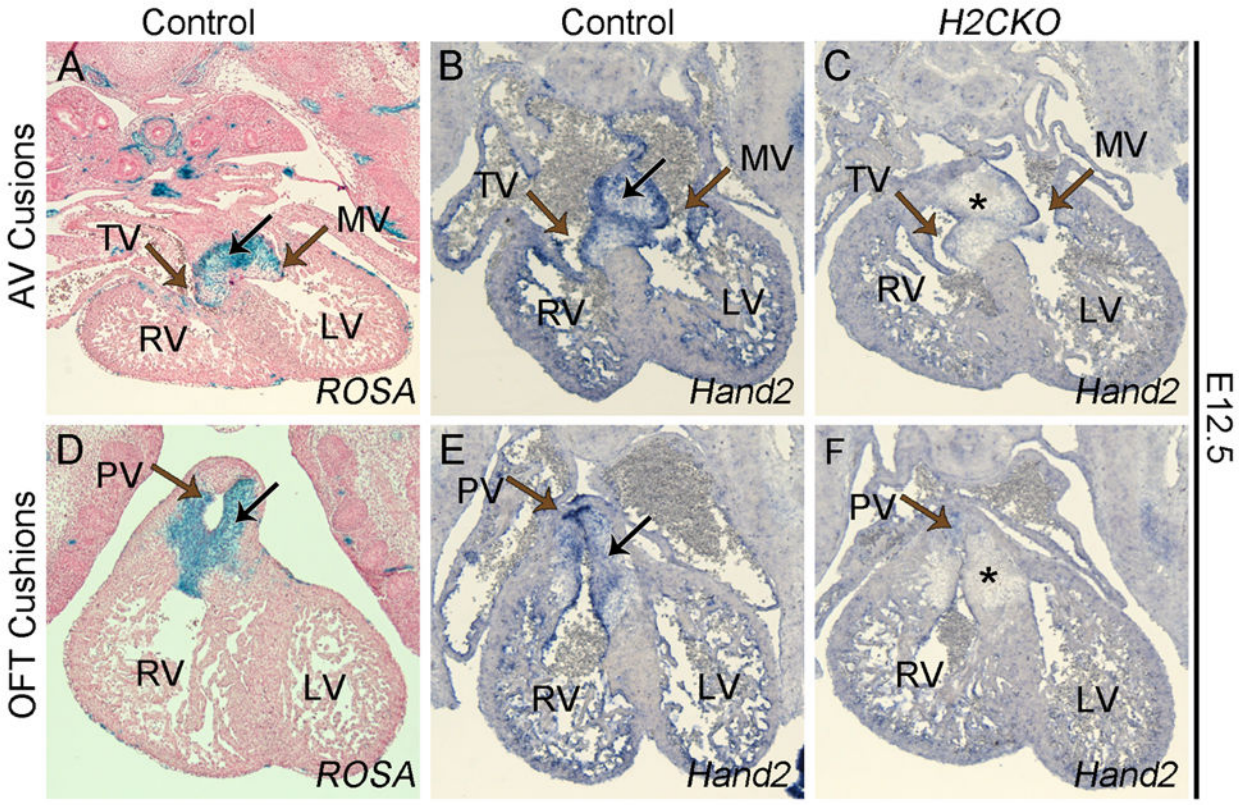


Figure 1. *Postn-Cre* mediated deletion of *Hand2* from AV and OFT cushions

Transverse section of *Postn-Cre(+)* lacZ stained control AV cushion at E12.5 (A). *Hand2* ISH of control (B) and *H2CKO* (C) AV cushion. Transverse section of *Postn-Cre(+)* β-galactosidase stained control OFT cushions at E12.5 (D). *Hand2* ISH of control (E) and *H2CKO* (F) OFT cushion. Black arrows indicate cushion mesenchyme, brown arrows indicate the primitive tricuspid valve (TV), mitral valve (MV), and pulmonary valve (PV). Asterisks indicate deletion of *Hand2*, right ventricle, RV; left ventricle, LV.

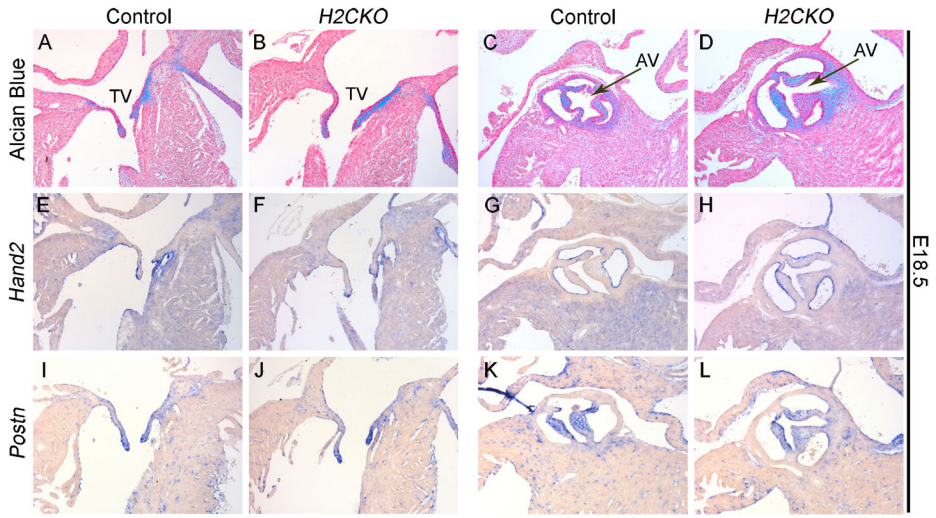


Figure 2. *Postn-Cre H2CKO* heart valves are normal at E18.5

Alcian blue stained section of control (A) and *H2CKO* (B) tricuspid valve. Alcian blue stained section of control (C) and *H2CKO* (D) aortic valve. *Hand2* ISH of control (E) and *H2CKO* (F) tricuspid valve. *Hand2* ISH of control (G) and *H2CKO* (H) aortic valve. *Postn* ISH of control (I) and *H2CKO* (J) tricuspid valve. *Postn* ISH of control (K) and *H2CKO* (L) aortic valve. TV, tricuspid valve; AV, aortic valve.

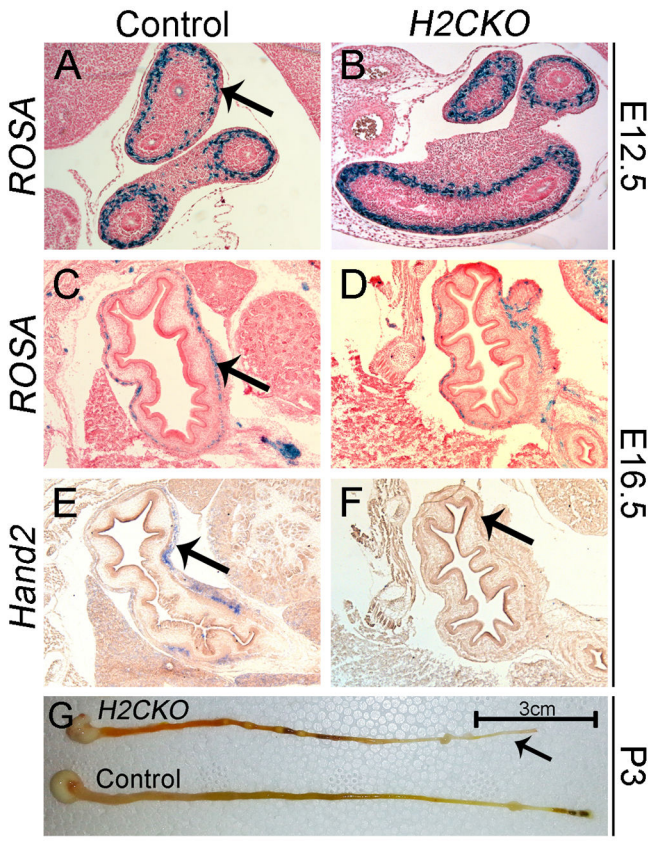


Figure 3. *Postn-Cre* is co-expressed with *Hand2* within the enteric nervous system

B-galactosidase stained sections of control (A) and *H2CKO* (B) intestines at E12.5. B-galactosidase stained sections of control (C) and *H2CKO* (D) stomachs at E16.5. *Hand2* section ISH of control (E) and *H2CKO* (F) stomachs at E16.5. Dissected out gastrointestinal tract of control and *H2CKO* P3 pups (G). Arrows in (A, C, E) indicate myenteric plexus, arrow in (F) indicates deletion of *Hand2*, arrow in (G) indicates lack of fecal matter posterior to the cecal appendage in *H2CKOs*.

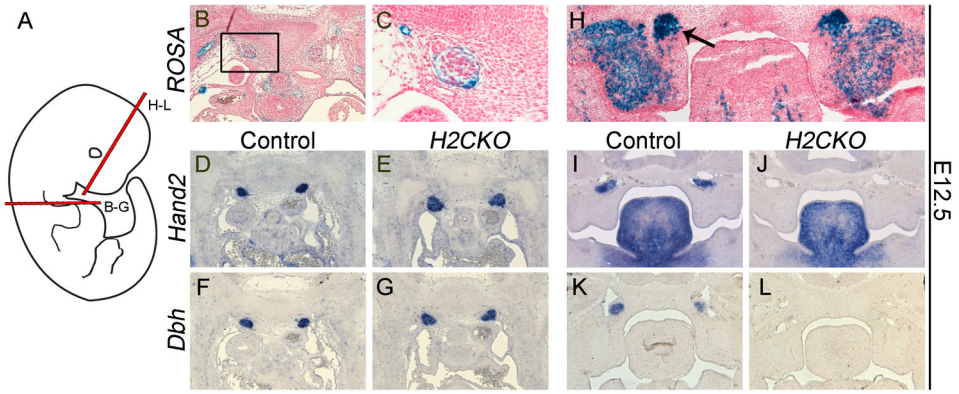


Figure 4. *Postn-Cre* mediates deletion of Hand2 within cells surrounding the sympathetic trunk and within the sphenopalatine ganglia at E12.5

Schematic showing planes of section (A). B-galactosidase stained sections of control sympathetic ganglia (B, C). *Hand2* ISH sections of control (D) and *H2CKO* (E) sympathetic ganglia. *Dbh* section ISH of control (F) and *H2CKO* (G) sympathetic ganglia. B-galactosidase stained frontal sections of sphenopalatine ganglia (arrow, H). *Hand2* ISH of sphenopalatine ganglia in control (I), and *H2CKO* (J) embryos. *Dbh* ISH in control (K), and *H2CKO* (L) embryos. B-galactosidase staining and ISH was conducted at E12.5

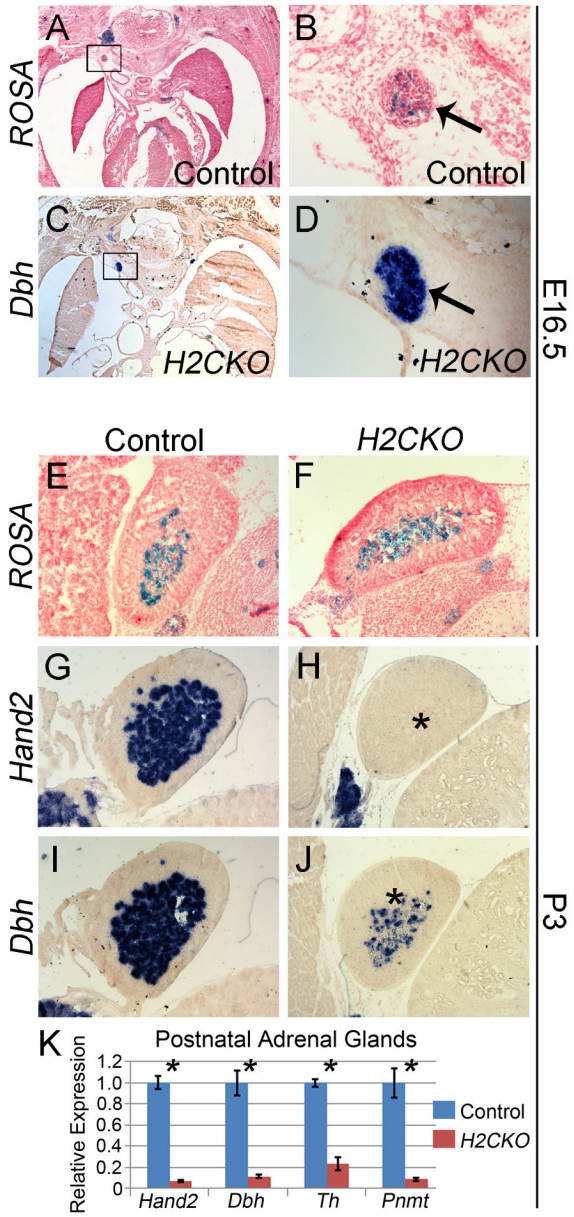
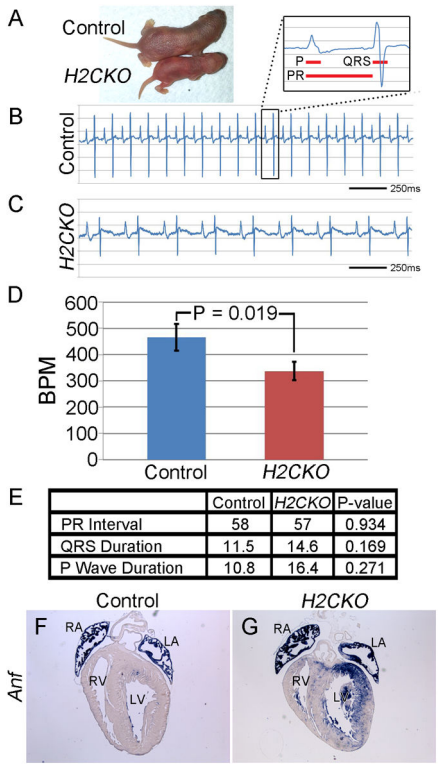


Figure 5. Expression of *Dbh* within sympathetic ganglia of *H2CKOs* is maintained at late stages of embryonic development, but *Postn-Cre* does ablate *Hand2* expression within the adrenal medulla, resulting in downregulation of *Dbh, Th, and Pnmt*

B-galactosidase stained sections of control sympathetic trunk ganglia (A) and magnification (B) at E16.5. *Dbh* ISH of *H2CKO* sympathetic trunk ganglia (C), and magnification (D) at E16.5. Arrows in (B, D) indicate ganglia. B-galactosidase stained sections of control (E), and *H2CKO* (F) adrenal medulla at E16.5. *Hand2* ISH of control (G) and *H2CKO* (H) adrenal medulla at P3. *Dbh* ISH of control (I) and *H2CKO* (J) adrenal medulla at P3. qRT-PCR of *Hand2*, *Dbh*, *Th*, and *Pnmt* in isolated P3 adrenal glands (K). Asterisks in (H, J) indicate reduction of *Hand2* and *Dbh* respectively. Asterisks in (K) indicate p-values less than 0.05.



	Control	*H2CKO*	P-value
PR Interval	58	57	0.934
QRS Duration	11.5	14.6	0.169
P Wave Duration	10.8	16.4	0.271

Figure 6. *Postn-Cre H2CKO* pups have significantly slower heart rates and expanded *Anf* expression

H2CKO pups that survive to P3 are noticeably smaller than littermates (A). Representative ECG trace for P3 control (B) and *H2CKO* (C) pups. Panel (B) insert represents one cycle of cardiac contraction. Red bars denote the portion of the trace measured for P-wave duration, PR interval, and QRS complex duration as labeled. Control pups averaged 466 bpm, while *H2CKO* pups averaged 337 bpm (D). P = p-value. Differences in average PR interval (ms), QRS complex duration (ms), and P wave duration (ms) between control and *H2CKO* P3 pups were not statistically significant (E). *Anf* ISH in P3 control (F) and *H2CKO* (G) hearts.

Table 1

H2CKOs die shortly after birth

Genotypes of embryos and pups collected at various stages of development. Expected number in parentheses.

	Postn-Cre(−)	***Postn-Cre(+);Hand2$^{fx/+}$***	***Postn-Cre(+);Hand2$^{fx/fx}$***
E12.5	21 (22)	11 (11)	13 (11)
E14.5	7 (5)	1 (3)	3 (3)
E16.5	9 (7)	4 (4)	1 (4)
E18.5	30 (29)	15 (15)	13 (15)
P10	29 (23)	16 (11)	0 (11)

Table 2

Penetrance of Cleft Palate in *Hand2$^{fx/fx}$* embryos

Embryos assessed from E14.5 to E18.5.

	Postn-Cre(−); Hand2$^{fx/+}$	***Postn-Cre(−); Hand2$^{fx/fx}$***	***Postn-Cre(+);Hand2$^{fx/+}$***	***Postn-Cre(+);Hand2$^{fx/fx}$***
Total Analyzed	10	12	10	10
# with Cleft Palate	0	2	0	2

Table 3

Hand2 function within the sympathetic nervous system.

Study	Model	Phenotype/Results	Conclusions
(Morikawa et al., 2005)	P19-embryonic carcinoma (P19-EC) cells stably expressing *Hand2* (P19-H2)	Retinoic acid treated P19-H2 cells but not P19-EC cells express peripherin (a peripheral nervous system marker), and a subset of these co-express Th.	Ectopic Hand2 expression is able to activate the sympathetic nervous system developmental program within P19-EC cells.
(Lucas et al., 2006)	Zebrafish *hand2* deletion mutant (*hands off*)	Sympathetic precursor cells aggregate to form normal sympathetic ganglion primordial, but *th* and *dbh* expression is strongly reduced	Generic neuronal differentiation is unaffected, but noradrenergic differentiation of sympathetic neurons is impaired
(Morikawa et al., 2007)	Mouse Wnt1-Cre Hand2 CKO	Death at E12.5 with cardiovascular and craniofacial defects. Sites of sympathetic development are populated by neural crest cells, which express pan-neuronal markers. *Th* and *Dbh* expression is dramatically reduced.	Hand2 permits sympathetic neurons to acquire a catecholaminergic phenotype
(Hendershot et al., 2008)	Mouse *Wnt1-Cre Hand2 CKO*	See above; *H2CKOs* exhibit a significant and progressive loss of sympathetic neurons.	Hand2 affects generation of the neural precursor pool by affecting proliferative capacity of progenitors and by regulating expression of transcription factors necessary for noradrenergic neuronal differentiation
(Morikawa and Cserjesi, 2008)	Mouse *Wnt1-Cre Hand2 CKO*	See above; early embryonic lethality could be rescued by administration of isoproterenol, a β-adrenoceptor agonist	Noradrenergic deficiency alone accounts for early embryonic lethality of *Wnt1-Cre H2CKOs*
(Schmidt et al., 2009)	Reduction/ablation of *Hand2* within differentiated sympathetic neurons by siRNA in cultured chick sympathetic neurons, and *Dbh-Cre* in *Hand2* conditional mice	Large decrease in *Th* and *Dbh* expression within *Hand2* siRNA treated chick sympathetic neurons. Pan-neuronal genes were not affected, while expression of cholinergic marker genes was enhanced. *Dbh-Cre H2CKO* mice showed decreased numbers of sympathetic neurons, and large reduction in Th expression	Hand2 plays a key role in maintaining noradrenergic properties in differentiated neurons