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Uncovering diversity in the development of central noradrenergic neurons and their efferents

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Abstract

Uncovering the mechanisms that underlie central noradrenergic neuron heterogeneity is essential to understanding selective subtype vulnerability to disease and environmental insult. Using recombinase-based intersectional genetic fate mapping we have previously demonstrated that molecularly distinct progenitor populations give rise to mature noradrenergic neurons differing in their anatomical location, axon morphology and efferent projection pattern. Here we review the findings from our previous study and extend our analysis of the noradrenergic subpopulation defined by transient developmental expression of *Hoxb1*. Using a combination of intersectional genetic fate mapping and analysis of a targeted loss of function mutation in *Hoxb1*, we have now uncovered additional heterogeneity based on the requirement of some noradrenergic neurons for *Hoxb1* expression. By comparing the distribution of noradrenergic neurons derived from the *Hoxb1* expression domain in wild-type and mutant mice, we demonstrate that *Hoxb1* expression is required by a subset of neurons in the pons. Additional fate mapping, using a *Hoxb1* enhancer element that drives Cre recombinase expression exclusively in rhombomere 4 of the hindbrain, reveals the existence of a subpopulation of noradrenergic neurons in the pons with more restricted axonal targets than the full *Hoxb1*-derived subpopulation. The unique projection profile of this newly defined subpopulation suggests that it may be functionally distinct. These analyses shed new light on the molecular determinants of noradrenergic identity in the pons and the overall complexity of the central noradrenergic system.

Keywords

norepinephrine; *Hoxb1*; fate map; recombinase-based; intersectional genetic fate mapping; efferents; rhombomere

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1. Introduction

It is now well accepted that central noradrenergic neurons are heterogeneous. Collectively defined by their ability to synthesize the neuromodulator norepinephrine, these neurons differ in their connectivity, molecular and physiological properties, and function (Berridge and Waterhouse, 2003; Chandler et al., 2014; Grzanna and Fritschy, 1991; Rinaman, 2011). Little is known about the ways in which this complexity arises, but such knowledge is critical to understanding the basis of selective noradrenergic neuron subtype vulnerability to disease and environmental insult. Variation in developmental gene expression is one source of heterogeneity in the nervous system (Brust et al., 2014; Champagnat et al., 2009; Dasen and Jessell, 2009), and the focus of our research has been the relationship between this early gene expression and mature noradrenergic neuron subtype identity.

To investigate how developmental gene expression differences generate diversity in the noradrenergic system, we adopted a recombinase-based intersectional genetic fate mapping approach in mice (Awatramani et al., 2003) (Fig 1). Recombinase-based fate mapping requires two genetically engineered alleles: a recombinase driver which expresses a site-specific recombinase such as Cre or Flp expressed in the cell population of interest, and an indicator allele which expresses a heritable marker, such as enhanced green fluorescent protein (eGFP), in response to recombinase activity (Jensen and Dymecki, 2014). For intersectional labeling, the indicator allele expresses a marker after recombination by two recombinases controlled by different driver alleles, so labeled cells represent the “intersection” or overlap of two independent gene expression domains (Fig 1, top). Recombination of the indicator allele is irreversible, so cells remain permanently labeled after transient driver expression. The labeled cells and their descendants share developmental history and constitute a genetically defined lineage that can be traced throughout development and into the adult brain, even after extensive cell migration.

In our initial analysis of the noradrenergic system, we exploited the unique gene expression profiles within rhombomeres (r), transient segmental units of the developing hindbrain. Four subpopulations of noradrenergic neurons were defined by intersectional expression of a noradrenergic specific Flp recombinase driver (*Dbh^{Flpo}*) (Robertson et al., 2013) and one of four rhombomere-specific Cre drivers (r1, *En1^{cre}* (Li et al., 2002); r2, *Hoxa2-cre* (Awatramani et al., 2003); r3&5, *Krox20^{cre}* (Voiculescu et al., 2000); and r4, *Hoxb1^{cre}* (O’Gorman, 2005)). In mice heterozygous for the dual-recombinase responsive indicator allele *RC::FrePe* (Bang et al., 2012; Engleka et al., 2012), *Dbh^{Flpo}*, and a rhombomere-specific Cre driver, noradrenergic neurons derived from that rhombomere (expressing both Flp and Cre) are labeled with eGFP, while noradrenergic neurons derived from other rhombomeres (expressing Flp alone) are labeled with red fluorescent mCherry (Fig. 1, top).

When we mapped the fates of the four genetically defined noradrenergic subpopulations, we found that their distribution in the adult brain varies significantly from their traditional anatomical subdivisions (Fig. 1 middle row) and reveals previously unappreciated lineal relationships between noradrenergic neurons in disparate anatomical locations. In addition, these genetically defined subpopulations have distinct axon morphology (Robertson et al., 2013) and efferent projection patterns (Fig. 1 bottom). The smallest subpopulations, derived

from the *Hoxa2-cre* and *Krox20* expression domains in r2 and r3&5, respectively, provide input to relatively limited targets. In sharp contrast, the *En1*- and *Hoxb1*-derived noradrenergic subpopulations, which comprise the largest genetic lineages, have axonal projections distributed throughout the brain. *En1*-derived noradrenergic neurons provide the highest input to areas of higher cognitive function such as the hippocampus and cortex, while the *Hoxb1*-derived noradrenergic neurons preferentially provide input to key central autonomic sites such as the hypothalamus, solitary nucleus, parabrachial nucleus and bed nucleus of the stria terminalis (BNST).

Our ability to map the axonal projections of each rhombomere-derived subpopulation across the entire brain in complete isolation enabled us to visualize the architecture of the central noradrenergic efferent system at a resolution that is difficult to achieve with other approaches. Notably, we discovered an unexpected projection from non-LoC *Hoxb1*-derived neurons to the prefrontal cortex, challenging the long held notion that the LoC is the sole source of noradrenergic projections to the cortex (Robertson et al., 2013). The distinct projection profile of each genetically defined subpopulation suggests that this method of reclassifying the noradrenergic system is functionally relevant, and the experimental access provided by fluorescent labeling will facilitate additional investigation at all levels from individual cells to intact animals.

Although rhombomere-based labeling has demonstrated that noradrenergic neurons are heterogeneous with regard to their embryonic origins, it did not reveal any of the molecular determinants of noradrenergic subtype development. It was previously proposed that *Hoxb1* is required for the development of medullary noradrenergic neurons (Gaufo et al., 2004). However, this conclusion was based on analysis of *Hoxb1* mutant embryos and not long-term fate mapping. By combining intersectional labeling with a targeted loss of function mutation in *Hoxb1*, we can now determine which, if any, of the noradrenergic neurons in the adult brain require *Hoxb1* expression. Here we present the results of further investigation into the *Hoxb1*-derived noradrenergic subpopulation. We demonstrate that *Hoxb1* expression is required by a subset of pontine noradrenergic neurons. In addition, we uncover further genetically defined diversity in the *Hoxb1* lineage.

2. Results

2.1. *Hoxb1* is required for the development of a subset of pontine central noradrenergic neurons

Based on the absence of dopamine β -hydroxylase (*Dbh*) expression within rhombomere 4 of the embryonic day (E) 11.5 *Hoxb1*-null mouse hindbrain, it has been speculated that *Hoxb1* expression is required for the differentiation of medullary A2 noradrenergic neurons (Gaufo et al., 2004). Given our previous finding that *Hoxb1*-derived noradrenergic neurons contribute to both the SubCV and A5 pontine nuclei and to caudal portions of the A1 and A2 medullary nuclei, we wanted to investigate whether *Hoxb1* expression is required by all *Hoxb1*-derived noradrenergic neurons or just neurons destined for the A2 medullary nucleus. Toward this goal, we exploited the fact that the *Hoxb1^{cre}* allele was generated by targeting cre to exon 1 of *Hoxb1*, resulting in a loss of function (*Hoxb1*-null) mutation (O'Gorman, 2005; Rottkamp et al., 2008). To visualize and map the fate of all noradrenergic

neurons in the context of a *Hoxb1*-null mutation, we crossed *Hoxb1*^{cre} mice with mice carrying *Dbh*^{Flpo} and *RC::FrePe* to generate triple transgenic *Hoxb1*^{cre};*Dbh*^{Flpo};*RC::FrePe* animals that are either heterozygous or homozygous for the *Hoxb1* mutation. In *Hoxb1*-null (*Hoxb1*^{cre/cre};*Dbh*^{Flpo/+};*RC::FrePe*) and heterozygous (*Hoxb1*^{cre/+};*Dbh*^{Flpo/+};*RC::FrePe*) mice, noradrenergic neurons with a history of *Hoxb1*^{cre} expression will be labeled with eGFP, while all other noradrenergic neurons will be labeled with mCherry (Fig. 2 CTR panels).

In *Hoxb1*-null and heterozygous mice, we tracked all noradrenergic neurons into the adult brain and mapped them onto the anatomically defined nuclei in the pons (LoC, SubC, A5) and medulla (A2, A1) according to an adult mouse brain atlas (Paxinos and Franklin, 2004). For each anatomically defined nucleus, we counted mCherry- and eGFP-positive noradrenergic neurons in male and female mice (Fig. 2, 3, and Supplemental Table 1). The number of noradrenergic neurons in each nucleus did not vary significantly according to sex. It is important to note that our intersectional approach is incapable of distinguishing *Dbh* positive adrenergic neurons in C1 and C2, from noradrenergic neurons in A1 and A2.

In the pons of *Hoxb1*-null animals we observed a striking deficit in the number of *Hoxb1*-derived noradrenergic neurons (Fig. 2, eGFP-labeled neurons). In the SubCV, 90% of the *Hoxb1*-derived noradrenergic neurons are absent in *Hoxb1* mutants. Similarly, 80% and 66% of the *Hoxb1*-derived noradrenergic neurons are missing in A5 and the SubCD of *Hoxb1*-null animals, respectively (Fig. 2). In the SubCV and A5, where *Hoxb1*-derived neurons constitute the majority of noradrenergic neurons (Fig 1.), the total number of neurons is also significantly decreased (Fig. 2, grey bars in graphs). The number of non-*Hoxb1*-derived (mCherry-positive) noradrenergic neurons was not significantly different in *Hoxb1*-null and heterozygous mice (Fig. 2, red neurons and bars).

In contrast, mapping of noradrenergic neurons onto the medullary A1 and A2 nuclei in *Hoxb1*-null mice revealed a significant *increase* in the number of *Hoxb1*-derived noradrenergic neurons (Fig. 3, eGFP-labeled neurons). In the C1/A1 nuclei, but not in C2/A2, an increase in the total number of labeled neurons was also observed (Fig 3, grey bars). We did not observe any significant change in the number of non-*Hoxb1*-derived (mCherry-positive) noradrenergic populations (Fig. 3, red neurons and bars), but we did detect co-expression of eGFP and mCherry in a few neurons. This co-labeling is never observed in heterozygous mice, because of the structure of the *RC::FrePe* allele (Fig 1) and the order in which the *Hoxb1*^{cre} and *Dbh*^{Flpo} driver alleles are expressed. The loxP-flanked mCherry-stop cassette of *RC::FrePe* is excised by the early, transient expression of *Hoxb1*^{cre} in progenitor cells (Freyer et al., 2011; Gaufo et al., 2000; Popperl et al., 1995). Later excision of the FRT-flanked stop cassette by *Dbh*^{Flpo} (Robertson et al., 2013) results in eGFP expression. Co-labeling can only be observed when Flp is expressed before Cre. In that situation, cells will express mCherry until Cre-mediated recombination deletes the mCherry-stop cassette and permits eGFP expression. The cells will transiently contain both fluorescent proteins until remaining mCherry mRNA and protein is turned over. Thus, co-expression of mCherry and eGFP in *Hoxb1*-null mice suggests that in at least a few cells, the cre-dependent excision of the mCherry-stop cassette was a recent event occurring in mature neurons.

Next, we wanted to determine the specific targets of the noradrenergic neurons that are missing in the *Hoxb1*-null mice. We exploited the ability of the *R/C::FrePe* allele to label axons with eGFP (Bang et al., 2012; Brust et al., 2014; Robertson et al., 2013) in order to compare the efferent projection profiles of the *Hoxb1*-derived noradrenergic population in *Hoxb1*-null and heterozygous mice. We did not observe a decrease in fiber density at any target site in *Hoxb1*-null mice relative to heterozygous controls. To the contrary, there are significantly more labeled axons at several target sites in *Hoxb1*-null mice, likely reflecting the increased number of eGFP-labeled cells in C2/A2 and C1/A1 (Fig. 4). Interestingly, this increase is not observed in the paraventricular thalamic (PV) nucleus, suggesting that the additional eGFP-labeled cells in *Hoxb1*-null mice do not project to the PV region.

The increase in the number of eGFP-positive noradrenergic neurons observed in C2/A2 and C1/A1 nuclei of *Hoxb1*-null mice may be due to the ectopic expression of cre observed in the hindbrain caudal to rhombomere 4 in *Hoxb1*-null embryos (Yang et al., 2008). However, ectopic cre expression does not explain the absence of a corresponding decrease in the number of mCherry labeled neurons and the statistically significant increase in the total number of noradrenergic neurons in C1/A1. Other possible explanations include increased proliferation of noradrenergic progenitors in the absence of *Hoxb1* expression or disrupted cell migration such that the noradrenergic neurons missing from the pons migrate to the medulla. However, the latter explanation is not supported by our cell counts: more cells are gained in the medulla than are lost in the pons. Also consistent with these data is the possibility that in the absence of *Hoxb1* expression, other cells in the brainstem may undergo a fate switch—becoming noradrenergic neurons, as defined by expression of *Dbh* and tyrosine hydroxylase (TH) — similar to what has been reported for motor neurons in *Hoxb1* mutants (Gavalas et al., 2003).

In summary, fate mapping on a *Hoxb1*-null genetic background demonstrates that *Hoxb1* expression is required for the development of a major subset of pontine SubCV and A5 noradrenergic neurons. These data reveal genetic and developmental complexity within the *Hoxb1*-derived noradrenergic neuron subpopulation and suggest that at least a portion of the pontine neurons may have a developmental history that is distinct from those of the medullary neurons. In the hindbrain, *Hoxb1^{cre}* expression is restricted to r4, but cre is also expressed in the spinal cord, caudal of the hindbrain (Freyer et al., 2011; Gaufo et al., 2000; Marshall et al., 1994; Popperl et al., 1995) and unpublished observations). Therefore, one plausible hypothesis is that the *Hoxb1^{cre}*-derived noradrenergic neurons consist of two lineages with distinct anatomic origins.

2.2. *b1r4*-cre defines a subset of *Hoxb1*-derived pontine noradrenergic neurons

To further investigate the developmental origins of *Hoxb1*-derived noradrenergic neurons, we used a *b1r4*-cre driver transgene, in which Cre expression is under the control of the *Hoxb1* r4 enhancer that restricts Cre expression exclusively to rhombomere 4 (Di Bonito et al., 2013; Studer et al., 1994). In *b1r4*-cre;*Dbh^{Flpo}*; *RC::FrePe* mice, noradrenergic neurons with a history of *b1r4*-cre expression (hereafter designated *b1r4*-cre-derived) express eGFP, while all non-*b1r4*-cre noradrenergic neurons express mCherry. We mapped the fate of *b1r4*-cre-derived noradrenergic neurons onto the mature anatomically defined noradrenergic

nuclei and found that *b1r4-cre*-derived noradrenergic neurons contribute to the SubCD, SubCV, and A5 pontine nuclei, but not to the medullary C2/A2, C1/A1 nuclei (Fig. 5). To determine the percent contribution to each nucleus we counted mCherry and eGFP positive neurons in male and female mice. We found that *b1r4-cre*-derived noradrenergic neurons comprise 2.8%, 46.5%, and 51.5% of the SubCD, SubCV, and A5 nuclei, respectively (Fig. 5). We also observed a small but significant increase in the number of *b1r4-cre*-derived noradrenergic neurons in the A5 nucleus of female mice compared to males (Supplemental Table 1). Regardless of this interesting sex difference, *b1r4-cre* labels fewer neurons than *Hoxb1^{cre}* in all three pontine nuclei.

These findings reveal the existence of at least two genetically distinct noradrenergic neuron subpopulations within the *Hoxb1* lineage: neurons that express both *b1r4-cre* and *Hoxb1^{cre}*, and those that express only *Hoxb1^{cre}*. The precise nature of these two subpopulations remains unclear. One possibility is that the *b1r4-cre* transgene is not expressed uniformly throughout rhombomere 4, indicating heterogeneity in the molecular programs that control development within the rhombomere. Alternatively, the non-*b1r4*-derived neurons may originate from a *Hoxb1* domain outside of rhombomere 4. Regardless of their origin, if these two genetically distinct subpopulations are also functionally distinct, they are likely to project to different target regions. Our genetic tools permit this hypothesis to be tested.

2.3. *b1r4-cre* defines a subpopulation of noradrenergic neurons with a distinct efferent projection pattern

To determine if the *b1r4-cre*- and *Hoxb1*-derived noradrenergic neurons project to the same or different target regions, we again exploited the ability of the *R/C::FrePe* allele to label axons with eGFP. We mapped the axonal projections of the *b1r4-cre*-derived noradrenergic subpopulation in *b1r4-cre;Dbh^{Flpo};R/C::FrePe* mice and those of the entire *Hoxb1*-derived noradrenergic subpopulation in *Hoxb1^{cre};Dbh^{Flpo};R/C::FrePe* mice.

Consistent with our previous findings (Robertson et al., 2013), noradrenergic neurons labeled by *Hoxb1^{cre}* provide input to key components of the central autonomic nervous system, including the insular cortex (Ctx), paraventricular thalamic (PV) nucleus, basolateral amygdala, bed nucleus of the stria terminalis (BNST), parabrachial nucleus, and solitary nucleus (Fig 6, left panels and Table 1). Upon mapping axonal projections from *b1r4-cre*-derived noradrenergic neurons, we found that these neurons project to many of the same targets, including the insular cortex (Fig 6, top right). As expected for a smaller subpopulation, the *b1r4-cre*-derived noradrenergic neurons appear to provide fewer projections at most target sites, although there are several exceptions (Table 1). In the PV thalamic nucleus, projections from the *b1r4-cre* and *Hoxb1*-derived subpopulations occur at approximately the same density (Fig 5), suggesting that the *b1r4-cre* subpopulation accounts for most, if not all, of the projections labeled by *Hoxb1^{cre}*. In contrast, projections from the *b1r4-cre*-derived subpopulation are virtually absent in basolateral amygdala and BNST, two regions that are heavily targeted by the full *Hoxb1*-derived population (Fig 6, right panels and Table 1). Thus, the *b1r4-cre*-derived noradrenergic lineage projects more heavily to a specific subset of the *Hoxb1*-derived targets, rather than constituting the same proportion of the projections at all target sites. This unique projection pattern suggests that *b1r4-cre*

expression may define a functionally distinct subpopulation of noradrenergic neurons within the *Hoxb1* lineage. These results highlight the role of early molecular programs in generating subtle differences in the neuronal circuitry.

3. Discussion

Our results underline the complex molecular and developmental heterogeneity of central noradrenergic neurons, and the value of intersectional labeling for identifying the different genetic lineages that make up broadly defined cell types. In addition to the four genetically defined noradrenergic subpopulations described in our recent study (Robertson et al., 2013), we have now demonstrated that the noradrenergic neuron population defined by transient developmental expression of *Hoxb1* consists of several distinct subpopulations. These new genetically defined subpopulations are correlated with, but do not precisely match, the anatomic division of the *Hoxb1*-derived population between the pons and medulla.

By combining intersectional labeling with a loss-of-function mutation of *Hoxb1*, we were able to assess whether *Hoxb1* is a molecular determinant of noradrenergic development for those neurons originating within the *Hoxb1* expression domain. Previous analysis of *Hoxb1*-null mice indicated that *Hoxb1* is required for the specification of medullary noradrenergic neurons in the solitary nucleus (Gaufo et al., 2004). This conclusion was based on observations in embryonic hindbrains, where ongoing developmental changes can make identification of cellular populations challenging. Our analysis, in which neurons in the adult brain are unambiguously labeled on the basis of developmental gene expression, demonstrates that *Hoxb1* is required for a subset of noradrenergic neurons in the pons. In the medulla of *Hoxb1*-null mice, the surprising increase in the number of labeled neurons hampers any attempt to determine whether *Hoxb1* expression is required for specification of a subset of neurons in A1 or A2.

Intersectional labeling of noradrenergic neurons in *b1r4-cre;DbhFlpo;RC::FrePe* mice has revealed the existence of another genetically defined subpopulation of noradrenergic neurons restricted to the pons. This population—defined by activity of a rhombomere 4-specific enhancer element in the context of a transgenic construct—has a unique axonal projection pattern, which hints that it may be functionally distinct. We have previously shown by retrograde labeling that axons from *Hoxb1*-derived noradrenergic neurons in the pons and medulla share some target sites (Robertson et al., 2013), but the near absence of *b1r4-cre*-derived axons in BNST and basolateral amygdala suggest that those regions receive many of their noradrenergic inputs from *Hoxb1*-derived neurons in the medulla.

Current genetic tools do not permit us to determine the degree of overlap between the *Hoxb1*-dependent noradrenergic subpopulation and *b1r4-cre*-derived subpopulation, but it may be significant that both of these newly defined noradrenergic subpopulations are restricted to the pons. Our lab is currently developing new indicator alleles that incorporate a third recombinase system, Dre/rox (Sauer and McDermott, 2004), and offer the ability to label axonal projections from both cre+ and cre-negative cells (unpublished observations). These new tools may make it possible in the future to label the *b1r4-cre*-derived subpopulation on a *Hoxb1*-null background and to specifically label fibers from the

subpopulation of *Hoxb1*-derived neurons that are not labeled by *b1r4-cre*. Future studies should also apply these new intersectional tools to explore noradrenergic efferents in regions beyond the forebrain such as the brainstem and spinal cord. Together, these analyses will shed new light on the *Hoxb1*-dependent noradrenergic neurons in the pons and the overall complexity of the noradrenergic system.

4. Experimental Procedure

4.1 Animal Care and Breeding

All animal procedures and breeding were carried out in accordance with the NIEHS Institutional Animal Care and Use Committee. Mice were maintained on a 12-hour light/dark cycle and given water and food *ad libitum*. *Hoxb1^{cre}* (O'Gorman, 2005; Rottkamp et al., 2008), *Dbh^{Flpo}* (Robertson et al., 2013), *RC::FrePe* (Bang et al., 2012; Engleka et al., 2012), and *b1r4-cre* (Di Bonito et al., 2013) colonies were maintained on a C57BL/6J background. Triple transgenic *Hoxb1^{cre};Dbh^{Flpo};RC::FrePe* animals were generated by crossing *Hoxb1^{cre/+}* to *Dbh^{Flpo/+};RC::FrePe*. Offspring were intercrossed to generate *Hoxb1*-null (*Hoxb1^{cre/cre};Dbh^{Flpo/+};RC::FrePe*), and heterozygous (*Hoxb1^{cre/+};Dbh^{Flpo/+};RC::FrePe*) mice. Triple transgenic *b1r4-cre;Dbh^{Flpo};RC::FrePe* animals were generated by crossing *b1r4-cre* to *Dbh^{Flpo/+};RC::FrePe*. *Hoxb1^{cre/cre}* and *Hoxb1^{cre/+}* mice were genotyped using primers 5' ATGGCCTGGGAGAGATCA (*Hoxb1*, forward), 5'-CCCGGTTACAAAGTGGGTACT (*Hoxb1*, reverse), and 5'-ATGTTTAGCTGGCCCAAATG (Cre, reverse) (<http://jaxmice.jax.org/strain/012373.html>). All other genotyping was performed as previously described (Robertson et al., 2013).

4.2 Immunohistochemistry

Adult *Hoxb1*-null (*Hoxb1^{cre/cre};Dbh^{Flpo/+};RC::FrePe*), heterozygous (*Hoxb1^{cre/+};Dbh^{Flpo/+};RC::FrePe*), *b1r4-cre;Dbh^{Flpo};RC::FrePe* and control mice were anesthetized with phenobarbital and transcardially perfused with 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were postfixed overnight in 4% PFA at 4 °C and then rinsed in PBS. Following equilibration in 30% sucrose in PBS brains were embedded and frozen in tissue-freezing medium (Triangle Biomedical Sciences). Brains were stored at -80°C until sectioned.

To label noradrenergic cell bodies, free-floating 40 µm coronal cryosections were incubated with chicken anti-GFP (1:10,000; ab13970 Abcam) and Rabbit anti-dsRed (1:1000; 632496 Clontech), or chicken anti-GFP, Rabbit anti-dsRed, and Mouse anti-tyrosine hydroxylase (1:500; GTX10372 GeneTex), followed by detection with Alexa 488 conjugated goat anti-chicken, Alexa 568 conjugated anti-rabbit, and Alexa 647 anti-mouse secondary antibodies (1:500; A11039, A11036, A21236 Life Technologies) as previously described (Robertson et al., 2013).

Immunoperoxidase labeling was used to achieve the highest-sensitivity detection of eGFP in axons. 40-µm free-floating coronal sections were incubated with chicken anti-GFP (1:10,000; ab13970 Abcam) followed by incubation with a biotinylated goat anti-chicken secondary (1:500, BA9010), Vectastain Elite ABC kit and DAB (all Vector Laboratories).

We identified eGFP positive projections throughout various brain regions according to an adult mouse brain atlas (Paxinos and Franklin, 2004) and collected representative images on a light microscope (Zeiss Axio Imager.Z2).

4.3 Cell Counts and Statistical Analysis

To quantify eGFP⁺ and mCherry⁺ noradrenergic neuron number in adult *Hoxb1*-null (*Hoxb1^{cre/cre};Dbh^{Flpo/+};RC::FrePe*), heterozygous (*Hoxb1^{cre/+};Dbh^{Flpo/+};RC::FrePe*), and *b1r4-cre;Dbh^{Flpo};RC::FrePe* mice, the anatomically defined nuclei (SubCD, SubCV, A5, A2 and A1) were counted as previously described (Jin et al., 2004; Robertson et al., 2013). Cell numbers are the sum of bilateral counts from 40 μ m sections spaced 120 μ m apart from the brainstem of a minimum of six mice for each strain of animals and a minimum of n=3 for each gender. We manually counted cells for each animal and anatomically matched the sections to an adult mouse brain atlas (Paxinos and Franklin, 2004). Data are expressed as the mean $\pm$ standard error (s.e.m.) and for each experiment all genotypes were represented. We compared noradrenergic neuron cell counts for each nucleus between *Hoxb1*-null (*Hoxb1^{cre/cre};Dbh^{Flpo/+};RC::FrePe*) and heterozygous (*Hoxb1^{cre/+};Dbh^{Flpo/+};RC::FrePe*) controls using unpaired, two-tailed *t* tests. Statistical tests were performed using GraphPad Prism (GraphPad Software). The exact animal numbers, gender distributions and the statistical analyses are also indicated in the figure legends. Data collection and analysis were not preformed blind to experimental conditions, because the noradrenergic neuron phenotype in *Hoxb1*-null mice is distinct enough for investigators to tell the genotype of the animals by observation of the tissue section.

4.4 Total projection pixel intensity quantification

We performed analysis of projection pixel intensity on sections labeled for eGFP by the immunoperoxidase methods described above. We photographed multiple fields from the region of interest (PV thalamus, BNST STMV, and BLP amygdala) at 20 $\times$ magnification. For quantification, we visualized the region of interest as a maximal projection of a z-stack using a Zeiss Axio Imager.Z2 light microscope (7 images for BLP amygdala, 5 images for BNST STMV, and 6 images for PV thalamus from three *Hoxb1*-null and control animals). Using NIH ImageJ software a manual threshold function was applied to identify and isolate projections with the eGFP label. Following isolation of the projections, the total area above threshold and the average pixel intensity was measured. The total projection pixel intensity was calculated by multiplying total area times the average pixel intensity. We statistically analyzed the total projection pixel intensity by an unpaired *t*-test using GraphPad Prism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Intersectional genetic fate mapping reveals noradrenergic neuron diversity
- *Hoxb1* is required for a subset of pontine noradrenergic neurons
- A subset of pontine *Hoxb1*-derived noradrenergic neurons are defined by *b1r4-cre*
- *b1r4-cre+* pontine noradrenergic neurons have a distinct efferent projection pattern

Required alleles

Potential outcomes

Norepinephrine projections from *En1*-, *Hoxa2*-, *Krox20*- and *Hoxb1*-derived norepinephrine neurons

Figure 1. Intersectional labeling reveals genetically defined subpopulations of noradrenergic neurons with distinct efferent projection patterns

(Top) Visualization of noradrenergic neurons derived from a specific rhombomere in the embryonic hindbrain requires a rhombomere-specific Cre driver, noradrenergic specific Flp driver, and dual-recombinase responsive fluorescent indicator allele, RC::FrePe. In mice that inherit all three alleles, noradrenergic neurons derived from the Cre expression domain will be labeled with eGFP. All other noradrenergic neurons will be labeled with mCherry.

(Middle) Four Cre driver lines expressed in the embryonic hindbrain (left, schematic sagittal view) define subpopulations of noradrenergic neurons that are distributed across the

anatomically defined noradrenergic nuclei in the adult brainstem (right). The locus coeruleus (LoC), subcoeruleus dorsal (SubCD) and ventral (SubCV), A7, A5, C2/A2 and C1/A1 are shown with color-coded contribution from each genetically defined subpopulation represented as colored circles on a schematic sagittal view of the adult brainstem compressed along the mediolateral axis. (Bottom) Prominent axonal projections from the four genetically defined noradrenergic subpopulations are illustrated as color-coded lines. The thickness of the open circle around each target region denotes the density of innervation. Olf bulb, olfactory bulb; Ctx ins, insular cortex; Ctx somato, somatosensory cortex; Hippo, hippocampus; BNST, bed nucleus of stria terminalis; Amyg, amygdala; Hippo, hippocampus; Thal, thalamus; Hyp, hypothalamus; PBN, parabrachial nucleus; Cereb, cerebellum; NTS, solitary nucleus. Adapted from Robertson et al., 2013.

Figure 2. Hoxb1 expression is required for the development of a subset of Hoxb1-derived noradrenergic neurons in the pontine SubC and A5 nuclei

(TOP) Coronal sections from adult mouse brainstem reveal the significant absence of *Hoxb1*-derived noradrenergic neurons in the SubC and A5 nuclei of *Hoxb1*-null animals (Null-*Hoxb1*^{cre/cre}; *Dbh*^{Flpo}; *RC::FrePe*) compared to control animals (CTR-*Hoxb1*^{cre/+}; *Dbh*^{Flpo}; *RC::FrePe*). *Hoxb1*-derived noradrenergic neurons are labeled with eGFP (green) and all other noradrenergic neurons are labeled with mCherry (red) in representative sections corresponding to the labeled areas in the schematic (top right). (Bottom) Counts of labeled neurons confirm a significant decrease in the number of eGFP-labeled neurons (***P*<0.01) and total noradrenergic neurons (**P*<0.05) in A5 of *Hoxb1*-null mice (top right graph). No significant change in mCherry-labeled A5 neurons was observed. In the SubCD of *Hoxb1*-null animals, a significant decrease in eGFP-labeled neurons (**P*<0.05), but no significant difference in mCherry or total noradrenergic neurons was observed (bottom left graph). In the SubCV of *Hoxb1* null animals, significant decreases in eGFP-labeled and total noradrenergic neurons were observed(****P*<0.001) (bottom right graph). Numbers are the sum of bilateral counts from 40-μm sections spaced 120 μm apart from the brainstems of seven mice for each fate map (n=7 with minimum of n=3 for each gender; error bars are mean ± s.e.m.). Scale bar represents 119 μm in all images.

Figure 3. *Hoxb1* expression is not required for the development of medullary *Hoxb1*-derived noradrenergic neurons

(Top) Coronal sections from adult mouse brainstem reveal an increase in *Hoxb1*-derived norepinephrine neurons in C1/A1 and C2/A2 nuclei of *Hoxb1*-null animals (Null-*Hoxb1*^{cre/cre}; *Dbh*^{Flpo}; *RC::FrePe*) compared to control animals (CTR-*Hoxb1*^{cre/+}; *Dbh*^{Flpo}; *RC::FrePe*). *Hoxb1*-derived noradrenergic neurons are labeled with eGFP and all other noradrenergic neurons are labeled with mCherry (red) in representative sections corresponding to the boxed areas in the schematic (left). Scale bar represents 100 μ m (C2/A2) or 75 μ m (C1/A1). (Bottom) Counts of labeled neurons confirm a significant increase of eGFP-labeled neurons (* $P < 0.05$) but no significant change in numbers of mCherry-labeled or total noradrenergic neurons in C2/A2 (left graph). In C1/A1, a significant increase of eGFP-labeled neurons (*** $P < 0.001$) and total noradrenergic neurons was observed (right graph). Numbers are the sum of bilateral counts from 40- μ m sections spaced 120 μ m apart from the brainstems of six mice for each fate map (n=6 with n=3 for each gender; error bars are mean $\pm$ s.e.m.) For the total count of noradrenergic neurons (grey bars), the number of neurons co-expressing mCherry and eGFP was subtracted from the GFP population to prevent double counting.

Figure 4. In *Hoxb1*-null mice, the number of noradrenergic axons is increased at target sites of the *Hoxb1*-derived noradrenergic subpopulation
 In *Hoxb1*-null (Null-*Hoxb1*^{cre/cre}; *Dbh*^{Ffpo}; *RC::FrePe*) and control (CTR-*Hoxb1*^{cre/+}; *Dbh*^{Ffpo}; *RC::FrePe*) mice, axons of *Hoxb1*-derived (eGFP-labeled) noradrenergic neurons are revealed by immunoperoxidase staining of representative sections from target sites of noradrenergic projections. Relative to controls, staining is heavier in *Hoxb1*-null sections indicating an increase in the number of labeled axons in the basolateral amygdala posterior part (BLP) and the bed nucleus of the stria terminalis (BNST) medial division ventral part (STMV) but not in the paraventricular (PV) thalamic nucleus. Grey boxes on coronal schematic diagrams indicate the approximate position of the imaged axons. Scale bar represents 136 μ m (Thalamus and BNST) or 47 μ m (Amygdala). The total projection pixel intensities (arbitrary intensity units) of eGFP-positive axon fibers from three *Hoxb1*-null and control mice are shown (BLP n=7, BNST STMV n=5, and PV n=6 images; error bars are mean $\pm$ s.e.m.). A two-tailed, unpaired *t*-test was used to determine significance. * $P = 0.01$ $t = 2.896$ df = 17 (BLP), *** $P = 0.0002$ $t = 5.111$ df = 13 (BNST STMV), Not significantly different $P = 0.389$ $t = 0.8878$ df = 15 (PV).

Figure 5. *b1r4-cre* expression defines a subpopulation of noradrenergic neurons in the pons

Coronal sections of brain from *b1r4-cre;Dbh^{Flpo};RC::FrePe* mice demonstrate that *b1r4-cre*-derived noradrenergic neurons (*b1r4-cre* and *Dbh^{Flpo}* expression; eGFP-labeled) populate the SubC dorsal, SubC ventral, and A5 noradrenergic nuclei in the pons. No *b1r4-cre*-derived noradrenergic neurons are observed in the pontine A7 nucleus and medullary C2/A2 and C1/A1 nuclei. Cell counts indicate that *b1r4-cre*-derived neurons constitute a smaller percentage of SubC and A5 neurons than those labeled by *Hoxb1^{cre}*. The number of labeled neurons in each anatomically defined noradrenergic nucleus (mean ± s.e.m.) is the sum of bilateral counts from 40-μm sections spaced 120 μm apart in the brainstems of seven mice (3 males and 4 females). Scale bar represents 128 μm (A7), 119 μm (LoC, SubC, C2/A2, C1/A1), or 77 μm (A5)

Figure 6. *b1r4-cre* defines a subpopulation of noradrenergic neurons with a distinct efferent projection pattern

At representative target sites, axons of eGFP-labeled *Hoxb1*-derived and *b1r4-cre*-derived noradrenergic neurons are revealed by immunoperoxidase staining. Projections from *Hoxb1*-derived noradrenergic subpopulation can be observed in insular cortex (Ctx), paraventricular (PV) thalamic nucleus, basolateral amygdala posterior part (BLP), and the BNST medial division ventral part (STMV). In contrast, projections from *b1r4-cre*-derived neurons are almost completely absent from the Amygdala and very sparse in the BNST. The representative images reflect observations from a minimum of five animals of each genotype. Grey boxes on coronal schematic diagrams indicate the approximate position of the imaged axons. Scale bar represents 136 μm (Thalamus and BNST), 56 μm (Ctx), or 47 μm (Amygdala).

Table 1Comparison of the efferent projection patterns from *Hoxb1*- and *b1r4*-cre-derived noradrenergic neurons

Brain region	<i>Hoxb1^{cre}</i>	<i>b1r4-cre</i>
Cerebral Cortex		
Insular Ctx	++	+
Orbital Ctx	+	+/-
Olfactory Bulb	+	+/-
Septum		
Lateral Septal Nucleus	+	+
Medial Septal Nucleus	+	+
Bed nucleus of the stria terminalis		
BNST, dorsal medial	++	+/-
BNST, dorsal lateral	+++	+/-
BNST, ventral medial	++++	+
BNST, ventral lateral	++++	+
Hypothalamus		
Paraventricular hypothalamic nucleus	++++	+
Dorsomedial hypothalamic nucleus	++++	++
Arcuate nucleus	+	+
Lateral hypothalamic area	+++	+
Amygdala		
Central Amygdaloid nucleus	+++	+/-
Basolateral amygdaloid nucleus	++++	+/-
Thalamus		
Central medial thalamic nucleus	+++	++
Paraventricular thalamic nucleus	+++	+++
Brainstem		
Dorsal raphe nucleus	++++	++
Median raphe nucleus	+	+/-
Locus Coeruleus	++++	+++
Parabrachial Nucleus	++++	+++
Parvicellular reticular nucleus	+++	++
Intermediate reticular nucleus	++++	++
Solitary nucleus	++++	++
Cerebellum	+	+

The relative density of noradrenergic innervation from *Hoxb1*-derived and *b1r4-cre*-derived neurons across various regions of the brain is shown from coronal sections immunostained for eGFP. The density of eGFP immunolabeling is graded as one of the following: undetectable in all animals (-), presence of rare fiber and not seen in all animals (+/-), sparse but consistent fibers in all animals (+), moderate presence with consistent fibers observed in all animals in multiple sections (++), strong innervation (+++), dense and heavy innervation (++++).