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Mutation in $LBX1/Lbx1$ precludes transcription factor cooperativity and causes congenital hypoventilation in humans and mice

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The respiratory rhythm is generated by the preBötzinger complex in the medulla oblongata, and is modulated by neurons in the retrotrapezoid nucleus (RTN), which are essential for achieving respiration in response to high CO$_2$. Here we identify a $LBX1$ frameshift ($LBX1$) mutation in patients with congenital central hypoventilation. The mutation alters the C-terminus but not the DNA-binding domain of $LBX1$. Mice with the analogous mutation recapitulate the breathing deficits found in humans. Furthermore, the mutation only interferes with a small subset of Lbx1 functions, and in particular with development of RTN neurons that coexpress Lbx1 and Phox2b. Genome-wide analyses in a cell culture model show that Lbx1$^{15}$ and wild-type Lbx1 proteins are mostly bound to similar sites, but that Lbx1$^{15}$ is unable to cooperate with Phox2b. Thus, our analyses on Lbx1$^{15}$ (dys)function reveals an unusual pathomechanism; that is, a mutation that selectively interferes with the ability of Lbx1 to cooperate with Phox2b, and thus impairs the development of a small subpopulation of neurons essential for respiratory control.

Significance

Maintaining low CO$_2$ levels in our bodies is critical for life and depends on neurons that generate the respiratory rhythm and monitor tissue gas levels. Inadequate response to increasing levels of CO$_2$ is common in congenital hypoventilation diseases. Here, we identified a mutation in $LBX1$, a homeodomain transcription factor, that causes congenital hypoventilation in humans. The mutation alters the C terminus of the protein without disturbing its DNA-binding domain. Mouse models carrying an analogous mutation recapitulate the disease. The mutation spares most Lbx1 functions, but selectively affects development of a small group of neurons central in respiration. Our work reveals a very unusual pathomechanism, a mutation that hampers a small subset of functions carried out by a transcription factor.


The authors declare no conflict of interest.

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protein without affecting its homeodomain. Homozygous mice carrying the analogous mutation (Lbx1<sup>FS/FS</sup>) displayed respiratory deficits that recapitulated the human phenotype. In Lbx1<sup>FS/FS</sup> mice, two Lbx1<sup>1/Phox2b</sup> neuronal subpopulations (in the RTN and the dorsal hindbrain) were severely affected, but in contrast to Lbx1 null mutants, second-order somatosensory neurons and limb skeletal muscle formed correctly. Genomewide DNA binding analysis of Lbx1<sup>FS</sup> in a cell culture model showed that the mutant variant mostly binds to similar sites as the wild-type protein. However, in contrast to the wild-type protein, Lbx1<sup>FS</sup> is unable to correctly cooperate with Phox2b, and instead overrides its function. Thus, the Lbx1<sup>FS</sup> protein is selectively impaired in a transcriptional cooperativity with Phox2b during neuronal development, but functions correctly in other contexts.

**Results**

A Homozygous LBX1 Frameshift Mutation Causes Recessive Congenital Central Hypoventilation Syndrome. We identified two male siblings, offspring from a consanguineous marriage, who displayed hypoventilation during the neonatal period. The parents were unaffected and had a healthy daughter (Fig. 1A). Two sisters from the father/mother side of the patients lost a child to cot death (SI Appendix, Fig. S1A). Both affected siblings studied here required continuous mechanical ventilation after birth because of respiratory insufficiency. They showed recurrent episodes of apnea and signs of central hypoventilation during sleep with no response to falling oxygen saturation or hypercapnia. The children were diagnosed with a severe pattern of classic CCHS. Sanger sequencing, microsatellite analysis, and multiplex ligation-dependent probe amplification of DNA from the children did not reveal any mutations in PHOX2B. Lbx1 ablation causes hypoventilation in newborn mice (6, 7). We therefore sequenced LBX1 in the affected individuals and identified a homoyzous frameshift mutation in its exon 2 (L BX1<sup>FS</sup> mutation; SI Appendix, Fig. S1 B and C). Sanger sequencing of the entire family confirmed that the L BX1<sup>FS</sup> mutation segregated with the phenotype. The mutation was predicted to alter the LBX1 protein at C terminus without affecting its homeodomain (SI Appendix, Fig. S1C). Furthermore, the L BX1<sup>FS</sup> variant was absent in control cohorts such as Exome Aggregation Consortium and 1000 Genomes.

Ablation of Lbx1 in mice results in a complex phenotype resulting from defects in the development of various hindbrain neuronal subtypes (db1–d84; see scheme in Fig. 1B), as well as deficits in the formation of dorsal spinal cord neurons and limb skeletal muscle (4–10, 23). However, the children carrying the L BX1<sup>FS</sup> mutation did not show any obvious change in limb musculature. We thus reasoned that the L BX1<sup>FS</sup> mutation might selectively impair neurons that participate in the central control of respiration.

**Similarities in the Genome-Wide Binding of Lbx1 and Lbx1<sup>FS</sup>.** To model alterations of Lbx1<sup>FS</sup> function and its binding to DNA in a neuronal context, we looked for a suitable neuronal cell line to use in this system. P19 murine embryonic teratocarcinoma stem cells differentiate into neurons that express Lbx1 and a HoxA gene code typical of the caudal hindbrain (rhombomeres 4–7) and anterior cervical spinal cord upon retinoic acid treatment (SI Appendix, Fig. S1 D and E) (24). In addition, they express Lmx1b, Pou4f1, and Prx1I, the latter at low levels (SI Appendix, Fig. S1F); this combination is indicative of excitatory somatosensory neurons of the spinal cord and hindbrain. We used this model to analyze Lbx1 and Lbx1<sup>FS</sup> binding on a genome-wide scale. The endogenous Lbx1 locus was first mutated in these cells using CRISPR-Cas9, and the resulting Lbx1 mutant cells were transduced with retroviruses encoding flag-tagged Lbx1 or Lbx1<sup>FS</sup> (referred to as Lbx1 and Lbx1<sup>FS</sup> cells). Cell clones that expressed comparable levels of Lbx1/Lbx1<sup>FS</sup> were chosen for ChIP-seq analysis. In neurons differentiated from such cells (named Lbx1 and Lbx1<sup>FS</sup> neurons), we identified 7,537 binding sites for Lbx1, but considerably more (n = 12,343) sites for Lbx1<sup>FS</sup>. A large fraction (59%) of the Lbx1 sites was also bound by Lbx1<sup>FS</sup> (SI Appendix, Fig. S1G). To analyze how the 1.6-fold increase in Lbx1<sup>FS</sup> binding sites related to binding strength, another important variable for transcription factor function, we performed read enrichment analysis combined with k-means clustering for Lbx1- and Lbx1<sup>FS</sup>-bound sites. In general, the mean read density for Lbx1<sup>FS</sup> was lower than for Lbx1 (Fig. 1C). Sites in which Lbx1<sup>FS</sup> bound more strongly than Lbx1 displayed, on average, low enrichment for both Lbx1<sup>FS</sup> and Lbx1 proteins (Fig. 1C). Together, our data show that the Lbx1<sup>FS</sup> mutant protein can bind to most Lbx1 sites; however, the binding is weaker and less specific than that of the wild-type protein.

Inspection of Lbx1 and Lbx1<sup>FS</sup> ChIP-seq tracks revealed occupancy of both factors on intronic and intergenic regions associated with the somatosensory genes Prdx1, Lmx1b, and Pou4f1 (Fig. 1D and SI Appendix, Fig. S2A). The occupancy of Lbx1/Lbx1<sup>FS</sup> on such loci was confirmed by ChIP-qPCR (Fig. 1E and SI Appendix, Fig. S2B). Similar gene expression levels for the three somatosensory genes were observed in neurons differentiated from Lbx1 and Lbx1<sup>FS</sup> cells, but they were not expressed in Lbx1 mutant neurons (SI Appendix, Fig. S2C). To test whether these intronic and intergenic regions correspond to enhancer elements, we performed ChIP-qPCR for H3K27ac and H3K27me3, two epigenetic marks associated with active or repressed enhancers, respectively (25). This showed strong enrichment for H3K27ac at the analyzed loci in neurons differentiated from Lbx1 and Lbx1<sup>FS</sup> cells, whereas H3K27me3 was not enriched (Fig. 1F and SI Appendix, Fig. S2D). Hence, Lbx1/Lbx1<sup>FS</sup> binding sites on the Lmx1b,

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**Fig. 1.** Genome-wide characterization of chromatin binding of a frameshift mutant LBX1 associated with CCHS in humans. (A) Pedigree of a family with two children diagnosed with CCHS (black). (B, Left) Transverse section of the developing brainstem stained with Olig3 (red) and Lbx1 (green) antibodies at E11.5. Olig3 is expressed in the dorsal brainstem, whereas Lbx1 is expressed in d81–d84 neurons. (B, Right) Scheme showing genes expressed in progenitor cells and neurons of the dorsal hindbrain. Lbx1 and Phox2b are coexpressed in d82 neurons. (C, Left) Heat maps showing read tracks at sites occupied by Lbx1 and Lbx1<sup>FS</sup> ± 5 kb around the binding sites. (C, Right) Lbx1 (blue) and Lbx1<sup>FS</sup> (pink) mean read densities (MRDs) for distinct classes of binding sites. (D) ChIP-seq tracks illustrating Lbx1 and Lbx1<sup>FS</sup> occupancy on intergenic and intronic regions of Prdx1. (E) ChIP-qPCR analysis using antibodies against flag-tag to validate Lbx1 and Lbx1<sup>FS</sup> occupancy on the highlighted regions displayed in D (n = 4 independent replicates). (F) H3K27ac (Left) and H3K27me3 (Right) ChIP-qPCR analysis performed on chromatin prepared from Lbx1 and Lbx1<sup>FS</sup> differentiated neurons (n = 4 independent replicates).
**Fig. 2.** The Lbx1\(^{FS}\) mutation causes central hypoventilation and loss of CO\(_2\) sensitivity in mice. (A) Plethysmographic traces of control and Lbx1\(^{FS}\) mice in normal air (0.04% CO\(_2\); Top traces) and high CO\(_2\)-containing air (hypercapnia, 8% CO\(_2\); Bottom traces). Numbers on the left of the traces indicate distinct individuals. (B) Quantification of V\(_{ei}\) of control (n = 9) and Lbx1\(^{FS}\) (n = 10) newborn mice in normal air and high CO\(_2\)-containing air (unpaired nonparametric Mann–Whitney U test). (C, Left and Middle) Histological analysis of RTN neurons (arrowheads) using Lbx1 (green) and Phox2b (red) antibodies; these neurons are present in control mice but not in Lbx1\(^{FS}\) mice at birth. Antibodies against ChAT (blue) were used to distinguish RTN (Lbx1\(^{−}\)/Phox2b\(^{+}\)/ChAT\(^{−}\)) neurons from facial (nVII) motor (Lbx1\(^{−}\)/Phox2b\(^{−}\)/ChAT\(^{+}\)) neurons. Confocal tissue scan modus was used to acquire photomicrographs and assembled using ZEN2012 software (10% overlap between tiles). (C, Right) Quantification of RTN neuron numbers in control and Lbx1\(^{FS}\) mice at E14.5 (n = 4 per condition; unpaired t test; t = 19.37; df = 6) and at birth (n = 4 per condition; unpaired t test; t = 26.08; df = 6). ***P < 0.0001.

The **Lbx1\(^{FS}\)** Mutation Does Not Preclude SpV and Limb Muscle Development. Next we analyzed inhibitory and excitatory somatosensory neurons of the spinal trigeminal (SpV) nucleus. These neurons are absent in Lbx1\(^{−}\)null mutant mice, where they instead assumed solitary tract nucleus and inferior olivary nucleus neuronal fates, respectively (7). Interestingly, the SpV was present in Lbx1\(^{1FS}\) mice (Fig. 3A). Furthermore, the solitary tract and inferior olivary nuclei appeared to have a normal size (Fig. 3A). Finally, limb muscle development is severely affected in Lbx1\(^{−}\)null mutant mice (8–10), but these muscle groups were present and appeared correctly formed in Lbx1\(^{1FS}\) mice (Fig. 3B). Together, our analyses demonstrate that the Lbx1\(^{FS}\) mutation selectively interferes with development of Lbx1\(^{+/+}\)/Phox2b\(^{+}\) RTN neurons, but in other contexts, the mutant protein functions correctly, as in development of somatosensory SpV neurons and limb muscles.

**dB2 Neurons Are Responsible for the Breathing Deficits Observed in Lbx1\(^{FS}\) Mice.** To assess whether the breathing deficits observed in Lbx1\(^{1FS}\) mice exclusively depend on dysfunction of dB2 derivatives, we conditionally restricted the Lbx1\(^{FS}\) mutation to the dB2 lineage by using Phox2b\(^{cre}\) (Phox2b\(^{cre}\);Lbx1\(^{FS}\lox\lox\), named dB2-Lbx1\(^{FS}\) mice; see SI Appendix, Fig. S6A for a scheme of the strategy). In such animals, neurons with a history of Phox2b expression carried an Lbx1\(^{FS}\) genotype, but other cells (Lbx1\(^{FS}\lox\)) retained one copy of a fully functional Lbx1\(^{lox}\) allele (SI Appendix, Fig. S4B). In dB2-Lbx1\(^{FS}\) animals, RTN neurons were absent (Fig. 4A). Plethysmographic recordings of dB2-Lbx1\(^{FS}\) animals showed a full recapitulation of the physiological phenotype observed in Lbx1\(^{1FS}\) animals (i.e., severe hypoventilation, lack of the hypercapnic reflex, frequent apneas; SI Appendix, Fig. S6 C–G;
summarized in Fig. 4B), as well as lethality (n = 12/12) within the first 2 h of life. Thus, all respiratory deficits associated with the Lbx1FS mutation are the result of a selective developmental deficit in the dB2 neuronal lineage.

RTN neurons arise from rhombomere 5 (29). We next restricted the Lbx1FS mutation to rhombomeres 3 and 5, using Egr2+ mice that only recombines in these rhombomeres (Egr2Cre;Lbx1Flox mice; see SI Appendix, Fig. S6B for a scheme of the strategy). As expected, RTN neurons were absent in Egr2-Lbx1FS animals (Fig. 4A). Plasmographic recordings of Egr2-Lbx1FS mice showed that they were unable to respond to high CO2 levels in the air (SI Appendix, Fig. S6 C–G). Nevertheless, Egr2-Lbx1FS mice did not display apneas and survived the postnatal period (n = 11/11), with a mild hypventilation that was observed in their early postnatal life (SI Appendix, Fig. S6 D and H). The response of Egr2-Lbx1FS mice to high levels of CO2 improved with maturation, but even adult mutants presented a blunted hypercapnic reflex (SI Appendix, Fig. S6H). This phenotype, largely similar to the one observed after conditional mutation of Phox2b in rhombomeres 3 and 5 (22), implies that several neuronal groups originating from dB2 precursors participate in the control of breathing.

We next used intersectional lineage tracing to specifically label dB2 derivatives with Tomato fluorescent protein, using Lmx1bCre; Phox2bFpox/;Ai65 mice (see SI Appendix, Fig. S7A for a scheme of the strategy). Tomato+/Lmx1b+/Phox2b+ cells were found, in addition to the RTN, around the trigeminal motor nucleus in rhombomeres 1 and 2 (a population known as periV neurons; see SI Appendix, Fig. S6B) and the trigeminal nucleus, respectively. NTS neurons express Lmx1b, and inferior olivary neurons express Foxp2 (blue). (B) Histological analysis of limb muscles in control, Lbx1+/+, and Lbx1FS newborn mice, using antibodies against laminin (Lam, red) and desmin (green). Confocal tile scan modus was used to acquire photomicrographs, and assembled using ZEN2012 software (10% overlap between tiles). Photomicrographs were mounted on a black frame to maintain figure panel proportions.

Ectopic Expression of Somatosensory Genes in Lbx1FS/Phox2b Expressing Neurons. To assess whether the absent dB2 neurons in Lbx1FS mice assumed an aberrant neuronal fate, we extended our intersectional genetic lineage tracing to Lbx1FS (Lbx1Cre;Phox2bFpox/Ai65Flox, see SI Appendix, Fig. S7A) mutant mice. This demonstrated that ectopic Tomato+ cells appeared in the somatosensory SpV nucleus of Lbx1FS mice, which were not observable in control animals (Fig. 5A and SI Appendix, Fig. S8A). These ectopic Tomato+ cells coexpressed markers of excitatory somatosensory neurons such as Prx1 or Lmx1b (Fig. 5A and B, SI Appendix, Fig. S8B). Thus, the Lbx1FS mutation selectively affects the development of an Lbx1+/Phox2b+ dB2 subpopulation that adopts an aberrant somatosensory fate.

We next modeled the (dys)function of Lbx1FS in Phox2b+ neurons, using our cell culture model. For this, Lbx1 mutant P19 mice were transduced with retroviruses encoding a HA-tagged version of Phox2b (hereafter Phox2b cells) alone or in combination with flag-tagged Lbx1 or Lbx1FS (Lbx1/Phox2b and Lbx1FS/Phox2b cells). We then sequenced the transcriptomes of neurons differentiated from these cells. Hierarchical expression clustering showed that Phox2b, Lbx1/Phox2b, and Lbx1FS/Phox2b neurons were clearly distinct from Lbx1 neurons and clustered separately (Fig. 5C). Nevertheless, Lbx1 and Lbx1FS/Phox2b neurons were more closely related to each other than to Lbx1/Phox2b or Phox2b neurons (Fig. 5C). Interestingly, the Prx1, Lmx1b, and Pou4f1 somatosensory genes were among the
most significant and differentially expressed genes in \( Lbx1^{FS} \)/\( Phox2b^{+} \) neurons compared with \( Phox2b \) or \( Lbx1/Phox2b^{+} \) neurons (Fig. 5D). Thus, \( Phox2b \) represses these somatosensory genes alone or even when \( Lbx1 \) is present, but this does not occur when \( Lbx1^{FS} \) and \( Phox2b \) are coexpressed, a change reminiscent of the one observed in vivo where \( Lbx1^{FS}/Phox2b^{+} \) (dB2) neurons assumed an aberrant somatosensory fate.

Next we analyzed chromatin modifications of the previously characterized enhancers of \( Prx1l1, Lmx1b, \) and \( Pou4f1 \) somatosensory genes. In \( Lbx1/Phox2b \) neurons, ChIP-qPCR showed a modest enrichment of \( Lbx1 \) and \( Phox2b \) at the analyzed loci (Fig. 5E and SI Appendix, Fig. S9). Moreover, the chromatin mark \( H3K27me3 \) was enriched in those sites, demonstrating that the enhancers are repressed. However, when the chromatin of \( Lbx1^{FS}/Phox2b \) neurons was used for ChIP-qPCR experiments, \( Lbx1 \), \( Phox2b \), and \( H3K27ac \) were significantly enriched at the \( Prx1l1, Lmx1b, \) and \( Pou4f1 \) enhancers (Fig. 5E and SI Appendix, Fig. S9). Thus, enhancer sequences of the \( Prx1l1, Lmx1b, \) and \( Pou4f1 \) genes are activated when \( Lbx1^{FS} \) and \( Phox2b \) are recruited to these sites, but repressed when \( Lbx1 \) and \( Phox2b \) are recruited.

**Discussion**

Respiratory disorders in humans range from irregular and unsteady respiration to the complete loss of breathing control. The most common causes of congenital hypventilation are dominant mutations in \( PHOX2B \) that affect the formation of the RTN. Here we show that a homozygous frameshift mutation in \( LBX1 \) causes severe congenital hypventilation that resembles classical CCHS. We used cell culture and mouse models to investigate the (dys)function caused by the frameshift mutation, which alters the C-terminal sequence of the protein but spares its homeodomain. In most developmental contexts, the mutant protein exerts its role correctly; that is, the mutation only interferes with small subsets of \( Lbx1 \) functions. Our analysis has thus revealed a very unusual pathomechanism of a transcription factor mutation that results in a severe respiratory disorder.

**\( Lbx1^{FS} \) Protein Correctly Functions in Most Developmental Contexts.**

Our cell culture modeling of \( Lbx1^{FS} \) binding showed that \( Lbx1^{FS} \) and \( Lbx1 \) largely bind to similar sites genome-wide, which is in agreement with conserved functionality of the \( Lbx1^{FS} \) protein in most developmental contexts. Motif analyses revealed subtle differences between the binding preferences of \( Lbx1 \) and \( Lbx1^{FS} \). In particular, a specific motif was present in \( Lbx1 \), but not in \( Lbx1^{FS} \) binding sites, which consists of a 16-bp-long nonpalindromic sequence that is composed of an \( Lbx1 \)-monodimer site combined with a half-site of another factor. Interestingly, the sequence that represents the second half-site corresponds to the preferred binding motif previously identified for \( Phox2b \) (27). This observation suggested a failure of \( Lbx1^{FS} \) to cooperate productively with \( Phox2b \).

\( Lbx1 \) and \( Phox2b \) are known to functionally repress each other: When \( Lbx1 \) is mutated, supernumerary \( Phox2b \) viscerosensory neurons arise (7). Vice versa, mutation of \( Phox2b \) results in the appearance of supernumerary somatosensory \( Lbx1 \) neurons (30). Remarkably, development of the dB2 lineage depends on both \( Lbx1 \) and \( Phox2b \) and relies on the repression of
the somatosensory genes (7, 13). Lbx1-dependent differentiation of somatosensory neurons can be modeled in vitro and occurs in the presence of Lbx1 and Lfx5. Interestingly, coexpression of Phox2b represses somatosensory genes in Lbx1+ but not Lbx1FS− mice. In the presence of Phox2b, the altered C-terminal sequence of Lbx1FS might impede the correct recruitment of coregulatory factors, thus accounting for the fact that Lbx1FS is unable to correctly cooperate with Phox2b.

**Lbx1** in CCHS. Here we demonstrate that the hypomorphic Lbx1FS mutation selectively interferes with the development of specific dB2 neuronal populations. Physiologically, Lbx1FS/FS mice display a plethora of respiratory deficits: slow and irregular breathing, lack of hypercapnic reflex, and frequent and pro-longed apneas. Together, these deficits appear to result in neonatal lethality. We observed that the conditional restriction of the Lbx1FS mutation to the dB2 lineage (dB2-Lbx1FS mice) fully recapitulates the physiological phenotypes observed in Lbx1FS/FS mice. In contrast, the conditional restriction of the Lbx1FS mutation to rhombomeres 3 and 5 (Egr2-Lbx1FS mice) impaired RTN neuron development, abolished the hypercapnic reflex, and caused mild hypoventilation, but not abnormal apneas or neonatal lethality. Interestingly, similar or even milder phenotypic abnormalities are observed when the Phox2b+27ala mutation is restricted to rhombomeres 3 and 5 (22). Thus, the Lbx1FS mutation causes respiratory deficits that are in part, but not completely, a result of the loss of RTN neurons.

Last, we report in this study that dB2 precursors produce, in addition to the RTN and periV cells, an additional not previously described group of Lbx1+/Phox2b+ neurons that locate dorsally in rhombomeres 3–6. Because of the complexity of the developmental deficits displayed by Lbx1 null mutant mice, the contribution of individual cell populations to respiratory deficits had previously not been assessable. We used here intersectional genetic strategies to show that the combined deficits in development of RTN and the dorsal Lbx1+/Phox2b+ population correlated with severe hypoventilation and neonatal lethality.

Further studies will be needed to define the connectivity and the exact function of this dorsal neuronal population.

**Materials and Methods**

**Research Involving Humans and Mice.** Venous blood and genomic DNA samples from humans were obtained by standard procedures. Written informed consent was obtained from all individuals. Experimental procedures and animal handling were conducted according to institutional protocols and guidance approved by the Max Delbrück Center (Berlin), CNRS (Gif sur Yvette), Max Planck Institute for Genetics (Berlin), and the Ethics Committee of the Charité Universitätsmedizin (Berlin). Details on mouse strains are provided in **SI Appendix, SI Materials and Methods**.

**Histology.** Development of dB2 neuronal derivatives was assessed on 20-μm transverse hindbrain sections from control and mutant mice. Details on antibodies and in situ probes used in this study are provided in **SI Appendix, SI Materials and Methods**.

**Cell Cultures.** P19 embryonic teratocarcinoma cells were obtained from ATCC (CRL-1825) and differentiated into neurons using 1 μm retinoic acid (Sigma), as described (31). Details on CRISPR-CAS9 mutation of Lbx1 in P19 cells, retroviral infection, ChiP, and deep sequencing experiments are provided in **SI Appendix, SI Materials and Methods**.

**Physiology.** Unrestrained plethysmographic recordings of individual mouse pups were carried out as described (32). Further details on plethysmographic recordings and Ca2+ imaging studies can be found in **SI Appendix, SI Materials and Methods**.

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