Supplementary Figure 1.

Expression of *Tshz3* in developing hindbrain and immunodetection of TSHZ3 in brainstem. *A*, *C*, Analysis of *Tshz3* expression by in situ Hybridization at E8.5 (*A*) and E10.5 (*C*). *B*, *D*, *E*, Analysis of β-galactosidase (β-gal) activity in the developing hindbrain of *Tshz3*$^{+/lacZ}$ embryos at E8.5 (*B*), E9.5 (*D*) and E10.5 (*E*). Note the restriction of *Tshz3* expression and β-gal activity along the rostrocaudal axis, with an anterior limit of expression that respects the rhombomere 3/4 boundary. *E*, flat-mounted hindbrain, viewed from the ventricular surface. Note the presence of two columns of Xgal-positive cells located on either side of the floor plate (red arrows). These ventral stripes are particularly thick in rhombomere 4 (r4) and in the caudal hindbrain. *F*, transverse section of E10.5 hindbrain, showing Xgal-positive cells located at positions where the cranial motoneuron precursors differentiated. *N*, Immunodetection of TSHZ3 in a coronal section of the medulla oblongata in WT E18.5 embryo. Abundant TSHZ3 positive cells were found throughout the ventrolateral medulla. Gi: gigantocellular reticular nucleus, IOM: inferior olive medial nucleus, IRt: intermediate reticular nucleus, nA: nucleus ambiguus, indicated by dotted line; nTS: nucleus of the solitary tract, PrebötC: prebötzinger complex, RP: raphe pallidus, SP5: spinal trigeminal nucleus. Scale bar, 500 µm (*C*, *D*, *F*, and *G*), 100 µm (*H-K*), 200 µm (*N*).

Supplementary Figure 2. Characterization of *Tshz3* mutants. *A-B*, Whole-mount *in situ* hybridization on E10.5 flat-mounted hindbrains of WT (*A*) and *Tshz3*$^{lacZ/lacZ}$ embryos (*B*) with *Phox2b* probe. *Phox2b* expression indicated that segmental boundaries in the hindbrain are preserved in *Tshz3*$^{lacZ/lacZ}$ embryos. In addition, the two ventral columns of *Phox2b* expressing precursors were not affected in *Tshz3*$^{lacZ/lacZ}$ embryos, suggesting that branchial and visceral motoneurons were correctly generated in the absence of *Tshz3*. *C-D*, Lateral view of E11.5

embryos stained with anti-neurofilament antibody. The branching pattern of the cranial nerves does not differ between WT (*C*) and *Tshz3*$^{lacZ/lacZ}$ embryos (*D*). Trigeminal (V), facial (VII), vestibuloacoustic (VIII), vagal (X), spinal accessory (XI) nerves. *E-F*: Analysis of the medullary 5-HT neurons by 5-HT immunocytochemistry on coronal sections of E16.5 in WT (*E*) and *Tshz3*$^{lacZ/lacZ}$ (*F*) embryos. The 5-HT neurons of the medullary raphe (MR) and those located in ventrolateral part of the medulla (VLM) were preserved in the mutants. *G*: The number of 5-HT+ cells did not differ significantly between WT and *Tshz3*$^{lacZ/lacZ}$ embryos (mean $\pm$ SEM, n=4). *H-I*: NK1R immunofluorescence on frontal sections through the raphe nuclei of WT and mutant embryos at E18.5. Scale Bar 200 µm.