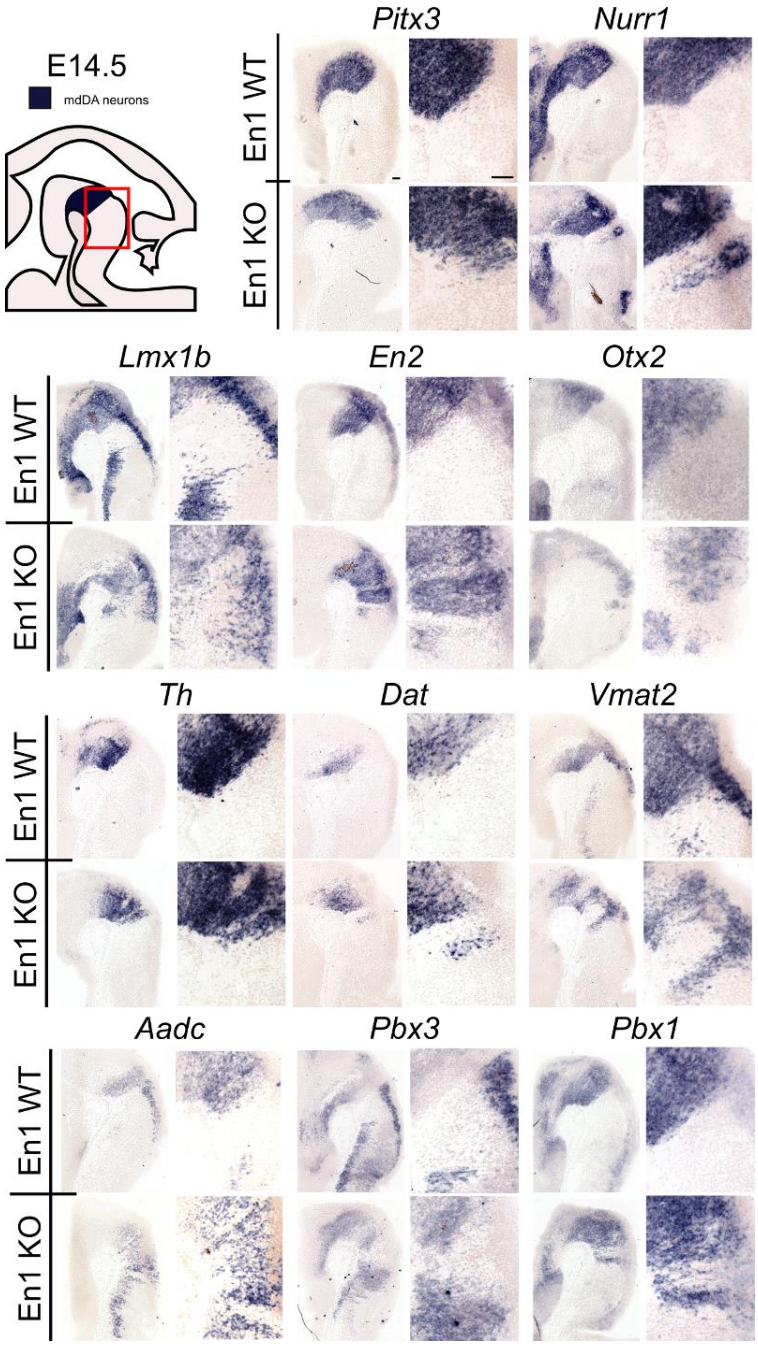

Fig. S1. Dopaminergic markers and transcription factors are ectopically expressed in the rostral hindbrain in embryonic (E14.5) En1 KO mouse. (**A-Z**) The expression patterns of transcription factors *Pitx3*, *Nurr1*, *Lmx1b*, *En2*, *Otx2* and dopaminergic markers (*Th*, *Dat*, *Vmat2*, *Aadc*, *Pbx3* and *Pbx1*) known to be expressed during the embryonic development of control mdDA neurons, are all caudally extended into the rostral hindbrain in En1KO [(para)medial sections as shown in schematic, area shown indicated by the red box in the schematic, scale bars indicates 100 μm].

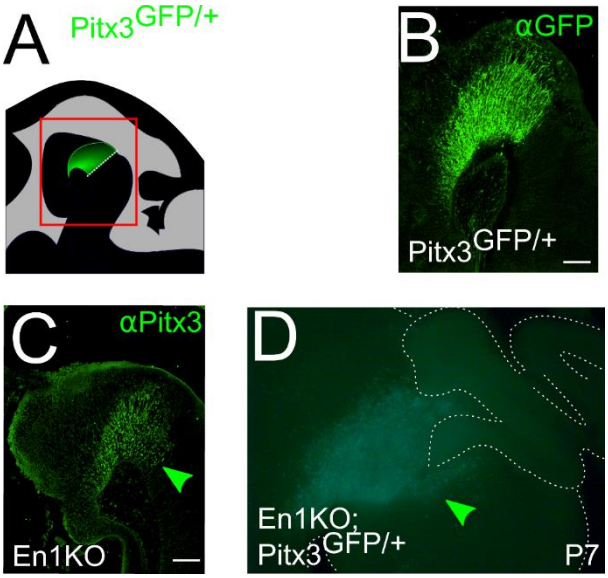

Fig. S2. Pitx3 and Pitx3-controlled GFP are present in eDA neurons in En1KO;Pitx3GFP/+ animals. (A-B) In Pitx3GFP/+ mice, GFP is uniquely expressed under the control of Pitx3. (**C**) Confirmation of presence of Pitx3 protein in eDA neurons in absence of En1. (**D**) En1KO;Pitx3GFP/+ animals reveal GFP expression in mdDA and eDA neurons at P7 [arrow, (para)medial sections as shown in schematic, area of higher magnification indicated by red box in schematic, scale bar indicates 100 μm].

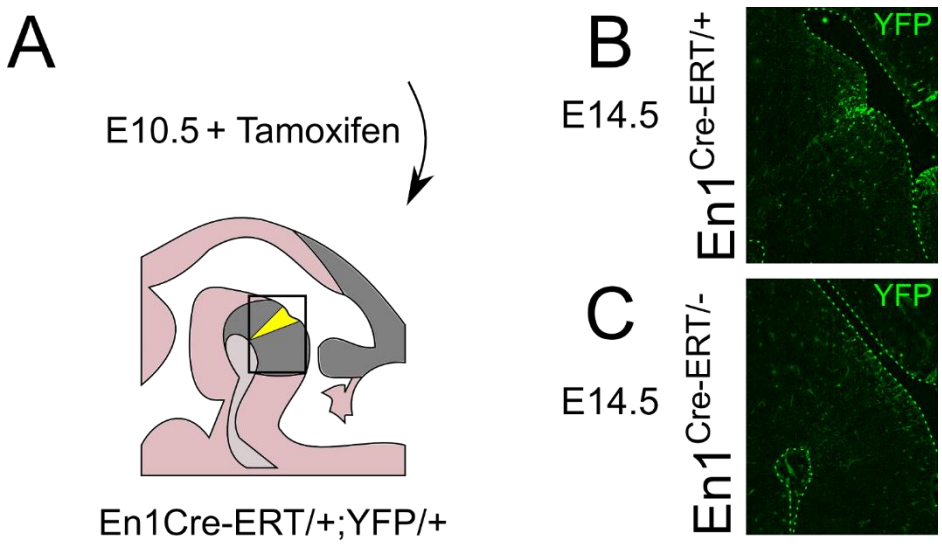

Fig. S3. Disorganized IsO area in En1Cre-ERTKO. (**A**) Tamoxifen induction at E10.5 resulted in En1Cre-controlled YFP expression of isthmic-restricted neurons. (**B-C**) The YFP-positive cells that are found in the En1Cre-ERT/KO are sparsely present and not localized at a specific location, compared to the En1Cre-ERT/WT animals in which the YFP-positive area is tightly restricted to the invagination of the IsO.

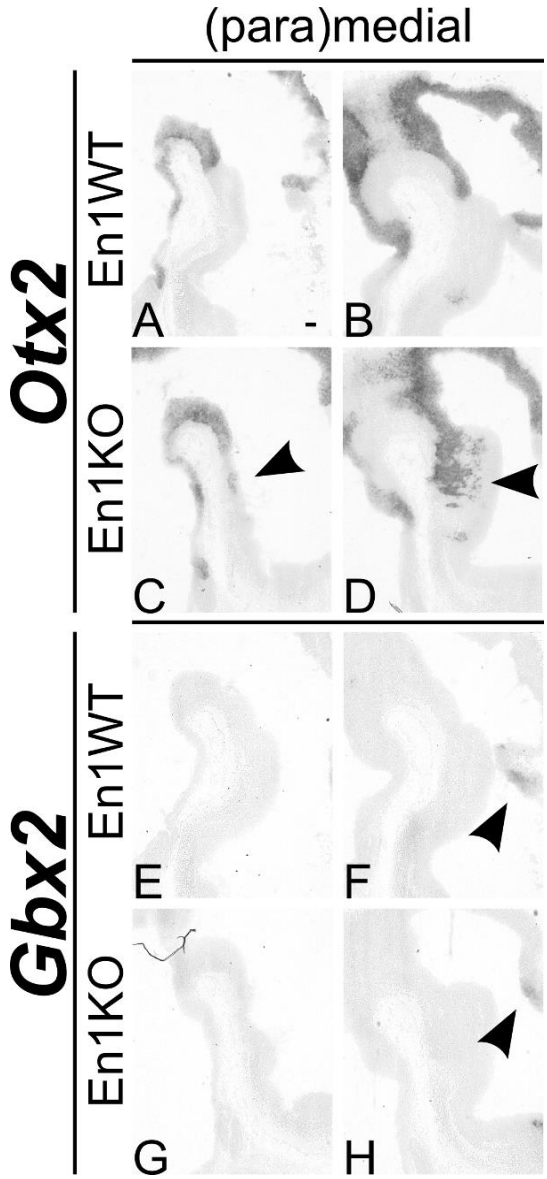

Fig. S4. *Gbx2* expression is unchanged at E12.5 in En1KO. (**A-D**) *Otx2* expression is present in midbrain at E12.5, but is caudally extended into the hindbrain in En1KO [arrow heads]. (**E-F**) *Gbx2* expression is absence at E12.5 in midbrain and R1, but is present in the cerebellar anlage [arrow head], and its expression remains unaltered in the En1KO, scale bar indicates 100 μm.

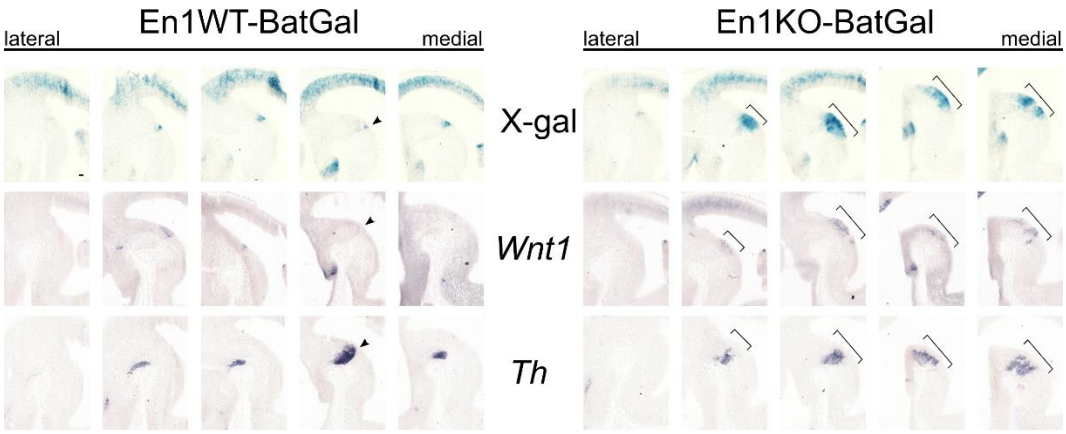

Fig. S5. Wnt1 and canonical Wnt-signaling are upregulated in R1 in absence of En1. β-catenin-β-galactosidase (BatGal) expression is present at the IsO at E14.5 in En1WT;BatGal+ animals [arrow], and is caudally extended in the En1KO;BatGal+ [brackets]. Adjacent sections of *Wnt1* mirror the β-galactosidase activity, both the restricted expression at the IsO in control [arrow] and the enlarged area in En1KO;BatGal+ animals [brackets]. Ectopic *Th* expression in En1KO;BatGal+ is present ventral to the area of enhanced Wnt-signaling and *Wnt1* expression [brackets], scale bar indicates 100 μm.