Supplementary Fig. 1. The midbrain floor plate, but not the hindbrain floor plate, is neurogenic.

(a-i) A comparison of Shh descendent cells (blue), their molecular profile, and the Shh mRNA expression (purple) in Shh::cre;R26R embryos during midbrain and hindbrain development. Coronal adjacent sections were labeled with a probe recognizing Shh mRNA (a, d, and g) and Xgal (b, e, and h); sections from another embryo were double labeled with antibodies against βgal and either Lmx1b (c), or TH (f), or NeuN/DAPI (i) at midbrain (mb; a-f) and hindbrain (hb; g-i) levels at 9.5 (a-c) and 12.5 dpc (d-i). Shh is initially expressed at the ventral midline at 9.5 dpc (a) but is downregulated at the midline by 12.5 dpc (d). On the other hand, in the hindbrain, Shh expression is maintained at the ventral midline at these embryonic stages (g; data not shown). At midbrain levels, Xgal labeling (Shh fate mapped cells; blue) is detected not only in the floor plate region (b and e) but also in the adjacent mantle layer (black arrow; e); in contrast, at hindbrain levels Xgal labeled cells are restricted to a tight ventral wedge at the ventral midline, extending processes (black arrowhead; see panel i) to the pial surface (h). A very few Xgal labeled cells are detected outside the hindbrain floor plate (empty arrowhead). At midbrain levels, early Lmx1b+ dopaminergic progenitors colocalize with βgal+ cells although, in isthmic regions, Lmx1b occupies a broader territory at 9.5 dpc (c; data not shown). During dopaminergic neurogenesis, all TH+ cells are situated medially within the βgal+ territory, and are βgal+. Immediately lateral to these dopaminergic cells is a TH-, βgal+ domain ( bracket) that predominantly produces non-dopaminergic descendents (Joksimovic, M. and Awatramani, R., in preparation; f). At hindbrain levels, βgal labeled cells extend processes (DAPI-/cytoplasmic βgal+; white arrowhead) to the pial surface, but rarely colocalize with NeuN+ (i), or 5-HT/Lmx1b+ serotonergic neurons (not shown).
Supplementary Fig. 2. Hindbrain serotonergic neurons are absent in Shh::cre;Shh cKO embryos
(a-b) Immunolabeling on 11.5 dpc coronal hindbrain sections with antibodies that recognize GATA3 (red) and 5-HT (green) in control (a) and Shh::cre;Shh cKO (b) mutant embryos. Note that expression of these serotonergic markers is virtually abolished in the mutant embryo. Control embryos harbor a floxed Shh allele (shown in a) or a Shh::cre allele (not shown).
Supplementary Fig. 3. Ectopic neurons in the spinal cord midline in Shh::cre;Shh cKO embryos

(a-h) Transverse sections of the spinal cord were labeled with a probe recognizing Shh mRNA (a and b) or antibodies against BrdU after 1 hour BrdU incorporation (c and d) at 11.5 dpc and Ngn2 (e and f) and Tuj-1 (g and h) at 10.5 dpc in control (a, c, e, and g) and Shh::cre;Shh cKO (b, d, f and h) embryos. Note that, in the mutant embryos, Shh expression is absent in the spinal cord (b) that correlates with an increased BrdU labeling (d), and prominent Ngn2 (f) and Tuj-1 (h; arrowhead) expression in comparison to the control (a, c, e, and g, respectively). Arrows in g and h indicate Tuj-1+ commissural interneurons crossing the ventral midline. Control embryos harbor a floxed Shh allele (shown in a, c, e, and g) or a Shh::cre allele (not shown).
Supplementary Fig. 4. Molecular characterization of the Shh::cre;beta-catenin cKO mutant

(A) Otx2 and Gbx2 expression in control (Shh::cre+;beta-catenin floxed/WT; a, b, e, and f) and Shh::cre;beta-catenin cKO (c, d, g, and h) mutant coronal sections at midbrain (mb; a-d) and midbrain-hindbrain boundary (mhb; e-h) levels at 11.5 dpc. Note a loss of Otx2 (red arrow) and lack of Gbx2 expression at mutant midbrain levels. However, faint Gbx2 expression was detected at the caudal most midbrain levels (not shown) in the proximity of the mhb. The mhb is intact in the mutant in comparison to the control (arrowheads).

(B) Lmx1a (red) and beta-catenin (green) expression on 12.5 dpc coronal midbrain sections in control (Shh::cre-;beta-catenin floxed/WT; a) and Shh::cre;beta-catenin cKO (b) mutant embryos. In mutants, beta-catenin immunoreactivity is diminished at the ventral midline as early as 10.5 dpc (not shown). Note that intact lateral Lmx1a+ dopaminergic progenitors in the mutant (see Fig. 5e-g) show an absence of beta-catenin immunoreactivity (asterisk). High magnifications of the left lateral side of the ventral midline are shown in a and b.
Supplementary Fig. 5. *Shh* expression inversely correlates with a production of TH+ midbrain dopamine neurons

(a-f) 11.5-12.0 dpc coronal midbrain sections were labeled with a probe specific to *Shh* (a, c, and e) and antibodies that recognize TH (b, d, and f) in control (a and b), *Shh::cre;beta-catenin cKO* (c and d), and *Nestin::cre;beta-catenin cKO* (e and f) mutant embryos. In *Shh::cre;beta-catenin cKO* embryos (an early, 9.0 dpc deletion), some sections reveal a gap between medially maintained *Shh* expression and the lateral *Shh* domain; in register with the gap in *Shh* expression, is the occurrence of TH+ neurons. Thus, there is a tight inverse correlation between *Shh* expression and TH+ neuron production (delineated by the white dotted lines). A late conditional deletion of *beta-catenin* (10.5-11.5 dpc) using *Nestin::cre* driver did not affect TH or *Shh* expression (e and f; this embryo is slightly younger). High magnifications of the left side of the ventral midline are shown in a-f. Control embryos shown are *Shh::cre+;beta-catenin floxed/wt* that showed consistent, wild-type, expression pattern of *Shh* and TH.
Supplementary Fig. 6. A model defining neurogenic potential of the ventral midline by the concerted action of Shh and Wnt signaling pathways

(left) In the developing hindbrain, high levels of Shh represses FP neurogenesis in part by inhibiting proliferation and proneural genes.

(right) In the early midbrain, Shh, in addition to midbrain specific factors (e.g. Otx2\(^1\)), is required for the expression of Lmx1a/b (\(^2\); Joksimovic and Awatramani, unpublished observations). Since Lmx genes can induce Wnt1 in the dorsal spinal cord and isthmus (\(^3,5\)), it is possible that Lmx genes are activators of Wnt1 in the midbrain FP; however, Otx2 may also have a direct role in Wnt1 expression \(^7,8\). Later, canonical Wnt signaling suppresses Shh levels, possibly by way of Msx1, thereby facilitating the appropriate rate of proliferation and proneural gene expression. In addition, canonical Wnt signaling promotes DA progenitor-specific characteristics by sustaining Otx2 and Lmx1a expression (it is possible that Lmx1a is downstream of Otx2). Together, this culminates in the production of the correct number of Nurr1+, Pitx3+, TH+ DA neurons during the appropriate neurogenic interval (10.5-14.5 dpc).