Supporting Information

Wataya *et al.* 10.1073/pnas.0803078105

SI Methods

Mouse ES Cell Culture. The maintenance medium contained G-MEM supplemented with 1% FCS, 10% KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, and 2000 U/ml LIF. For SFEBq differentiation, the day on which ES cells were seeded to differentiate was defined as differentiation day 0. No medium change was necessary before day 7. For the analysis of insulin's effect, ES cells were first aggregated in the indicated differentiation medium. Then, the medium was changed by transferring the aggregates to new plates on the indicated day, followed by rinsing with PBS. Recombinant mouse Shh-N, nodal, Lefty, Wnt3a, Dkk1, BMP7, FGF8b, and BDNF were purchased from R&D Systems, and CNTF from Sigma. Cyclopamine was purchased from Toronto Research Chemicals and LY294002, PD98059, Akt inhibitor VIII from Calbiochem.

Dissociation Neuronal Culture. For observation of axon and synapse formation, cells were cultured in 96-well plates until day 13, then dissociated from aggregates by 0.25% trypsin-EDTA and plated onto polyD-lysine/laminin-coated coverslip at a density of 8.5×10^4 cells/cm^2 in DFNB + 10% serum, 10 ng/ml CNTF, 50 ng/ml BDNF, and 50 ng/ml NT3. For the dissociation culture of Shh-treated cells, cells were dissociated from aggregates using the Neural Tissue Dissociation kit (Sumilon) and plated onto polyD-lysine/laminin/fibronectin-coated culture slides (BD) at a density of 2.0×10^4 cells/cm^2 in DFNB + 50 ng/ml BDNF on day 13.

Generation of Knockin ES Cell Lines. The gene targeting strategy is illustrated in Fig. S2*C*. To generate the targeting construct, the 5′ arm (6.4 kbp) and 3′ arm (3.1 kbp) were amplified by PCR from 129J mouse genomic DNA (from EB5 ES cells). The cDNA of enhanced *GFP* (*EGFP*; BD Biosciences) was fused in-frame into the first exon of the *Rax* gene at the initial ATG. A *PGK* promoter-driven neomycin-resistance selection cassette flanked by loxP sites was inserted downstream of *EGFP*. After electroporation of the linearized vector into EB5 ES cells, homologous recombinant ES cells were selected with neomycin and then screened by PCR. Three targeted clones (20, 116, and 122) were confirmed by Southern blot analysis with the 3′, 5′, and *neo* probes (Fig. S2*D* and data not shown). The floxed PGK-neo cassette was removed from 20 and 116 by transient transfection with Cre-expressing plasmid (kindly provided by Dr. K. Yamamura) using Lipofectamine 2000 (Invitrogen). The resultant subclones (clone 20-10, 20-18, 116-2, and 116-18) exhibited indistinguishable abilities to differentiate into Rax$^+$ hypothalamic progenitors, and subclone 20-10 was mainly used for the experiments shown in this report.

FACS Sorting. Cells were counted with FACSAria (BD) and the data were analyzed with the FACSDiva software (BD). For apoptosis analysis, SFEBq-cultured ES cells were dissociated on day 4 and labeled by Annexin V-PE Apoptosis Detection kit (MBL) by manufacturer's instruction.

For cell preparation, cells were dissociated to single cells by 0.25% trypsin-EDTA treatment and analyzed at 4°C. For isolation, cells were sorted on day 7 following dissociation with 0.25% trypsin-EDTA and filtration through Cell Strainer (BD Biosciences). GFP$^+$ cells and GFP$^-$ cells were gated by referring to scattered plots of the EB5 population to avoid cross-contamination. The sorted cells were collected in ice-cold DFK medium. Sorted GFP± cells were reanalyzed by FACS to confirm the quality of sorting.

Human ES Cell Culture. Human ES cells were maintained and cultured as described (8). DF/KSR medium for SFEBq was DMEM/F12 medium (Sigma) supplemented with 20% KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, and 0.1 mM 2-mercaptoethanol. For SFEBq culture, hES cells were pretreated for 1 h with 10 μM Y27632 (Tocris), dissociated with trypsin/EDTA, and reaggregated using 96-well low cell-adhesion plates (5000 cells/well, 150 μl) in medium containing 10 μM Y27632. As described previously (8), to block endogenous Nodal signals, 5 μg/ml SB431542 (Tocris) was added to the differentiation medium from day 0 to day 15. Akt inhibitor VIII (2 μM) was added from day 3 to day 30 when indicated. On day 30, qPCR was performed. Data were normalized by beta-actin expression. Primers used are listed in Table S1.

Immunohistochemistry and quantitative PCR. Immunohistochemistry was performed as described previously (4, 8, 14) with the primary antibodies described below. For statistical analysis, 15–40 aggregates were examined for each experiment, which was repeated at least four times.

The antibodies were used at the following dilutions: Bf1 [rabbit/polyclonal/1:500 (4)], N-cadherin (mouse/monoclonal/1:2000/BD pharmingen), Six3 (rabbit/polyclonal/1:1000, guinea pig/polyclonal/1:1000), Rax [mouse/polyclonal/1:200, rabbit/polyclonal/1:200 (14)], Nestin (mouse/monoclonal/1:400, BD PharMingen), Nkx2.1 [mouse/monoclonal/1:100/Zymed, rabbit/polyclonal/1:2000/Biopad, guinea pig/polyclonal/1:3000 (4)], Pax6 (mouse/monoclonal/1:100/DSHB), Otp (guinea pig/polyclonal/1:3000), SF1 (mouse/monoclonal/1:100/Perseus, rabbit/polyclonal/1:400/Affinity BioReagents), Foxb1 (goat/polyclonal/1:400/Abcam), Tuj1 (mouse/monoclonal/1:500/Babco), Brn2 (goat/polyclonal/1:100/Santa Cruz) neurophysin II (goat/polyclonal/1:400/Santa Cruz), VGLUT2 (guinea pig/polyclonal/1:2000/Chemicon), tyrosine hydroxylase (TH; sheep/polyclonal/1:500/Chemicon), Agouti-related protein (AgRP; goat/polyclonal/1:400/neuromics), Neuropeptide Y (NPY; rabbit/poluclonal/1:500/Chemicon), Sox1 [chickin/polyclonal/1:500/Chemicon, rabbit/polyclonal/1:1000 (4)], Crx [rat/polyclonal/1:800 (14)], Chx10 (rabbit/polyclonal/1:2000/Exalpha), Tau (rabbit/polyclonal/sigma/1:400), MAP2(mouse/sigma/1:1000), Synaptophysin (rabbit/polyclonal/Zymed/1:400), and PSD95 (mouse/monoclonal/Affinity BioReagents/1:400).

For antibody production, cDNA encoding the carboxy-terminal portion of mouse *Six3* (residues 292–353) was amplified by PCR and subcloned into *pGEX-4T-3* (Amersham Biosciences). The fusion protein was expressed in *Escherichia coli* strain BL21 and purified with glutathione sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. Antibodies against Six3 were obtained by immunizing rabbits and guinea pigs with purified GST-Six3. The antisera against Six3 were preabsorbed with GST-sepharose and then affinity purified with a Six3-bound GST-sepharose column. The antiserum against Otp was raised in guinea pigs against a synthetic peptide (RKALEHTVSMSFT; reference) and was affinity purified.

The immunostaining specificity of each antibody was confirmed by immunostaining with the appropriate embryonic tissues as a positive control under the same conditions. Counter nuclear staining was performed with DAPI (Molecular Probes).

Quantitative PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instruction and data were normalized by GAPDH expression. Primers used for qPCR are listed in Table S1.

Statistical Analysis. Values shown on graphs represent the mean ± SE unless otherwise described. Statistical tests for significance in mean values are described in the corresponding legends. Statistical analysis was performed using the Prism program (GraphPad).

1. Lin X, *et al.* (1999) Identification, chromosomal assignment, and expression analysis of the human homeodomain-containing gene Orthopedia (OTP). *Genomics* 60:96–104.

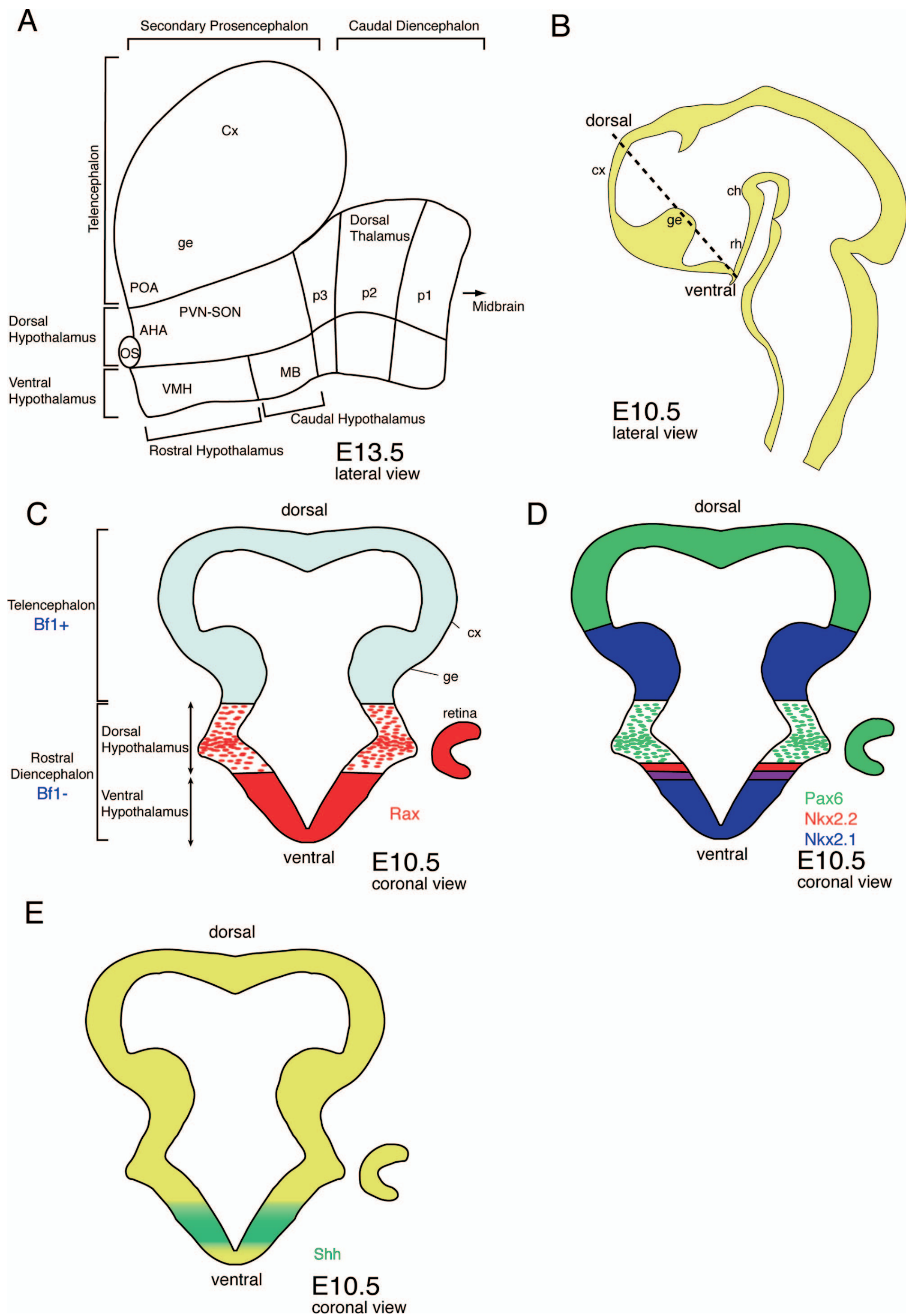

Fig. S1. Schematic of anatomical subdivision and marker expression in the rostral forebrain. (*A*) Schematic (lateral view; modified from ref. 1) of the prosomeric domains in the E13.5 mouse forebrain. (*B*) Lateral view of the E10.5 mouse forebrain. The section line for panels *C* and *D* (also for Fig. 1*F*, 3*B*, and Fig. S2*K*) is indicated by the dotted line. (*C*) Coronal view of the E10.5 mouse forebrain. Rax expression is illustrated in red. (*D*) Expression of Nkx2.1 (blue), Nkx2.2 (red), and Pax6 (green) in the E10.5 mouse forebrain. The exact boundary of the dorsal and ventral domains is still under some debate, but it is likely to coincide with Nkx2.2 expression at the upper border of the Nkx2.1^{+} area. (*E*) Expression of Shh (green) in the E10.5 mouse forebrain. Little Shh expression is present in the floor of the third ventricle. cx, cortex; ge, ganglionic eminence; POA, preoptic area; AHA, anterior hypothalamic area; OS, optic stalk; VMH, ventral medial hypothalamus anlage; MB, mammillary body; PVN, paraventricular nucleus; SON, supraoptic nucleus; p1-p3, prosomeres 1–3.

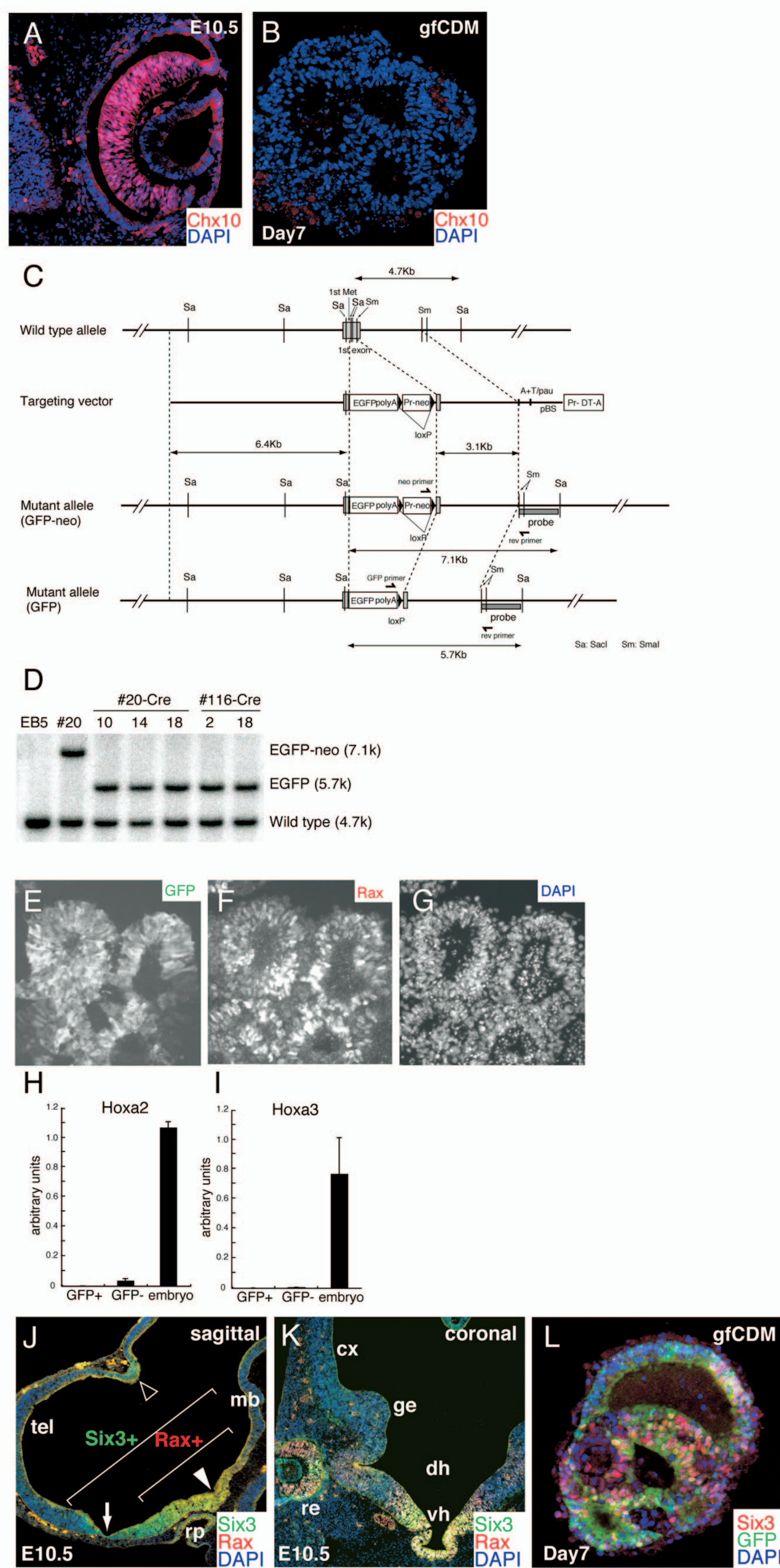

Fig. S2. Generation and differentiation of Rax–GFP knockin ES cell lines and marker expression in SFEBq/gfCDM culture. (*A* and *B*) The frozen sections of an E10.5 mouse retina (*A*) and SFEBq/gfCDM-cultured ES cells (*B*; day 7) were stained with antibodies against Chx10. DAPI was used for counter staining. (*C*) Construction of the targeting vector for Rax–GFP knockin. (*D*) Southern blot analysis with the 3′ probe. The neo cassette was removed from two Rax–GFP-knocked-in clones (20 and 116). (*E*–*G*) Expression of GFP (*E*) and endogenous Rax (*F*) in Rax–GFP ES cells cultured by SFEBq/gfCDM. DAPI was used for counter staining (*G*). (*H* and *I*) qPCR analysis of Rax–GFP positive and negative factions with *Hoxa2* and *a3* primers. (*J*–*L*) Immunostaining of Six3. Six3 expression in the E10.5 mouse embryo is restricted to parts of the forebrain, including the hypothalamus, retina, epithalamus, and part of the subpallium (rather weakly) (*J* and *K*). (*L*) Immunostaining showed that Six3^{+} cells were fewer in day 7 aggregates than in day 5 aggregates (Fig. 1*D*) and most of them expressed Rax–GFP (either weakly or strongly). Arrow, lamina terminalis; arrowhead, infundibulum; open arrowhead, epithalamus; tel, telencephalon; mb, mammillary body; cx, cortex; ge, ganglionic eminense; dh and vh, dorsal and ventral hypothalamus, respectively; and re, retina.

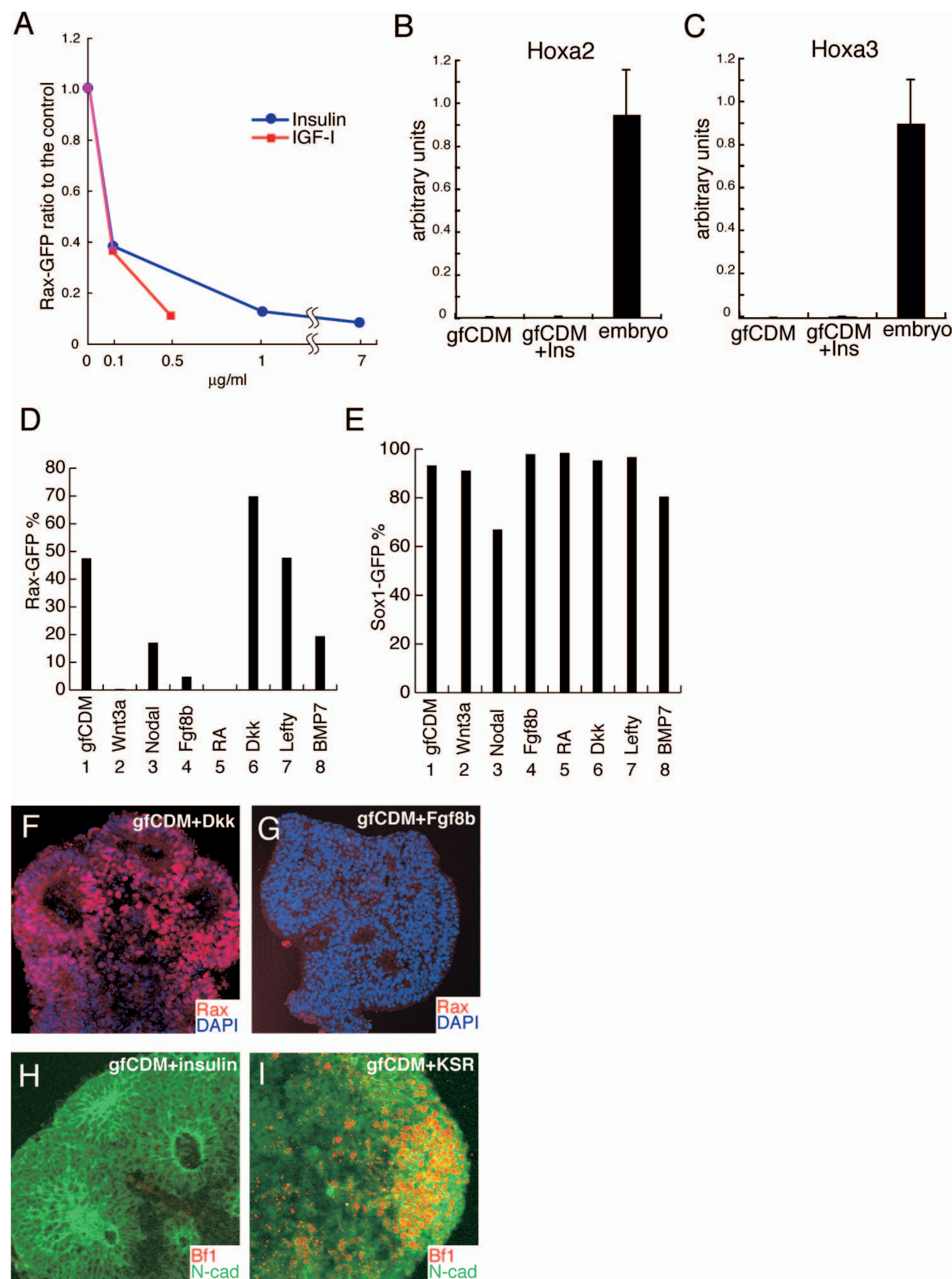

Fig. S3. Insulin and other caudalizing factors suppresses Rax expression on SFEBq culture. (*A*) Dose-response analysis of the effects of insulin and IGF1 on SFEBq/gfCDM-cultured Rax–GFP ES cells. Cells were cultured with insulin or IGF1 at the indicated concentration from the beginning of the differentiation culture, and the percentages of Rax–GFP$^+$ cells were analyzed by FACS on day 7. The Rax–GFP$^+$ percentage of the control (insulin-free gfCDM; as in Fig. 2*A*) was referred to as 1.0 and the relative ratios are shown. Effects of growth factors on Rax–GFP$^+$ percentages in SFEBq/gfCDM culture on day 7. (*B* and *C*) qPCR analysis on day 7 for the expression of *Hoxa2* (*B*) *and Hoxa3* (*C*). Total RNA from E10.5 whole embryo was used as a control. (*D* and *E*) Growth factors or their inhibitors were added on day 4 in SFEBq/gfCDM culture, and Rax expression and neural induction were analyzed by FACS. Rax–GFP$^+$ percentages were analyzed on day 7 (*D*) and Sox1–GFP$^+$ on day 5(*E*). Final concentrations were: Nodal (1 μg/ml), retinoic acid (200 nM), Lefty (1 μg/ml), Wnt3a (200 ng/ml), Dkk1 (200 ng/ml), BMP7 (500 ng/ml), and Fgf8b (250 ng/ml). Immunostaining of day 7 aggregates stained by Rax and DAPI (*F* and *G*) shows fundamentally the same results as panel *D*. (*F* and *G*) Immunostaining showed that Dkk treatment in gfCDM culture increased the number of Rax$^+$ cells moderately (*F*), whereas Fgf8b treatment diminished them (*G*). (*H* and *I*) Effects of additives on Bf1 induction in gfCDM culture. Few Bf1$^+$ cells were found in insulin-added gfCDM (*H*), while substantial Bf1 induction was seen in KSR-added (final 10%) gfCDM (*I*).

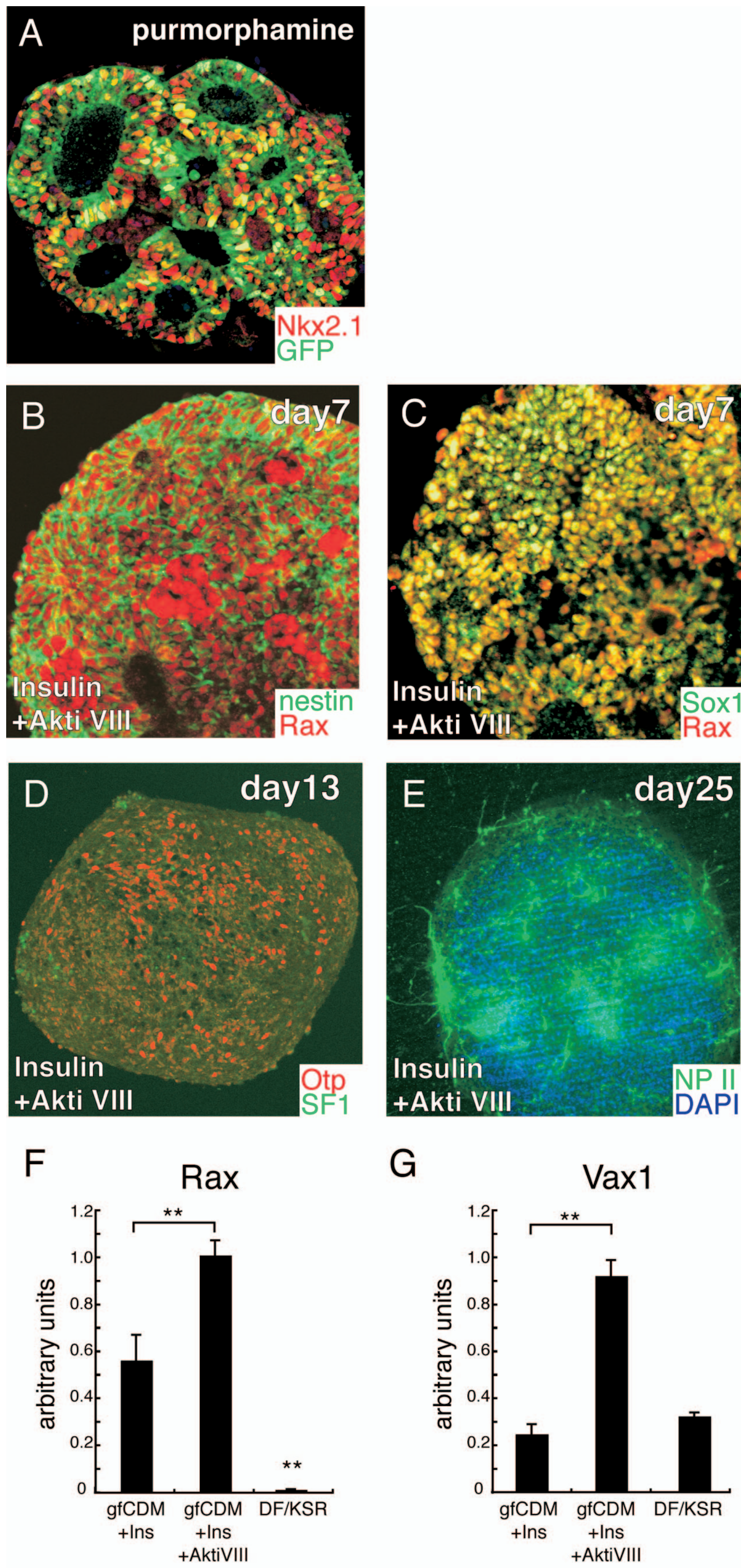

Fig. S4. Chemical agonist of Shh for rostral-ventral hypothalamic induction and rostral hypothalamic differentiation by using Akt inhibitor with insulin-added gfCDM. (*A*) A frozen section of day 7 SFEBq/gfCDM-cultured aggregates treated with purmorphamine (5 μg/ml) on day 4. Immunostaining of Nkx2.1 (red) and Rx-GFP (green) indicated rostral–ventral hypothalamic differentiation. (*B–E*) Immunostaining of SFEBq-cultured ES cells in insulin-contained CDM supplemented with Akt inhibitor VIII. (*B* and *C*) Frozen sections of day 7 aggregates were immunostained with Rax (*B* and *C*), nestin (*B*), and Sox1 (*C*). Most of Rax^+ cells were both $nestin^+$ and $Sox1^+$. (*D*) Sections of a day 13 aggregate of sorted GFP^+ fraction were immunostained by Otp and SF1. Numerous Otp^+ cells were observed while few cells expressed SF1, as seen in SFEBq/gfCDM-cultured ES cells. (E) Immunostaining of a day 25 aggregate. The Akt inhibitor-treated SFEBq cells differentiated into neurophysin II^+ neurons. (*F* and *G*) qPCR analysis of SFEBq/CDM-cultured human ES cells. Human ES cells were cultured as described in *SI Methods*. Medium used for this SFEBq culture was insulin-added gfCDM with/without Akt inhibitor or DMEM-F12/KSR (*SI Methods*). Expression of *Rax* (*F*) and *Vax1* (*G*) were analyzed on day 30. Statistical significance vs. the control (gfCDM + Insulin) was evaluated by Bonferroni's test. **, $P < 0.01$

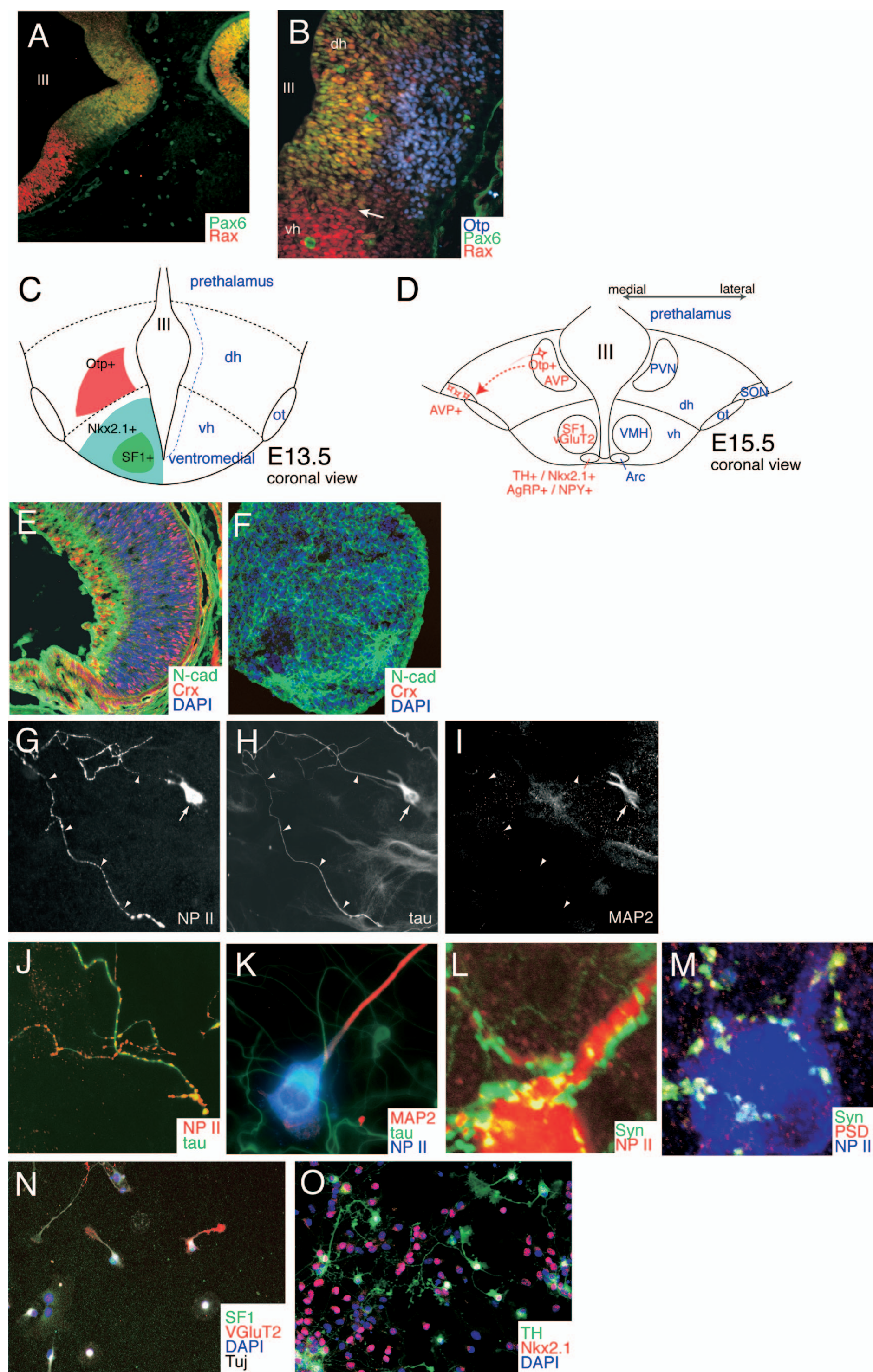

Fig. S5. Rostral hypothalamic neurons (*A*) Expression of Rax in the dorsal and ventral hypothalamus at E10.5 (coronal section). Strong Rax expression is observed in a portion (≈60%) of the neuroepithelial cells in the rostral–dorsal hypothalamus while it is detected in the majority (>90%) of the neuroepithelial cells in the rostral–ventral region. (*B*) Accumulation of Otp^{+} neuronal precursors (blue) in the subventricular zone adjacent to the $Pax6^{+}/Rax^{+}$ ventricular zone of the dorsal hypothalamic domain. Rax (red); Pax6 (green); III, third ventricle; arrow, the approximate position of the dorsal–ventral border. (*C* and *D*) Schematic of anatomical subdivisions and marker expression in the rostral hypothalamus (coronal view) at E13.5 (*C*) and E15.5 (*D*). (*C*) E13.5 hypothalamus. The majority of the cells in the ventral domain (vh) are $Nkx2.1^{+}$. Otp^{+} neuronal precursors are generated predominantly in the dorsal domain (dh) while $SF1^{+}$ cells in the ventral domain give rise to neurons in the VMH. (*D*) E15.5 hypothalamus. In the dorsal domain, Otp^{+} precursors generate AVP neurons in the PVN and SON. SON-AVP neurons migrate radially (dotted arrow) along the radial glia to the lateralmost region dorsal to the optic tract (ot). In the hypothalamus, the anatomical medial–lateral axis corresponds to the extent of radial migration and the birth dates of neurons, rather than to the primary embryonic axes. (*E* and *F*) Expression of Crx (red) and N-cadherin (green) in the mouse neural retina (*E*), and SFEBq/gfCDM-cultured ES cells (*F*; day 15). (*G–M*) Dissociation culture of FACS-purified Rax–GFP^{+} cells. Immunostaining with neurophysin II (*G*, *J*, and *K*), the axonal marker tau (*H*, *J*, and *K*) and the dendritic marker MAP2 (*I* and *K*) on day 25. Arrow, soma; arrowhead, axon. Immunostaining of synaptophysin (Syn; *L* and *M*), PSD95 (*M*) and neurophysin II (*L* and *M*) was performed on day 35. (*L*) High magnification view of Fig. 5*G*. Numerous synaptic-like punctae immunoreactive for synaptophysin cover the surface of somata and dendrites. Part of synaptophysin staining overlapped with the postsynaptic marker PSD95 (*M*). (*N* and *O*) Dissociation culture of FACS-purified Rax–GFP^{+} cells treated with 30 μM Shh. (*N*) Immunostaining for SF1 (green), VGluT2 (red; located mainly in the growth cones), and TuJ1 (white) on day 25 (dissociation neuronal culture). SF1/VGluT2 staining is characteristic of mature VMH neurons. (*O*) Immunostaining for TH (green) and Nkx2.1 (red). This marker combination is characteristic of A12 neurons in the arcuate nucleus.

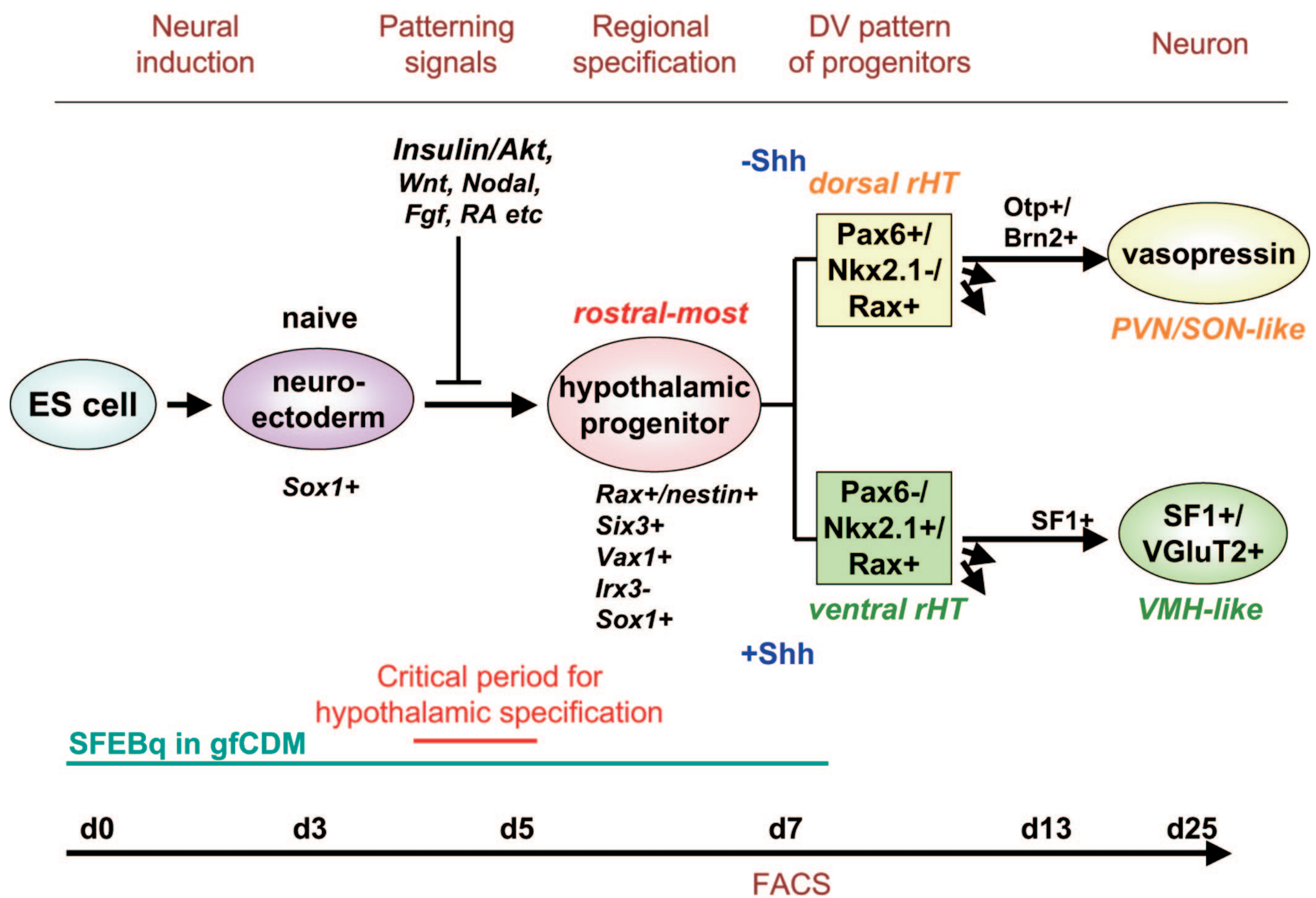

Fig. S6. ES cell differentiation into hypothalamic progenitors in SFEBq/gfCDM culture. Schematic summary of the findings in this study. When cultured in the chemically defined medium containing no additional growth factors such as insulin, Wnt, Nodal, Fgfs, BMP, and RA, ES cells frequently differentiate into Sox1$^+$ naïve neuroectomdermal cells that are fated to become rostral hypothalamic progenitors (Rax$^+$/nestin$^+$/Six3$^+$/Vax1$^+$/Irx3$^-$). Without Shh treatment, these progenitors have the characteristics of dorsal hypothalamic progenitors (Pax6$^+$/Nkx2.1$^-$) while Shh treatment promotes ventral hypothalamic differentiation (Pax6$^-$/Nkx2.1$^+$). ES cell-derived dorsal hypothalamic progenitors can generate vasopressin neurons, presumably via Otp$^+$/Brn2$^+$ intermediate precursors, as embryonic progenitors do. ES cell-derived ventral hypothalamic progenitors (Shh treated) give rise to neurons characteristic of the ventral hypothalamus (e.g., SF1$^+$ glutamatergic neurons in the VMH, A12 dopaminergic neurons and AgRP/NPY neurons in the arcuate nucleus).

Table S1. Primers for qPCR

	Forward	Reverse
mouse-Otx2	CGTTCTGGAAGCTCTGTTTG	TTTTCAGTGCCACCTCTTCC
mouse-Rax	TTCGAGAAGTCCCACTACCC	TTCATGGACGACACTTCCAG
mouse-Vax1	CGCCAGGGTCTCGAAGAAC	CCGCTGAGGAATTGGATTTACT
mouse-Six3	GGTTTAAGAACCGGCGACAG	TACCGAGAGGATCGAAGTGC
mouse-Irx3	CAACGAGCACCGCAAGAA	TGGTGATGATGGCCAACATG
mouse-En2	ATGGGACATTGGACACTTCTTC	CCCACAGACCAAATAGGAGCTA
mouse-Hoxa2	TACGAATTTGAGCGAGAGATTGG	GTCGAGGTCTTGATTGATGAACT
mouse-Hoxa3	TCAGCGATCTACGGTGGCTA	GAGGCAAAGGTGGTTCACCC
mouse-Hoxb1	GCCCCAACCTCTTTTCCCC	GACAGGATACCCCGAGTTTTG
mouse-Hoxb9	GGAAGCGAGGACAAAGAGAG	TTGAGGAGTCTGGCCACTTC
mouse-GAPDH	TGACCACAGTCCATGCCATC	GACGGACACATTGGGGGTAG
human-Rax	GGCAAGGTCAACCTACCAGA	CTTCATGGAGGACACTTCCAG
human-Vax1	CTCTCCGAGACCCAGGTG	GACACCACCGAGCGTAGC
human -beta-actin	CAATGTGGCCGAGGACTTTG	CATTCTCCTTAGAGAGAAGTGG