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Supplemental Information

Deterministic *HOX* Patterning in Human Pluripotent Stem Cell-Derived Neuroectoderm

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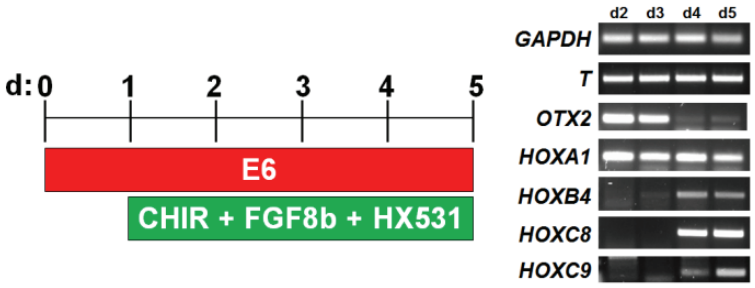

Figure S1. Inhibition of RA signaling by 1 μM HX531 does not disrupt *HOX* progression, related to Figure 1. RT-PCR data indicates that CHIR/FGF8b effects are not indirectly mediated by endogenous RA signaling.

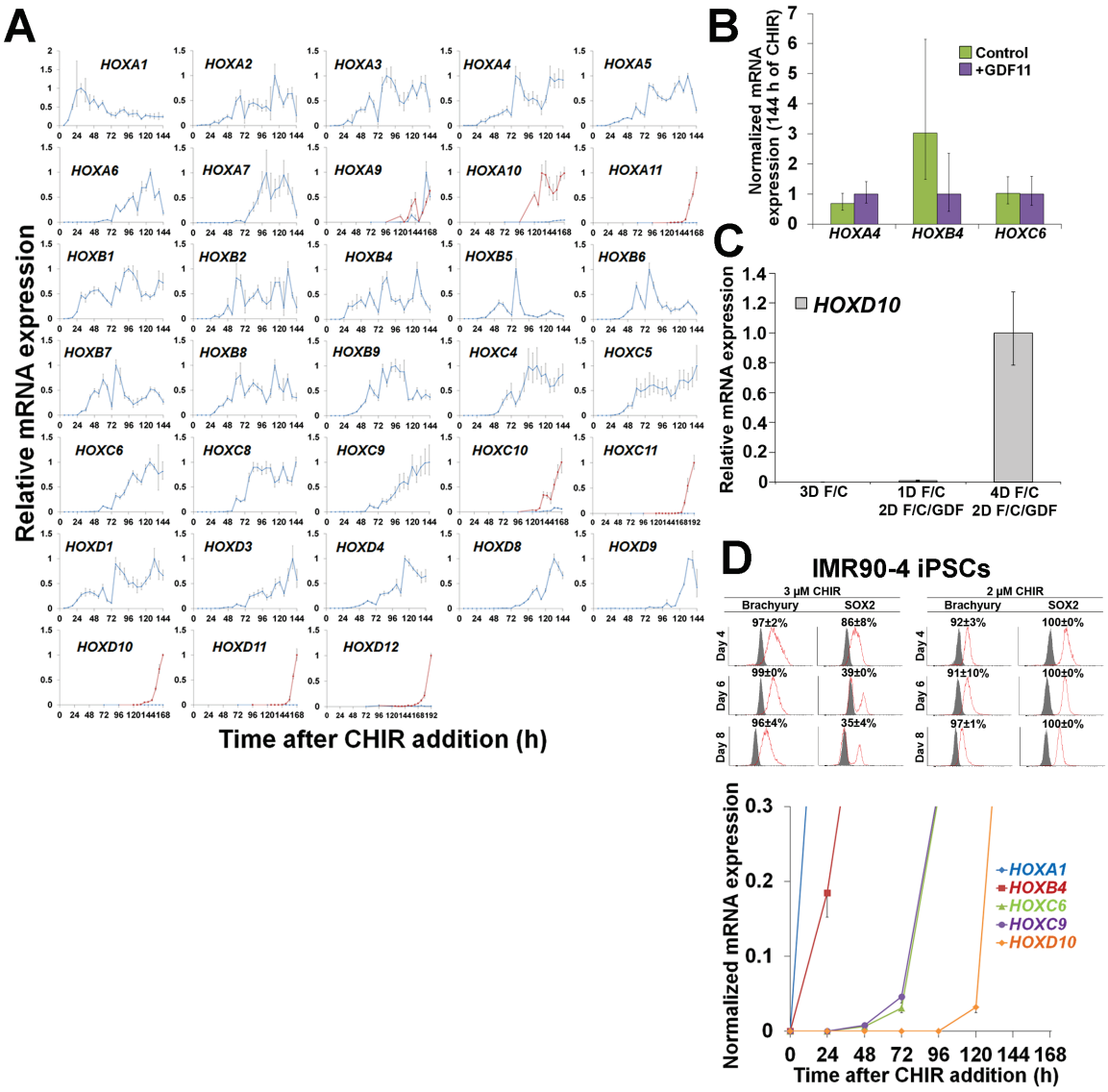

Figure S2. *HOX* regulation in the neuromesodermal state, related to Figure 2. (A) qPCR analysis of *HOX* expression during neuromesodermal propagation in H9 hESCs. Experimental timing is found in Figure 2B. Data are presented as mean ± S.D. from technical duplicates normalized to maximum expression for each gene. Blue lines = samples not receiving GDF11; red lines = samples receiving GDF11 according to Figure 2B. *HOXC11* and *HOXD12* required 192 h of CHIR treatment to be appreciably detected. **(B)** Rostral *HOX* expression is unaffected by GDF11 treatment during H9 hESC neuromesodermal propagation. qPCR data are presented as mean ± S.D. from technical duplicates normalized to the GDF11-treated sample for each

gene. **(C)** Premature treatment with GDF11 during neuromesodermal propagation does not effectively induce *HOXD10*. qPCR data are presented as mean ± S.D. from technical duplicates normalized to the condition of maximum *HOXD10* expression. **(D)** Evaluation of neuromesodermal propagation and *HOX* expression in IMR90-4 iPSCs. Differentiation was conducted according to Figure 2B. The neuromesodermal phenotype was assessed during differentiation in 2 or 3 μM CHIR. Grey histograms, IgG control. Red histograms, label of interest. Whereas 2 μM CHIR maintained the neuromesodermal state, 3 μM CHIR resulted in a mesodermal shift exemplified by a reduction in SOX2 expression. Data were collected by flow cytometry and presented as mean ± S.D. from biological duplicates. During neuromesodermal propagation, progressive *HOX* activation was also verified by qPCR. Data are presented as mean ± S.D. from technical duplicates normalized to maximum expression for each gene.

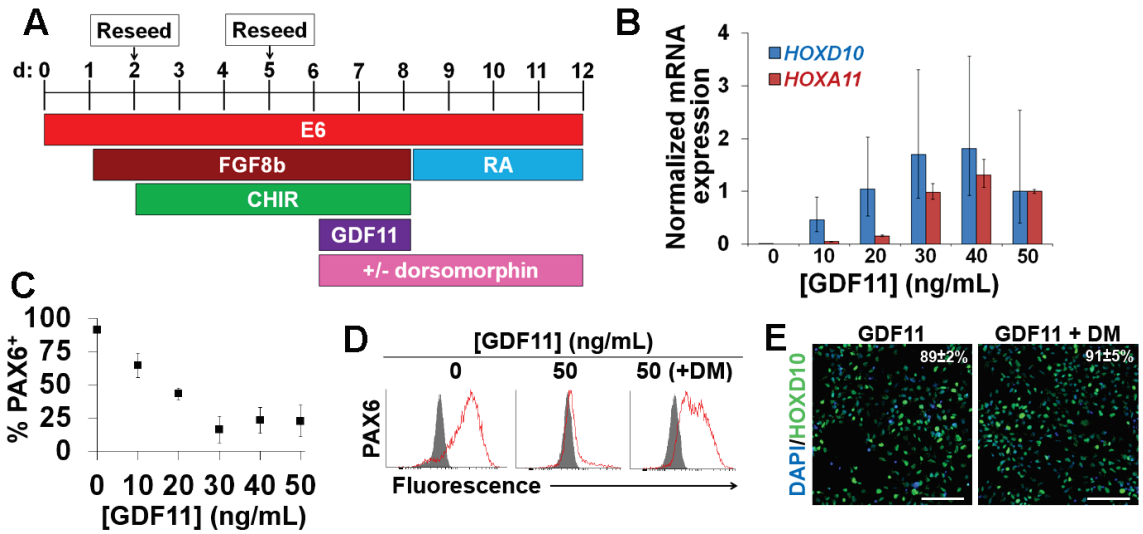

Figure S3. Dorsomorphin is required during GDF11 treatment to achieve Pax6 expression after RA treatment, related to Figure 3. (A) Timeline of differentiation. All analyses were conducted at day 12. **(B)** qPCR assessment of lumbosacral gene expression. Data are presented as mean ± S.D. from technical duplicates normalized to the maximum GDF11 dose. **(C)** Expression of PAX6 in response to increased GDF11 concentrations. Data were collected by flow cytometry and presented as mean ± S.D. from biological duplicates. SOX2 was uniformly expressed regardless of GDF11 treatment (data not shown). **(D)** Flow cytometry histograms demonstrating that the addition of dorsomorphin (DM) is sufficient to recover PAX6 expression (83±4% $PAX6^{+}$). Grey histograms, IgG control; Red histograms, PAX6. **(E)** Immunocytochemistry at day 12 indicates dorsomorphin does not affect the acquisition of HOXD10. Percentages are listed as mean ± S.D. calculated from 2 fields per sample (technical replicates, minimum 2000 cells counted). Scale bars, 100 μm.

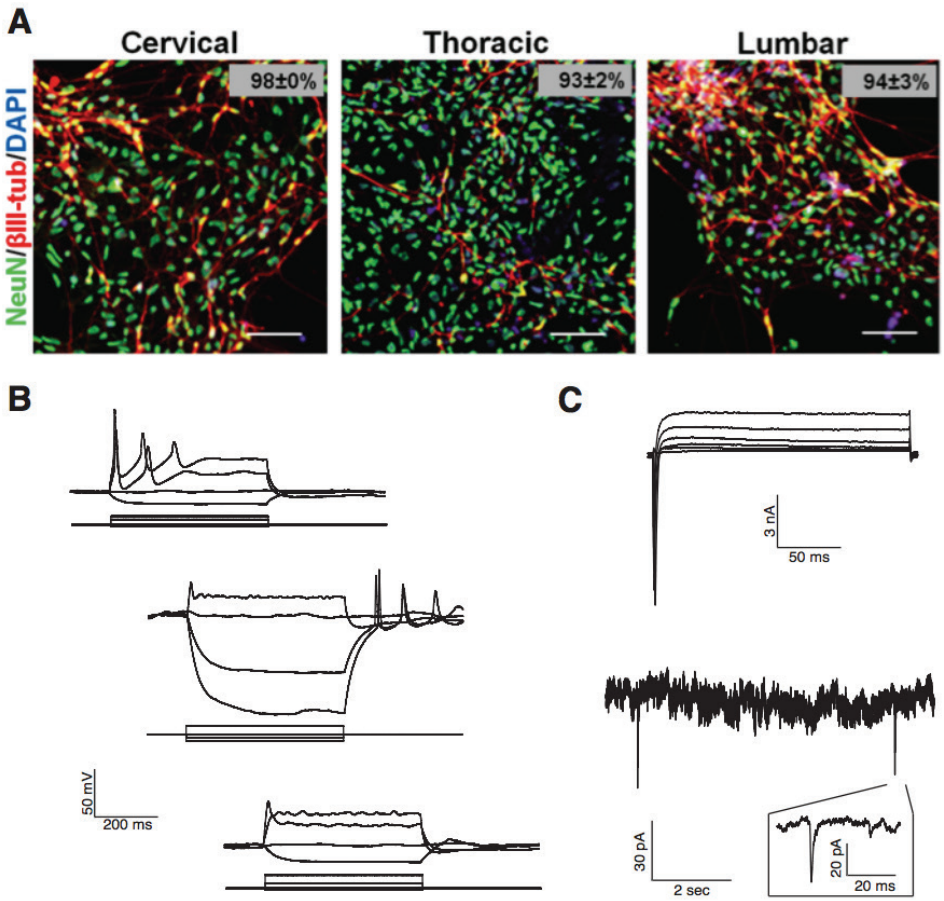

Figure S4. NeuN quantification and electrophysiology after neuronal maturation, related to Figure 4. (A) Samples were differentiated according to Figure 4C. Scale bars, 100 μm. Percentages are mean ± S.D. calculated from 2-4 fields per sample (technical replicates, minimum 1000 cells counted). **(B)** Samples were differentiated for >2 months prior to current clamp characterization of membrane properties (see Experimental Procedures for details). Examples of the three classes of action potentials (APs) were observed. Top: Mature APs (observed in 4/18 cells). Middle: Rebound APs triggered at offset of hyperpolarization (observed in 4/18 cells). Bottom: "Abortive" AP indicative of an immature neuron ((Belinsky et al., 2014); observed in 7/18 cells). APs were observed across all conditions (cervical, thoracic, lumbar), with no noticeable difference in basic membrane properties. Resting membrane potentials: -

31±3.9mV. Input resistance: 1±1.6 GΩ. Specific capacitance: 14.38±1.8pF. **(C)** Voltage clamp characterization of ionic and synaptic currents. Examples of whole-cell currents in response to incrementing 500ms voltage steps (top). Spontaneous quantal synaptic currents (bottom) were observed in 6/11 cells tested across all conditions.

Table S1. Proteins identified at a 1% FDR and TMT quantitation values. This information is located in the attached spreadsheet.

Table S2. Primary antibody list.

Antigen	Host species	Clone or product #	Dilution	Vendor
SOX2	Mouse	10H9.1	1:1000 (FC) 1:500 (ICC)	Millipore
Brachyury	Goat	AF2085	1:200 (FC) 1:500 (ICC)	R&D Systems
PAX6	Mouse	N/A	1:200 (FC)	DSHB
HOXB1	Sheep	AF6318	1:100 (ICC)	R&D Systems
HOXB4	Rat	I12	1:50 (ICC)	DSHB
PAX6	Rabbit	PRB-278P	1:500 (ICC)	Covance
N-cadherin	Mouse	32	1:500 (ICC)	BD Biosciences
OTX2	Goat	AF1979	1:500 (ICC)	R&D Systems
HOXD10	Goat	N/A	1:500 (ICC)	R&D Systems
βIII-tubulin	Rabbit	PRB-435P	1:1000 (ICC)	Covance
NF-H	Mouse	SMI-32R	1:1000 (ICC)	Covance
Synapsin	Rabbit	AB1543	1:250 (ICC)	Millipore
NeuN	Mouse	A60	1:100 (ICC)	Millipore
HB9	Mouse	81.5C10	1:50 (ICC)	DSHB
ISL1	Mouse	39.4D5	1:100 (ICC)	DSHB
ISL1	Goat	AF1837	1:500 (ICC)	R&D Systems
FOXP1	Rabbit	ab16645	1:20,000 (ICC)	Abcam

Table S3. Primer sequences used for RT-PCR.

Gene	Primer sequence
GAPDH	F: CACCGTCAAGGCTGAGAACG R: GCCCCACTTGATTTTGGAGG
SOX2	F: TGGACAGTTACGCGCACAT R: CGAGTAGGACATGCTGTAGGT
T	F: CTTCCCTGAGACCCAGTTCA R: CAGGGTTGGGTACCTGTCAC
OTX2	F: CCAGACATCTTCATGCGAGAG R: GGCAGGTCTCACTTTGTTTTG
HOXA1	F: AAATCAGGAAGCAGACCCAC R: GTAGCCGTACTCTCCAACTTTC
HOXB4	F: TACCCCTGGATGCGCAAAGTTC R: TGGTGTTGGGCAACTTGTGG
HOXC5	F: ACAGATTTACCCGTGGATGAC R: AGTGAGGTAGCGGTTAAAGTG
HOXC6	F: GAATGAGGGAAGACGAGAAAGAG R: CATAGGCGGTGGAATTGAGG
HOXC8	F: TTTATGGGGCTCAGCAAGAGG R: TCCACTTCATCCTTCGGTTCTG
HOXC9	F: AGCACAAAGAGGAGAAGGC R: CGTCTGGTACTTGGTGTAGG

Table S4. Taqman primers used for qPCR.

Gene	Life Technologies Assay ID
RPS18	Hs01375212_g1
CTNNB1	Hs00355049_m1
HOXA1	Hs00939046_m1
HOXA2	Hs00534579_m1
HOXA3	Hs00601076_m1
HOXA4	Hs01573270_m1
HOXA5	Hs00430330_m1
HOXA6	Hs00430615_m1
HOXA7	Hs00600844_m1
HOXA9	Hs00266821_m1
HOXA10	Hs00172012_m1
HOXA11	Hs00194149_m1
HOXB1	Hs00157973_m1
HOXB2	Hs00609873_g1
HOXB4	Hs00256884_m1
HOXB5	Hs00357820_m1
HOXB6	Hs00980016_m1
HOXB7	Hs00270131_m1
HOXB8	Hs00256885_m1
HOXB9	Hs00256886_m1
HOXC4	Hs00205994_m1
HOXC5	Hs00232747_m1
HOXC6	Hs00171690_m1
HOXC8	Hs00224073_m1
HOXC9	Hs00396786_m1
HOXC10	Hs00213579_m1
HOXC11	Hs00204415_m1
HOXC12	Hs00545229_m1
HOXD1	Hs00707081_s1
HOXD3	Hs00232506_m1
HOXD4	Hs00429605_m1
HOXD8	Hs00251905_m1
HOXD9	Hs00610725_g1
HOXD10	Hs00157974_m1
HOXD11	Hs00360798_m1
HOXD12	Hs00706957_s1

Supplemental Experimental Procedures

Mass spectrometry

Pelleted cells were lysed in 8 M urea, 50 mM Tris (pH 8.0), 50 mM sodium chloride, and protease inhibitor (Roche) by glass bead milling (Retsch) for 40 min (8 cycles of 4 min shaking, 1 min resting). The lysate was centrifuged at 10,000 x g for 5 min to remove cell debris. Protein content was measured using a BCA assay (Thermo Pierce). Disulfide bonds were reduced and alkylated using 5 mM dithiothreitol and 10 mM iodoacetamide. The alkylating reaction was then quenched with 5 mM dithiothreitol. Samples were diluted to 1.5 M urea and digested with trypsin (Promega) overnight at a 1:100 enzyme to protein ratio. Peptides were then desalted using tC18 solid-phase extraction columns (SepPak, Waters) and dried under vacuum.

Peptides were labeled with TMT 10plex isobaric labeling reagents (Thermo Scientific). Briefly, 0.8 mg of reagent was re-suspended in 50 uL acetonitrile and added to 200 µg peptides re-suspended in 100 µL of 200mM TEAB, and shaken for 2 h. To ensure each sample contributed the same amount of protein, aliquots of each sample were mixed at a 1:1:1:1:1:1:1:1:1 ratio (9plex) according to BCA assay results and reporter ions from this mixture were used to inform the subsequent mixture ratio. Labeling reactions were then quenched with hydroxylamine. Samples were mixed according to reporter ion ratios, desalted, and dried under vacuum.

Peptides were re-suspended in reverse phase buffer A (20 mM ammonium formate; pH10). Buffer B contained 20 mM ammonium formate in 80% acetonitrile at pH 10. Separations were performed using a Surveyor liquid chromatography pump (Thermo Scientific) with a Gemini C18 column (250 mm x 4.60 mm; Phenomenex). Peptides were eluted using the following gradient: 0-4 min, 100% buffer A, 4-8 minutes 2-16% buffer B, 8-12 minutes 16-30% buffer B, 12-32 minutes 30%-55% buffer B, all at a flow rate of 0.8 mL min^{-1}. Samples were collected from 10 to 40 min. Finally, the column was held at 100% buffer B for 10 min and then

re-equilibrated. Fractions were dried under vacuum and re-suspended in 0.2% formic acid for mass spectrometry analysis.

LC-MS was performed on a Thermo Orbitrap Fusion coupled to a nanoAcquity UPLC (Waters). Mobile phase A comprised water, 0.2% formic acid, and 5% DMSO and mobile phase B comprised acetonitrile, 0.2% formic acid, and 5% DMSO. Peptides were separated on a 75 µm inner diameter fused silica capillary packed with 1.7 um diameter, 130 A pore size Bridged Ethylene Hybrid C18 particles (Waters) heated to 60 °C. Samples were loaded onto the column for 12 min at 0.35 µl/min. Mobile phase B increased to 4% at 0.1 min, 12% at 32 min, 22% at 60 min, and 30% at 70 min. The column was then washed for 5 minutes at 70% B and re-equilibrated for 20 min at 0% B. Eluting peptides were converted to gas phase ions by electrospray ionization.

The mass spectrometer instrument method consisted of isolation at 0.7 Th with the quadrupole, HCD fragmentation with normalized collision energy of 37, and analysis with the orbitrap at 60K resolution at 200 *m/z*. Automatic Gain Control targets were set at 10^5 with max injection time of 60 ms. Precursors with a charge state of 2-8 could be sampled for MS (Nesvizhskii and Aebersold, 2005). Monoisotopic precursor selection was enabled. Dynamic exclusion was set to 30 s with a 10 ppm tolerance around the precursor.

Raw data were analyzed using Coon OMSSA Proteomic Analysis Software Suite (COMPASS) (Wenger et al., 2011). Briefly, DTA files were generated by extracting peak information from .Raw files. Spectra were searched against Uniprot human reference proteome using Open Mass Spectrometry Search Algorithm. Search parameters include a 75 ppm precursor mass tolerance, 0.03 da product ion tolerance, and up to three missed cleavages. Carbamidomethylation of cysteines and TMT modification of lysines and the N terminus were set as fixed modifications while oxidation of methionines was set as a variable modification. Peptides and proteins were filtered to a 1% FDR. Peptide and protein quantification was performed using TagQuant as described previously. Briefly, extracted reporter ion intensities

were purity corrected and normalized such that the total signal from each channel is equal. A list of all detected proteins is provided in Table S1.

Flow cytometry

Flow cytometry was conducted as previously described (Lippmann et al., 2014). Primary antibodies are listed in Table S2. Samples were run on a FACSCalibur (BD Biosciences) and data were analyzed using Cyflogic software. Positive events were quantified by gating above the top 1% of species-matched IgG controls.

Immunocytochemistry

Immunocytochemistry was conducted as previously described (Lippmann et al., 2014). Primary antibodies are listed in Table S2. Samples were visualized on a Nikon Ti-E epifluorescence microscope or a Nikon A1R confocal microscope. For $HOXD10^+$ cells in Figure 3D and Figure S3E, positive labeling was quantified relative to DAPI nuclear stain by manual counting using ImageJ software. For $FOXP1^+$ motor neurons in Figure 4B, images were quantified by manually counting the number of $ISL1^+$ or $HB9^+$ cells that co-expressed FOXP1 per field using ImageJ. For HOXB1 and HOXB4 in Figure 3C, positive labeling was quantified relative to DAPI using CellProfiler software (Carpenter et al., 2006). HOXB1 and HOXB4 expression were quantified within $HB9^+$ motor neurons in Figure 4A by manual counting using ImageJ software.

RT-PCR and qPCR

Total RNA was extracted from cultured cells using Trizol (Life Technologies) according to the manufacturer's instructions. DNase (Roche) was used to eliminate genomic DNA during the isolation process. After isolation, 1-5 µg of total RNA was immediately subjected to reverse transcription using the Thermoscript RT-PCR kit (Life Technologies) in a 20 µL reaction according to the manufacturer's instructions. Resultant cDNA was diluted and utilized for RT-PCR (conducted as previously described (Lippmann et al., 2014); see Table S3 for primer sequences) or qPCR on a BioRad CFX96 detection unit using Taqman Gene Expression

Master Mix (Life Technologies) and Taqman primers (Life Technologies; see Table S4 for primer list). $\Delta\Delta C_t$ values for each gene were calculated relative to *RPS18* and converted to fold difference assuming 100% primer efficiency.

Electrophysiology

Cells were moved to a recording chamber perfused with a bath solution containing 128 mM NaCl, 5 mM KCl, 25 mM HEPES, 30 mM D-glucose, 1 mM $MgCl_2$ and 2 mM Ca^{2+} (pH 7.4, adjusted to 300-310 mOsm with D-glucose). Borosilicate glass pipettes were pulled to a resistance of 4-6 MΩ and filled with an intracellular solution (pH 7.4) containing 130 mM K-gluconate, 10 mM HEPES, 5 mM Na-phosphocreatine, 2 mM Mg-ATP, 1 mM EGTA, and 0.3 mM Na-GTP. All experiments were conducted at room temperature. Whole-cell patch-clamp recordings were made with an Axon MultiClamp 700b amplifier (Molecular Devices). Target cells were identified visually by their neuron-like morphology. Typical seal resistances after achieving the patch were 15MΩ, neurons showing large (+10%) fluctuations in seal quality over the course of an experiment were excluded from the analysis. Measurements of resting membrane potentials (RMPs) were made immediately after achieving whole-cell mode. Liquid junction potentials were not corrected. To measure action potentials, membrane voltage was monitored in response to 500ms current injections, beginning at -100pA, and increasing in 10pA increments to 500pA (or when the membrane response to successive injections began to clearly plateau). Rapidly depolarizing spikes crossing 0mV were considered APs. Cells were held in voltage clamp at -60mV for measurement of spontaneous synaptic activity. To measure whole-cell currents, cells were held at -60mV and current responses were measured following 500ms voltage steps incrementing from -110mV to 110mV in 10mV steps.

Supplemental References

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