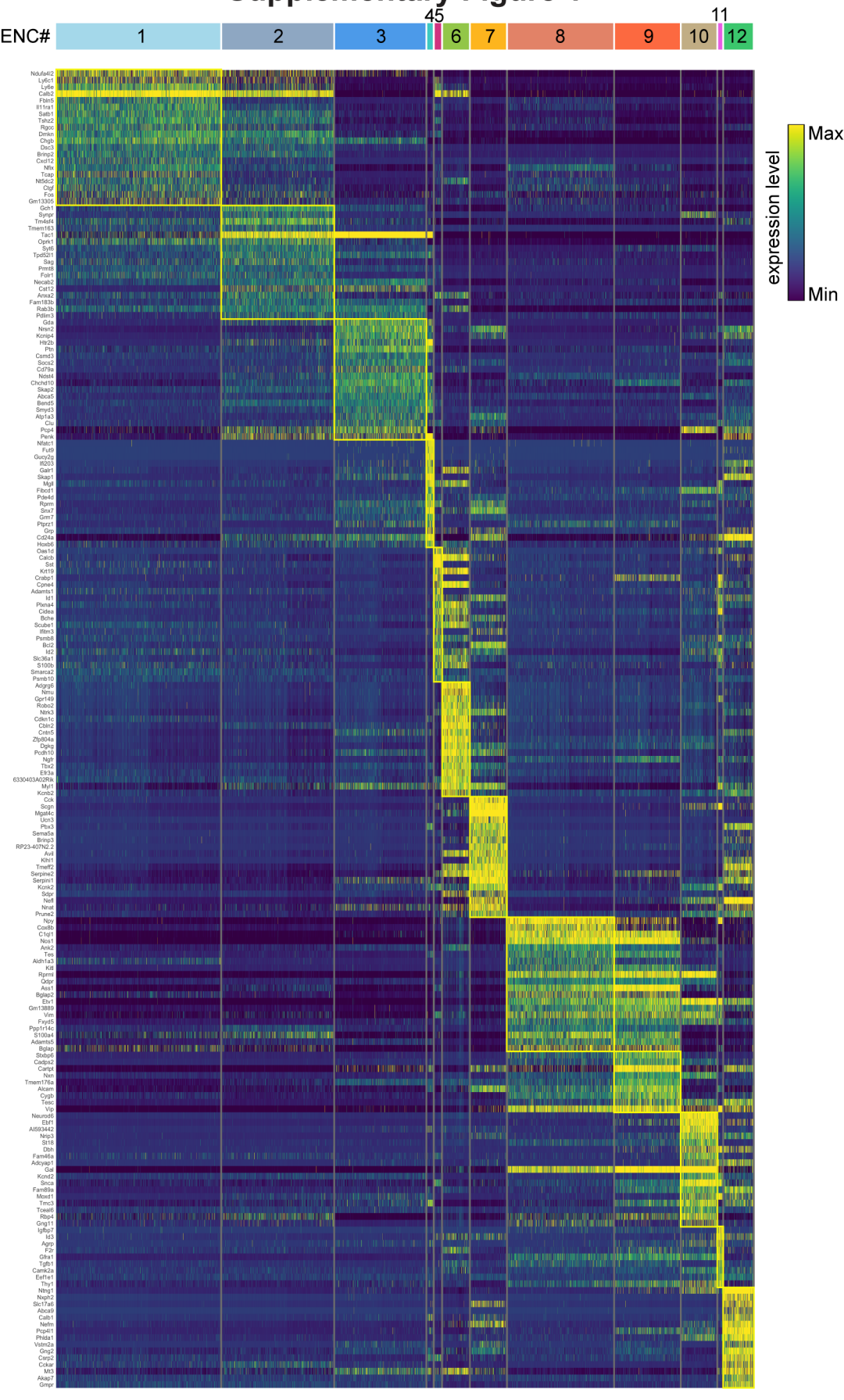
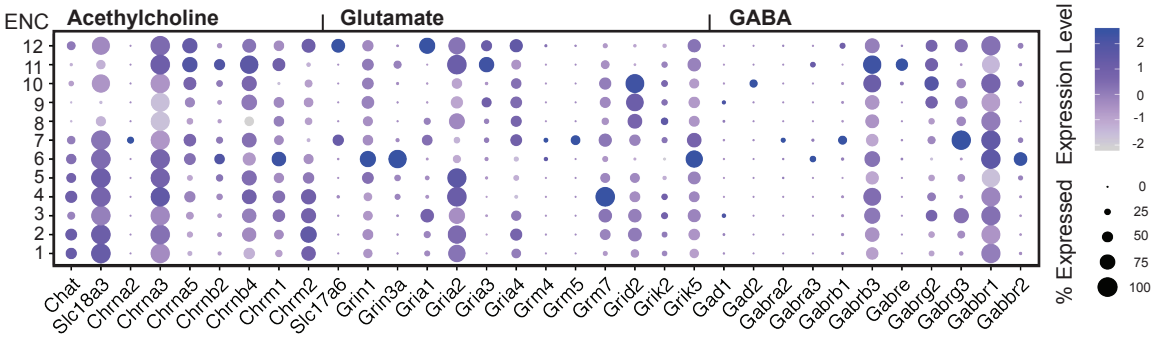
Supplementary Figure 1

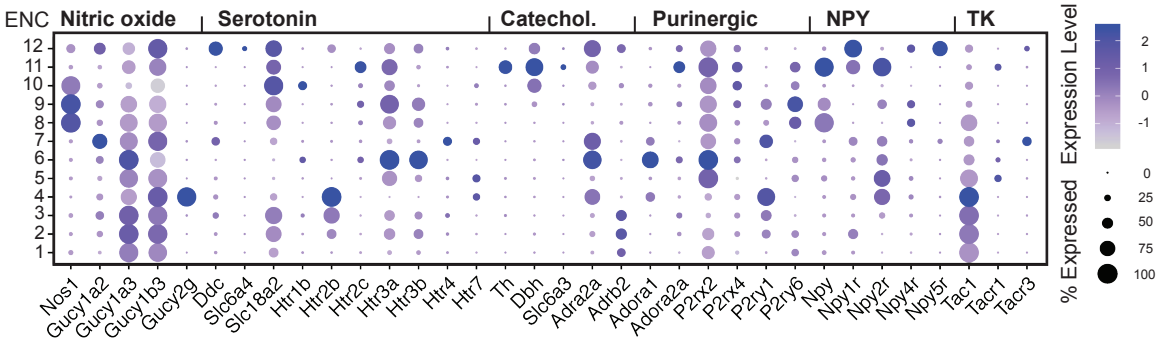


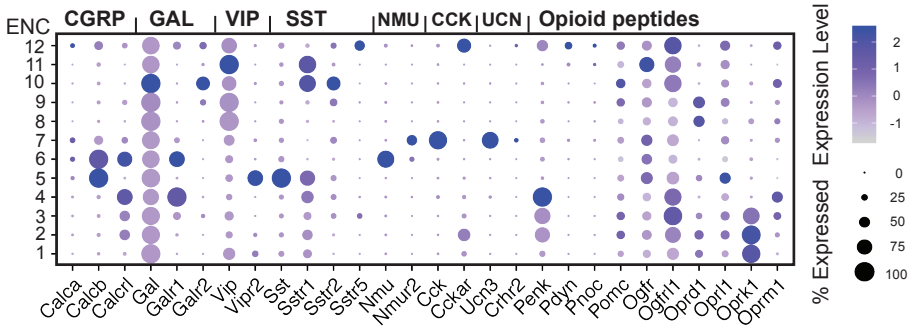
Supplementary Figure 1: Heatmap representing top-20 (by average logFC) differentially expressed genes of each ENC. Related to Figure 1.
ENC: Enteric Neuron Class

Supplementary Figure 2

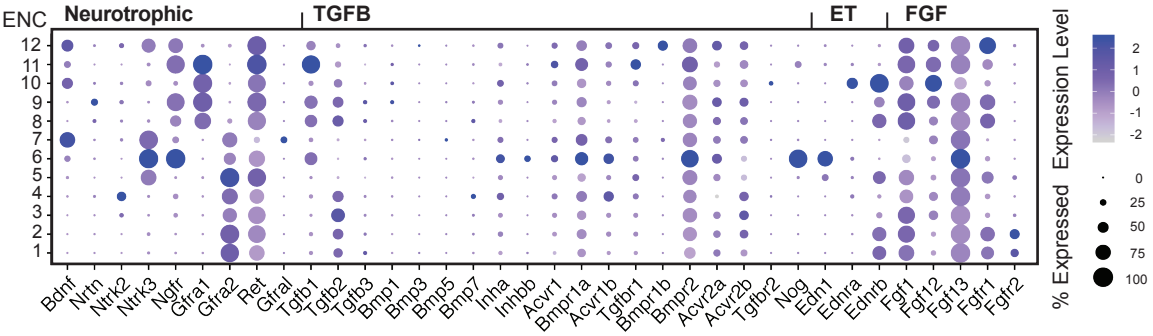
a) Neurotransmission

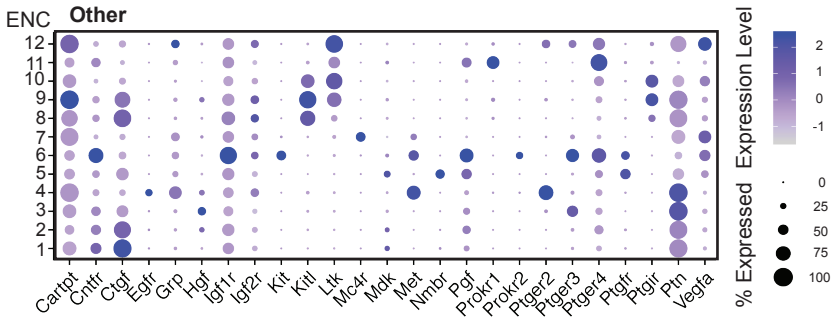




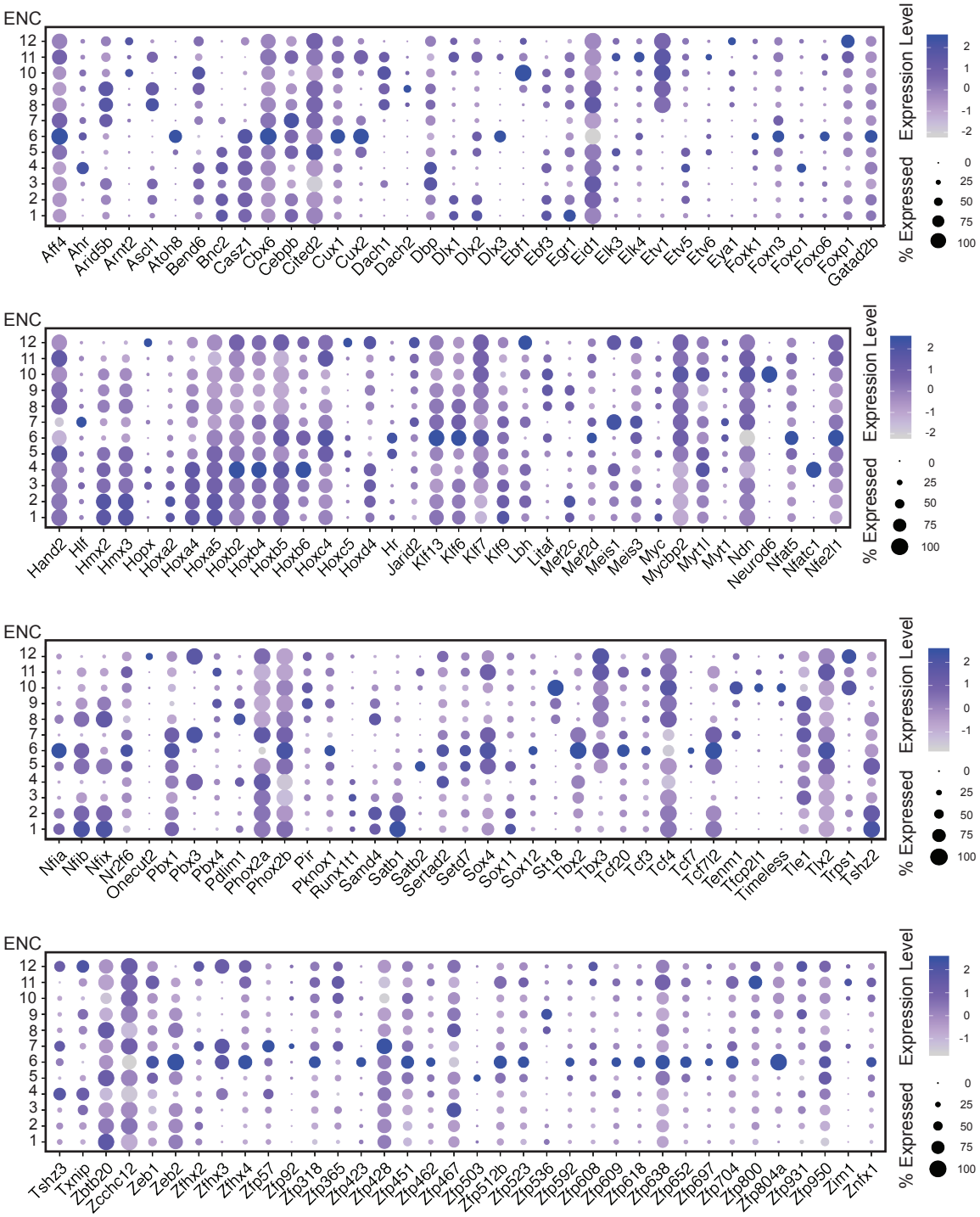


b) Cell-Cell Signaling

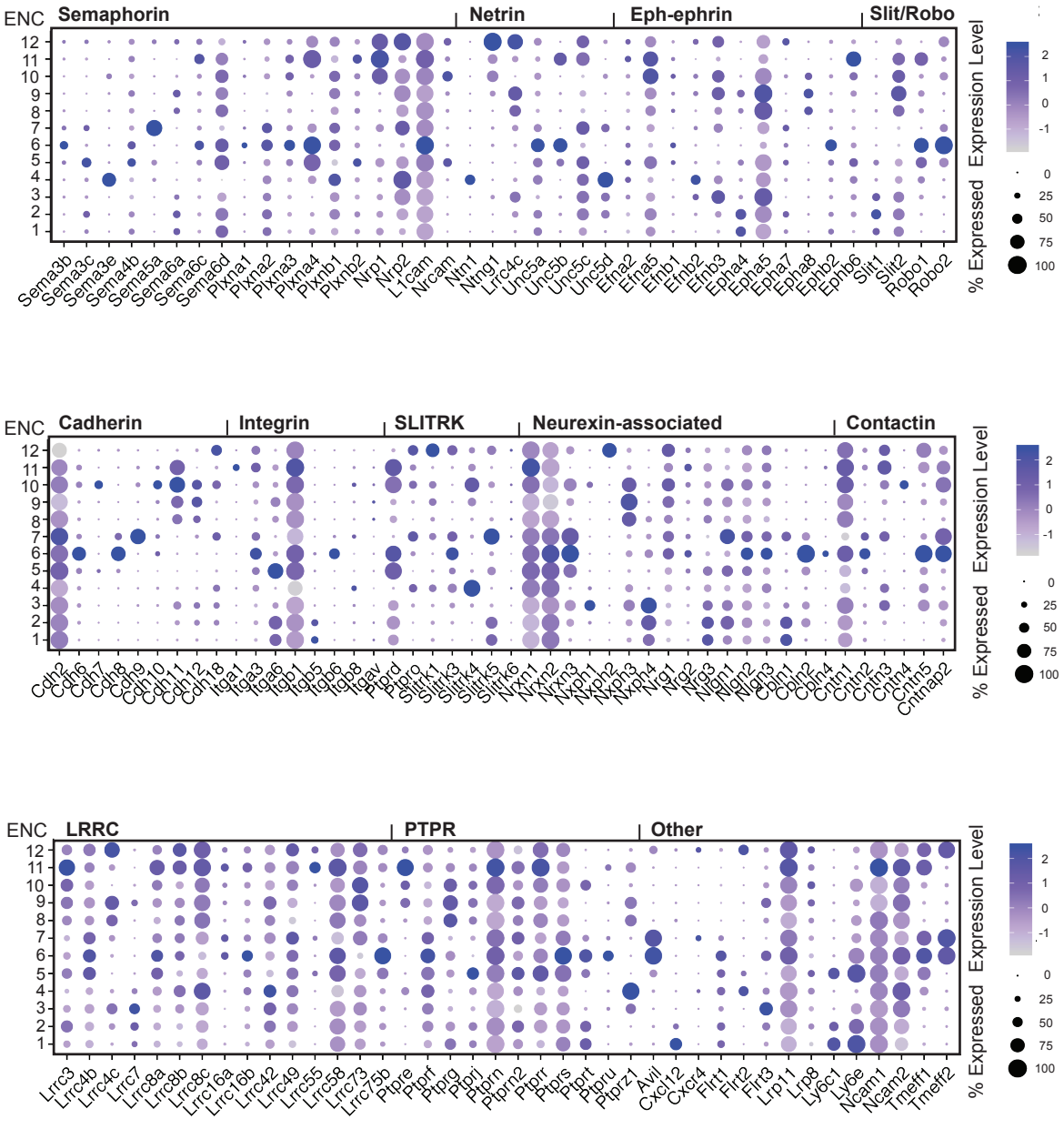




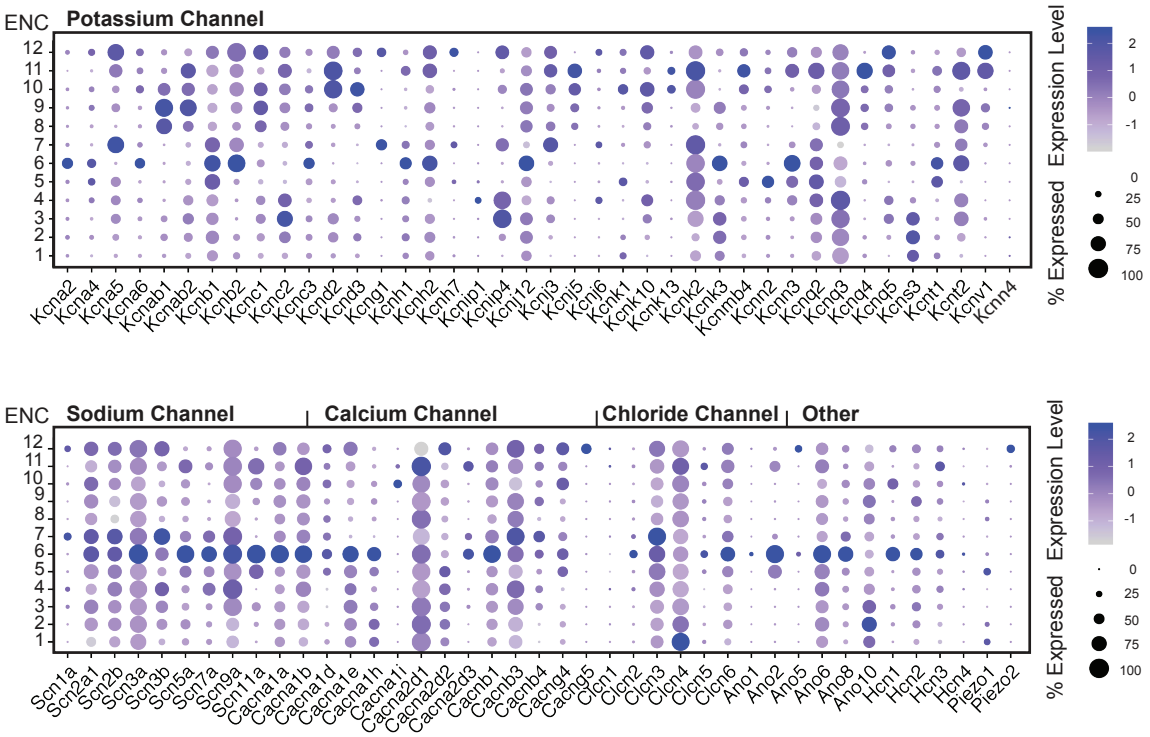
c) Transcription Factors



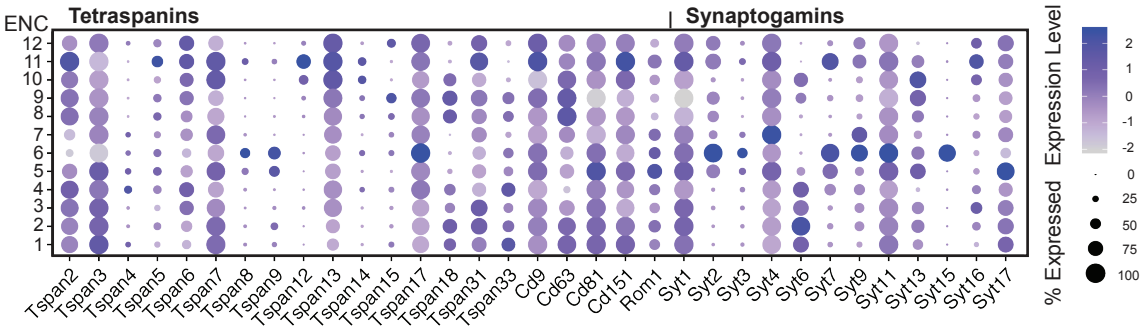
d) Adhesion Molecules



e) Ion Channels

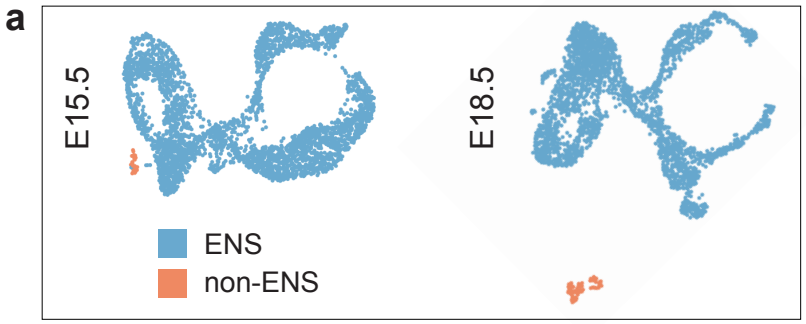


f) Membrane Trafficking



Supplementary Figure 2. Dot plots displaying expression of genes conferring neuronal phenotypes in ENC1-12.
Gene categories involved in a) neurotransmission, b) other cell-cell signaling, c) transcription, d) adhesion e) ion transport, and f) membrane trafficking. Color scale represents z-score and dot size represent percent of cells with non-zero expression within a given class.
ENC: Enteric Neuron Class; GABA: Gamma-aminobutyric acid; NPY: Neuropeptide Y; TK: Tachykinin; CGRP: Calcitonin gene-related peptide; GAL:Galanin;VIP: Vasoactive intestinal peptide; SST: Somatostatin; NMU: Neuromedin U; CCK: Cholecystokinin; UCN: Urocortin; TGFB: Transforming growth factor beta; ET:Endothelin; FGF: Fibroblast growth factor; LRRC: Leucine Rich Repeat Containing; PTPR: Protein tyrosine phosphatase receptor.

Supplementary Figure 3



b

Morarach, Mikhailova et al	
non-ENS (E15.5)	non-ENS (E18.5)
Col1a1	Col1a1
Lum	Ifitm1
Col6a1	Lum
Tgfbi	Tgfbi
Ifitm1	Nkx2-3
Nkx2-3	Col6a3
Foxf1	Meis2
Tcf21	Foxf1
Htra3	Kcnj8
Islr	Abi3bp
Aldh1a2	Tgm2
Col6a3	Smoc2
Tgm2	Tcf21
Colec10	Tmem45a
Wisp1	Igf1
Hlx	Oaf
Gm266	Col6a1
Tmem45a	Mylk
Foxf2	Aspn
Aspn	Mgp
Pdgfra	Spon2
Angptl1	Ogn
Smoc2	Gm11627
Fendrr	Loxl1
Dcn	Colec10
Ogn	Col6a2
Kcnj8	Pamr1
Oaf	Ptgis
Rgs5	Col1a2
Shisa3	Anxa1
Cxcl12	Wisp1
Bmp4	Ccdc80
Cped1	Tnfrsf11b
Col3a1	Gstm2
Ccdc80	Pltp
Col14a1	Plagl1
Snai2	Sparcl1
Ptrf	Lmod1
Mylk	Htra3
Il33	Foxf2
Mgp	Lox
Pitx2	Gm266
Igfbp7	Dcn
Colec12	Plac8
Gm11627	Hlx
Adamdec1	Cped1
Crispld2	Mmp23
Igf1	Lrrc17
Sdpr	Rbpms
Lrrc17	Angptl1

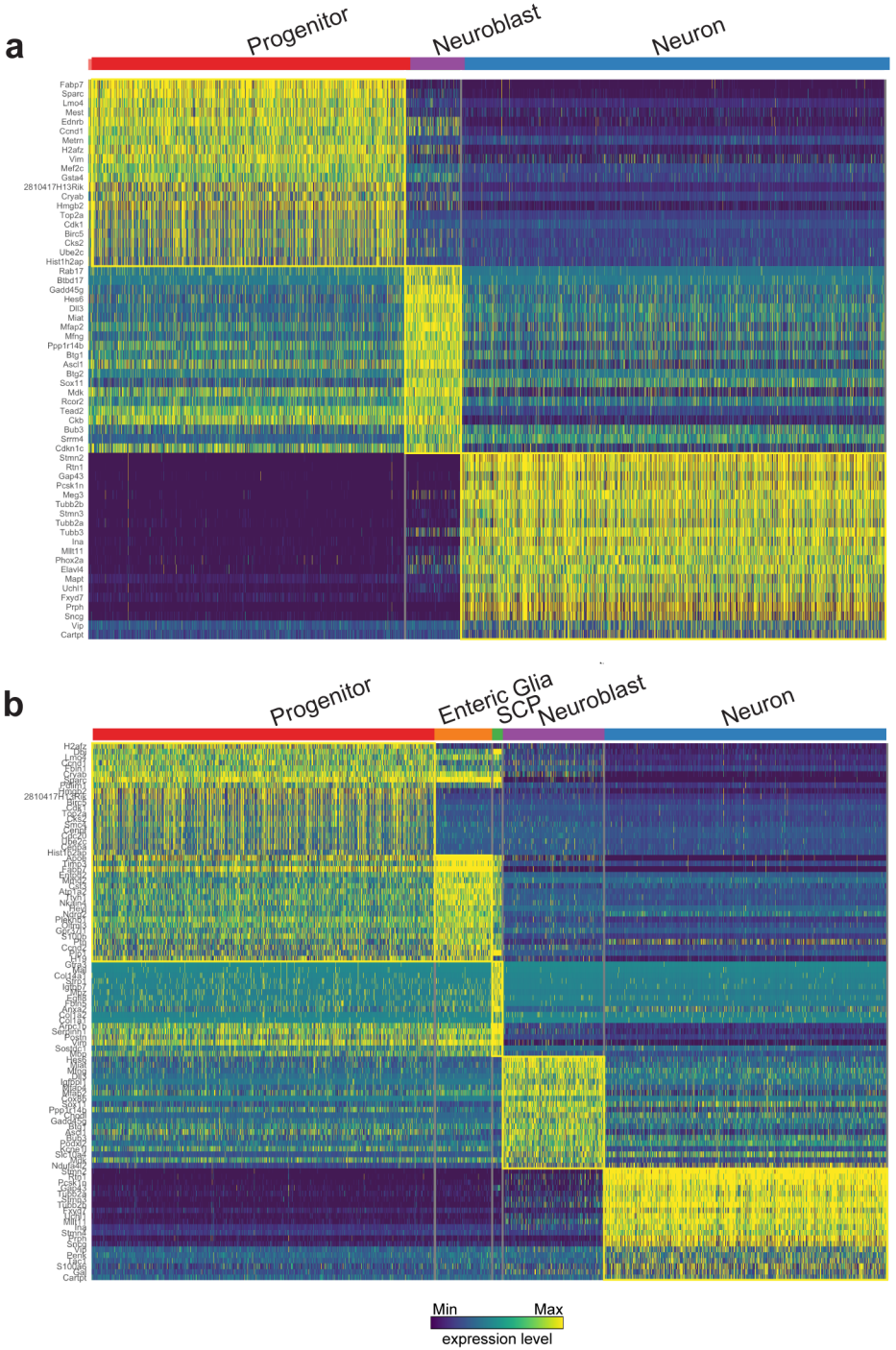
c

Ling and Sauka-Spengler, 2019
Chicken non-ENS cells (HH18)
Prrx1
Twist1
Col1a2
Cytl1
Dcn
Cald1
Lum
Cdh11
Ebf3
Id1
Olfml3
Gja1
Mfap2
Colec12
Prrx2
Lpar1
Ednra
Mmp2
Six1
Socs2
Cg-16
Snai1
Wnt5a
Tpm1
Ebf2
Dlx2
Col4a1
Limd2
Mab21l1
Mdk
Vegfd
Inka1
Sparc
Sema3f
Homer2
Selenoh
Rps2
Serbp1
Fkbp7
Fbln1
Fstl1
Plpp3
Prelid1
Fn1
Vcan
Dlx1
Arid3a
Gpc4
Meis2
Elk3

Supplementary Figure 3: Non-ENS populations are fibroblast-like cells.
a) Non-ENS versus ENS cells indicated on UMAPs at E15.5 and E18.5.
b) Top 50 differentially expressed genes at each stage.The gene expression profiles indicate a precursor stage of previously published ENMFB[1] cells (*Dpt+/Spon2+/Lum+/Dpt+*). Together, the data is in agreement with a contribution of mesenchymal fibroblast-like cells from neural crest. c) Table with top 50 differentially expressed genes in neural crest-derived mesenchymal cells captured from the developing chicken gut[2] at HH18 (approximately E11.5 in the mouse). Genes that were enriched in our non-ENS populations are marked in orange (46% of the genes). HH: Hamburger-Hamiton stage; UMAP: Uniform Manifold Approximation and Projection.

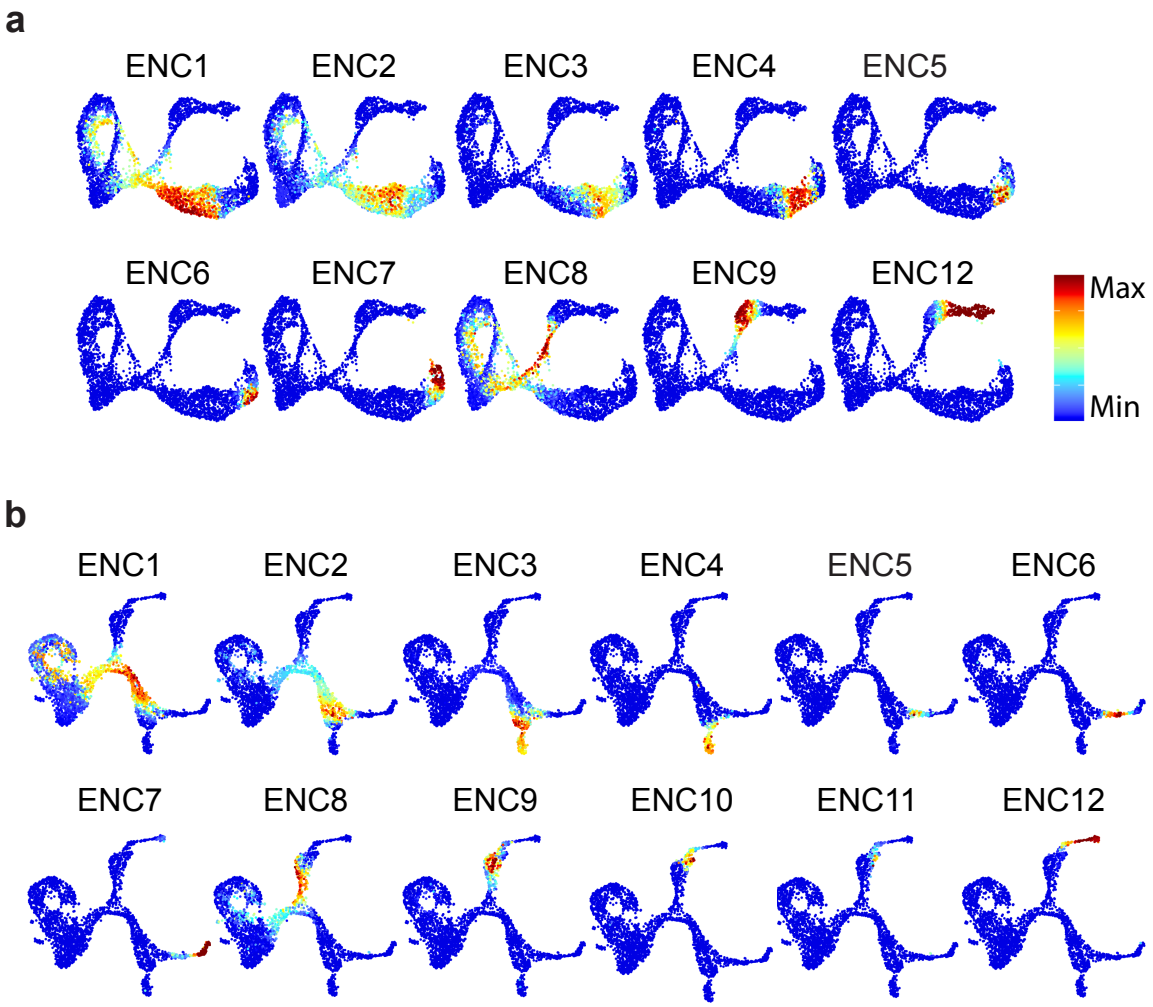
References:
1) Zeisel, A. et al. Molecular Architecture of the Mouse Nervous System. Cell 174, (2018)
2) Ling, ITC and Sauka-Spengler, T. Early chromatin shaping predetermines multipotent vagal neural crest into neural, neuronal and mesenchymal lineages. Nat Cell Biol 12 (2019)

Supplementary Figure 4



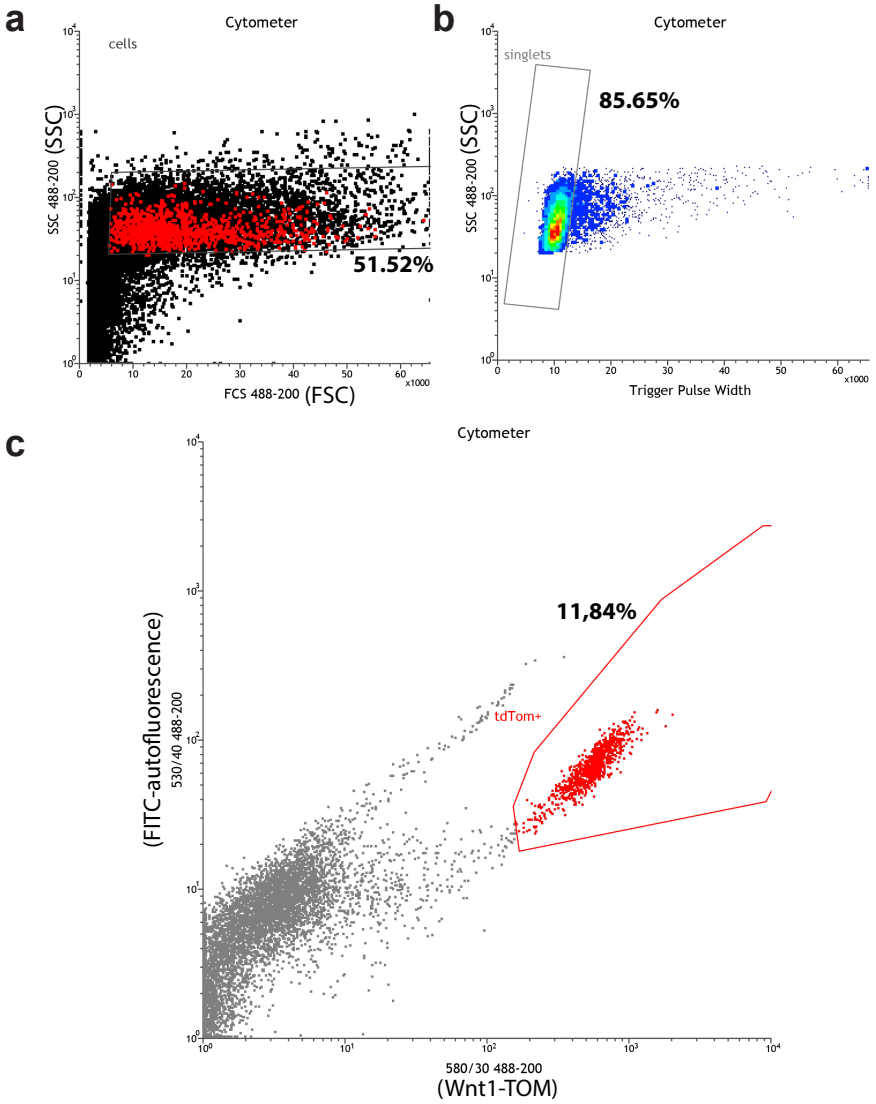
Supplementary Figure 4: Heatmaps showing top marker genes for clusters representing generic cell states of the developing ENS.
Related to Figure 5a and b. a) E15.5 b) E18.5. SCP: Schwann Cell Precursor

Supplementary Figure 5



Supplementary Figure 5: Individual identity transfers of ENC1-12 onto developing ENS. Prediction scores shown on UMAPs at **a)** E15.5 and **b)**18.5**.** Related to Figure 6a and b. UMAP: Uniform Manifold Approximation and Projection

Supplementary Figure 6



Supplementary Figure 6: Representative Flow Cytometry Data Plots.
Plots show gating strategy for sorting TOM+ cells from *Wnt1-Cre;R26R-Tom* mice at E18.5. a) Forward vs Side Scatter plot gating on cells b) Side Scatter vs Trigger Pulse Width to gate out doublets and multiplets c) green fluorescence (530/40) vs red fluorescence (580/30) to distinguish true TOM+ cells from autofluorescent cells. Sorted cells are shown in red. SSC: Side-scattered light; FSC: Forward-scattered light

SUPPLEMENTARY TABLE 3

Primary Antibodies used in the study

Target	Host	Dilution	Source	Comment:
5-HT	Goat	1:500	Abcam ab66047	
ANO1	Rabbit	1:1,000	Abcam ab64085	
ANO2	Mouse	1:1,000	Santa Cruz sc-390956	
CALB	Rabbit	1:1,000	Chemicon AB1778	recognises Calretinin
CALB	Rabbit	1:500	Swant CB38a	recognises Calretinin
CALB	Mouse	1:500	Swant CB300	
CALB	Goat	1:500	R&D AF3320	recognises Calretinin
CALR	Mouse	1:500	Santa Cruz sc-365956	
CALR	Rabbit	1:1,000	Swant 7696	
CCK	Rabbit	1:300	Abcam ab83180	enhanced with colchicine
CCK	Rabbit	1:16,000	Immunostar 20078	enhanced with colchicine
CGRP	Goat	1:1,500	AdBsero 1720-9007	enhanced with colchicine
CGRP	Rabbit	1:5,000	Sigma C8198	enhanced with colchicine
DBH	Rabbit	1:1,000	Immunostar 22806	
ENK	Rabbit	1:3-6,000	St John's STJ98660	
ENK	Mouse	1:300	Abcam ab150346	
FUT9	Rabbit	1:300	Novus Bio NBP2-57960	
GAD2	Rabbit	1:50,000	Kind gift from J.Kaltschmidt; see Betley et al, Cell, 2009 https://doi.org/10.1016/j.cell.2009.08.027	
GAL	Goat	1:200	Abcam ab99452	Weak staining in cell bodies
GDA	Rabbit	1:1000	Atlas Antibodies HPA024099	
GFP	Goat	1:1,000	Abcam ab6662	FITC-conjugated
HuC/D	Mouse	1:300	Molecular Probes A21271	
NDUFA4L2	Rabbit	1:200	St John's STJ94374	Weak staining in cell bodies
NDUFA4L2	Rabbit	1:5,000	St John's STJ116500	Weak staining in cell bodies
NEUROD6	Rabbit	1:100	Abcam ab85824	
NF-M	Mouse	1:500	Abcam ab7794	
NMU	Mouse	1:200	Santa Cruz sc-398600	Variable weak
NMU	Rabbit	1:200	St John's STJ116312	Variable weak, only on sections
NOS1	Goat	1:1,000	Abcam ab1376	
NOS1	Rabbit	1:200	Santa Cruz sc-648	
NOS1	Mouse	1:300	Santa Cruz sc-5302	
NPY	Rabbit	1:3,000	Immunostar 22940	
NTNG1	Rabbit	1:200	Abcam ab221456	
NTNG1	Mouse	1:400	Santa Cruz sc-271774	
NXPH2	Rabbit	1:200	Atlas Antibodies HPA034759	
PBX1	Rabbit	1:1000	Cell Signaling #4542	
PBX3	Rabbit	1:200	Sigma AV32070	
PGP9.5	Rabbit	1:1,000	ThermoFisher PA5-29012	

PGP9.5	Mouse	1:300	Novus Bio NB600-1160	
PIEZO2	Rabbit	1:200	LifeSpan LS-C180178	mainly in processes
Pro-CCK	Rabbit	1:800	Frontiers Institute AB2571674	Extremely weak
RFP/Tomato /Ruby	Rat	1:1,000	Chromotek, 5F8	
RPRML	Rabbit	1:3,000	Atlas Antibodies HPA062668	
SOM	Rat	1:300	Merck MAB354	
Sox10	Goat	1:2,000	R&D Systems AF2864	
Sox2	Rabbit	1:4,000	Seven Hills WRAB-1236	tissue becomes dotty
Substance P	Rabbit	1:1,000	Chemicon 1566	
Substance P	Rat	1:100	Biorad 8450-0505	
TH	Rabbit	1:500	Pelfreeze P40101	
TH	Sheep	1:300	Novus Bio NB300-110	
UCN3	Mouse	1:200	Santa Cruz sc-517449	strong background
UCN3	Rabbit	1:1,000	Biomatik CAU23545	
VGLUT2	Guinea pig	1:5,000	Millipore AB2251	Enhanced by colchicine
VIP	Rabbit	1:200	AbD Serotec 9535-0204	

Good staining
OK staining
Weak staining
Unspecific/undetected staining

Secondary antibodies used in the study

Alexa Fluor 488/FITC (dilution 1:400)	**Alexa Fluor 555/594 (dilution 1:1,000)**	**Alexa Fluor 647/Cy5 (dilution 1:250)**
Donkey anti-goat 488 ThermoFisher A11055	Donkey anti-goat 555 ThermoFisher A21432	Donkey anti-goat 647 ThermoFisher A21447
Donkey anti-guinea pig 488 Jackson 706-545-148	Goat anti-guinea pig 555 ThermoFisher A21435	Donkey anti-guinea pig Cy5 Jackson 706-175-148
Donkey anti-mouse 488 ThermoFisher A21202	Donkey anti-mouse 555 ThermoFisher A31570	Donkey anti-mouse 647 ThermoFisher A31571
Goat anti-mouse IgG1 488 ThermoFisher A21121	Goat anti-mouse IgG1 555 ThermoFisher A21127	Goat anti-mouse IgG1 647 ThermoFisher A21240
Goat anti-mouse IgG2a 488 ThermoFisher A21131	Goat anti-mouse IgG2a 555 ThermoFisher A21137	Goat anti-mouse IgG2a 647 ThermoFisher A21241
Goat anti-mouse IgG2b 488 ThermoFisher A21141		Goat anti-mouse IgG2b 647 ThermoFisher A21242
Rat anti-mouse IgG1 FITC ThermoFisher 11-4015-82		
Donkey anti-rat 488 ThermoFisher A21209	Donkey anti-rat 594 ThermoFisher A21208	Goat anti-rat 647 Cell Signaling 4418S
Donkey anti-rabbit 488 ThermoFisher A32790	Donkey anti-rabbit 555 ThermoFisher A31572	Donkey anti-rabbit 647 ThermoFisher A31573
Donkey anti-sheep 488 ThermoFisher A11015	Donkey anti-sheep 555 ThermoFisher A21436	