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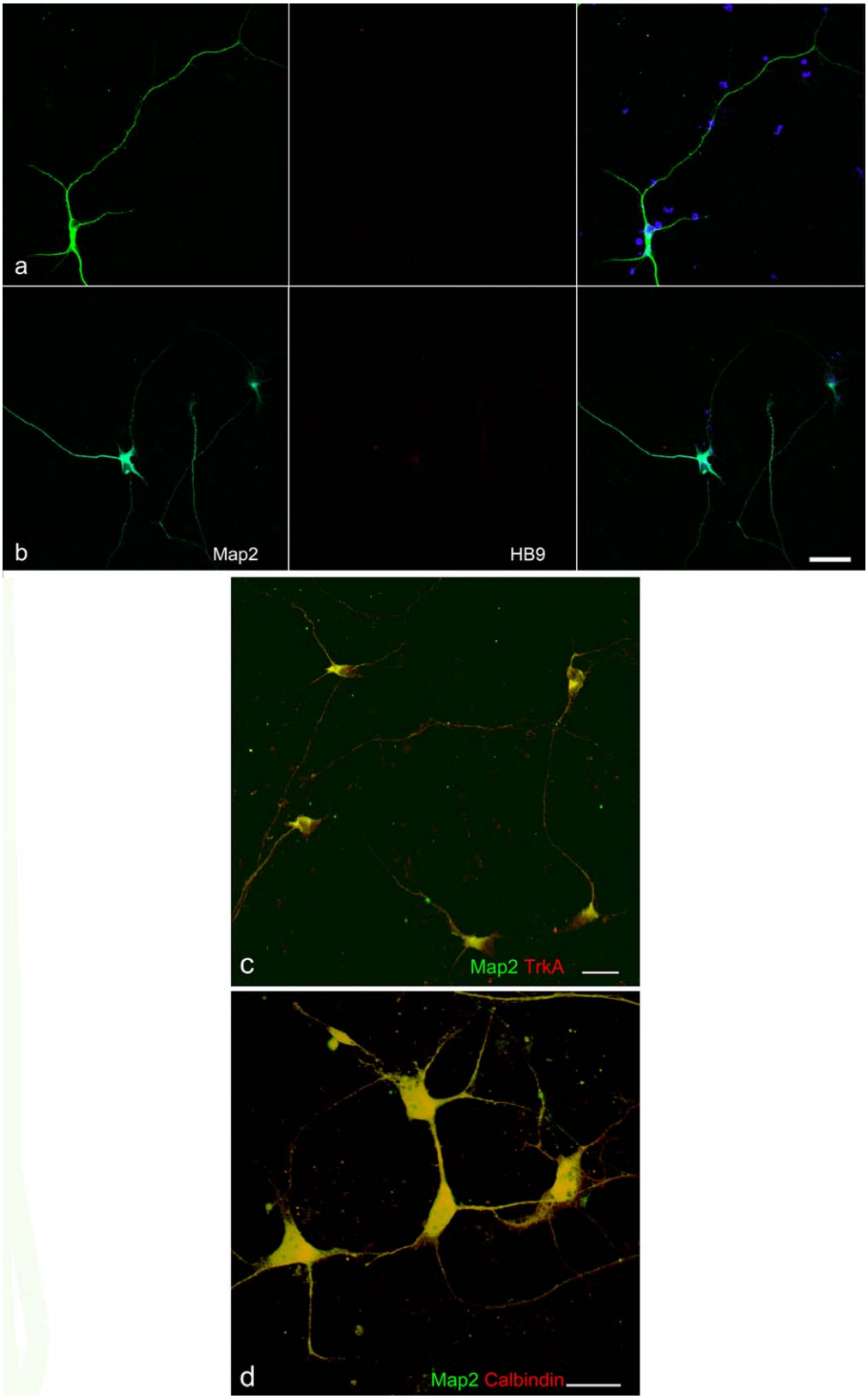
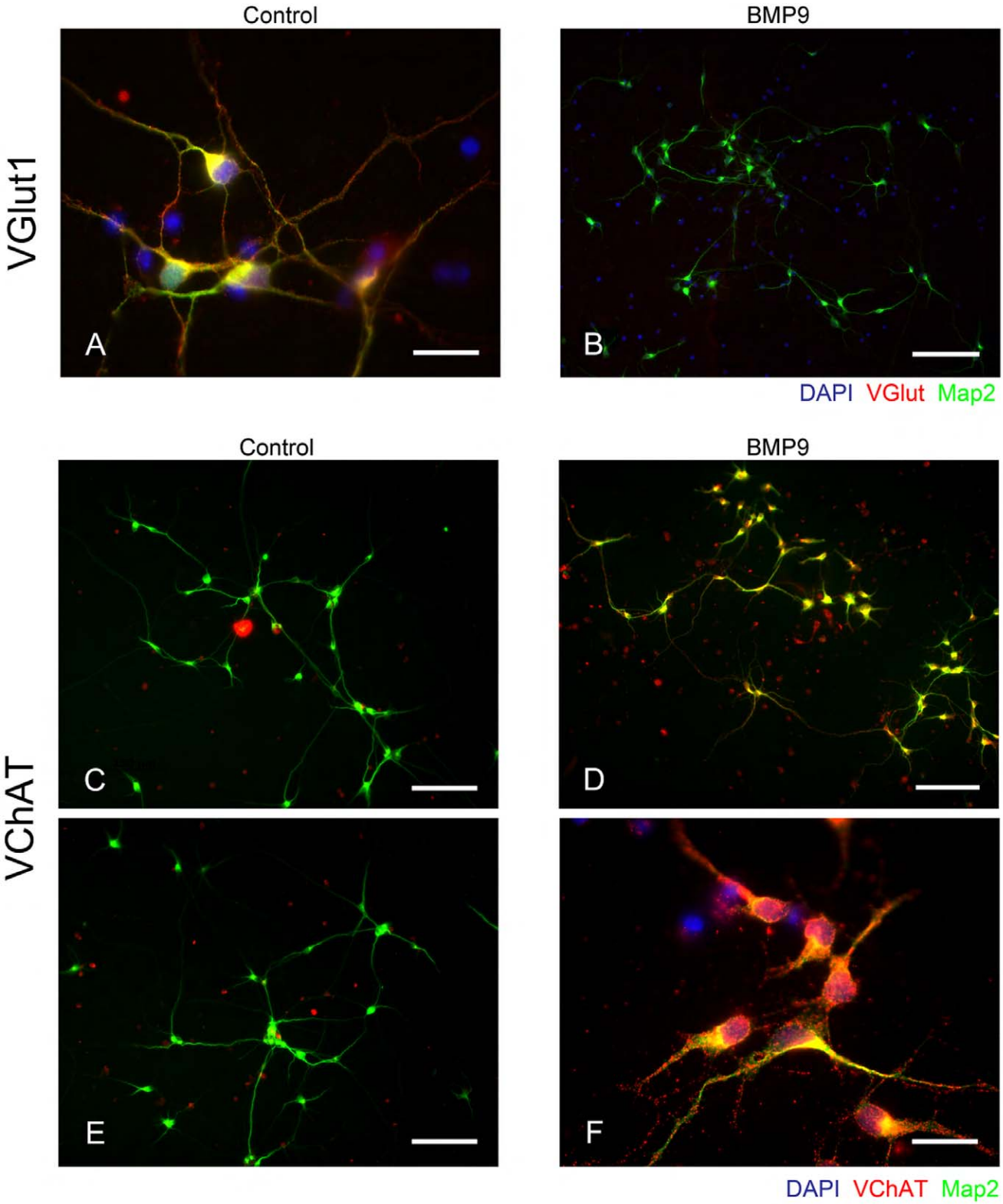


Supplemental Figure 1 Additional Histochemistry

a,b: At maximum 594nM laser intensity on a confocal microscope, presumptive BFCN generated through BMP9 treatment are not HB9 [HLXB9] immunopositive, indicating that they are not cholinergic motor neurons. Scale bar =20μM

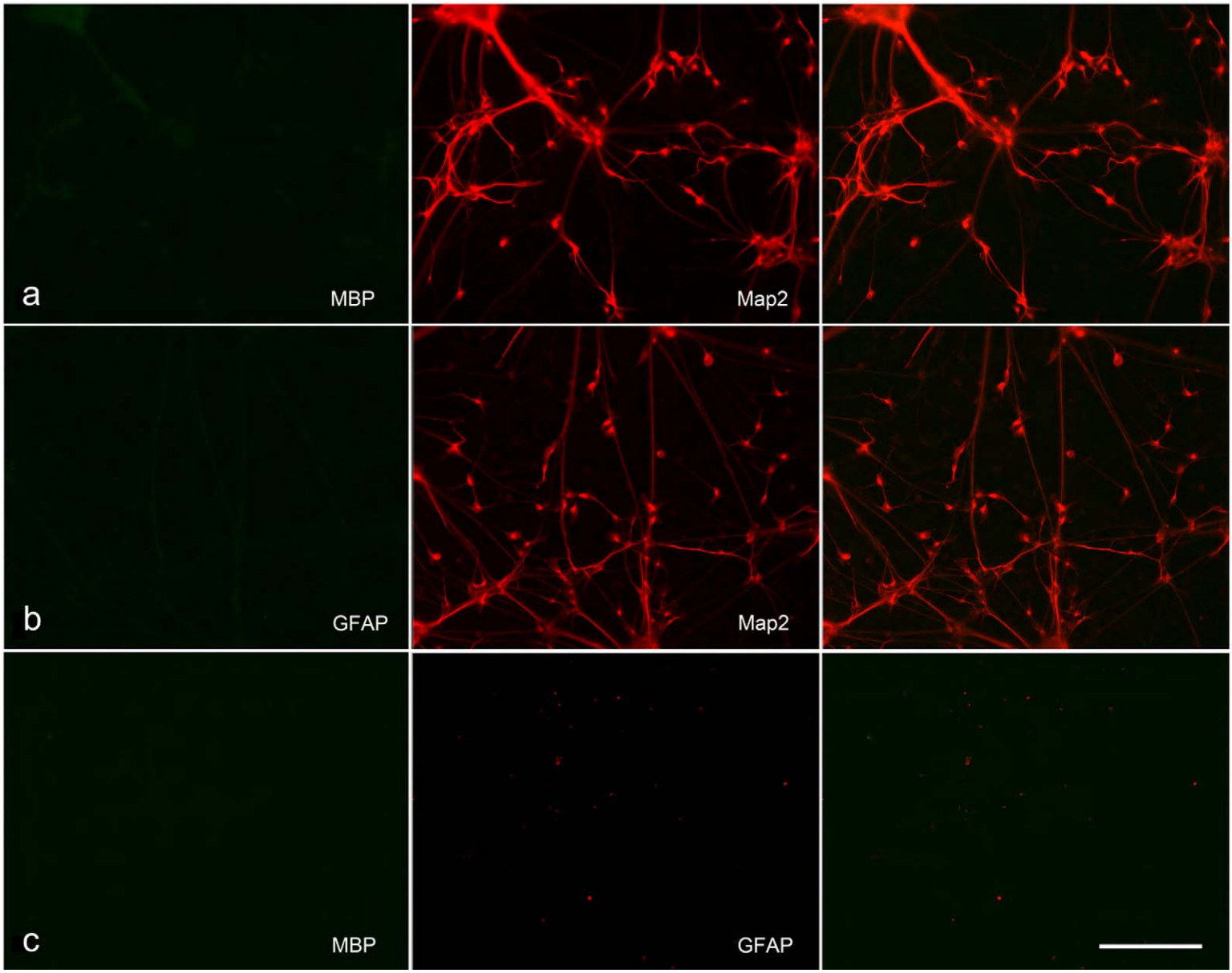
c: Confocal analysis shows the neurons to express high levels of TrkA, a marker specific in the cortex to the BFCN population. Scale bar =20μM

d: Confocal analysis shows the neurons to express high levels of calbindin, a marker expressed in BFCN. Scale bar =20μM



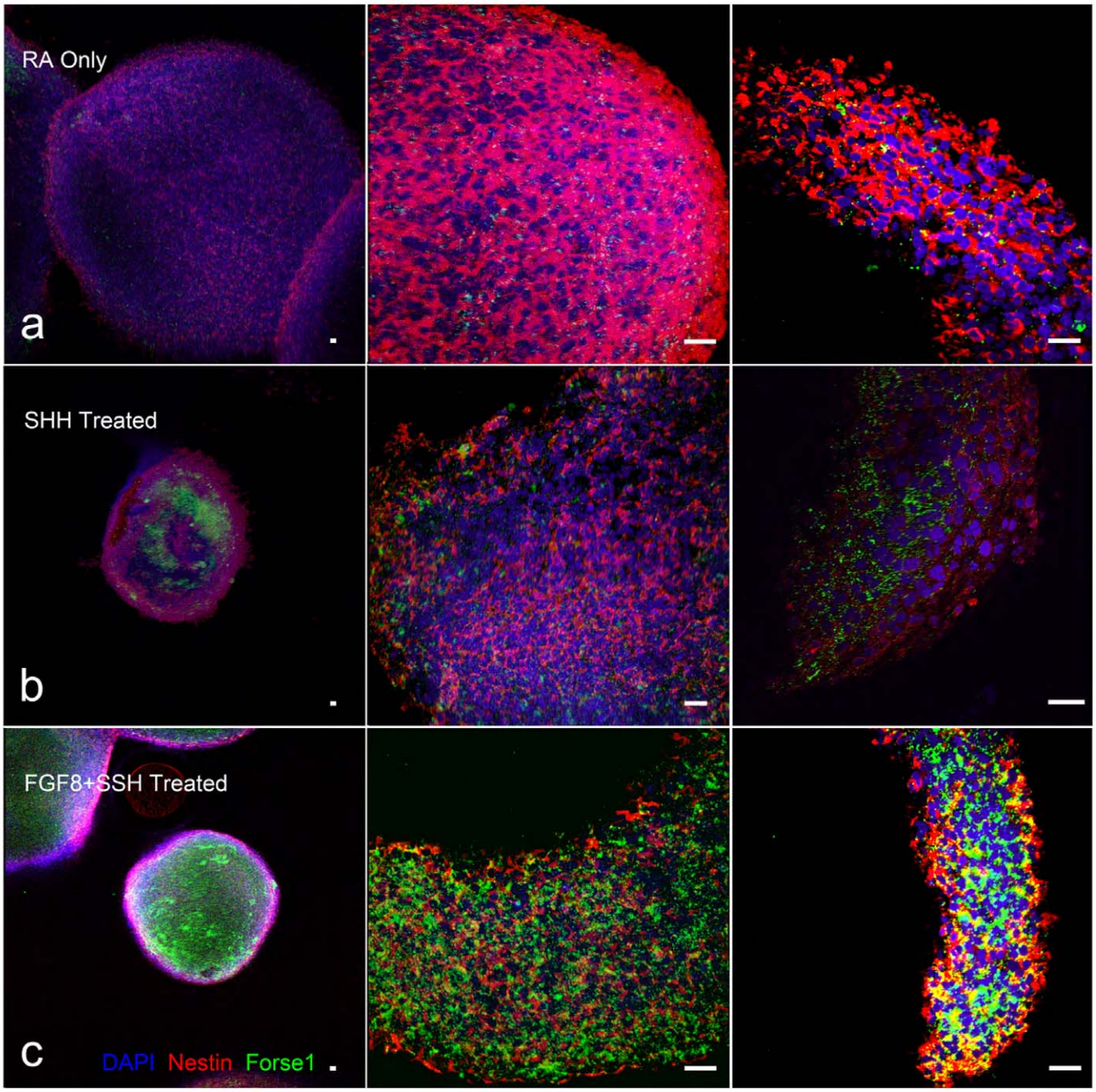
Supplemental Figure 2 BMP9 signaling changes hNSC-derived neurons from a glutamatergic to cholinergic phenotype

Control neurons express high levels of VGlut1 [a] but low levels of VChAT [c,e], while 72 hours of 10 ng ml^{-1} BMP9 treatment after SHH/FGF8 pretreatment results in an opposite phenotype, with low levels of Vglut1 [b] and high expression of VChAT [d,f]. These data suggest that the control neurons generated in these experiments default to a glutamatergic phenotype, and BMP9 is able to alter this default lineage commitment towards a cholinergic phenotype. Scale bars: a,f = 20μM, b-e = 100μM



Supplemental Figure 3 Culture conditions utilized only support neuronal growth

Combinatorial staining for MBP and MAP2 [a], GFAP and MAP2 [b], or GFAP and MBP [c] show that the cultures generated through the BMP9 treatment paradigm contain only neurons after 16 days in these media conditions, and are free of oligodendroglial [MBP] or astroglial [GFAP] cells. Scale bar =100μM



Supplemental Figure 4 FORSE1 immunohistochemistry demonstrates the generation of forebrain progenitor cells

FORSE1 is an antibody specific to forebrain progenitor cells which binds to the Le^x phosphacan, a brain-specific chondroitin sulfate proteoglycan.

a: Neurospheres generated using our culture system are almost uniformly nestin positive [red immunofluorescence], but the control retinoic acid-derived cells do not express FORSE1 [green immunofluorescence], which correlates with their inability to be driven towards a BFCN lineage.

b: Cells pretreated for 72 hours in 200 ng ml^{-1} SHH alone show a small increase in FORSE1 staining, and some ability to be made into BFCN [Not Shown].

c: Neurospheres pretreated for 72 hours in 100 ng ml^{-1} FGF8 and 200 ng ml^{-1} SHH show a marked increase in both FORSE1 expression and the ability to become BFCN [Figures 1,2,4]. Scale bars = 20μM

ATGAGTCCGGAGGGCGACACCCCCAGCCCGCCTGCTCGCCCGCCCCCTCCTTATGAGAGAG
AGGGAGCGCGGCGCCGGAGCCACACTGCGCCGAGCCCGCGCCCCGCCGCCACCTCGGCCCG
GGAGCCAGGGAGCGAGCCCTGCGTGTCCGCGCGGGGCGCCCGAGCCGCGGGGCGCACGGA
GGCGCCCAGAGAGGAGCGCCCCGGGGCGGCCGCAGCTCCGAACAAGATGCAGCGGGCCGG
AGGCGGTAGCGCCCCTGGGGGCAACGGCGGGGGCGGCGGCGGGGGCCCGGGCACTGCCTT
CTCCATCGACTCCCTAATCGGGCCGCCGCCGCCGCGCTCCGGCCACTTGCTGTACACCGGC
TACCCCATGTTCATGCCCTACCGGCCGCTCGTGCTGCCGCAGGCGCTGGCCCCTGCGCCGC
TGCCCGCTGGCCTCCCGCCCCTCGCCCCGCTAGCCTCTTTCGCCGGCCGCCTTACCAACAC
CTTCTGCGCGGGGCTGGGTCAGGCTGTGCCCTCGATGGTGGCGCTGACCACCGCGCTGCC
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GCCGCCGCCGCCACTGCCGCCCGAAACAACCCCGAGCCAGGCGGCCGACGCCCAGAGGGT
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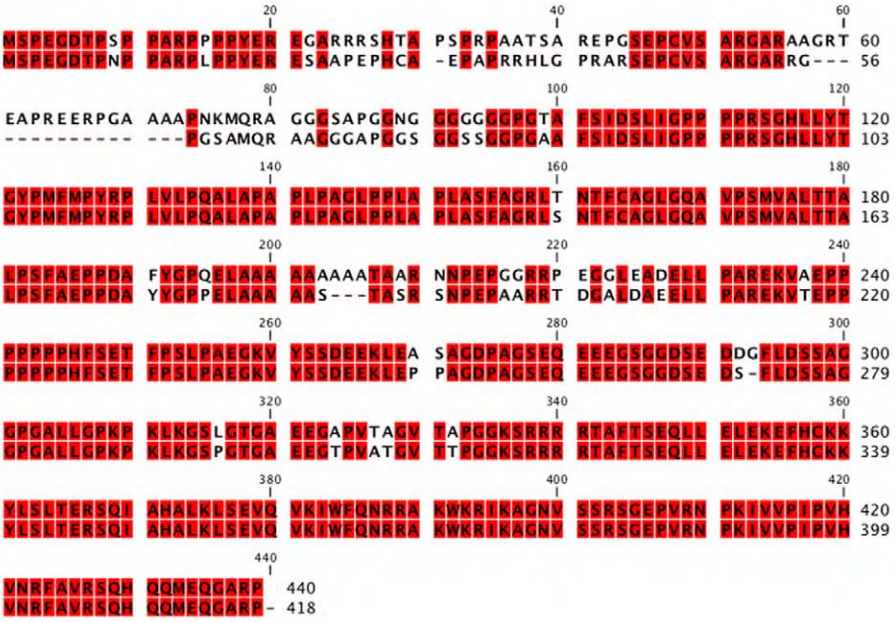
a

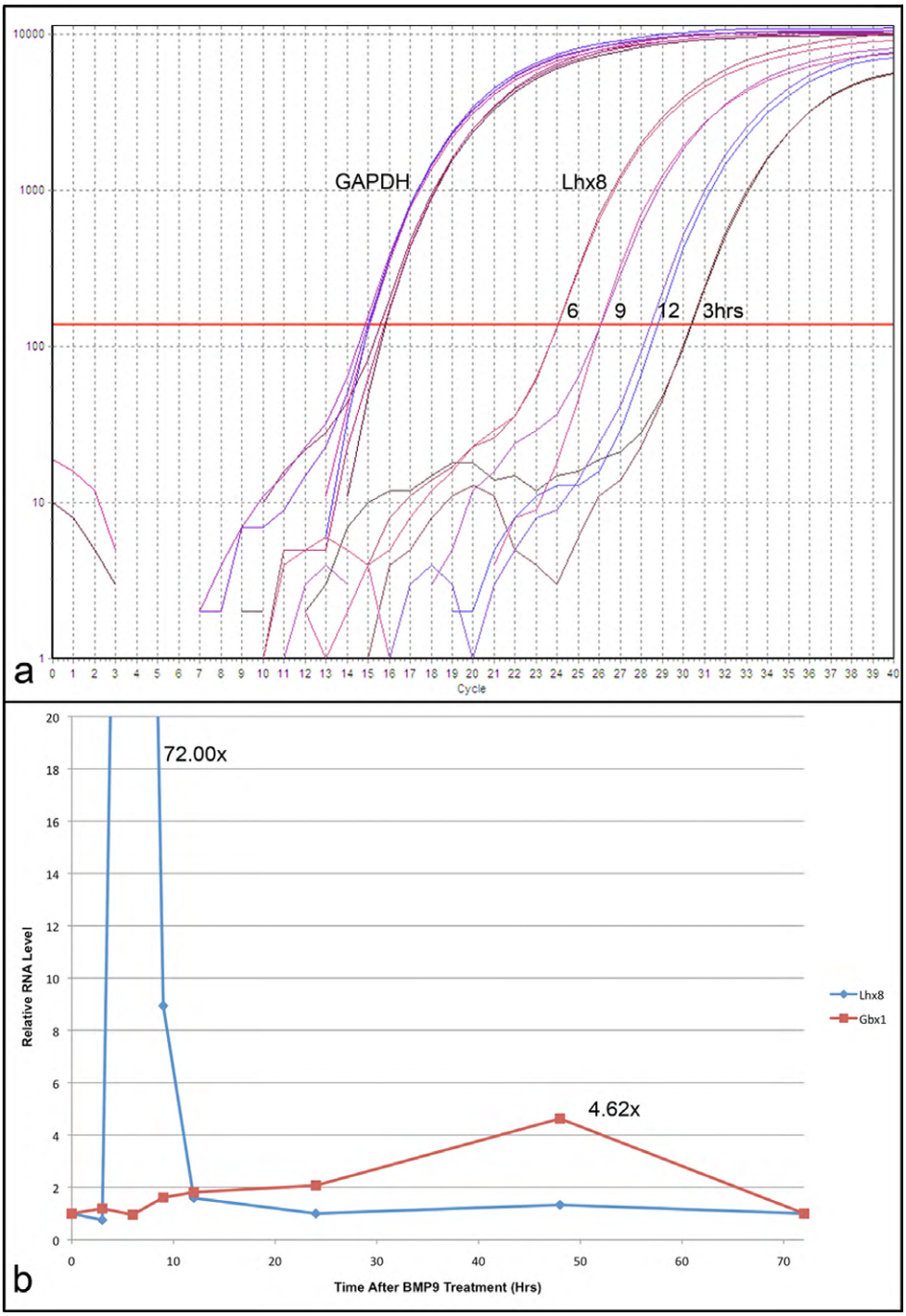


b

Supplemental Figure 5 Sequence of full length *Gbx1* determined through RACE experiments

a: Nucleotide sequence of the novel human *Gbx1* sequence

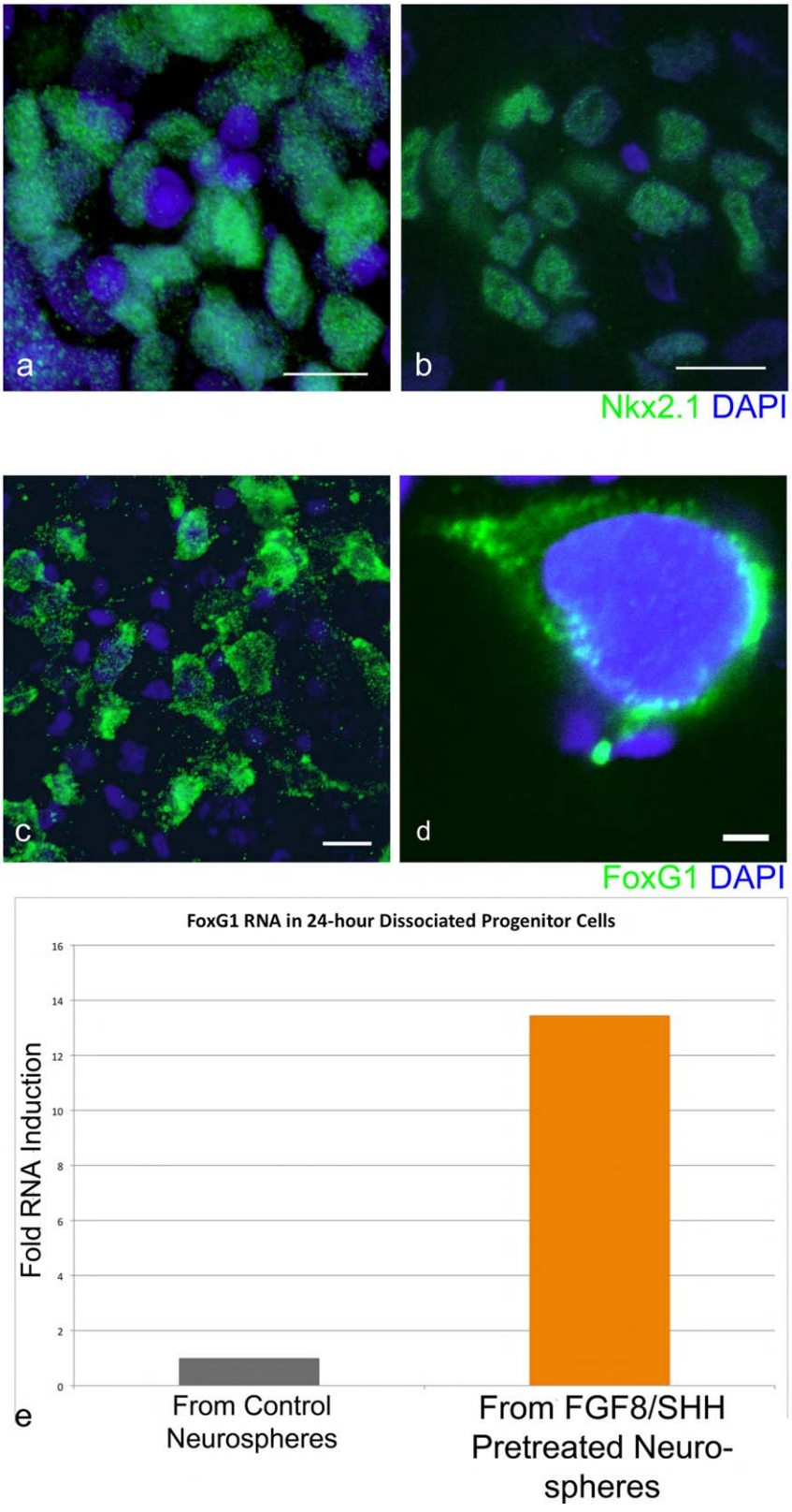
b: The human (upper sequence) and murine (lower sequence) Gbx1 proteins have a high level of homology



Supplemental Figure 6 Transcription factor induction following BMP9 treatment

a: Representative qRT-PCR curves showing the distinct upregulation of *Lhx8* expression between 3 and 12 hours after 10 ng ml^{-1} BMP9 treatment of human neurospheres.

b: Time course of transcription factor response to BMP9 treatment. *Lhx8* levels increased to 72x baseline at 6 hours, while *Gbx1* levels gradually increased to 4.62x baseline over 48 hours. Related factors, such as *Lhx6* and *Isl1* were unchanged following BMP9 treatment.



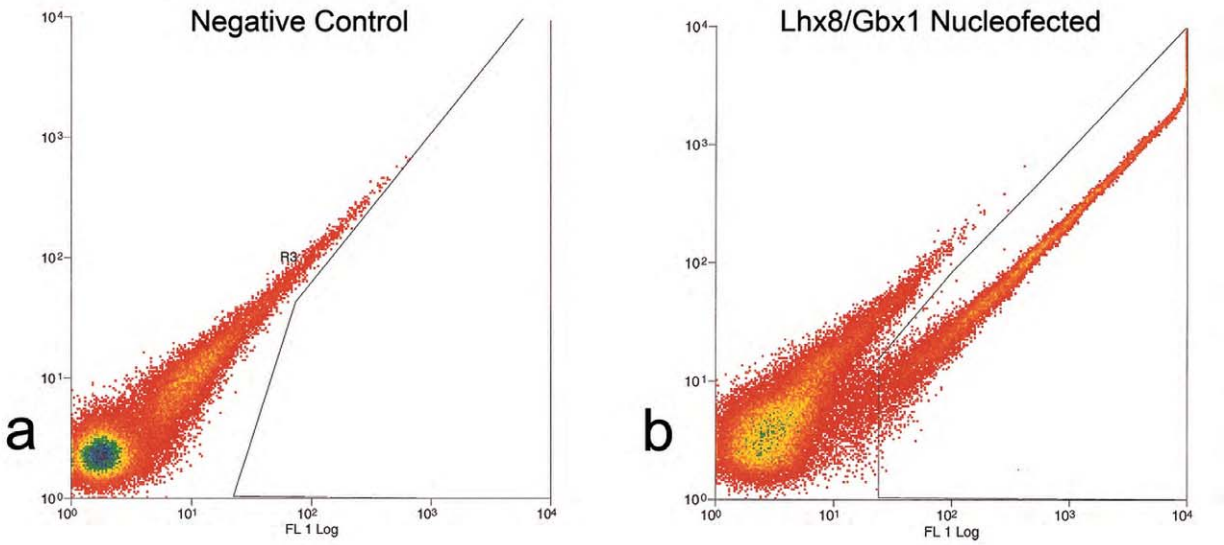
Supplemental Figure 7 Progenitor populations express additional forebrain-specific markers

a,b: 24 hours after dissociation neural progenitors pretreated with FGF8 and SHH for 72 hours have high levels of nuclear Nkx2.1 expression. Scale bar =10μM

c: Layered confocal z-stacks show the robust expression of FoxG1 24 hours after the dissociation neural progenitors pretreated with FGF8 and SHH for 72 hours. Scale bar =10μM

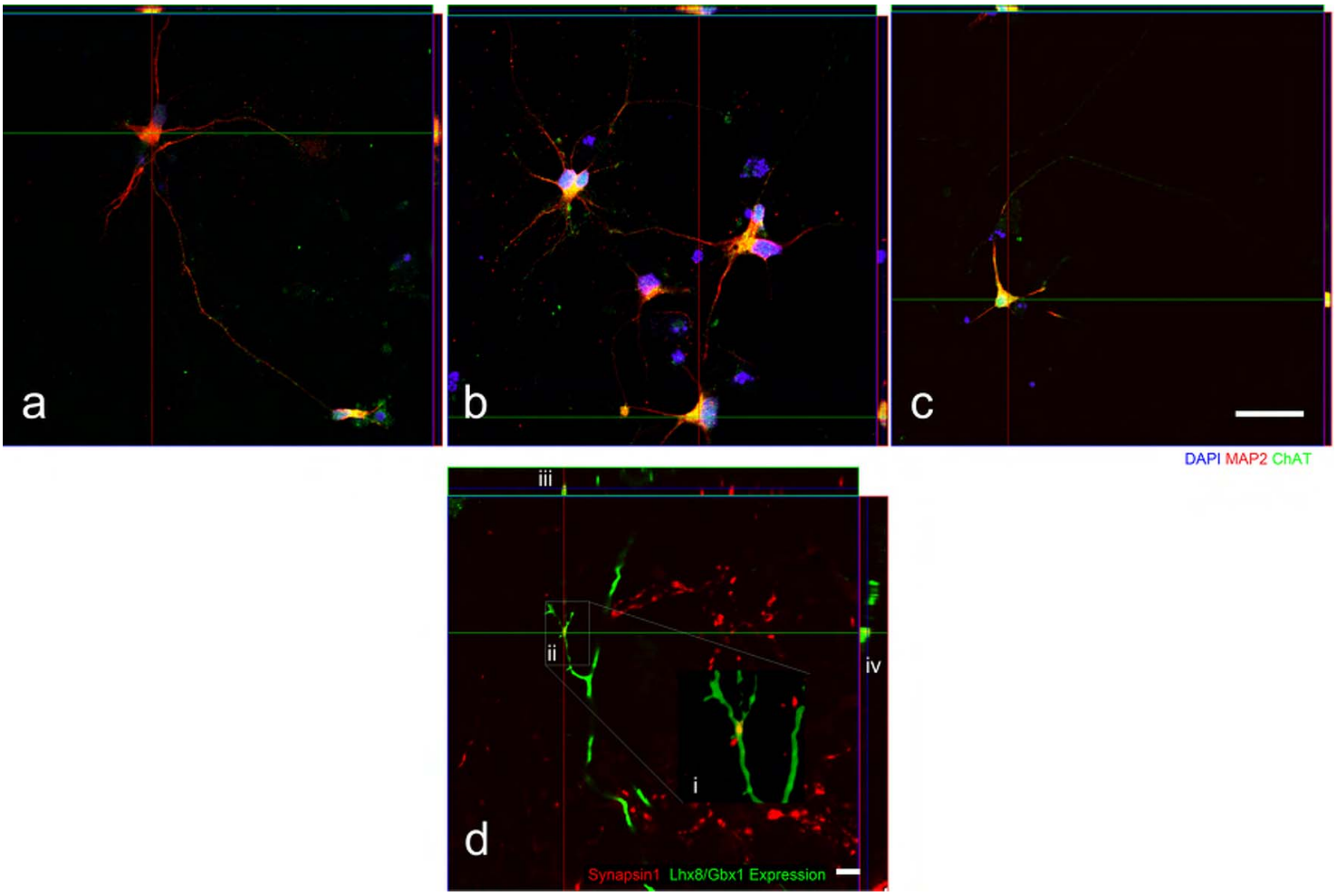
d: A closer analysis of single confocal sections clarifies that the FoxG1 expression is properly localized in the perinuclear cytoplasm, as appropriate for differentiating progenitor cells. Scale bar =2μM

e: 24 hours after dissociation neural progenitors pretreated with FGF8 and SHH for 72 hours have far higher levels of *FoxG1* expression than untreated control neural progenitors.



Supplemental Figure 8 Representative FACS data

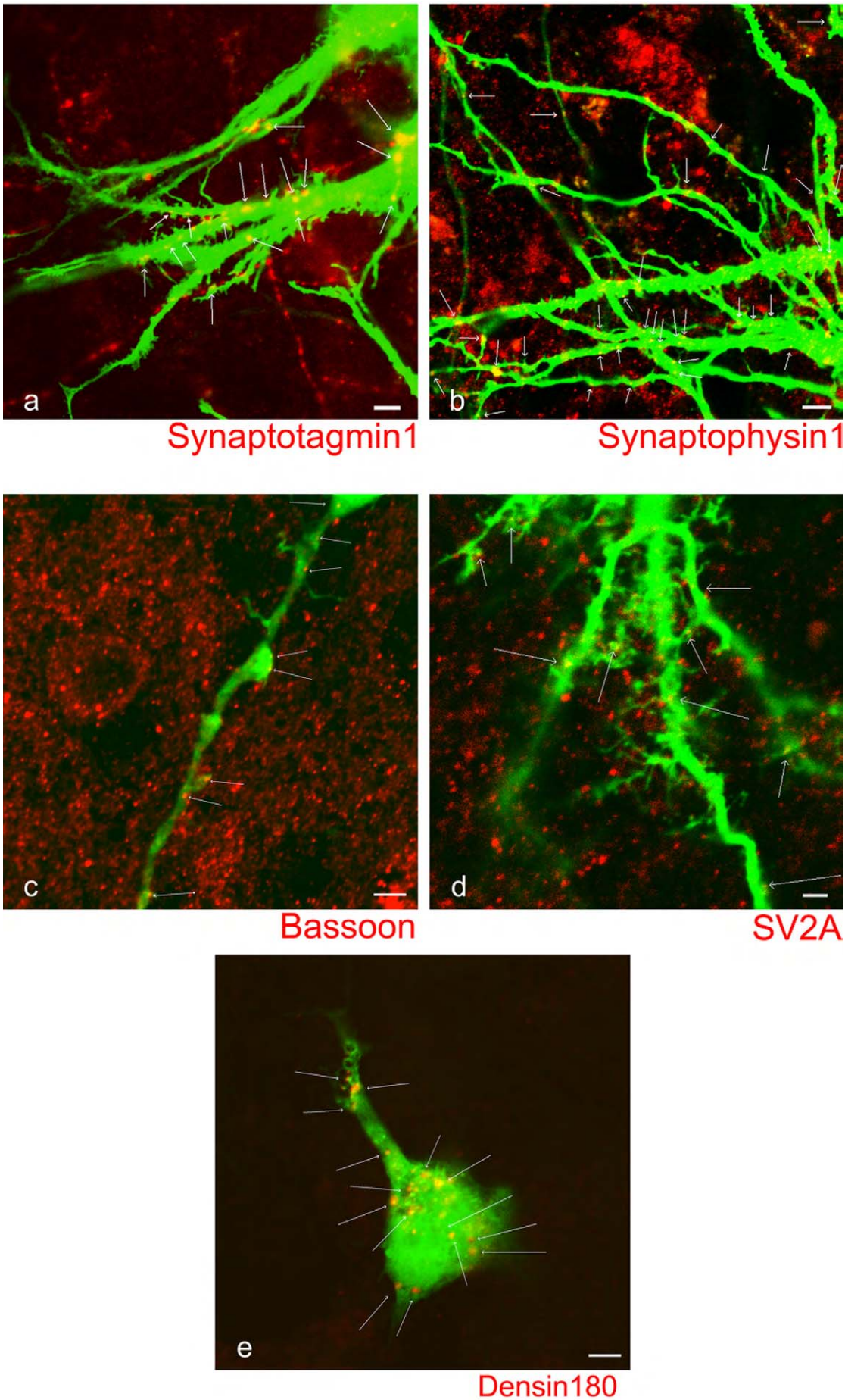
After gating to remove debris and doublets, neurons were FACS-purified based on GFP expression; only cells with expression levels markedly above the negative control were kept while minimally-expressing cells were discarded.



Supplemental Figure 9 Orthagonal views of z-stacked confocal images

a,b,c: Orthagonal views of z-stacked confocal images indicate ChAT immunopositivity [green channel] is entirely encapsulated within MAP2 immunopositivity [red channel], demonstrating that ChAT immunopositivity is localized within the neurons and is neither background staining within the cells nor on their surface [side/top bar beside each image]. Scale bar = 20μM

d: An orthagonal view of z-stacked confocal images confirms the generation of presynaptic terminals in nucleofected neurons expressing *Lhx8* and *Gbx1* after engrafting into murine hippocampal slice cultures. An enlarged and flattened rendering (i) of a synapse (ii), showing the total inclusion of synapsin1 within the axon. Inclusion is confirmed with individual x-axis (iii) and y-axis (iv) renderings of the synapse showing complete overlap of the punctate synapsin1 staining [red channel] within the eGFP-expressing engrafted axon. Scale bar = 5μM.


a
Synaptotagmin1
b
Synaptophysin1
c
Bassoon
d
SV2A
e
Densin180

Supplemental Figure 10 Additional synaptic markers

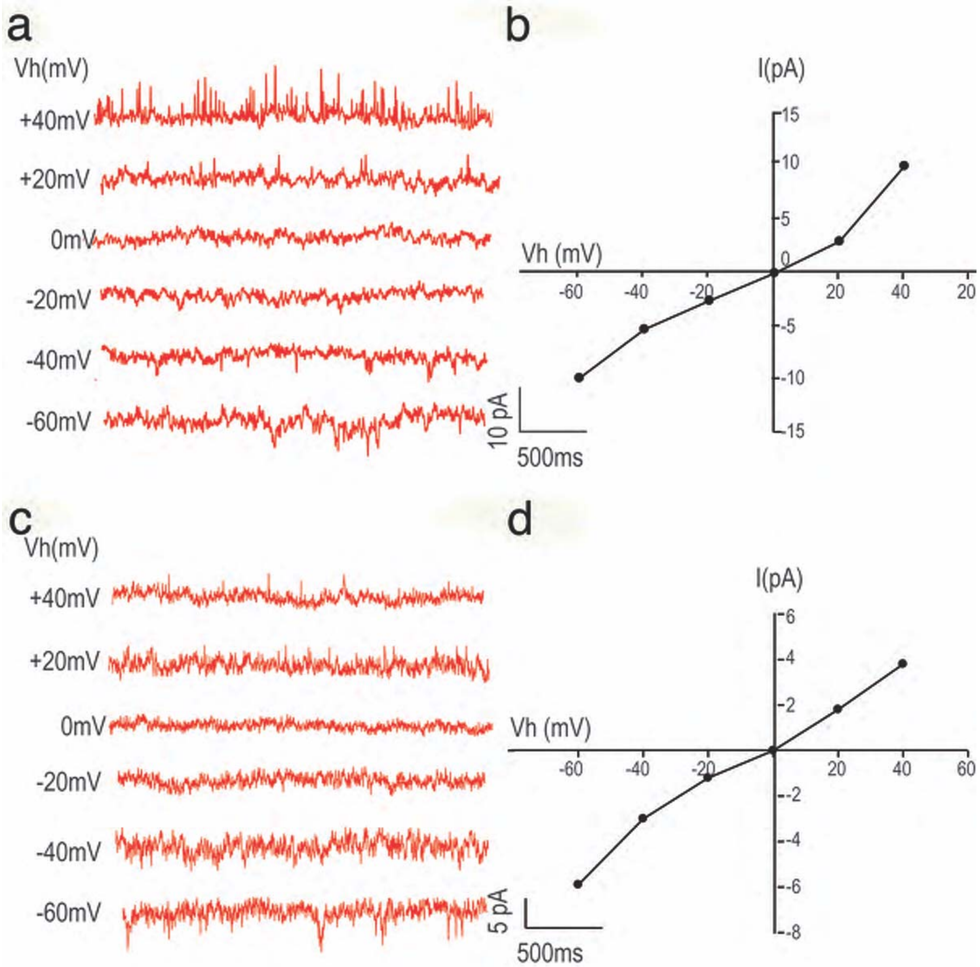
a: Immunohistochemistry for Synaptotagmin1 demonstrating the inclusion of immunopositive presynaptic densities within the axons of 14 day old neurons following engraftment into *ex vivo* murine hippocampal cultures. All green fluorescence in Supplemental figure 10 is the eGFP expression from the adenovirally-labeled FACS-purified *Lhx8*/*Gbx1* transiently overexpressing neurons. All scale bars in Supplemental Figure 10 =5μM

b: Immunohistochemistry for Synaptophysin1 demonstrating the inclusion of immunopositive presynaptic densities within the axons of 14 day old neurons following engraftment into *ex vivo* murine hippocampal cultures.

c: Immunohistochemistry for Bassoon demonstrating the inclusion of immunopositive presynaptic densities within the axons of 14 day old neurons following engraftment into *ex vivo* murine hippocampal cultures.

d: Immunohistochemistry for SV2A demonstrating the inclusion of immunopositive presynaptic densities within the axons of 14 day old neurons following engraftment into *ex vivo* murine hippocampal cultures.

e: Immunohistochemistry for Densin180 demonstrating the inclusion of immunopositive postsynaptic densities within the axons of 14 day old neurons following engraftment into *ex vivo* murine hippocampal cultures.



Supplemental Figure 11 Additional electrophysiology. !

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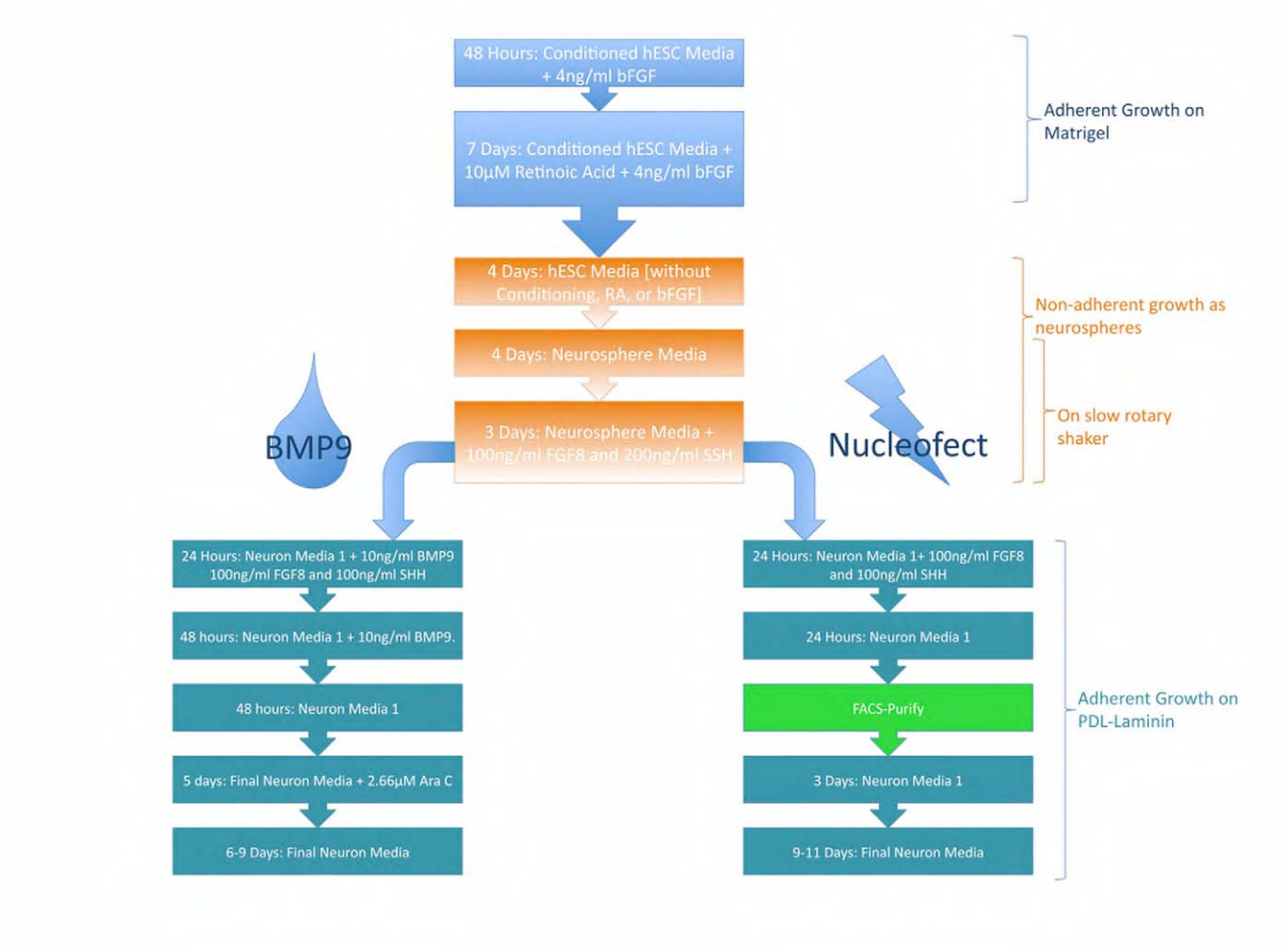
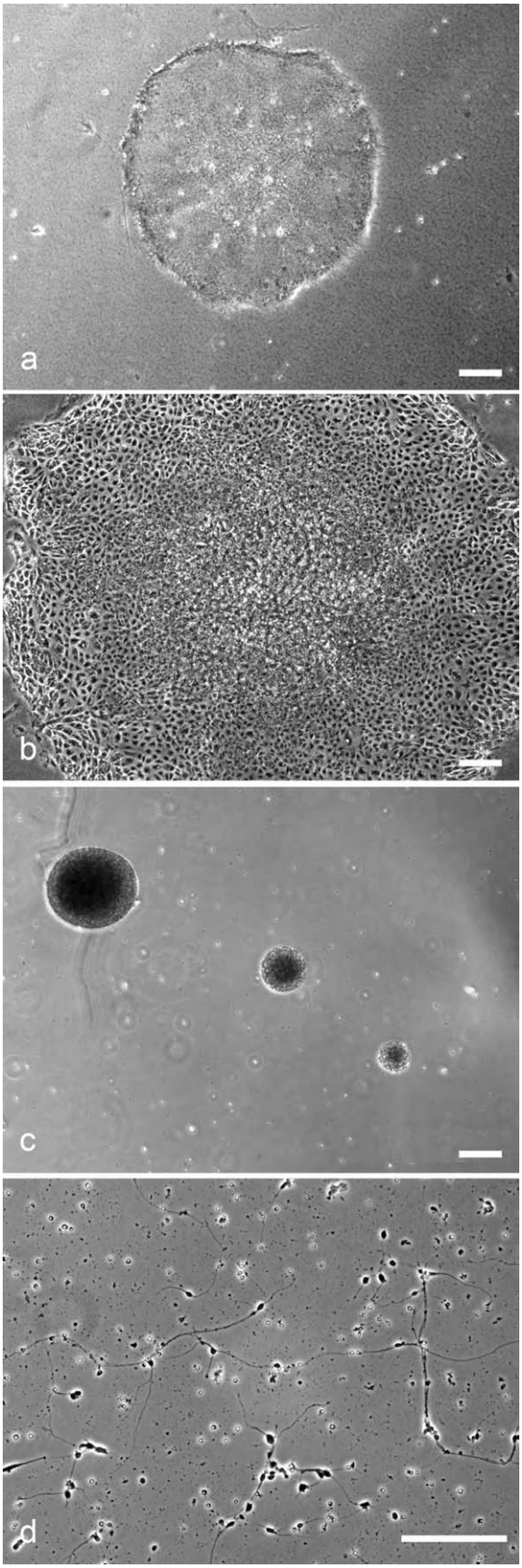
Culture Stage	Cell Number
hESC: Beginning RA treatment	100
hESC: 7 days RA treatment (hESC have ~2 1/3 day doubling time in RA)	800
Beginning neurospheres following hESC dissociation (70% survival)	560
End of Neurosphere Culture (3x sphere radius)	21000
Cells surviving 2 days following nucleofection (70% survival)	14733
FACS Positive Cells (50% average GFP+ cells)	7366
BFCN at 19 days (94% ChAT+ / Map+ cells)	6900

Supplemental Table 1 Approximate Cell Number

Approximate number of cells at each step of the protocol for each one hundred starting hESC at the beginning of RA treatment.



Supplemental Figure 12 Schematic of the timeline of media transitions



Supplemental Figure 13 Representative images of cells during differentiation

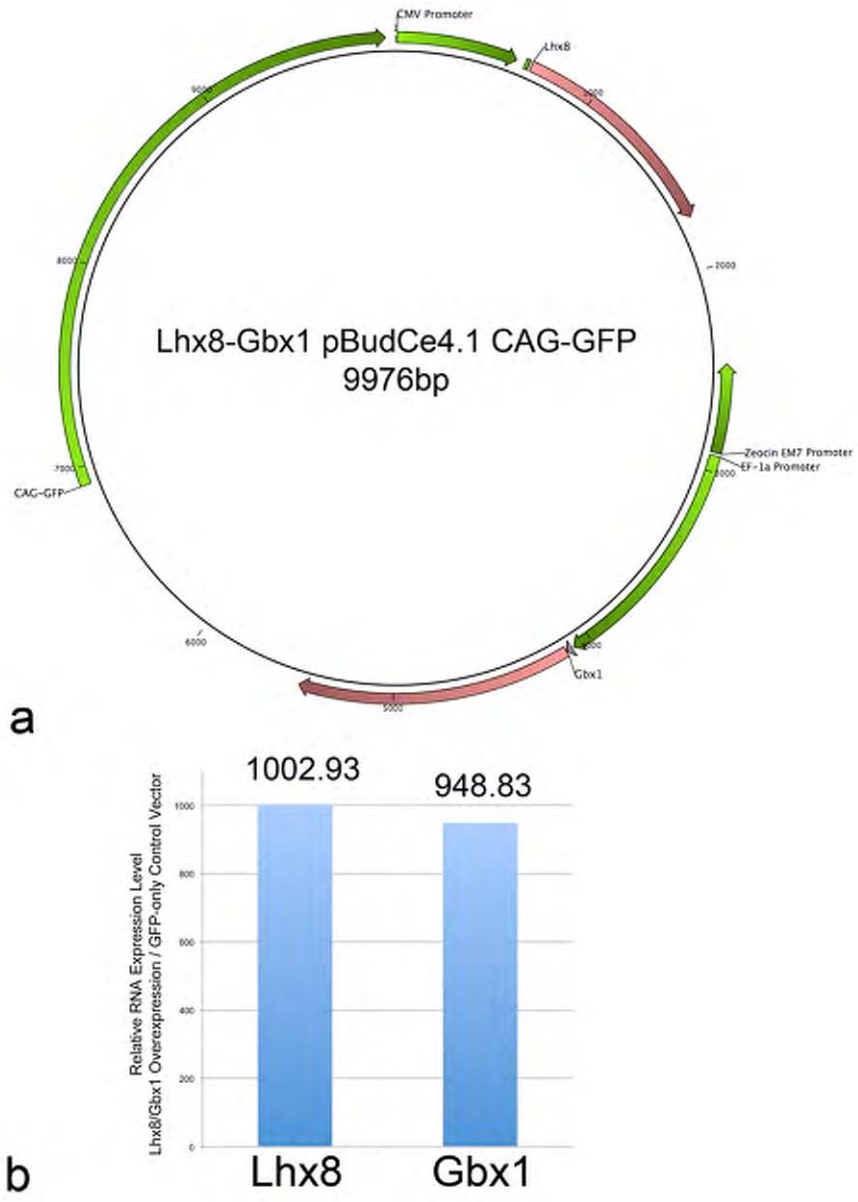
a: Typical untreated hESC colony morphology on matrigel. Colonies with a rim of differentiating cells, internal separation between the hESC, or non-circular colony shape were discarded.

b: 10μM retinoic acid treatment alters the morphology of the hESC, generating cells with both larger nuclei and expanded cytoplasm.

c: Neurospheres after 4 days in neurosphere media.

d: Neurons 5 days after FACS-purification.

All scale bars 200μM（



Supplemental Figure 14 Expression vector used for transcription factor overexpression

a: A vector based on the pBudCe4.1 backbone [Invitrogen] was generated to express *Lhx8* from the CMV promoter and *Gbx1* from the EF-1a promoter. A constitutively active CAG-GFP cassette was added into the vector backbone to allow FACS-purification, as either a GFP-fusion construct or IRES sequence could have altered gene function or expression levels.

b: 24 hours after nucleofection, expression levels of *Lhx8* and *Gbx1* increase by 1,002.93x and 948.83x when compared with nucleofection of the GFP-only empty vector control, indicating that all three promoters cause functional transcription in the hNSC used for these experiments.

	Forward	Reverse
Acetylcholinesterase	GGAACCGCTTCCTCCCCAAATTG	TGCTGTAGTGGTCGAACTGGTTCTTC
ß-III Tubulin	ATCAGCGTCTACTACAACGAGGCC	CAAAGATGAAATTGTCAGGCCTGAAGAGATGT
CNPase	AAGATGGACTTGGTCACCTACTTTGGAAAG	CGTCTTGGGTGTCACAAAGAGGG
ChAT 5'	TGCCGCCTACTGAGAGCAA	GTGGCAGGAGTCAAGGTTGGT
ChAT 3'	CATGAAGCAATACTATGGGCTCTTCTCCTC	GACGGCGGAAATTAATGACAACATCCAAG
FoxG1	AGAAGAACGGCAAGTACGAGA	TGTTGAGGGACAGATTGTGGC
GAD1	CCAGAAAACTGGGGCTCAAGATCTG	GCAAACAGATTAGAGAAGTCAGTCTCTGTGC
GAPDH	GAGCACAAGAGGAAGAGAGAGACCC	GTTGAGCACAGGGTACTTTATTGATGGTACATG
Gbx1	GCTGGAAGCTGATGAGCTGCT	CTTCTCCTCATCTGAGCTGTACACCTTC
Gbx1 Endogenous	GAAACCCCAAGATTGTTGTCCCCATAC	CAGATCCCTCGCCTTCCTAAGTTCTTG
GFAP	CTGGATCTGGAGAGGAAGATTGAGTCG	CTCATACTGCGTGCGGATCTCTTTCA
Islet1	TGAAATGTGCGGAGTGTAATCAGTATTTGGAC	CACACAGCGGAAACACTCGATGTG
Lhx8	GTTTCAGAATTGTAGAGCACGCCACAAG	CTATGCAGCGCAGTTAACATCGTTCC
Lhx8 Endogenous	GTTACCCCATTCAATGACACAACTGCC	CAGCAAAGTGATGTTGGAAATGCTTTAGGTG
Lhx6	CACGGCTACATCGAGAGTCAGGTAC	CAATCTGGCTCCATTTACCTTCTCAC
Nkx2.1	CTACTGCAACGGCAACCTGGG	CCATGAAGCGGGAGATGGCG
NOS	CAGGCTGTGACTGATGACCACATC	AGGTCATGTTTGGAGATGACCCTTGAG
P75NTR	GGAGAAAAACTCCACAGCGACAGTG	AGAGCCGTTGAGAAGCTTCTCCAC
Somatostatin	CAGACTCCGTCAGTTTCTGCAGAAG	CTTCAGGTTCCAGGGCATCATTCTC
TrkA	GAGGTCTCTGTTCAGGTCAACGTCT	CTCAGTGAAGATGAAGCTGGTCTCATTGA
Tyrosine Hydroxylase	AGTGTCATCACCTGGTCACCAAGTTC	CTTCAGCGTGGTGTAGACCTCCTT
VGlut1	GCTACATTGTCACTCAGATTCCAGGAGG	ATCCTCACGAAGATGACACAGCCATAG

Supplemental Table 2 qRT-PCR primer sequences

All primers have a melting temperature of $60^0 \pm 0.3^0$. All primer sets [except GAPDH] span an intron.