Supplemental Information

Embryonic Stem Cell-Derived Peripheral Auditory Neurons Form Neural Connections with Mouse Central Auditory Neurons *In Vitro* via the α2δ1 Receptor

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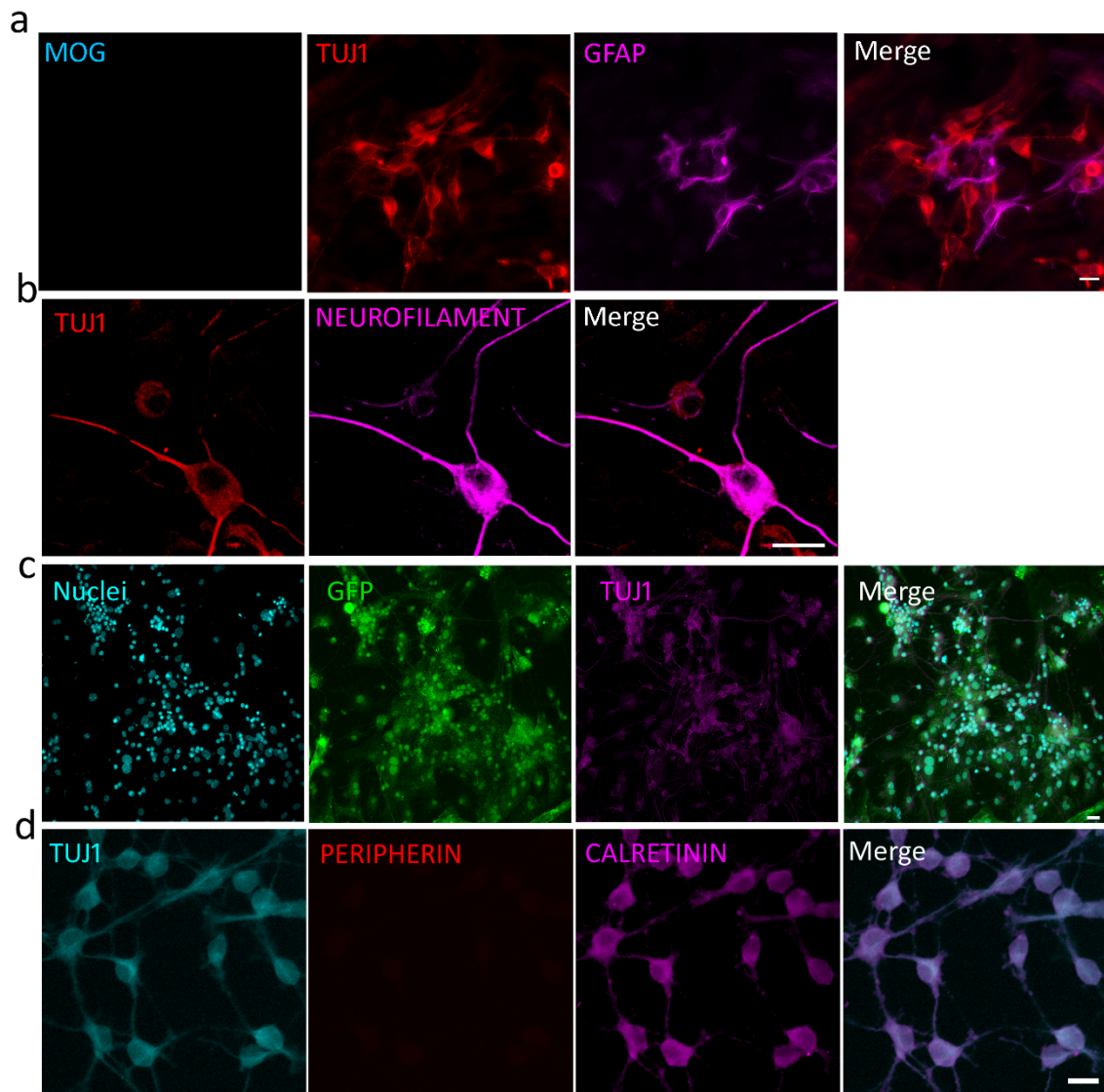
Supplemental Information

Figure S1. Neuronal differentiation of 4C2 ES cells, related to Figure 2.
(a) Immunofluorescence shows ESNs express TUJ1 and GFAP, but not MOG.
(b) Immunofluorescence shows TUJ1-postivie cells are also labeled by NEUROFILAMENT.
(c) Immunofluorescence shows that all 4C2-derived cells express GFP and some of them are double-labeled with TUJ1.
(d) Immunofluorescence shows all TUJ1-postivie cells are labeled by anti-CALRETININ but not anti-PERIPHERIN antibodies.
Data shown in all panels represent eight pooled independent biological experiments.
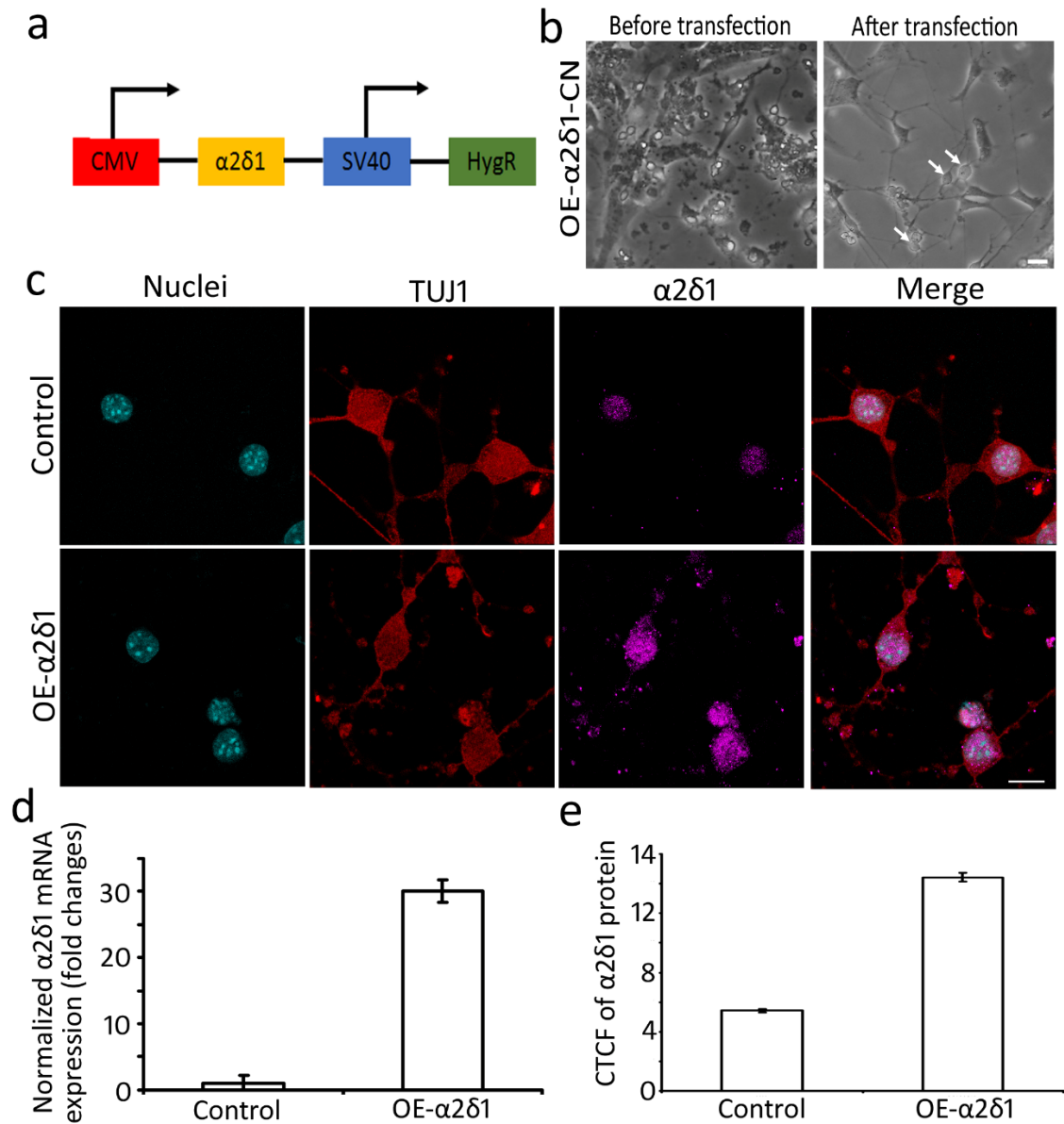Scale bar: 20 μm in a, b, d and f; 50 μm in c.

Figure S2. Overexpression of *α2δ1* in CN neurons, related to Figure 5.

(a) A diagram designates the α2δ1-plasmid for CN neurons overexpression.

(b) Phase contrast images show CN cells before (left panel) and after transfection of α2δ1-plasmids followed by hygromycin antibiotic selection (right panel). After antibiotic selection, a few neuron-like cells are observed (arrows).

(c) Immunofluorescence shows that CN neurons overexpressing *α2δ1* (OE-α2δ1*)* expresses more α2δ1 proteins than that of wildtype CN neurons (control).

(d) Compared to the control group, qPCR shows approximately 30-fold up-regulation of *α2δ1* expression in the OE-α2δ1 group.

(e) Compared to the control group, quantification corrected total cell fluorescence (CTCF) study shows approximately 3-fold up-regulation of α2δ1 protein expression in the OE-α2δ1 group.

Data shown in all panels represent four pooled independent biological experiments.

Scale bar: 20 μm in b; 10 μm in c.

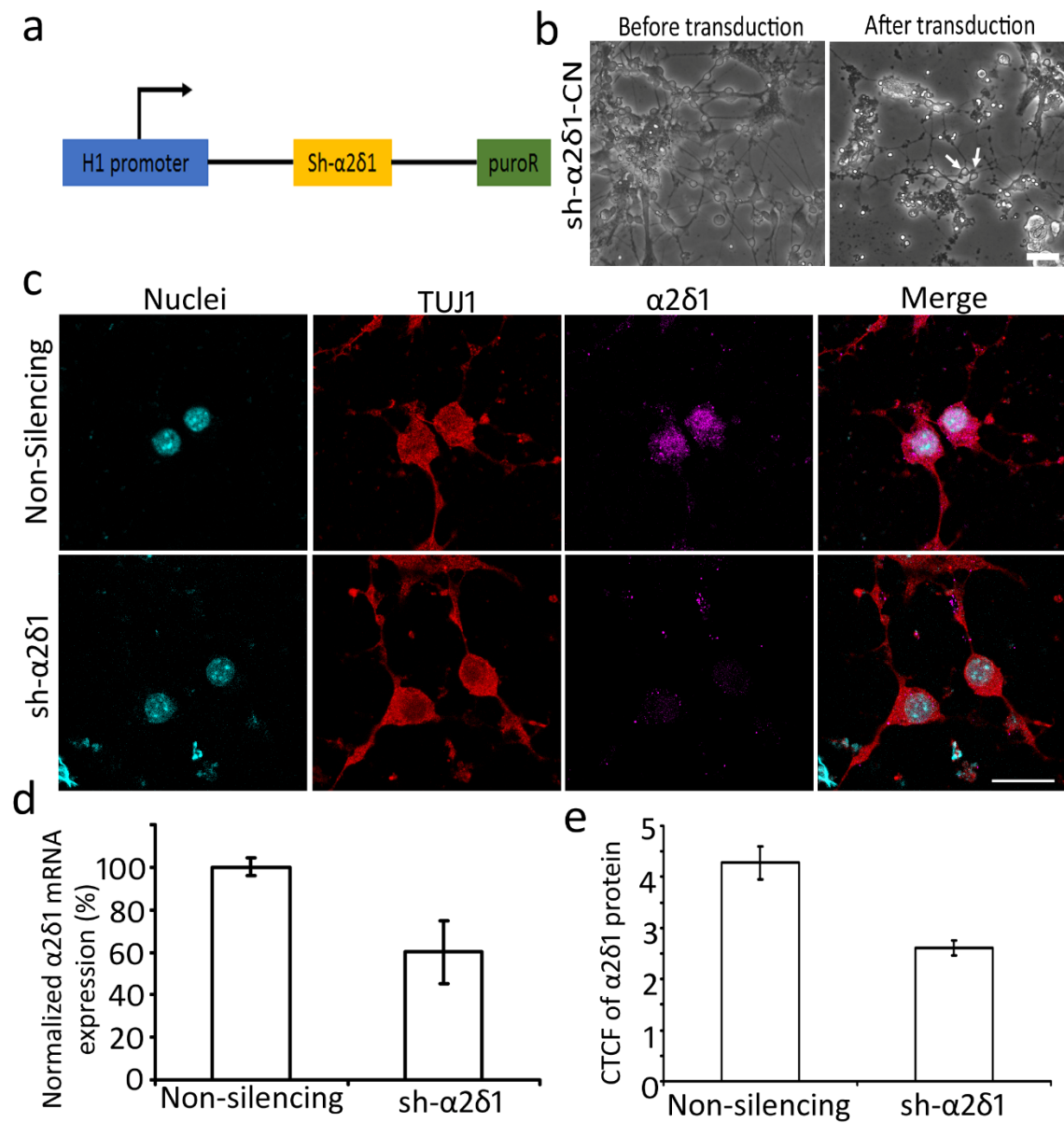**Figure S3. Silencing of *α2δ1* in CN neurons, related to Figure 5.**

(a) A diagram designates the sh-α2δ1-plasmid for CN neurons silencing.

(b) Phase contrast images show CN cells before (left panel) and after transduction of virus containing sh-α2δ1-plasmids followed by puromycin antibiotic selection (right panel). After antibiotic selection, a few neuron-like cells are found (arrows).

(c) Compared to the non-silencing group, wildtype CN neurons treated with virus containing the sh-*α2δ1* vector (sh-*α2δ1*) express a reduced level of α2δ1 proteins.

(d) Compared to the non-silencing group, qPCR showed approximately 40% down-regulation of *α2δ1* expression in the sh-α2δ1 group.

(e) Compared to the control group, quantification corrected total cell fluorescence (CTCF) study shows approximately 50% down-regulation of α2δ1 protein expression in the sh-α2δ1 group.

Data shown in all panels represent four pooled independent biological experiments.

Scale bar: 50 μm in b; 20 μm in c.

a

EPSC
(From CN to ESN)

500 pA

1 ms
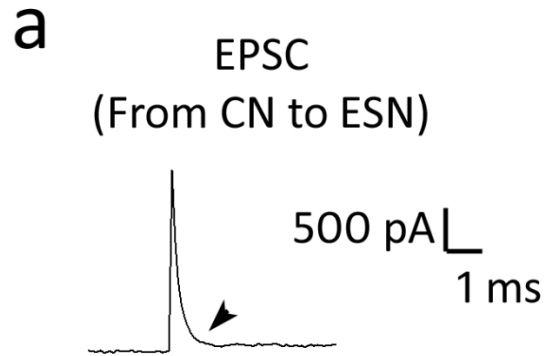
Figure S4. Evaluation of EPSCs in the co-culture, related to Figure 7.
Pair recording EPSC electrophysiology is used to study the function of new synapses. In the same sample that observes EPSCs on the CN neuron in response to ESN depolarization (Fig. 7d), inward currents are not found on ESN following CN depolarization, suggesting that the neural activity is directional, and that the ESN is pre-synaptic and the CN neuron is post-synaptic neurons.
Data shown in all panels represent six pooled independent biological experiments.

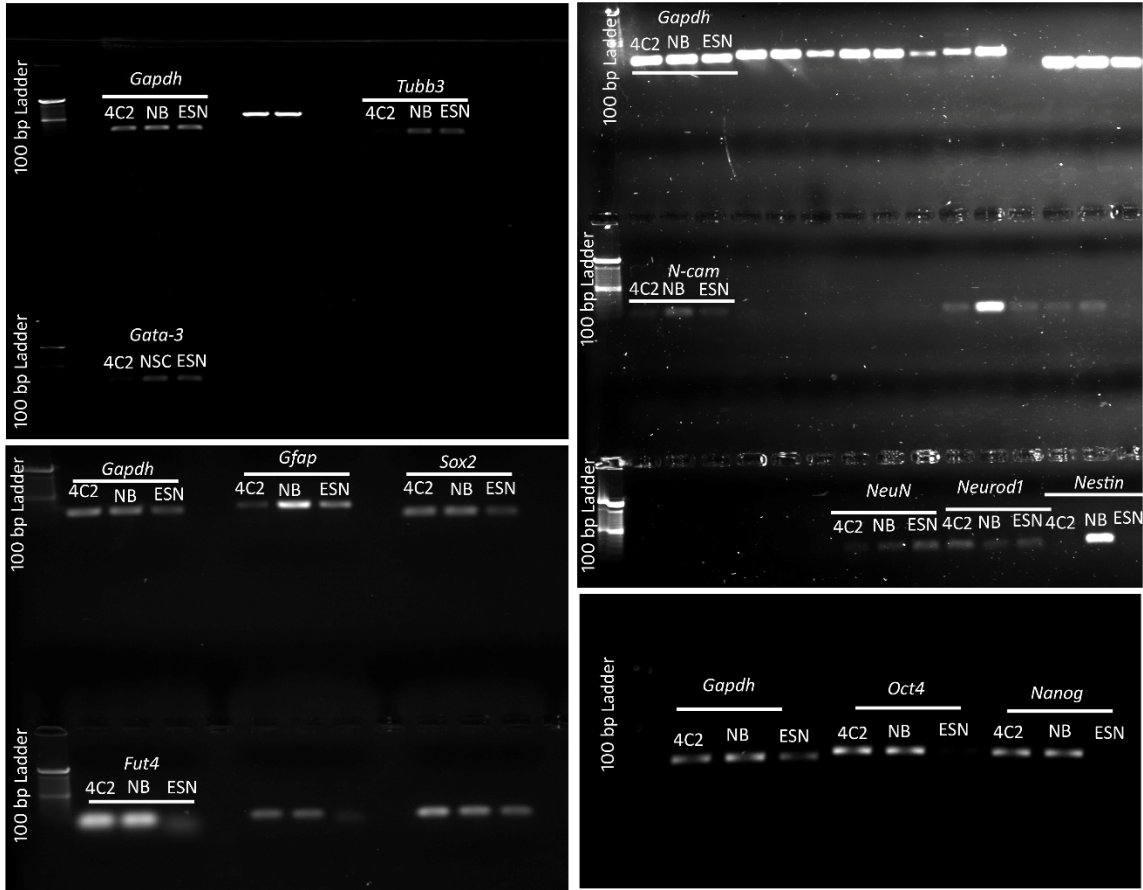**Figure S5. Original RT-PCR electrophoresis gel of Fig. 1d.**

Figure S6. Original RT-PCR electrophoresis gel of Fig. 2b1.
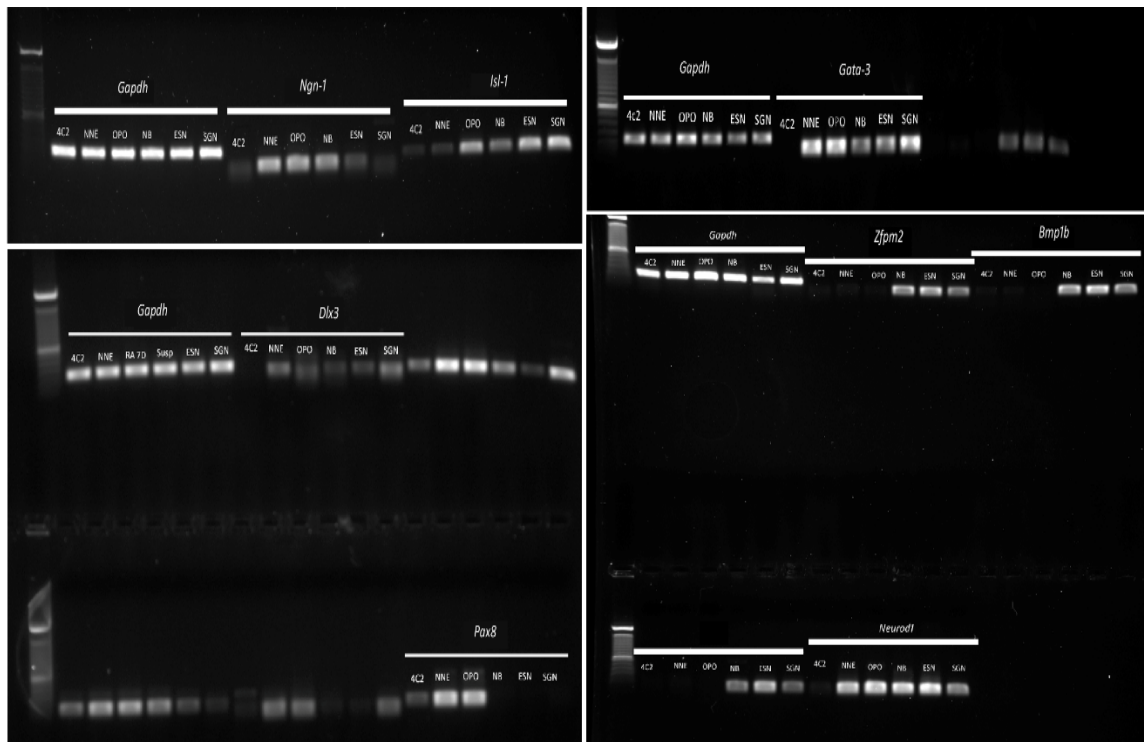
Figure S7. Original RT-PCR electrophoresis gel of Fig. 2b2.

Table S1. ANOVA analysis of TSP1 treatment in co-cultures, related to Figure 3

The number of puncta

Source of Variation	SS	df	MS	F	P-value
Treatment	53.4321	5	10.6864	27.5234	3.02E-12
Error	16.3072	42	0.3883		
Total	69.7393	47			

The area of puncta

Source of Variation	SS	df	MS	F	P-value
Treatment	32.2317	5	6.4436	21.1483	1.66E-10
Error	12.8023	42	0.3048		
Total	45.034	47			

Table S2. ANOVA analysis of gabapentin treatments in co-cultures, related to Figure 4

The number of puncta

Source of Variation	SS	df	MS	F	P-value
Treatment	1421.18	6	236.86	9.6812	1.34E-07
Error	1590.32	65	24.466		
Total	3011.50	71			

The area of puncta

Source of Variation	SS	df	MS	F	P-value
Treatment	39.839	6	6.6398	10.498	3.77E-08
Error	41.7459	66	0.6325		
Total	81.5849	72			

Table S3. Primers of RT-PCR and quantitative RT-PCR in Figures 1 and 2

Gene	Forward	Reverse	Product length (bp)
Gapdh	GGCCGCATCTTCTTGTGCAGT	TTCTCGGCCTTGACTGTGCCGTT	229
Pou5f1	CGAACCTGGCTAAGCTTCCA	TCCACCTTCTCCAACTTCACG	219
Nanog	ACGCTGATGACCTTATCTGCG	AAGCAGAAGATGCGGACTGTG	226
Fut4	CGCACGGATAAGGCGCTGGT	CGGGTCCCGTCCGACCAAAC	409
Sox2	ACGCCTTCATGGTATGGTCC	TCATGCTGTAGCTGCCGTTG	219
Cop-gfp	ATGGAGAGCGACGAGAGCG	GCGAGATCCGGTGGAGC	669
Nes	GCCTGGATCTGGAAGTCAACA	TCTGGCATTCCCTGAGCAAC	306
N-cam	GACAGAACCCGAAAAGGGC	GTTGGGGACCGTCTTGACTT	94
Gfap	CGCTTCTCCTTGTCTCGAATG	GCTCGAAGCTGGTTCAGTTCA	212
Tubb3	CCTTGTGTCTGCCACCATGA	CATCGAACATCTGCTGCGTG	206
Neurod1	AGCCCTGATCTGGTCTCCTT	AAAGTCCGAGGGTTGAGCTG	101
Neun	ATCGTAGAGGGACGGAAAATTGA	GTTCCCAGGCTTCTTATTGGTC	72
Slc17a7	TGGCTGTGTCATCTTCGTGA	CCAGCCGACTCCGTTCTAAG	113
Gata3	CTCGGCCATTCGTACATGGAA	GGATACCTCTGCACCGTAGC	134
α2δ1	CTTCGCCCGTCACTATCAAGT	CCAGTTGGCGTGCATTGTTG	153
Dlx3	TTTTTGAACTTGGAGCGGCG	TTCTGTTCAGTTCCGGGTCG	225
Bmpr1b	CTCCCTCTGCTGGTCCAAAG	GCTTCCTCCGTGGTGAAGAA	143
Zprm2	GTGACATGGCAAGGAGTGGA	CAAAGTCCACCACAAAGGCG	130
Pax8	AGACTACAAGCGGCAGAACC	GAAGGTGCTTTCGAGGACCA	153
Trkc	GCAATGCCAGTGTTGCTCTC	ACGCACCACAAACTCAATGC	101

Table S4. Primary antibodies in Figures 1-6

Primary antibody	Company	Cat #	Dilution
OCT4	R&D	MAB1759	1:200
NANOG	R&D	AF2729	1:200
SSEA1	R&D	MAB2155	1:200
SOX2	R&D	AF2018	1:200
NESTIN	DSHB	RAT-401	1:100
N-CAM	STCZ	SC-106	1:100
A2B5	R&D	MAB1416	1:400
GFAP	STCZ	SC-6170	1:200
TUJ1	AVES	TUJ	1:500
NEUN	Millipore	MAB377	1:100
NEUROD1	STCZ	SC46684	1:200
VGLUT1	Neuromab	73-066	1:50
MOG	Millipore	AB5680	1:200
GATA3	Sigma	HPA029731	1:200
TRKB	R&D	MAB1494	1:200
TRKC	R&D	AF1404	1:200
NA-V	Millipore	AB5210	1:100
SV2	DSHB	SV2-a	1:50
α2δ1	Sigma	C5105	1:1000
TGFP	Thermofisher	PA5-22688	1:2000
PERIPHERIN	Millipore	MAB1527	1:200
CALRETININ	Chemicon	AB1550	1:200
CTBP2	BD	612044	1:500
NEUROFILAMENT	AVES	NFL	1:1500
MYOSIN VIIA	DSHB	138-1	1:200

Table S5. Secondary antibodies in Figures 1-6

Secondary antibody	Company	Cat #	Dilution
AMCA Donkey anti-Chicken IgY	Jackson IR	703-156-155	1:500
Alexa Fluor 488 Donkey anti-Rabbit IgG	Jackson IR	711-546-152	1:500
Cy3 Donkey anti-Chicken IgY	Jackson IR	703-166-155	1:500
Cy3 Donkey anti-Mouse IgG	Jackson IR	715-166-150	1:500
Cy3 Donkey anti-Rabbit IgG	Jackson IR	711-165-152	1:500
Cy3 Donkey anti-Goat IgG	Jackson IR	705-166-147	1:500
Cy3 Fab Fragment Goat anti-Mouse IgG	Jackson IR	115-167-003	1:500
Alexa Fluor 647 Donkey anti-Chicken IgY	Jackson IR	703-496-155	1:500
Alexa Fluor 647 Donkey anti-Mouse IgG	Jackson IR	715-496-150	1:500
Alexa Fluor 647 Donkey anti-Goat IgG	Jackson IR	705-496-147	1:500
Alexa Fluor 647 Donkey anti-Rabbit IgG	Jackson IR	711-496-152	1:500