Figure S1

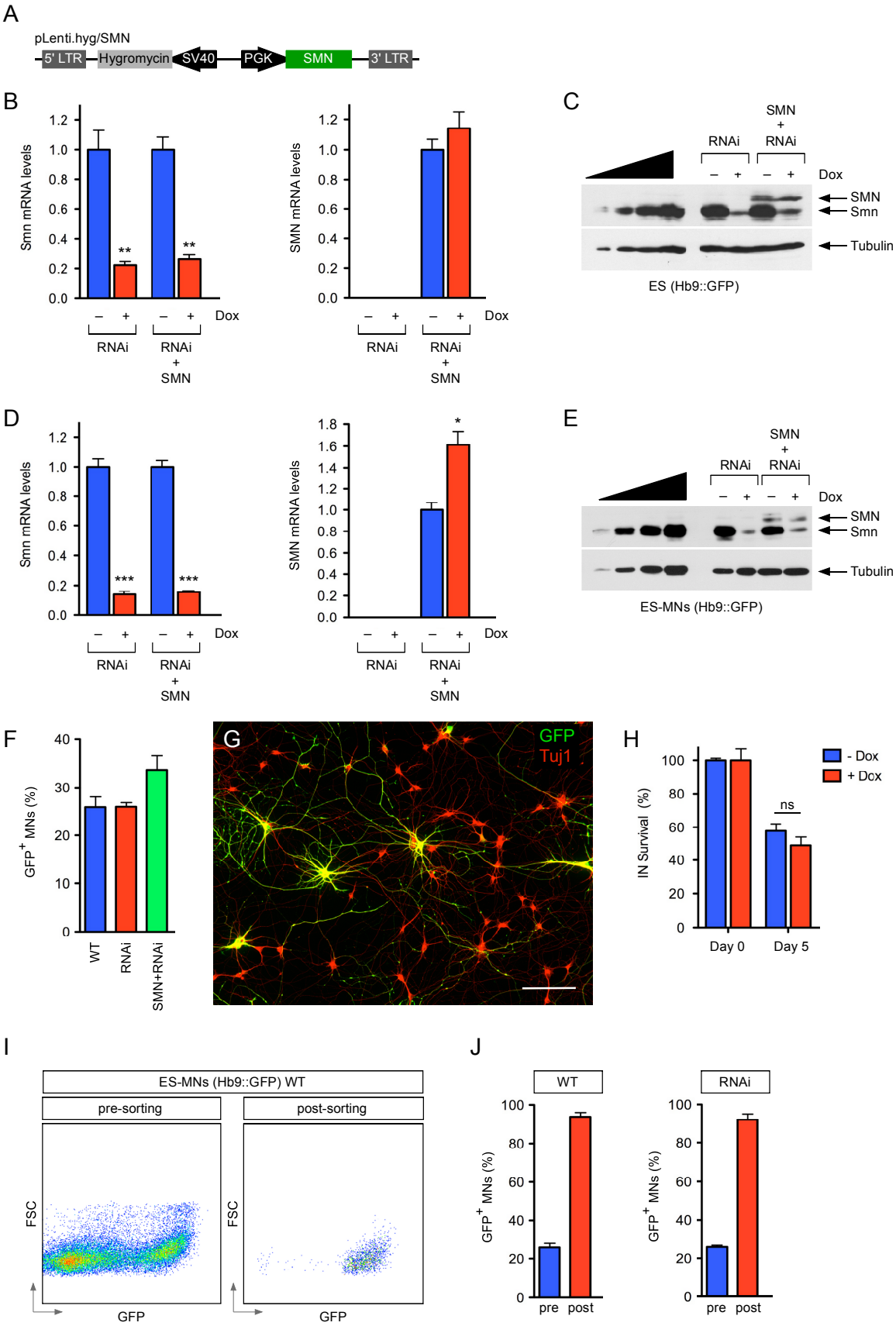
A
pLenti.hyg/SMN
5' LTR
Hygromycin
SV40
PGK
SMN
3' LTR
B
Smn mRNA levels
SMN mRNA levels
Dox
RNAi
RNAi + SMN
C
SMN + RNAi
RNAi
Dox
SMN
Smn
Tubulin
ES (Hb9::GFP)
D
Smn mRNA levels
SMN mRNA levels
Dox
RNAi
RNAi + SMN
E
SMN + RNAi
RNAi
Dox
SMN
Smn
Tubulin
ES-MNs (Hb9::GFP)
F
GFP+ MNs (%)
WT
RNAi
SMN+RNAi
G
GFP
Tuj1
H
IN Survival (%)
- Dox
+ Dox
ns
Day 0
Day 5
I
ES-MNs (Hb9::GFP) WT
pre-sorting
post-sorting
FSC
GFP
J
WT
RNAi
GFP+ MNs (%)
pre
post

Figure S1. Characterization of ES cell lines with inducible Smn knockdown, Related to Figure 1.
(A) Schematic representation of the lentiviral vector used to generate the ES(Hb9::GFP)-SMN/Smn_{RNAi} cell line. (B) RT-qPCR analysis of mouse Smn and human SMN mRNA levels in Smn_{RNAi} (RNAi) and SMN/Smn_{RNAi} (RNAi+SMN) ES cells cultured with and without Dox for 4 days. RNA levels in Dox-treated cells are expressed relative to those in untreated cells. Data are represented as mean and SEM from independent experiments (n=3). (C) Western blot analysis of SMN protein levels in RNAi and RNAi+SMN ES cells cultured with and without Dox for 4 days. A two-fold serial dilution of RNAi ES cell extract is analyzed on the left. (D) RT-qPCR analysis of mouse Smn and human SMN mRNA levels in FACS-purified MNs differentiated from RNAi and RNAi+SMN ES cells cultured with and without Dox for 5 days. RNA levels in Dox-treated cells are expressed relative to those in untreated cells. Data are represented as mean and SEM from independent experiments (n=3). (E) Western blot analysis of SMN protein levels in mixed cultures of ES-MNs cultured with and without Dox for 5 days after differentiation from RNAi and RNAi+SMN ES cells. A two-fold serial dilution of the extract from RNAi ES-MNs is analyzed on the left. (F) Quantification of the efficiency of GFP^+ MN differentiation from WT, RNAi and RNAi+SMN ES(Hb9::GFP) cells by FACS. Data represent mean and SEM from independent experiments ($n \geq 3$). (G) Representative image of mixed ES-MN cultures differentiated from RNAi ES cells and immunostained with anti Tuj1 antibodies (red) at 5DIV. GFP^+ MNs are shown in green. Scale bar=150mm. (H) Survival analysis of $Tuj1^+/GFP^-$ INs differentiated from RNAi ES cells and cultured with or without Dox for 5 days. For each group, the number of INs was measured from immunostaining experiments as in G at 0DIV and 5DIV. Data normalized to 0DIV are shown as mean and SEM from independent experiments (n=3). (I) Representative FACS profiles of ES-MNs differentiated from WT ES cells pre and post sorting are shown. (J) FACS analysis of the percentage of GFP^+ MNs differentiated from WT and RNAi ES cells pre and post sorting. Data represent mean and SEM from independent experiments ($n \geq 3$).

Figure S2

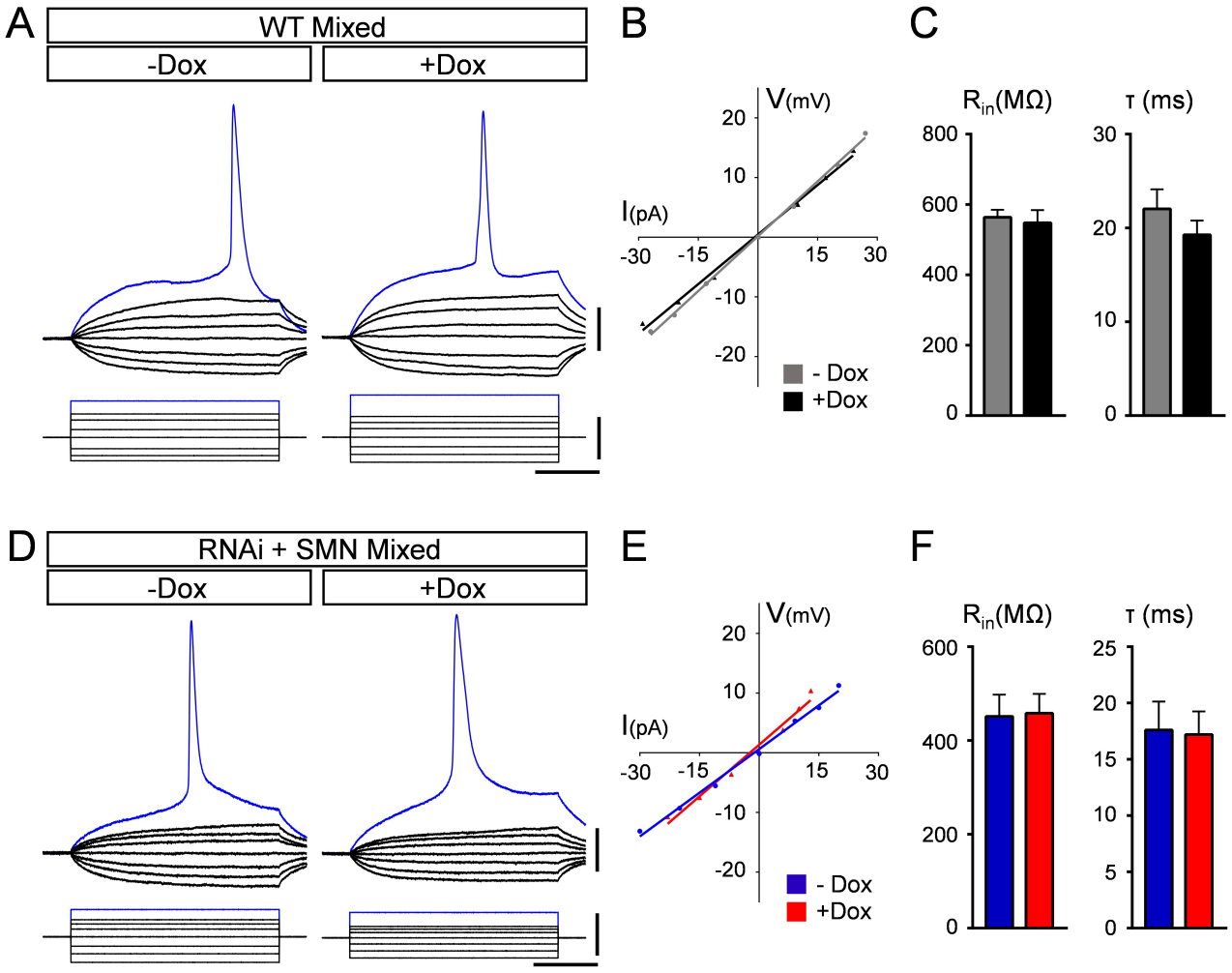
A
WT Mixed
-Dox
+Dox
B
V(mV)
I(pA)
20
10
-10
-20
-30
-15
15
30
- Dox
+Dox
C
R_in(MΩ)
τ (ms)
800
600
400
200
0
30
20
10
0
D
RNAi + SMN Mixed
-Dox
+Dox
E
V(mV)
I(pA)
20
10
-10
-20
-30
-15
15
30
- Dox
+Dox
F
R_in(MΩ)
τ (ms)
600
400
200
0
25
20
15
10
5
0

Figure S2. Expression of human SMN rescues the hyperexcitability phenotype induced by Smn deficiency in MNs, Related to Figure 2.

(A) Superimposed membrane responses (upper traces) following current injection (lower traces) in WT MNs cultured with and without Dox for 5 days. Scale bars=20mV, 40pA, 40ms. (B) Current/voltage relationships for the MNs shown in A. Input resistance: -Dox=602MΩ, +Dox=563MΩ. (C) Mean and SEM of input resistance (R_{in}) and time constant (τ) of WT MNs cultured with (n=11) and without (n=11) Dox at 5DIV. (D) Superimposed membrane responses (upper traces) following current injection (lower traces) in control (-Dox) and Smn-deficient (+Dox) MNs differentiated from RNAi+SMN cells at 5DIV. Scale bars=20mV, 40pA, 40ms. (E) Current/voltage relationships for the MNs shown in D. (F) Mean and SEM of input resistance (R_{in}) and time constant (τ) of control (n=10) and Smn-deficient (n=10) MNs differentiated from RNAi+SMN cells at 5DIV.

Figure S3

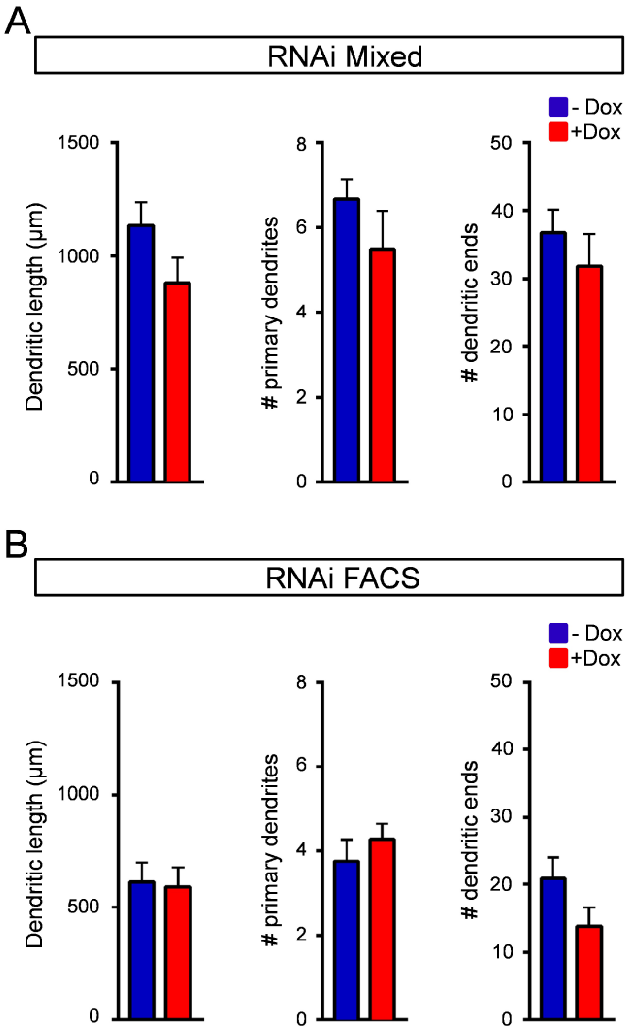
A
RNAi Mixed
- Dox
+Dox
Dendritic length (µm)
1500
1000
500
0
primary dendrites
8
6
4
2
0
dendritic ends
50
40
30
20
10
0
B
RNAi FACS
- Dox
+Dox
Dendritic length (µm)
1500
1000
500
0
primary dendrites
8
6
4
2
0
dendritic ends
50
40
30
20
10
0

Figure S3. Effects of Smn deficiency on the dendritic trees of MNs, Related to Figure 3.
(A) Neurolucida analysis of dendritic length, number of primary dendrites and number of dendritic ends of MNs differentiated from RNAi ES cells and cultured in mixed conditions with (n=13) or without (n=12) Dox at 5DIV. (B) Same analysis as in A using FACS purified MNs differentiated from RNAi ES cells.

Figure S4

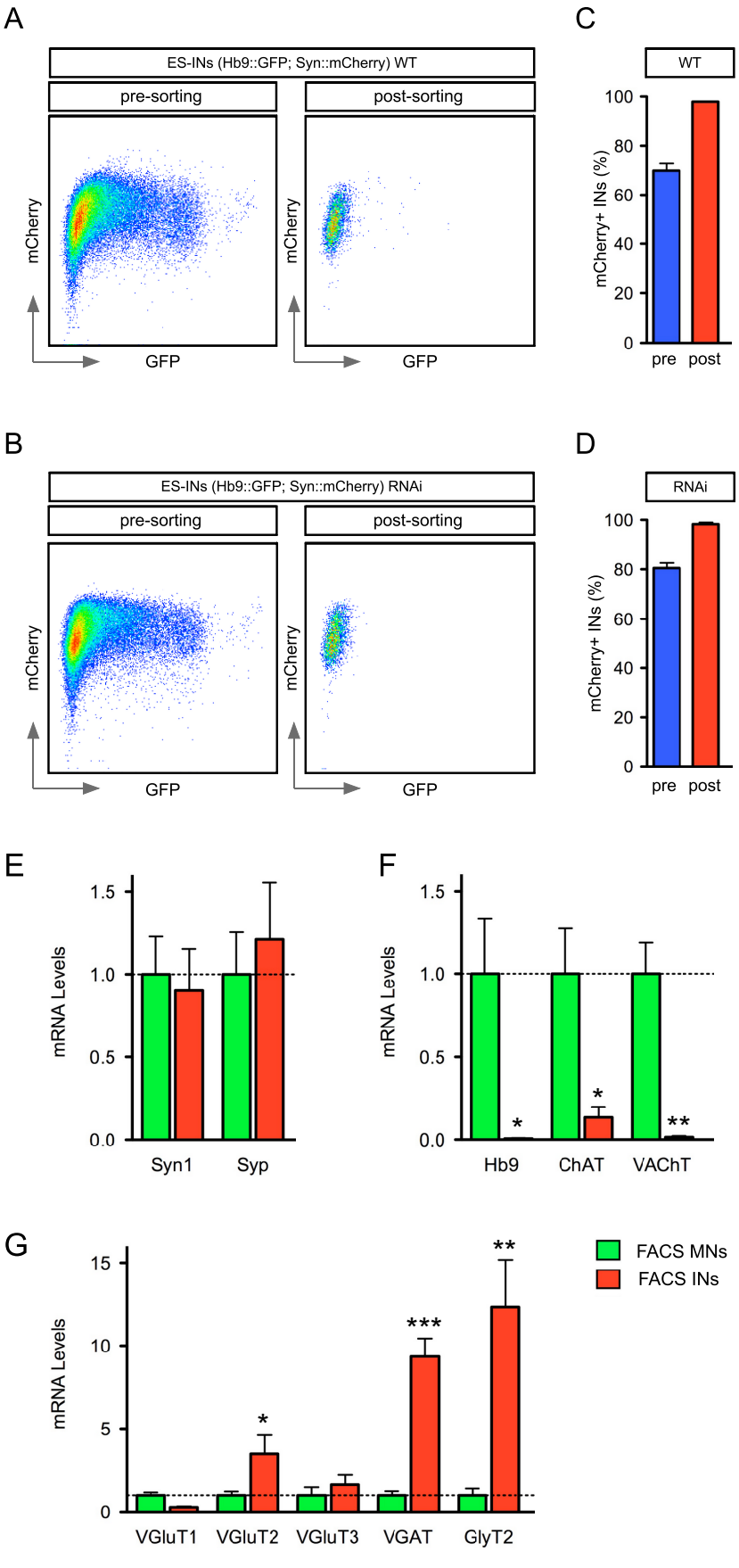
A
ES-INs (Hb9::GFP; Syn::mCherry) WT
pre-sorting
post-sorting
mCherry
GFP
C
WT
mCherry+ INs (%)
pre
post
B
ES-INs (Hb9::GFP; Syn::mCherry) RNAi
D
RNAi
E
mRNA Levels
Syn1
Syp
F
Hb9
ChAT
VAChT
G
VGluT1
VGluT2
VGluT3
VGAT
GlyT2
FACS MNs
FACS INs

Figure S4. Characterization of INs differentiated from ES cell lines with inducible Smn knockdown, Related to Figure 4.

(A) Representative FACS profiles of ES-INs differentiated from WT ES cells pre and post sorting are shown. (B) Representative FACS profiles of ES-INs differentiated from RNAi ES cells pre and post sorting are shown. (C) FACS analysis of the percentage of $mCherry^+$ MNs differentiated from WT ES cells pre and post sorting. Data represent mean and SEM from independent experiments (n=3). (D) FACS analysis of the percentage of $mCherry^+$ MNs differentiated from RNAi ES cells pre and post sorting. Data represent mean and SEM from independent experiments (n=3). (E) RT-qPCR analysis of Syn1 and Syp mRNA expression in FACS-purified MNs and INs. (F) RT-qPCR analysis of the MN markers Hb9, ChAT and VAChT mRNAs in FACS-purified MNs and INs. (E) RT-qPCR analysis of VGluT2, VGAT and GlyT2 mRNAs in FACS-purified MNs and INs. All RT-qPCR data represent mean and SEM from independent experiments ($n \geq 3$).

Figure S5

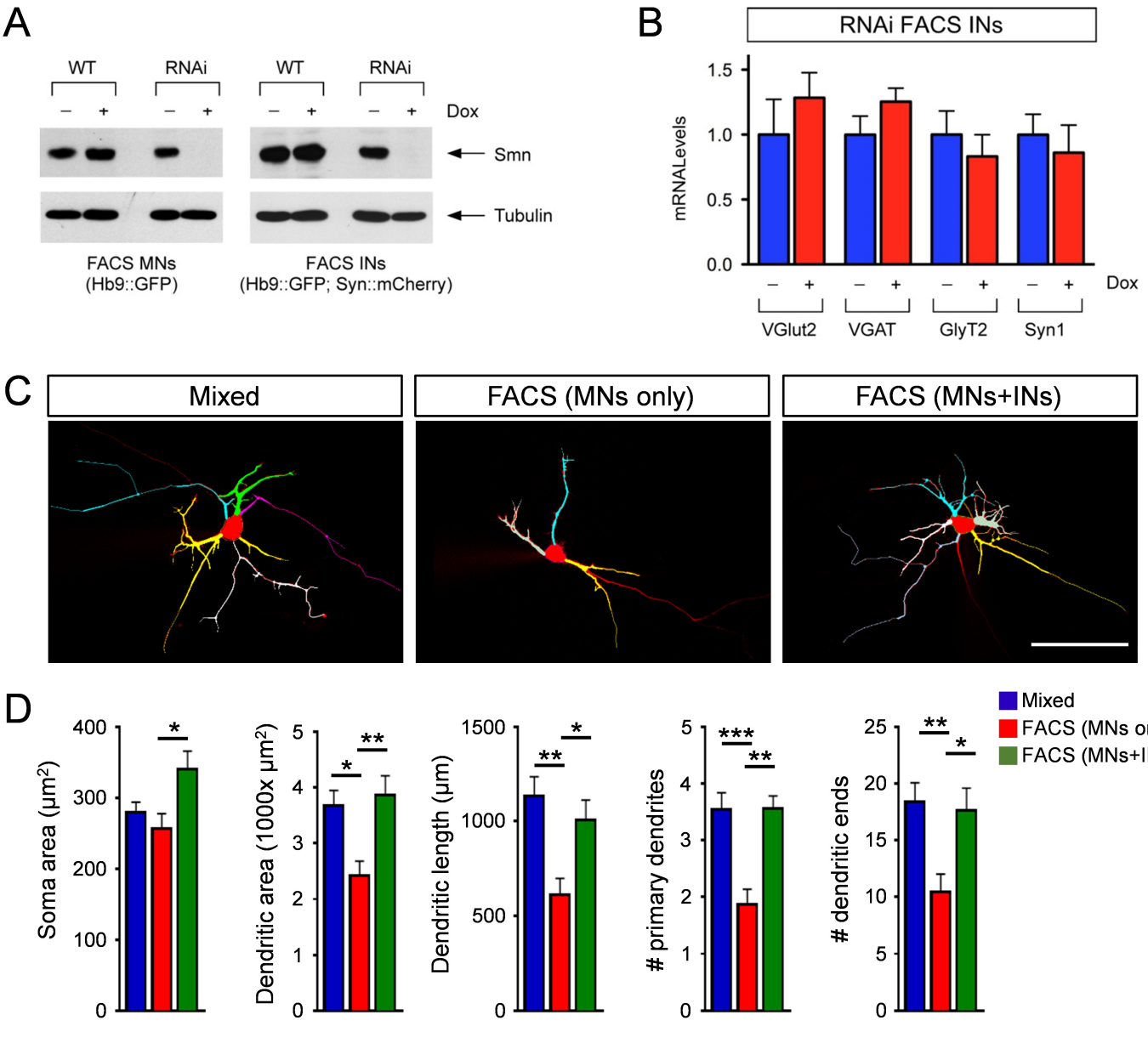
A
WT
RNAi
WT
RNAi
– + – + – + – +
Dox
Smn
Tubulin
FACS MNs
(Hb9::GFP)
FACS INs
(Hb9::GFP; Syn::mCherry)
B
RNAi FACS INs
mRNALevels
1.5
1.0
0.5
0.0
– + – + – + – +
Dox
VGlut2
VGAT
GlyT2
Syn1
C
Mixed
FACS (MNs only)
FACS (MNs+INs)
D
Mixed
FACS (MNs only)
FACS (MNs+INs)
Soma area (µm²)
400
300
200
100
0
*
Dendritic area (1000x µm²)
5
4
3
2
1
0
*
**
Dendritic length (µm)
1500
1000
500
0
**
*
primary dendrites
5
4
3
2
1
0
***
**
dendritic ends
25
20
15
10
5
0
**
*

Figure S5. Molecular and morphological characterization of MNs and INs in culture, Related to Figure 5.
(A) Western blot analysis of Smn protein levels in FACS-purified MNs differentiated from WT and RNAi ES(Hb9::GFP) cells as well as FACS-purified INs differentiated from WT and RNAi ES(Hb9::GFP; Syn::mCherry) cells. Both MNs and INs were cultured with or without Dox for 5 days. (B) RT-qPCR analysis of VGluT2, VGAT, GlyT2 and Syn1 mRNA expression in FACS-purified INs differentiated from RNAi cells and cultured with or without Dox for 5 days. Data represent mean and SEM from independent experiments (n=4). (C) Neurolucida reconstructions of the somato-dendritic tree of Alexa-555 intracellularly-filled MNs from mixed cultures and from FACS-purified MNs cultured either alone or with FACS INs at 5DIV. Scale bar=100μm. (D) Quantification of soma area, dendritic area, dendritic length, number of primary dendrites and number of dendritic ends for MNs in the same groups as in (C) at 5DIV.

Figure S6

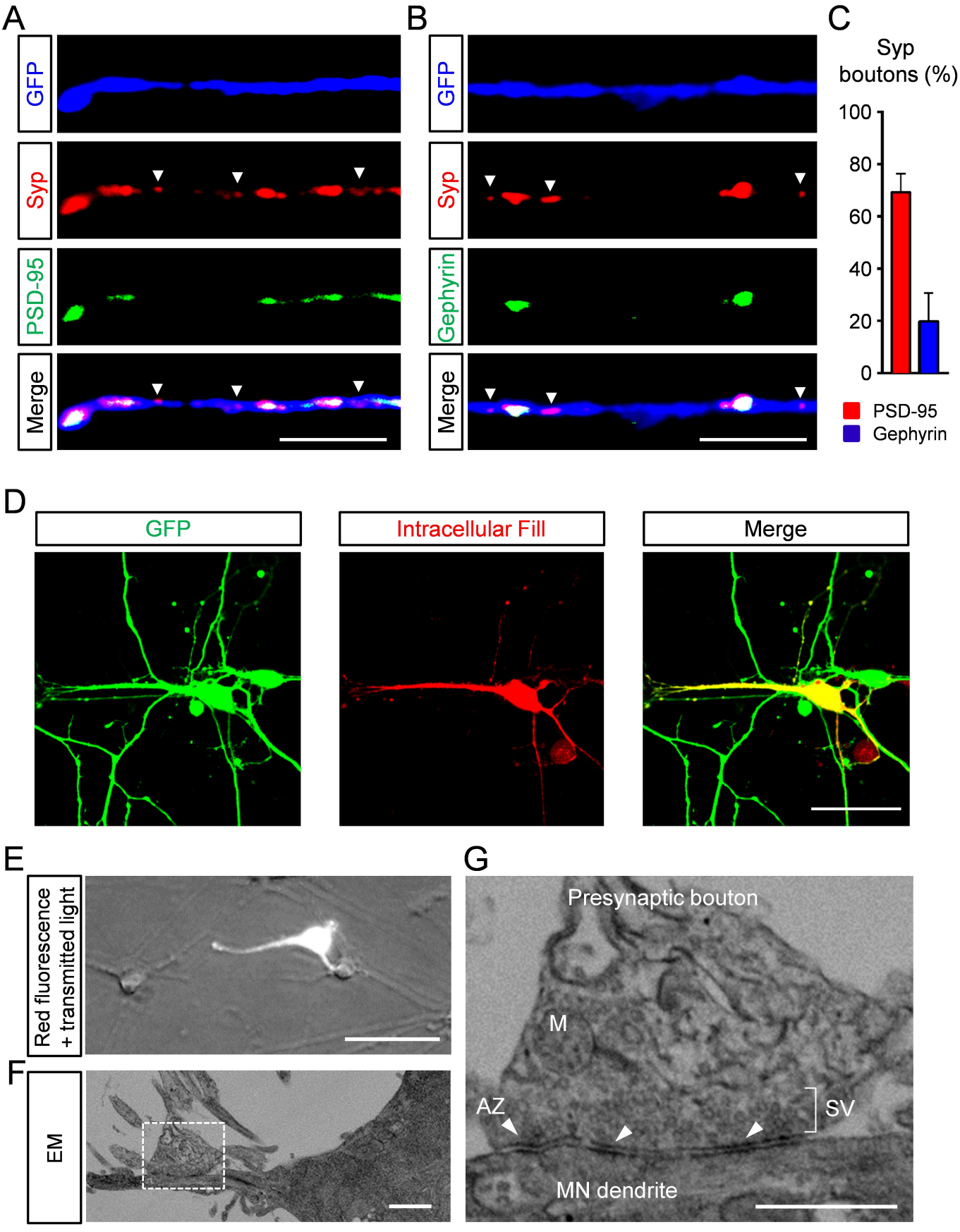
A
GFP
Syp
PSD-95
Merge
B
GFP
Syp
Gephyrin
Merge
C
Syp boutons (%)
100
80
60
40
20
0
PSD-95
Gephyrin
D
GFP
Intracellular Fill
Merge
E
Red fluorescence + transmitted light
F
EM
G
Presynaptic bouton
M
AZ
SV
MN dendrite

Figure S6. Post-synaptic specialization and ultrastructural characterization of synapses onto MN dendrites, Related to Figure 6.

(A) Confocal images of Syp (red) and PSD-95 (green) immunoreactivity on a GFP^+ motor neuron dendrite (blue) from MN(WT)+IN(WT) co-cultures at 5DIV. Arrowheads indicate presynaptic Syp^+ boutons that are not opposed to PSD-95. Scale bar=5μm (B) Confocal images of Syp (red) and Gephyrin (green) immunoreactivity on a GFP^+ motor neuron dendrite (blue) from MN(WT)+IN(WT) co-cultures at 5DIV. Arrowheads indicate presynaptic boutons that are not opposed to Gephyrin. Scale bar=5μm (C) Quantification of the number of Syp^+ boutons opposed to PDS-95 and Gephyrin relative to all Syp^+ synapses onto MNs from MN(WT)+IN(WT) co-cultures at 5DIV (n=8). (D) Images of GFP^+ MNs (green) and an intracellular filled MN (red) from MN(WT)+IN(WT) co-cultures. Scale bar=50μm. (E) Fluorescence and transmitted light image of the intracellularly labeled MN in (D). Scale bar=50μm. (F) Image from an ultra-thin section of the MN in (E) showing a synapse on the MN dendrite (white dotted box). Scale bar=1μm. (G) Higher magnification view of the synapse in (F). Scale bar=500nm. M: mitochondrion; SV: synaptic vesicles; AZ: active zone (arrowheads).

Figure S7

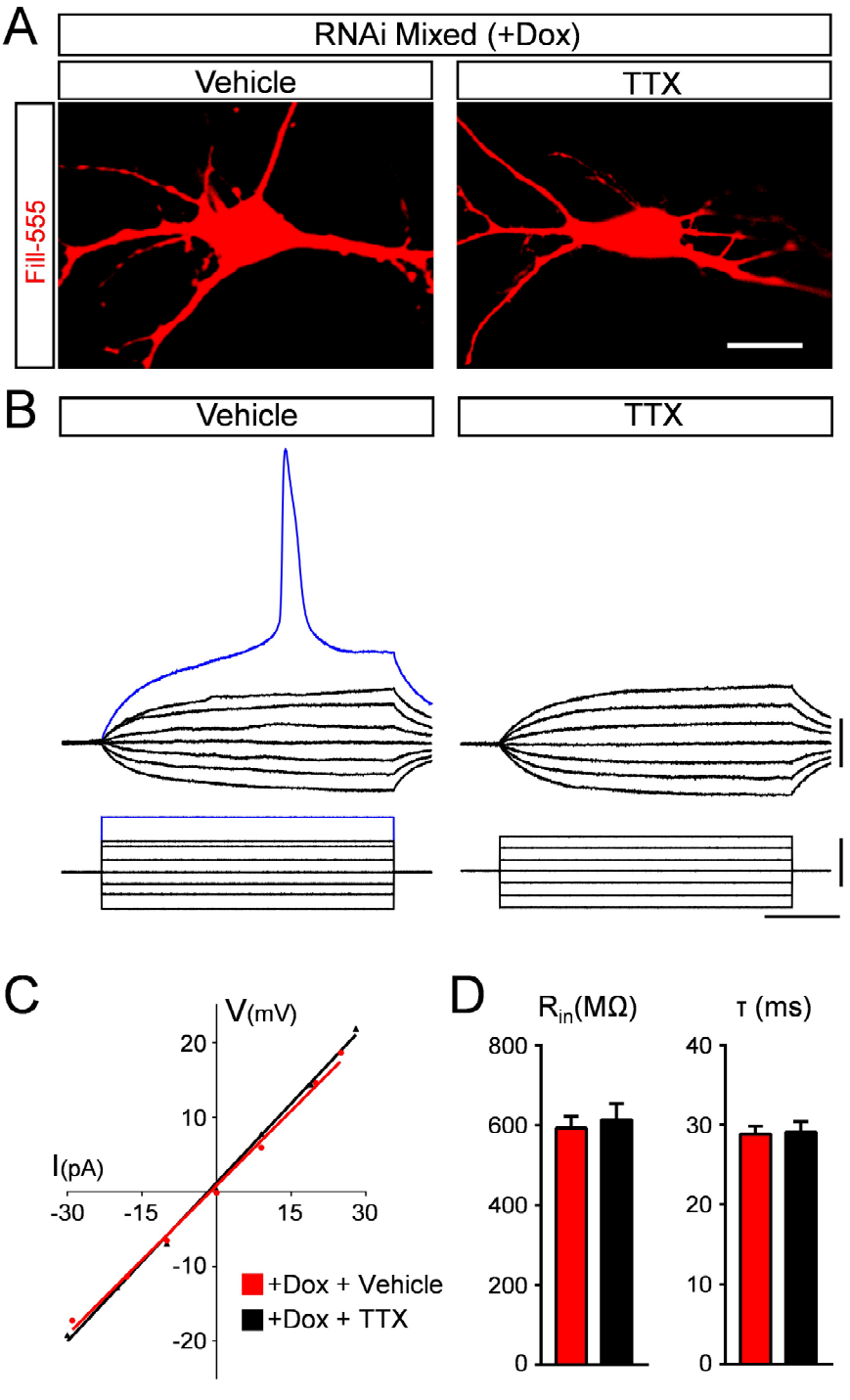
A
RNAi Mixed (+Dox)
Vehicle
TTX
Fill-555
B
Vehicle
TTX
C
V(mV)
20
10
-10
-20
I(pA)
-30
-15
15
30
+Dox + Vehicle
+Dox + TTX
D
Rin(MΩ)
800
600
400
200
0
τ (ms)
40
30
20
10
0

Figure S7. TTX treatment does not increase excitability of Smn-deficient MNs, Related to Figure 7.
(A) Representative images of Alexa-555 intracellularly-filled Smn-deficient (+Dox) MNs differentiated from RNAi ES cells and cultured under mixed conditions in the presence of either vehicle or TTX for 5 days. Scale bar=20μm. (B) Superimposed membrane responses (upper traces) following current injection (lower traces) in Smn-deficient MNs cultured with either vehicle or TTX as in A. Scale bars=20mV, 40pA, 40ms. (C) Current/voltage relationships for the MNs shown in B. Input resistance: Vehicle=667MΩ, TTX=708MΩ. (D) Mean and SEM of input resistance (R_{in}) and time constant (τ) of Smn-deficient MNs cultured with vehicle (n=11) or TTX (n=11) for 5 days.

Table S1. Summary of all parameters from recorded MNs, Related to Figure 2.

Condition	n	Treatment	RP (mV)	R_{IN} (mΩ)	τ (ms)	C (pF)	Soma (μm^2)
Mixed (RNAi)	15	-Dox	-47.3±2.0	489.8±36.0	25.4±2.1	52.8±3.2	300.0±12.9
	14	+Dox	-47.1±1.8	633.3±22.7**	30.7±1.5*	49.1±2.7	291.6 ±18.8
FACS (RNAi)	15	-Dox	-47.8±1.7	563.7±23.3	33.8±2.6	61.2±5.2	317.9±24.0
	15	+Dox	-48.3±1.9	537.7±39.7	33.8±2.9	63.9±4.5	335.6±16.5
Blockers							
Mixed (RNAi)	24	Control	-45.1±1.1	489.5±19.7	22.2±1.3	45.5±2.2	320.8±12.3
	15	TTX	-45.0±1.2	653.6±36.3***	29.4±1.9*	48.3±2.6	281.3±11.7
	12	ACh R	-45.0±1.6	522.7±41.5	22.4±2.2	42.6±1.9	320.4±16.5
	13	GABA+Gly R	-42.9±2.2	538.2±25.3	22.9±1.8	42.7±2.7	298.9±13.4
	11	Glut R	-46.5±1.5	698.3±25.3***	34.4±2.9***	50.4±4.4	341.5±20.6
	11	All blockers	-46.6±1.5	669.0±28.6***	31.3±2.0**	47.5±3.0	291.6±12.9
	11	Control +Dox	-44.5±1.2	592.9±30.3**	28.8±1.0*	50.3±3.3	291.6±20.9
	11	TTX +Dox	-45.6±0.9	613.8±31.67*	29.1±1.3*	48.3±2.6	270.0±13.3
FACS co-plated							
MN(WT)+IN(WT)	12	+Dox	-45.1±1.2	584.2±43.3	26.3±1.8	48.0±3.6	294.9±16.0
MN(RNAi)+IN(RNAi)	13	+Dox	-42.2±1.7	736.4±22.8**	34.3±2.5*	47.2±4.1	305.8±16.8
MN(RNAi)+IN(WT)	18	+Dox	-46.7±1.4	552.8±28.5	22.4±1.3	46.2±2.2	300.4±44.1
MN(WT)+IN(RNAi)	17	+Dox	-42.7±0.8	756.7±31.6**	34.9±2.2***	41.3±2.2	311.9±15.9
Mixed RNAi+SMN	10	-Dox	-39.0±1.2	451.6±46.4	17.6±2.5	39.0±3.2	304.0±13.2
	10	+Dox	-40.7±1.3	458.3±41.3	17.2±2.1	38.5±4.0	307.5±16.4
Mixed WT	11	-Dox	-42.5±1.1	563.8±21.1	22.0±2.1	39.0±3.2	282.7±20.5
	11	+Dox	-43.1±1.2	548.0±36.6	19.3±1.5	35.6±2.7	290.8±21.8

Table S2. List of primers used in this study, Related to Figure 4.

Name	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
Smn	TGCTCCGTGGACCTCATTTCTT	TGGCTTTCCTGGTCCTAATCCTGA
GAPDH	AATGTGTCCGTCGTGGATCTGA	GATGCCTGCTTCACCACCTTCT
ChAT	CGGTCCTCTTAAAAGACTCCACCAAC	TGTGTGTGTCACTGAGGTCTCCA
GFP	CGTCTATATCATGGCCGACAAGCA	TTGTGGCGGATCTTGAAGTTCACC
mCherry	GCGCCTACAACGTCAACATCAA	GTTCGTACTGTTCCACGATGGTGT
Synapsin I	ACAAGGCCTACATGAGGACATCAG	CGTGTCTACCCACAACTTGTACCT
Synaptophysin	TGCAGTGGGTCTTTGCCATCTT	ACTTCGATGTTGAGGGCACTCT
VGluT1	TATGGCAGCTTCGGGATCTTTTGG	TCCCCGATGGCATCCTCAATGTAT
VGluT2	ATGCCCTTAGCTGGTATCCTTGTG	ACATGTACCAGACCATGCCAAAGC
VGluT3	CTGGAATGGTTTGTCCCCTCATTG	AGATGACTCCGCTGTAGTGTACCA
GlyT1	GCAACCTGTCTCACCTGTTCAACT	CGTCTGACAGCTTCAGCACATACA
GlyT2	ACATTTGTCAGCGGGAGTGAAGAG	ACCCAAGCCAAGAAAAGGCAGAAG
VGAT	AATCTCAAGGCCGTGTCCAAGTTC	GAGACAGTAAGCGATGACCAGGAT
VAChT	CCACGTATCAGTCTATGGCAGTGT	GCTCAAAGCCAAGTGAGTGAACGA
Hb9	CTCACCGAGACTCAGGTGAAGATT	AGCCTGCTCTTTGGCCTTTT
hSMN exon 4	AAGCCCAAATCTGCTCCATGGAAC	TGGCTTTCCTGGTCCCAGTCTT

Table S3. List of antibodies used in this study, Related to Figure 6.

Name	Company	Cat #	Host	Application	Dilution
SMN clone 8	BD Transduction laboratories	610646	Mouse	WB	1:10,000
Tubulin DM 1A	Sigma	T9026	Mouse	WB	1:10,000
GFP	Abcam	13970	Chicken	IF	1:2000
Tuj1	Covance	MMS-435P	Mouse	IF	1:500
Synaptophysin I	Synaptic Systems	101-004	Guinea Pig	IF	1:500
VGluT2	Synaptic Systems	135-403	Rabbit	IF	1:500
VGAT	Synaptic Systems	131-011	Mouse	IF	1:500
DsRed	Clontech	632496	Rabbit	IF	1:500
PSD-95	Millipore	MABN68	Mouse	IF	1:250
Gephyrin	Synaptic Systems	147021	Mouse	IF	1:100

Supplemental Experimental Procedures

Lentiviral Constructs and Viral Production

All lentiviral constructs were generated by standard cloning techniques using the pRRLSIN.cPPT.PGK-GFP.WPRE vector (Addgene plasmid 12252) as a backbone (Dull et al., 1998; Zufferey et al., 1998). The pLenti.neo/TR vector constitutively expresses the tetracycline-dependent repressor (TetR) protein under the control of the PGK promoter as well as the neomycin resistance gene following an IRES element. The pLenti.pur/Smn_{RNAi} vector expressing an shRNA targeting mouse Smn mRNA and the pLenti.hyg/SMN vector constitutively expressing an RNAi-resistant, epitope-tagged human SMN were previously described (Ruggiu et al., 2012). The pLenti.hyg/Syn-mCherry vector contains the mCherry coding region, which was PCR amplified from pRSET-B.mCherry (Addgene) using the following primers: CGGCGGATCCGCCGCCACCATGGTGAGCAAGGGCGAGG (Forward) and TCGACTCGAGTCGACTACTTGTACAGCTCGTCC (Reverse), driven by the mouse Syn1 promoter. The Syn1 promoter was PCR amplified from genomic DNA of NIH3T3 cells using the following primers: GTCCGATATCGGACAAGAACCCCACCCC (Forward) and TCCGCGGCCGCGGATCCGCTCTCTGGCACGACGCGACTC (Reverse). The construct also expresses the hygromycin resistance gene driven by the SV40 promoter.

Lentiviral stocks were prepared by transient co-transfection of 293T cells with lentiviral constructs and helper plasmids pLP1, pLP2 and pLP/VSVG using calcium phosphate (CalPhos Mammalian Transfection Kit, Clontech). Medium was changed the following day. Supernatant was collected 48 and 72 hours post transfection. Lentivirus was concentrated by ultracentrifugation at 19,500 rpm for 2.5 hours at 20°C, reconstituted in PBS, and stored in aliquots at -80°C. The viral titer was determined with the Lenti-X^{TM} qRT-PCR Titration Kit (Clontech).

ES Cell Lines and Tissue Culture

ES cells were grown either on gamma-irradiated MEFs (for expansion) or on gelatin-coated dishes (for differentiation) in ES cell medium EbryoMax DMEM (Millipore); 15% FBS (HyClone); 2mM L-glutamine (Invitrogen); pennicilin/streptomycin (GIBCO); 1x non-essential amino acids (Millipore); 1x nucleosides (Millipore); 0.1mM 2-mercaptoethanol (Millipore); and 1000U/ml leukemia inhibitory factor (LIF) (Millipore).

To generate ES cell lines through lentiviral transduction, ES cells were thawed on a monolayer of mouse embryonic fibroblasts (MEFs) DR4 (GlobalStem). Following overnight transduction with lentivirus, the medium was changed and the appropriate antibiotics added at the following final concentrations: 125µg/mL Neomycin (Invitrogen), 1µg/ml Puromycin (Sigma), and 125µg/ml Hygromycin B (Invitrogen). Following antibiotic selection, individual ES clones were grown into 96-well replica plates and further characterized for transgene expression.

Directed Differentiation of ES Cells into MNs and INs

ES cells were trypsinized with 0.05% Trypsin/EDTA (GIBCO) and $2x10^6$ cells transferred to a 10cm plate in differentiation medium: 45% ADMEM/F12 (Invitrogen); 45% Neurobasal medium (Invitrogen); 10% Knockout Serum Replacement (Invitrogen); 2mM L-glutamine (Invitrogen); penicillin/streptomycin (GIBCO), and 0.1mM 2-mercaptoethanol (Millipore). The following day floating embryoid bodies (EBs) were collected, spun down (500 rpm, 2 minutes) and transferred to a 10cm non-adherent plate in differentiation medium. For MN differentiation, floating EBs were collected the next day and divided into five 10cm plates with differentiation medium supplemented with 1µM retinoic acid (RA, Sigma) and 0.25µM smoothened agonist (SAG, Calbiochem). For differentiation of INs, EBs were divided into two 10cm plates with differentiation medium supplemented with 1µM RA only. Differentiation medium was changed every 2 days after induction. Five days following induction, EBs were collected, washed twice in PBS/0.4% glucose and trypsinized for 5 minutes in trypsin supplemented with DNase I (50 µg/ml) and $MgCl_2$ (3mM). EBs were then mechanically dissociated in PBS supplemented with 0.4% glucose, 50µg/ml DNase I and 3mM $MgCl_2$. The dissociated cells were transferred into a new falcon tube, centrifuged (800 rpm, 5 minutes) and re-suspended in neuronal plating medium: Neurobasal supplemented with B27 (GIBCO), 2% horse serum FCS (GIBCO), 0.5mM L-glutamine (Invitrogen), 0.025mM glutamate (Sigma), 2-mercaptoethanol (Millipore), penicillin/streptomycin (GIBCO), and neurotrophic factors [10ng/ml each CNTF, BDNF, and GDNF (GIBCO)]. Dissociated cells were plated on dishes pre-coated for 20 minutes at room temperature with 15mg/ml poly-L-ornithine (Sigma), followed by overnight coating at 37°C with 3mg/ml laminin in L-15 supplemented with 0.2% sodium bicarbonate (GIBCO). For survival assays, 3,000 (mixed MNs) or 1,500 (FACS-purified MNs) cells in 100ul were plated in each well of 96-well plates. For recordings and immunofluorescence analysis, 50,000 cells were plated in each well of 4-well plates containing 12mm No. 1.5 glass coverslips (Fisher). For WB and RT-qPCR experiments, up to $1x10^6$ cells were plated in each well of 6-well plates.

To induce Smn knockdown, Dox (Fisher) was added at 100ng/ml to the culture medium of ES cells or ES-derived neurons.

Immunohistochemistry and Confocal Microscopy

MNs and INs cultured on 12mm glass coverslips in 4-well plates either in the presence or in the absence of Dox for 5 days were washed briefly with PBS, fixed for 30 minutes at room temperature with 4% formaldehyde-PBS made fresh before each use from 1ml ampules of 16% formaldehyde (Thermo Scientific), and permeabilized with 0.1% Triton X-100-PBS for 5 minutes on ice. Blocking was performed using 10% Normal Donkey Serum (NDS) (Millipore) in PBS for 1 hour at room temperature. Fixed cells were incubated with primary antibody diluted in 10% NDS-PBS for 1 hour at room temperature. Cells were then washed and incubated with the appropriate secondary antibody conjugated to fluorescent dye (Jackson ImmunoResearch Laboratories) diluted at 1:250 in 10% NDS-PBS for 1h at room temperature in the dark. DAPI was added with the secondary antibodies. Cells were washed with PBS before coverslips were mounted on glass slides using Prolong Gold Antifade reagent (Invitrogen) and left to dry overnight in the dark at room temperature. For studies of IN survival (Fig. S1G-H) and mCherry expression in $Tuj1^+$ INs (Fig. 4B-C), images were acquired with a 10x objective with a fluorescent microscope (Olympus IX71 with a Hamamatsu digital camera) and analyzed off-line using ImageJ.

For synaptic counts, cells were imaged using an SP5 Leica confocal microscope. Synapses were counted off-line from Z stack images captured at 0.2 µm z-steps to include the whole cell body and dendrites of each MN that was filled with a fluorescent dye (Cascade Blue dextran, Alexa-555). Images were scanned using a 63x glycerol objective. Synapses were counted over each filled MN surface using the Leica LASAF software. To obtain density estimates, we measured all Syp^+ boutons on dendritic segments at 50 µm from the cell body and divided this number by the total linear length of all dendritic segments in each compartment.

Electrophysiology

Glass coverslips containing neuronal cultures were transferred to a customized recording chamber placed under the objective of a confocal microscope (SP5 Leica) equipped with 4 single laser lines (405, 488, 543, 650nm) and a 2-photon laser (Chameleon XR, Coherent). The chamber was filled with HEPES buffer containing 145mM NaCl, 3mM KCl, 1mM $MgCl_2$, 1.5mM $CaCl_2$, 10mM glucose, and 10mM Hepes adjusted to pH 7.4 with NaOH. Electrodes were pulled with a P1000 puller (Sutter) to resistance of 10-15MΩ. Electrodes were filled with intracellular solution containing: 10mM NaCl, 130mM K-Gluconate, 10mM HEPES, 1mM EGTA, 1mM $MgCl_2$, 0.1mM $CaCl_2$, 1mM Na_2ATP, and fluorescent hydrazide (AlexaFlour-555, Cascade Blue), pH adjusted to 7.2-7.3 with KOH (the final osmolarity of the intracellular solution was ~305-309 mOsm).

EM Analysis

FACS-purified, ES-derived WT MNs and INs were co-cultured on 12mm plastic coverslips and GFP^+ MNs were intracellularly filled with fluorescent hydrazide (AlexaFlour-555) at 5DIV and processed by correlative light and serial section electron microscopy and image acquisition as described previously (Tapia et al., 2012). The cultures were fixed with 4% PFA and 2% glutaraldehyde overnight at 4ºC and transferred into PBS. Intracellular labeled MNs were then imaged using an SP5 Leica confocal microscopy equipped with a 543He-Ne laser line and 20x water immersion objective (zoom 2, 0.5NA). Placement of fiducial markers helped to record the position of the dye labeled cells in the electron microscope for further processing as described previously (Tapia et al., 2012). Following ROTO treatment, the dye labeled cells were alcohol dehydrated and flat embedded with Epon resin (60^oC, 2 days). Resin blocks were sectioned (~40nm) with a diamond knife (Diatome) in a Leica Ultramicrotome (UC7), and the serial sections containing the cells of interest collected with ATUM (Tapia et al., 2012). After a short incubation with lead citrate (1%), the serial sections were placed on silicon wafers (Universal Wafers), and imaged using a 10kV electron beam in a SIGMA field emission scanning electron microscope (Zeiss). Serial sections (approximately 200-300 sections per cell) were acquired, aligned and segmented using TrakEM2 in FIJI/NIH ImageJ (Cardona et al., 2010).

Supplemental References

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