Supplemental information

A Small Molecule Screen in Stem Cell-derived Motor Neurons Identifies a Kinase Inhibitor as a Candidate Therapeutic for ALS

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Supplemental Data

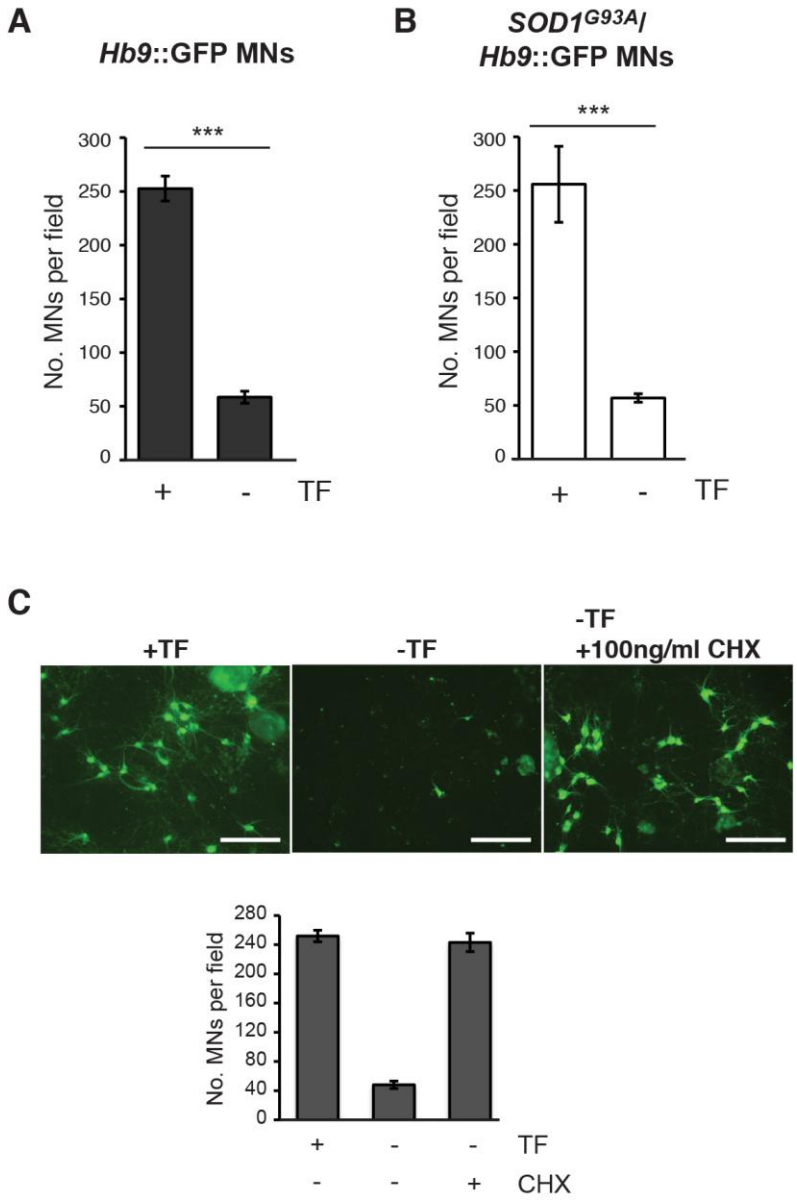


Figure S1, related to Figure 1. Trophic factor withdrawal-induced MN death.

(A and B) Trophic factor (TF) withdrawal induced about 80% MN death, giving about a five-fold screening window, in *Hb9*::GFP (A) and *SOD1*^{G93A}/*HB9*::GFP (B) MN cultures. Freshly dissociated EBs were plated at 8,000 GFP^{+} cells per well in poly-*D*-lysine coated 384-well plates. TFs were removed four days later, and plates were fixed after another three days. Surviving MN

numbers were obtained by counting GFP^+ cells. This trophic factor-induced death was relatively specific to MNs since the total numbers of non-MNs (determined by counting $Hoechst^+$ nuclei) did not change substantially after trophic factor withdrawal (data not shown). Data are presented as mean ± SEM. ★★★$p<0.001$ (student's t-test, two-tailed).

(C) Cycloheximide (CHX), a protein synthesis inhibitor, was used as the positive control for the screen. CHX at concentration of 100ng/ml showed no toxicity and was able to prevent MNs from dying in the –TF condition. Scale bar represents 200μm.

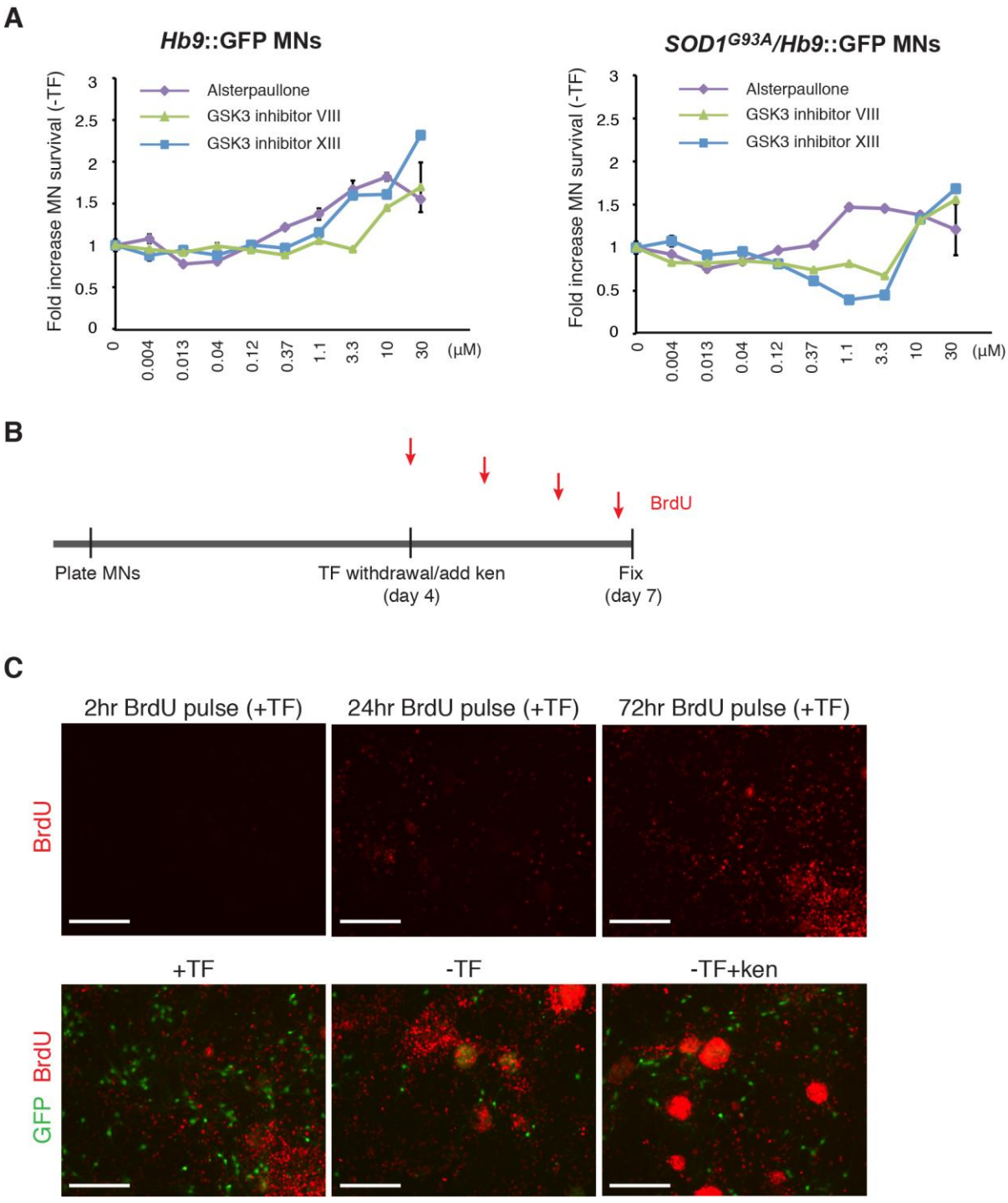


Figure S2, related to Figure 2. Survival effect of additional GSK-3 inhibitors.

(A) Dose curves of other GSK-3 inhibitors that had more variable activities than kenpaullone. Data are represented as average ± SEM.

(B) and (C) MNs surviving after kenpaullone (ken) treatment were not generated from BrdU-

labeled progenitor cells. (B) Overview of BrdU labeling method. Trophic factors were removed and kenpaullone was added to *HB9*::GFP MNs on day 4. BrdU was added to cultures 2hrs, 24hrs, 48hrs and 72hrs before fixing on day 7. (C) (Upper panel) Images of +TF conditions from different lengths of BrdU incubation. (Lower panel) Images of +TF, -TF and –TF+ken cells after 72 hours of BrdU incubation. There were few, if any, $BrdU^{+}/GFP^{+}$ double-positive cells in any of the cultures examined, suggesting that none of the surviving MNs were newly generated during the course of the experiment. Kenpaullone-treated cultures had many BrdU-positive clusters of cells that were not seen under other conditions, suggesting that kenpaullone may also stimulate progenitor cell proliferation or support survival or proliferating progenitors. Scale bar represents 200μm.

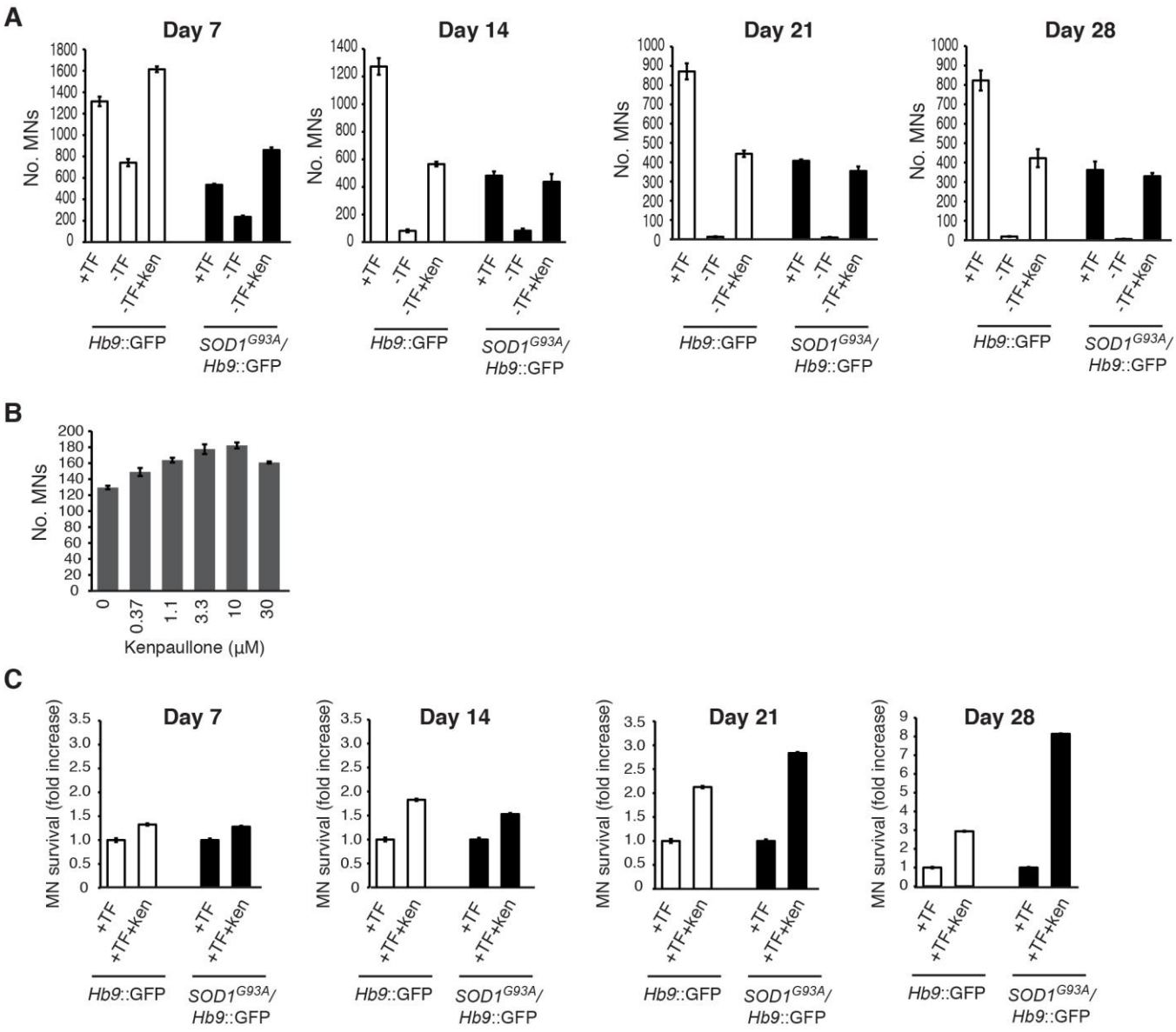


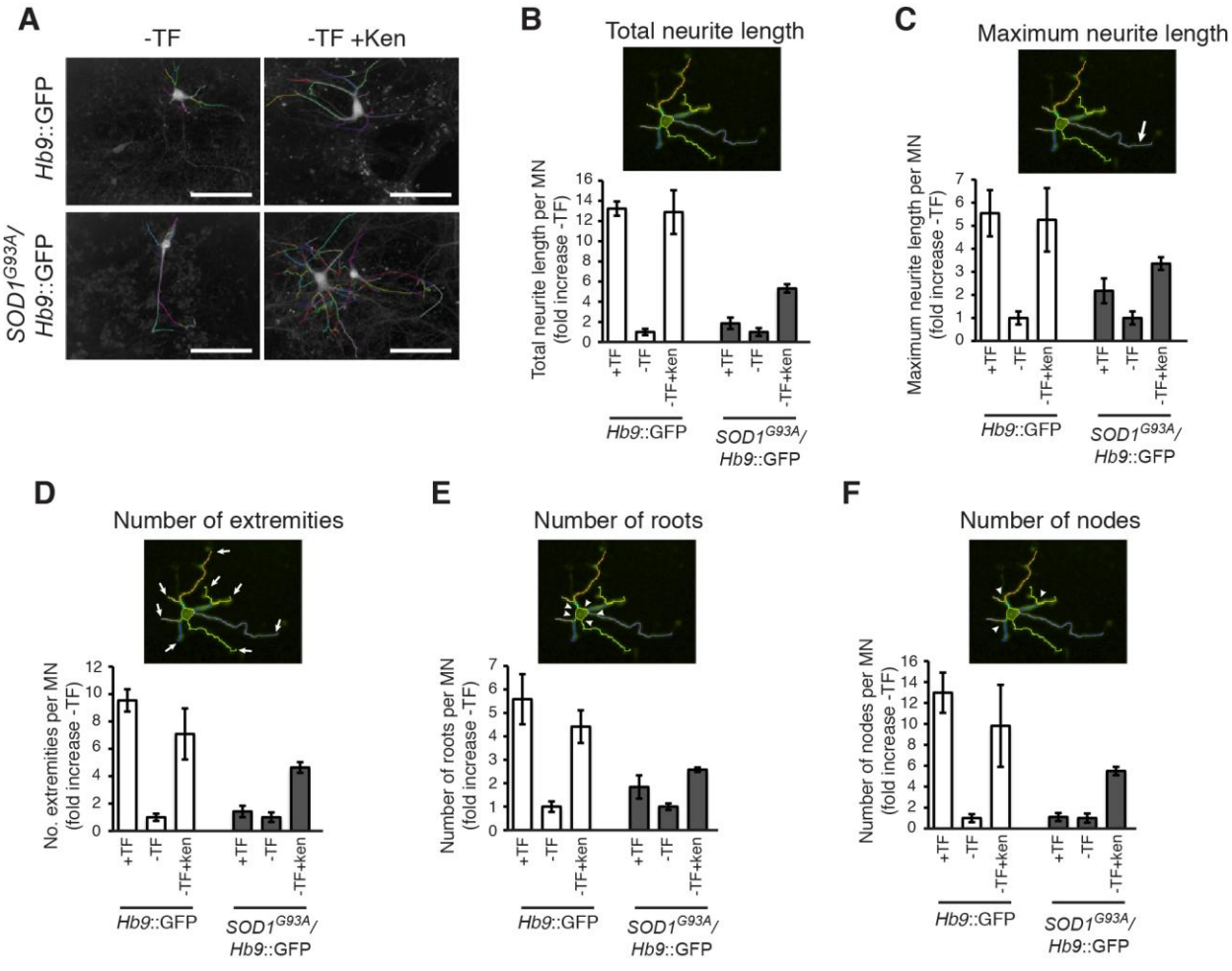
Figure S3, related to Figure 3. Kenpaullone promotes long-term survival of both types of MNs in medium with and without trophic factors.

(A) Number of MNs was determined at various times after TF withdrawal. Cells were fed and kenpaullone was refreshed every two days. TF= trophic factors; ken = kenpaullone.

(B) Three days of kenpaullone treatment has a small effect on MN survival even in the presence of trophic factors.

(C) Long-term survival effect of kenpaullone in complete medium (+TF). Kenpaullone was added to cells on day 4, and survival was quantified at the indicated times. Cells were refed with fresh medium and compound every two days.

Data are presented as mean ± SEM.



G

	+TF	-TF/+kenpaullone	*p*
Capacitance (pF)	57.3 ± 5.4 (*n*=5)	52.4 ± 3.6 (*n*=5)	0.48
Resting Vm (mV)	60.1 ± 3.0 (*n*=5)	58 ± 1.6 (*n*=5)	0.56
Input Resistance (MΩ)	191 ± 61 (*n*=5)	192.4 ± 74 (*n*=5)	0.99
Na (pA)	10644 ± 246 (*n*=5)	13710 ± 3747 (*n*=5)	0.55
K (pA)	9340 ± 1097 (*n*=5)	7299 ± 806 (*n*=5)	0.19
Kainate (pA)	3186 ± 1168 (*n*=4)	1552 ± 262 (*n*=4)	0.21
GABA (pA)	14933 ± 498 (*n*=4)	12601 ± 1274 (*n*=4)	0.70

Figure S4, related to Figure 4. Morphological and electrophysiological analyses.

(A)~(F) Kenpaullone (ken) enhances MN neurite arborization. Trophic factors (TFs) were removed and kenpaullone (5μM) was added on day 4, and neuronal arborization was analyzed by

imaging on day 21. (A) Overview of traces of neuronal processes as defined by imaging software. Kenpaullone increases all aspects of neuronal arborization analyzed, including (B) total neurite length per MN, (C) maximum neurite length per MN, (D) numbers of extremities per MN, (E) numbers of roots per MN, and (F) numbers of nodes per MN. Data are presented as mean ± SEM. Scale bar represents 100μm.

(G) Quantification of electrophysiological properties of *HB9*::GFP MNs in +TF and in –TF/+kenpaullone conditions analyzed after three days of kenpaullone treatment. The two types of cells were similar by these measurements. p values all >0.1 by two-tailed t-test. pF - picofarads, mV - millivolts, and pA – picoamperes. Data are presented as mean ± SD.

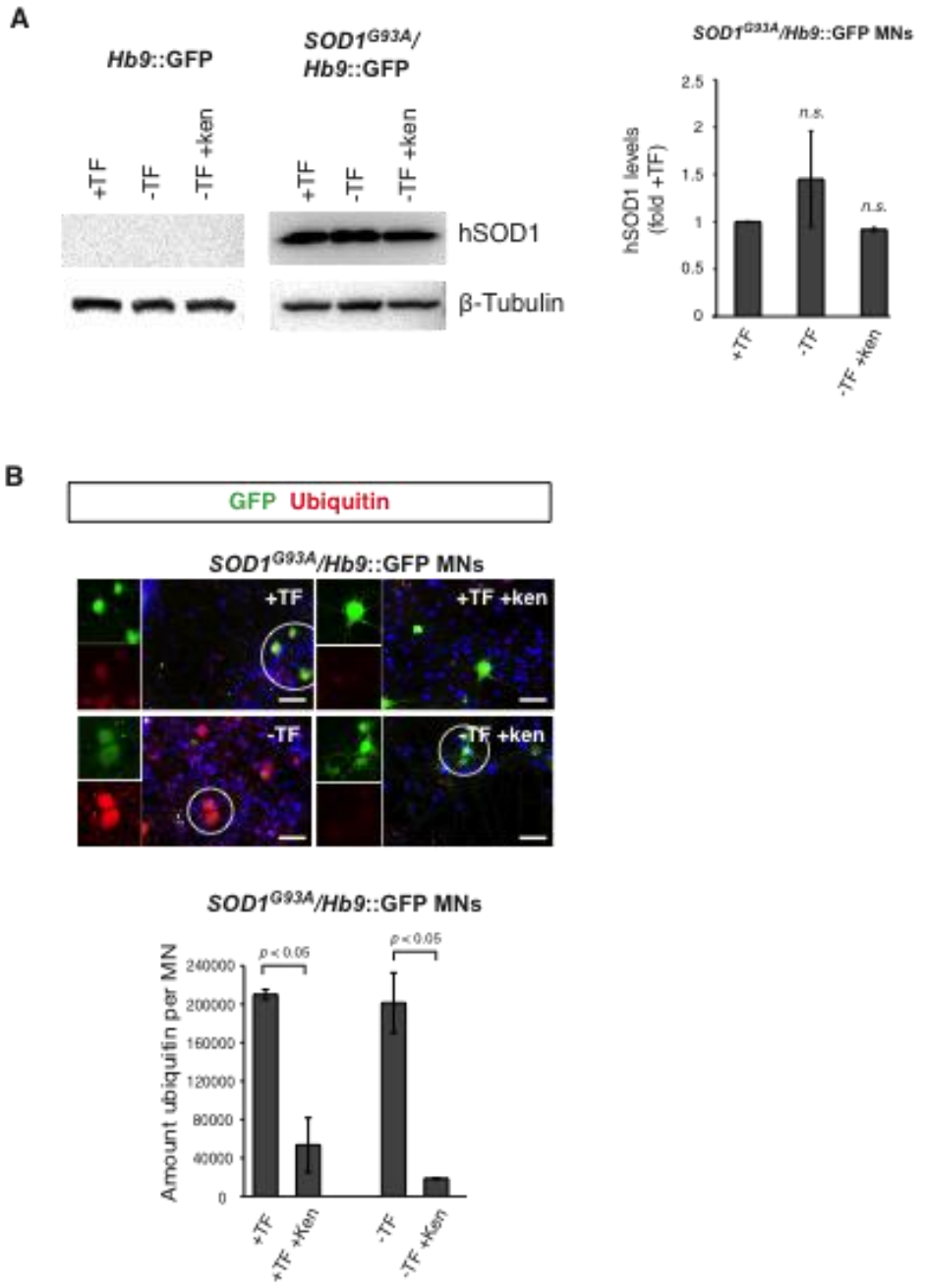


Figure S5, related to Figure 5. Kenpaullone reduces ubiquitin protein levels at later times.

(A) No significant decreases in SOD1^{G93A} protein levels in cultures were observed by Western blotting on day 15.

(B) Image analysis demonstrates that kenpaullone treatment reduces ubiquitin levels in *SOD1^{G93A}/HB9*::GFP MNs on day 21. Insets show higher magnification of cells contained within

white circles. Data are presented as mean ± SEM. *p* values were calculated with a two-tailed Student's t test. *n.s.*, not significant. Scale bar represents 50μm.

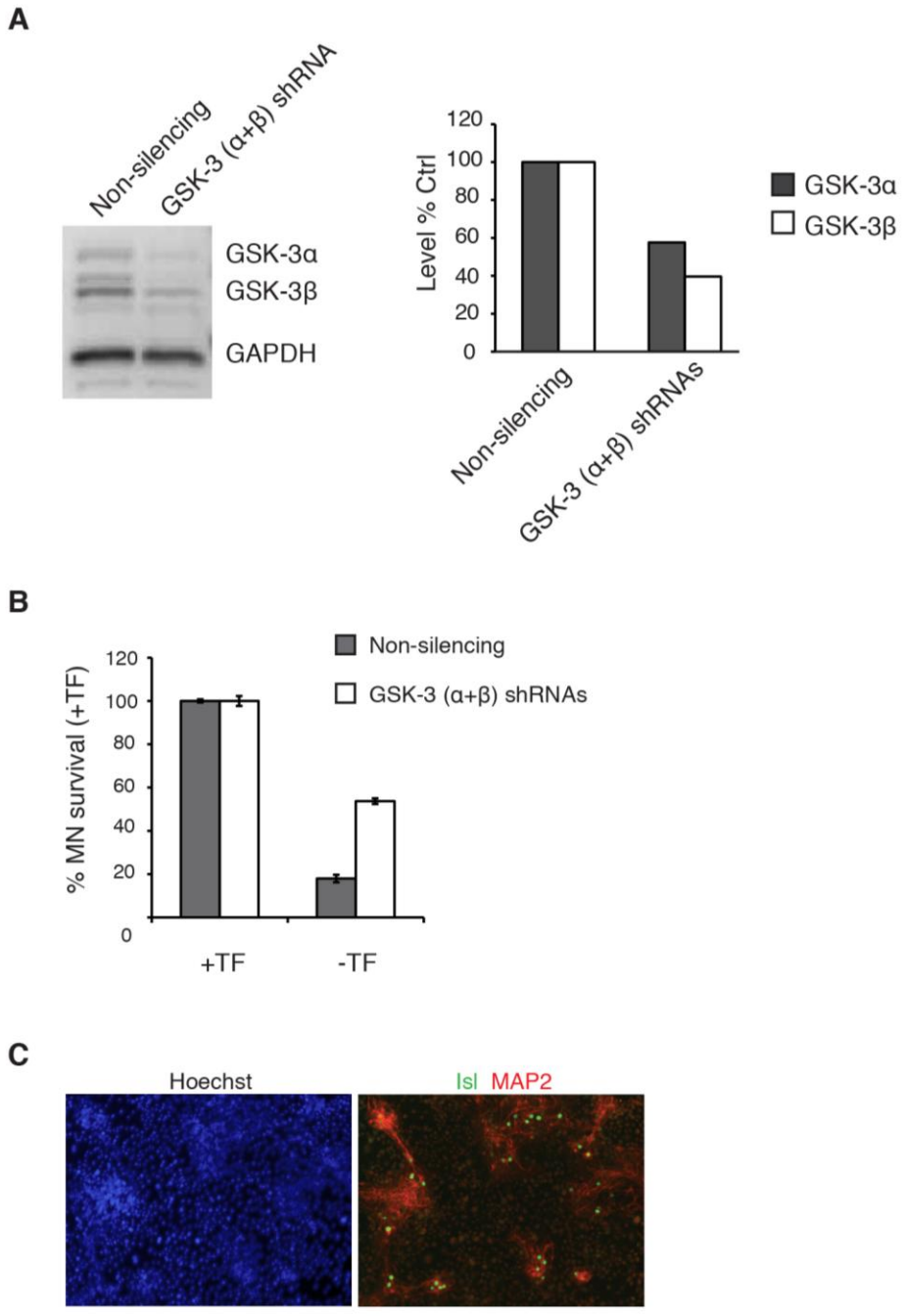


Figure S6, related to Figure 6. Survival effects of GKS-3α and GSK-3β knockdown.

(A) A representative Western blot shows the levels of knockdown of GSK-3α and GSK-3β in *HB9*::GFP MN cultures after infection with either non-silencing shRNA or shRNAs against both GSK-3α and GSK-3β. Two days after virus addition, puromycin was added to eliminate non-infected cells, and four days later the cultures were harvested for Western blotting. At the time of harvest approximately 20~30% of all cells were MNs (data not shown). About 43% knockdown

of GSK-3α and 60% knockdown of GSK-3β were generated by these shRNAs. Data are presented as mean ± SEM.

(B) MN survival after GSK-3 knockdown by shRNAs. *HB9*::GFP MN cultures were infected with lentivirus on day 2. Trophic factors (TFs) were removed on day 4, and survival was analyzed on day 7. MN survival was quantified with respect to that in cultures kept in +TF conditions. Data are presented as mean ± SEM.

(C) Islet/MAP2 staining on GSK-3$\alpha^{+/-}$/GSK-3$\beta^{-/-}$ (3/4 KO) MN culture. Scale bar represents 100μm.

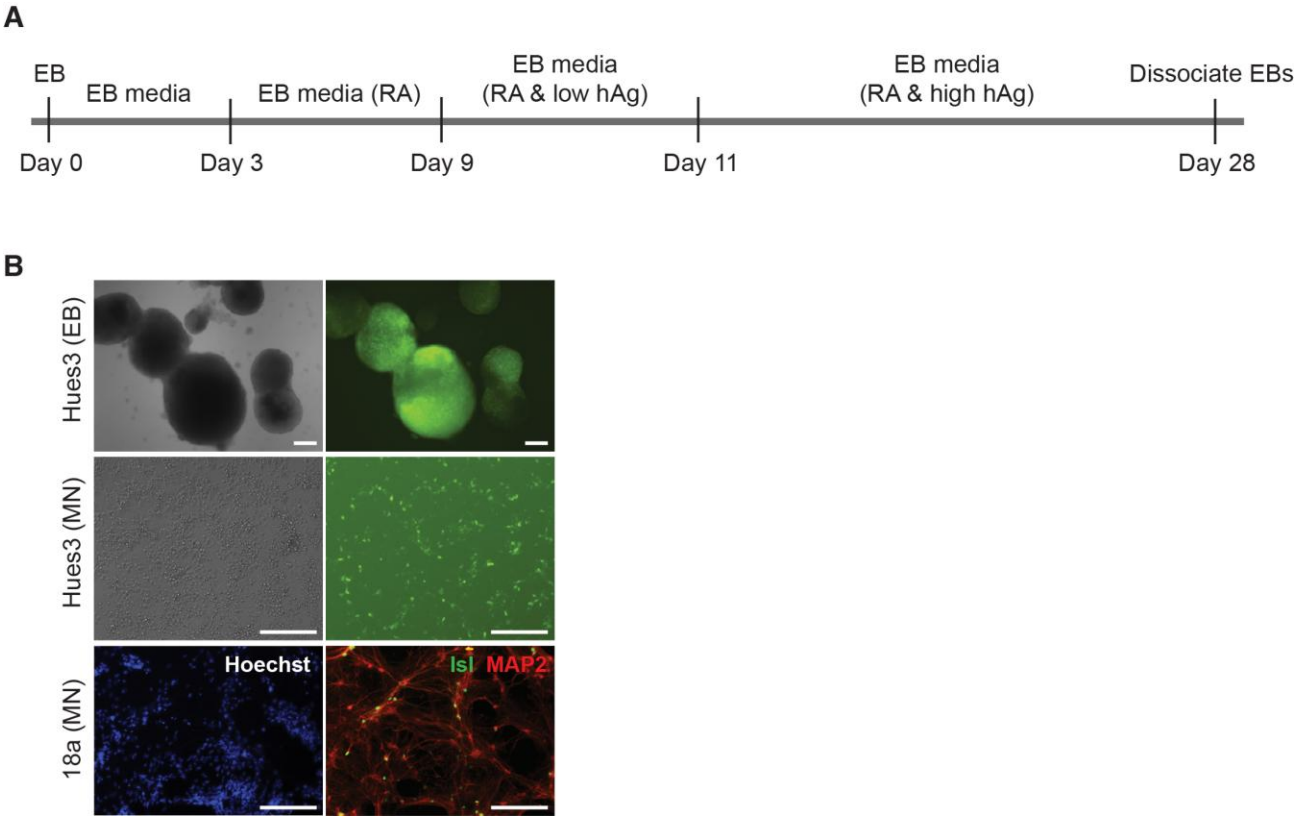
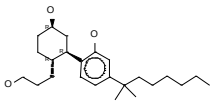
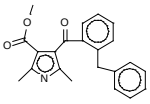
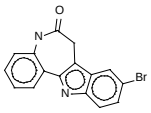
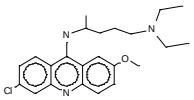
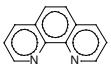
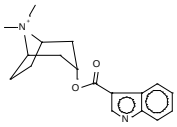
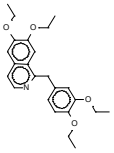
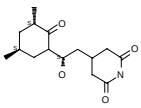
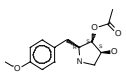
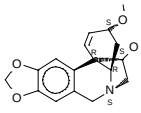


Figure S7, related to Figure 7. Differentiation of MNs from human ESCs and iPSCs

(A) Overview of differentiation protocol for human MNs. Also see experimental procedures.

(B) Images of EBs (top panel) and MNs (middle panel) from Hues-3/*Hb9*::GFP cells and MNs derived from human healthy control iPSCs (bottom panel). Islet and MAP2 staining were used to mark MNs derived from iPSCs. Scale bar represents 200μm.

Supplemental Tables

	Compound Name	CAS Number	Molecular Structure
1	CP55940	83002-04-4	
2	FPL-64176	120934-96-5	
3	Kenpaullone	142273-20-9	
4	Quinacrine dihydrochloride dihydrate	6151-30-0	
5	1,10-Phenanthroline monohydrate	5144-89-8	
6	3-Tropanylindole-3-carboxylate methiodide	89565-68-4	
7	Ethaverine hydrochloride	985-13-7	
8	Cycloheximide	66-81-9	
9	Anisomycin	22862-76-6	
10	Crinamine	639-41-8	

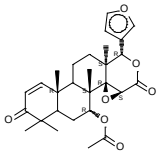
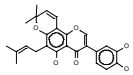
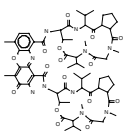
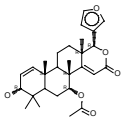
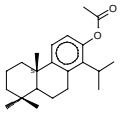
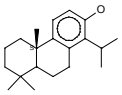
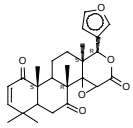
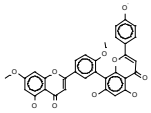
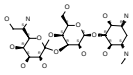
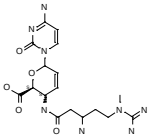
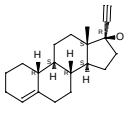
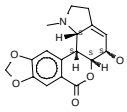
11	Gedunin	2753-30-2	
12	Pomiferin	572-03-2	
13	Dactinomycin	50-76-0	
14	3-alpha-hydroxydeoxygedinin	30437-71-9	
15	Totarol acetate	15340-82-6	
16	Totarol	511-15-9	
17	Deacetoxy-7-oxisogedunin	5506-92-3	
18	Ginkgetin K salt	481-46-9	
19	Hygromycin B	31282-04-9	
20	Blasticidin S	2079-00-7	
21	Lynestrenol	52-76-6	
22	Hippeastrine hydrobromide	22352-41-6	

Table S1, related to Figure 1. List of validated hit compounds.

List of the twenty two hits that promoted the survival of both *HB9*::GFP and *SOD1*^{G93A}/*HB9*::GFP MNs.

Supplemental Experimental Procedures

Small Molecule Libraries for Motor Neuron Survival Screens

The small molecules we used in these screens were: LOPAC1280 Collection (Sigma-Aldrich), Spectrum Collection (Microsource Discovery Systems), and Prestwick Chemical Library. A selected additional set of individual kinase inhibitors and GPCR ligands was also included.

Hit Identification

In the primary screen, we selected the top ranked compounds in each plate that also scored greater than 3 standard deviations above the low ctrl in terms of the numbers of GFP^+ MNs. Compounds that scored as hits but gave rise to abnormal MN morphology (for example, cells with markedly reduced soma size) were excluded. All primary hits were subsequently tested in a nine-point dose-response assay, using the same screening protocol.

Image Analyses for Quantification of Synapses and Neurite Arborization

For synapse analysis, we quantified imaging data using the PerkinElmer Acapella software spot detection algorithm. Synapses were defined as the overlap of presynaptic and postsynaptic spots on MNs. On each GFP^+ cell, we first drew a circle around the cell body with a diameter of approximately two cell body diameters, similar to that described by Ullian et al. (Ullian et al., 2004), as the area for spot detection. Within each circle, presynaptic sites were detected using a primary antibody against synapsin and a secondary antibody conjugated with Alexa-632. Candidate spots that met an average intensity threshold were accepted as legitimate presynaptic punctae. Then we expanded the radius of each presynaptic puncta by 1 pixel, since the pre- and

post-synaptic spots often were slightly offset, especially at higher magnifications, and measured the intensity in the postsynaptic channel, using a PSD95 primary antibody and a secondary antibody conjugated with Alexa-546. Postsynaptic punctae that exceeded the threshold mean intensity threshold were accepted, permitting us to define "colocalized" spots, or synapses.

The neurite tracing method in Acapella software is based on methods developed at CSIRO (http://www.perkinelmer.com/pages/020/cellularimaging/assays/neuriteoutgrowth.xhtml). The cell bodies of MNs were located by expression of *HB9*::GFP, and the neurites were also detected by GFP fluorescence. The algorithm measured the lengths and fluorescence intensities of each neurite and the branching level (primary, secondary, etc). The total lengths of neurites and the numbers, lengths and fluorescent intensities of primary and secondary branches were measured based on selected threshold parameters.

Adjustment of Calculations for MN Survival Using GSK3 Knockout Lines

In these cultures, approximately 70% of $Isl^+/MAP2^+$ cells were GFP^+ (i.e., MNs). However, after trophic factor withdrawal, because MNs die selectively under these conditions, only about 50% of $Isl^+/MAP2^+$ cells were GFP^+ MNs. This means that counting $Isl^+/MAP2^+$ cells could overestimate the amount of survival after trophic factor withdrawal by approximately 40%. When deprived of neurotrophins, 35% of wild-type $Isl^+/MAP2^+$ cells survived, while 44%, 57% and 54% of $Isl^+/MAP2^+$ cells in *GSK3*α-KO, *GSK3*β-KO and 3/4-KO cultures survived. Using the correction factor for overcounting surviving MNs, this would mean that wild-type MN survival would be approximately 25%, while the KO MNs would have 31%, 41% and 39% survival, respectively.

Phospho-Kinase Antibody Array

Freshly dissociated *HB9*::GFP EBs were plated at a density of 3 million GFP^+ cells/10 cm dish and trophic factor withdrawal/compound addition was performed on day 4. Protein lysates were generated 6 and 24h after treatment using RIPA buffer supplemented with protease and phosphatase inhibitors (Pierce Biotechnology). Equal amounts of total protein from treatment groups were added to Phospho-Kinase Antibody Arrays (R&D Systems, #ARY003). Arrays were prepared following the manufacturer's instructions and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). Background signal was subtracted and duplicate spot pixel densities were averaged using QuantityOne software (BioRad).

JNK/c-Jun Signaling Pathway Analysis

For JNK/c-Jun signaling pathway analysis, freshly dissociated *SOD1*^{G93A}/*HB9*::GFP and *HB9*::GFP EBs were plated at a density of 30,000 GFP^+ cells/well in 96-well plates and trophic factor withdrawal/compound addition was performed on day 4. Cells were fixed at 1, 6, 24 and 48h after treatment, stained with appropriate antibodies, and imaged using an automated microscope (Perkin Elmer Operetta) at 20x magnification. GFP^+ MNs were identified using Columbus analysis software (Perkin Elmer). Nuclear and cytoplasmic compartments were defined using Hoechst and GFP. Mean intensity of fluorescent staining across nuclear or cytoplasmic compartments was measured for each MN. Intensities were corrected for background staining levels (secondary antibody only) and the number of positive MNs per condition quantified.

Kinase activity assays

For kinase activity assays, 0.4μg of MLK3 and 0.1μg of HGK active kinases (SignalChem) were

incubated for 35 minutes at 30°C with 0.5µg of MBP (SignalChem) in the presence of [γ-32]ATP and Kenpaullone/CHIR99021. The enzymatic reaction was stopped with Laemmli buffer and samples were separated in a polyacrylamide gel. Finally, the gel was exposed to a Storage Phosphor Screen (GE Healthcare) and scanned in a Typhoon 9400 scanner (GE Healthcare). Signal density was estimated with Quantity One software (Bio-Rad).

Supplemental References

Ullian, E.M., Harris, B.T., Wu, A., Chan, J.R., and Barres, B.A. (2004). Schwann cells and astrocytes induce synapse formation by spinal motor neurons in culture. Mol Cell Neurosci *25*, 241–251.