Novel combinatorial screening identifies neurotrophic factors for selective classes of motor neurons

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Supporting Information (SI) Appendix

Supplemental Fig. S1
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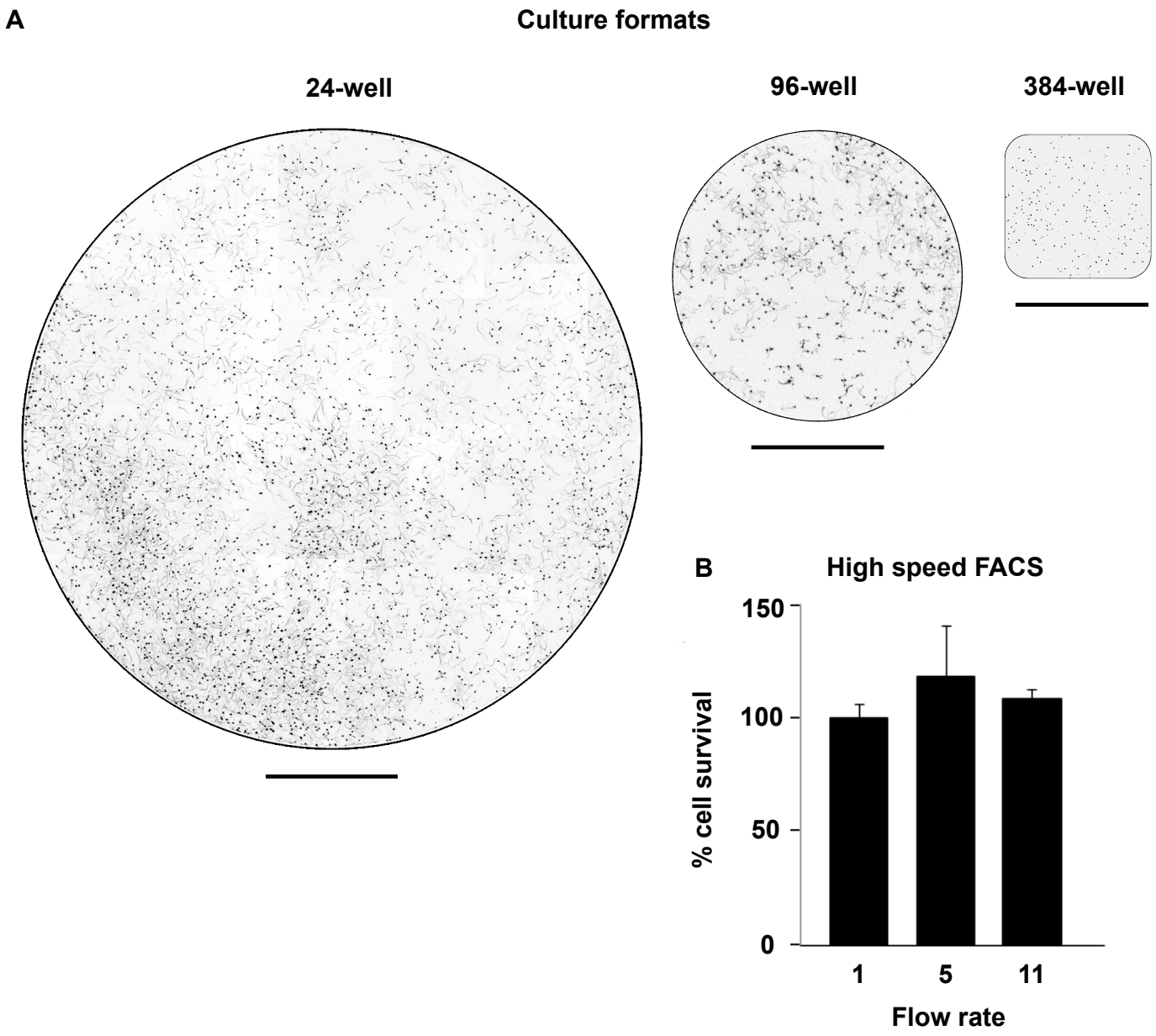

Supplemental Figure S1. High speed FACS-isolation of motor neurons.

A. Images of FACS-isolated motor neurons after 3 DIV culture. Cells are seeded on 24-well plates fitted with 14 mm coverslips (10.000 cells per well), 96-well plates (1.500 cells per well) or 384-well plates (500 cells per well). Scale bar 3 mm.

B. High speed FACS. Motor neurons are isolated at flow rates 1, 5 and 11 corresponding respectively to 14.3, 48.4 and 104 ml/min. Cell survival (mean ± sd) at three days in vitro is indistinguishable after FACS at high flow rate (5, 11) or low flow rate (1). Shown is one out of three representative cultures each done in the presence of 12 NTFs. Differences are not significant by Kruskal Wallis test.

Supplemental Fig. S2
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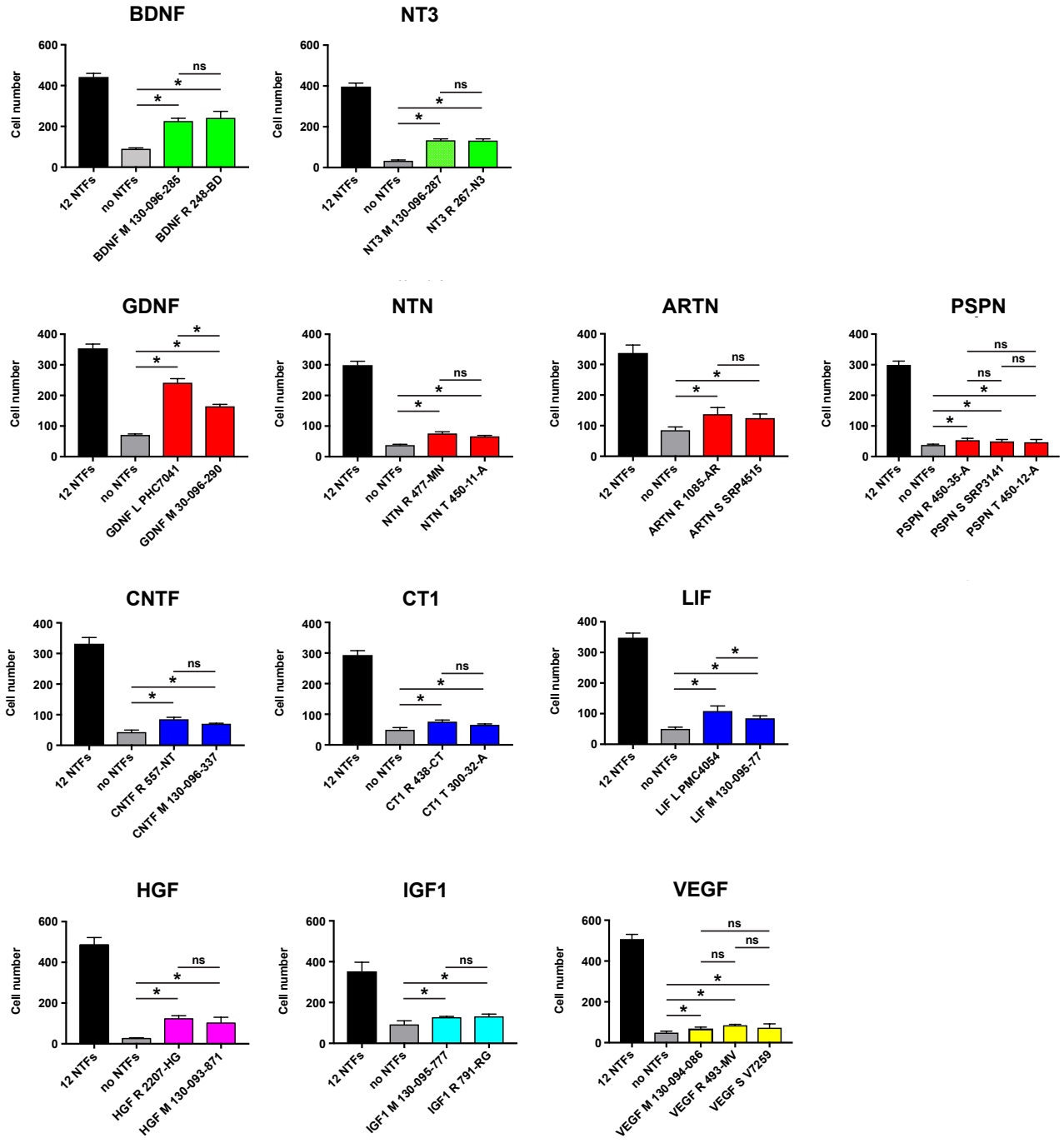
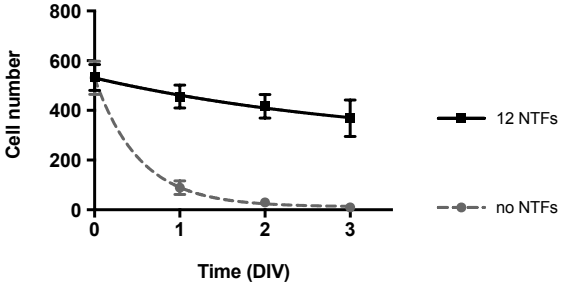
A Biological activity of NTFs



B Survival kinetics of lumbar MNs



Supplemental Figure S2. Biological activity of neurotrophic factors on motor neuron survival.

A. Full biological activity of all 12 neurotrophic factors (NTFs) was verified by comparing side by side the effects of different commercial batches purchased from Life Technologies (L), Miltenyi (M), R&D Systems/Bio-Techne (R), Sigma (S) and Tebu (T). NTFs were prepared according to the supplier's recommendations and diluted to their reported optimal concentration (Table 4) in chemically defined medium. Motor neurons (MN) were isolated by high speed FACS from mouse lumbar spinal cord, automatically seeded into 96-well cell culture dishes, and cultured in the presence of a single NTF, all 12 NTFs or no NTFs. Analysis of motor neuron cell survival (mean ± sd) at 3 DIV revealed significant biological activity of single NTFs in comparison to no NTFs. NTFs from different suppliers had similar biological activities, except batches of GDNF and LIF. Statistical significance was tested by Kruskal-Wallis test and Tukey post hoc test, n = 6 wells per condition : * $p < 0.01$; ns: not significant.

B. Survival kinetics of lumbar motor neurons (mean ± sd) cultured for up to 3 DIV in the presence or absence of 12 NTFs. Data were fitted by the non-linear least-squares method (GraphPad Prism) using a one phase exponential decay equation.

Supplemental Fig. S3
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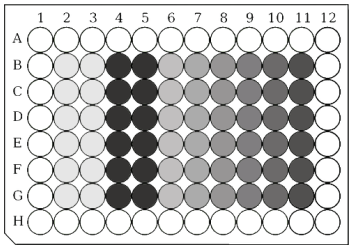
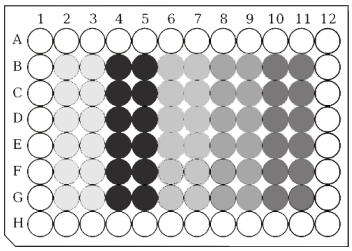
A Plate design

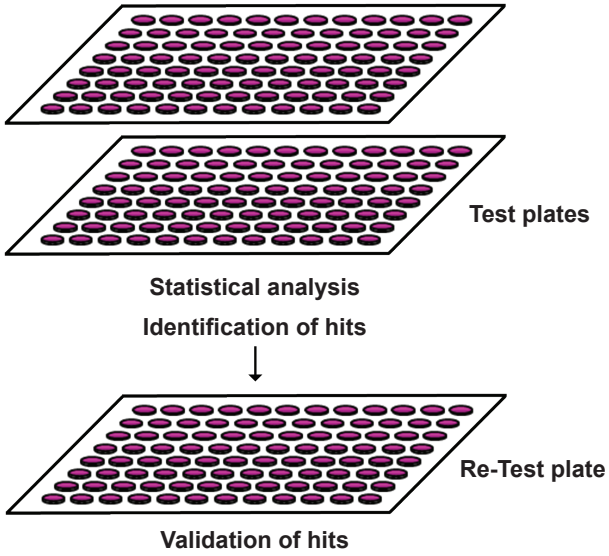


NTFs no all 1 2 3 1+2 1+3 2+3



NTFs no all 1 2 1+2

B Flow chart



C NTF assay (mean data)

	% Survival NTF	% Survival NTF combination										
GDNF	40,3											
BDNF	25,0	64,4										
HGF	21,8	61,7	50,6									
NTN	21,0	56,9	35,6	33,1								
NT3	12,5	53,9	32,8	19,0	18,1							
ARTN	10,3	49,0	44,6	40,0	16,6	12,2						
LIF	8,0	50,4	30,5	28,7	29,1	27,2	24,3					
IGF1	7,9	46,0	29,3	35,2	37,4	19,2	10,9	20,5				
CNTF	7,8	49,6	34,4	35,9	25,1	21,5	21,7	10,7	10,9			
VEGF	6,7	45,6	37,2	31,0	30,4	9,0	12,6	20,0	12,0	12,0		
PSP	4,8	37,5	35,7	20,8	17,2	18,6	8,6	4,6	11,9	14,9	10,2	
CT1	2,0	35,0	35,3	18,0	24,3	13,2	8,9	10,1	9,1	4,5	5,0	4,5
		40,3	25,0	21,8	21,0	12,5	10,3	8,0	7,9	7,8	6,7	4,8	2,0
		GDNF	BDNF	HGF	NTN	NT3	ARTN	LIF	IGF1	CNTF	VEGF	PSP	CT1

D NTF assay (median data)

	% Survival NTF	% Survival NTF combination										
GDNF	43,0											
BDNF	23,2	58,1										
HGF	20,6	62,2	49,6									
NTN	20,1	56,7	37,2	34,0								
NT3	12,2	52,3	31,1	14,1	17,2							
ARTN	11,7	45,6	41,8	41,3	14,3	11,9						
LIF	7,0	46,3	31,2	31,7	28,4	30,6	24,7					
IGF1	7,0	46,5	29,0	34,6	34,6	19,6	10,7	19,6				
CNTF	6,9	50,3	35,4	37,6	26,6	21,6	25,3	13,3	10,8			
VEGF	5,4	44,5	35,4	31,6	33,8	9,2	10,7	20,4	10,4	12,4		
PSP	4,4	35,9	35,6	21,3	19,1	17,9	10,9	4,2	8,3	12,9	13,7	
CT1	3,7	34,8	35,0	18,0	29,9	13,4	8,1	10,7	10,8	7,4	4,1	4,9
		43,0	23,2	20,6	20,1	12,2	11,7	7,0	7,0	6,9	5,4	4,4	3,7
		GDNF	BDNF	HGF	NTN	NT3	ARTN	LIF	IGF1	CNTF	VEGF	PSP	CT1

E NTF assay (range of data)

	% Survival NTF	% Survival NTF combination										
GDNF	33,6 - 47,6											
BDNF	17,0 - 30,7	55,9 - 61,6										
HGF	12,4 - 30,9	61,6 - 68,6	47,4 - 58,0									
NTN	13,8 - 27,6	51,7 - 60,4	29,0 - 41,9	32,2 - 34,4								
NT3	7,8 - 17,3	50,8 - 56,8	28,2 - 38,4	11,3 - 26,2	14,8 - 21,6							
ARTN	9,0 - 16,1	44,3 - 56,8	39,6 - 51,9	38,9 - 43,1	8,5 - 22,5	7,7 - 12,1						
LIF	3,5 - 10,7	43,6 - 51,9	28,0 - 32,4	28,2 - 35,2	26,3 - 32,6	25,3 - 32,0	23,2 - 26,1					
IGF1	3,8 - 8,9	41,4 - 51,5	28,4 - 29,9	30,1 - 39,9	32,2 - 40,4	17,6 - 24,7	7,4 - 14,0	18,8 - 22,2				
CNTF	1,6 - 11,5	45,6 - 52,4	32,8 - 40,1	30,7 - 43,6	21,4 - 28,2	16,1 - 26,3	24,3 - 26,3	9,4 - 17,1	10,1 - 12,1,			
VEGF	1,7 - 11,8	36,1 - 52,0	27,8 - 43,6	23,0 - 36,8	23,2 - 35,7	8,8 - 10,4	4,6 - 14,5	15,3 - 22,1	10,4 - 15,4	10,3 - 15,9		
PSP	2,1 - 9,3	28,7 - 44,4	24,2 - 40,9	17,8 - 23,3	14,2 - 20,9	12,7 - 22,4	5,8 - 12,6	0,6 - 5,0	7,8 - 17,2	9,1 - 20,1	2,9 - 16,8	
CT1	0,1 - 7,4	31,4 - 41,9	34,9 - 38,4	17,2 - 18,9	26,9 - 34,7	12,4 - 15,2	2,3 - 14,4	7,9 - 13,4	4,8 - 13,5	1,3 - 8,2	-3,3-15,5	4,0 - 5,0
		33,6 - 47,6	17,0 - 30,7	12,4 - 30,9	13,8 - 27,6	7,8 - 17,3	9,0 - 16,1	3,5 - 10,7	3,8 - 8,9	2,6 - 11,5	1,7 - 11,8	2,1 - 9,3	0,1 - 7,4
		GDNF	BDNF	HGF	NTN	NT3	ARTN	LIF	IGF1	CNTF	VEGF	PSP	CT1

Supplemental Figure S3. Screening NTF combinations.

A. Design of test and re-test plates. 96-well test plates (upper panel) contain rows with negative controls (culture medium only), positive controls (all 12 NTFs in culture medium), three individual NTFs (1, 2, 3) and all three pairwise NTF combinations (1+2, 1+3, 2+3). Re-test plates (lower panel) are used to confirm the effects of two NTFs in combination. Plates are prepared prior to FACS by manual pipetting of culture medium (135 µl) and addition of NTFs or NTF combinations (15 µl) at 10-fold final concentration in culture medium. Wells on the edges are filled with water.

B. Flow chart for data analysis and processing, ref. (1). Statistical differences between conditions on each plate are tested by Kruskal Wallis test and Dunn's post hoc test.

C. NTF assay showing mean values of motor neuron survival. Survival data are expressed relative to no NTFs (set 0%) and all 12 NTFs (set 100%).

D. NTF assay showing median values of motor neuron survival.

E. NTF assay showing range of data (25% - 75% percentiles).

Supplemental Fig. S4
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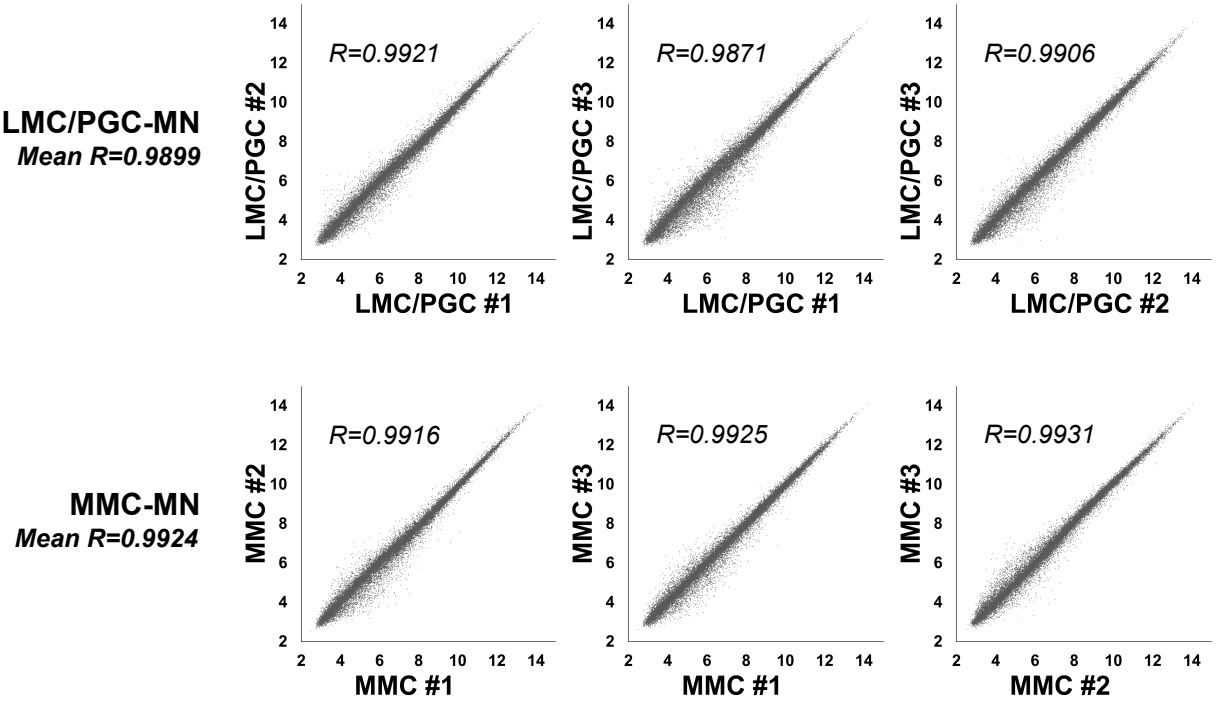
A Marker gene expression in motor neuron subsets

			Fold change LMC/PGC vs MMC						
Gene	**Synonyme**	**GSM**	**mean**	**sd**	**#1**	**#2**	**#3**	**p-value**	**MN marker**
Pou3f1	Oct6, Scip	GSMG0027043	-2,78	0,274287	-2,62	-2,63	-3,1	9.41E-4	MMC
Lhx3		GSMG0022555	-9,73	2,046485	-9,22	-8,21	-12,15	2.95E-4	MMC
Lhx4		GSMG0002258	-4,89	0,228108	-8,21	-8,61	-8,22	2.71E-3	MMC
Aldh1a2	Raldh2	GSMG0039772	24,25	5,461734	18,6	26,3	29,16	1.1E-3	LMC
Lhx1		GSMG0007133	5,6	0,653682	6,31	5,01	5,54	1.2E-3	LMC
Foxp1	FoxP1	GSMG0033453	2,18	0,542248	2,82	2,06	1,77	3.67E-2	LMC/PGC
Nos1	nNos	GSMG0029706	3,26	0,40129	3,5	3,51	2,81	4.51E-3	PGC
Hnf6	Onecut1	GSMG0039805	3,68	0,633798	4,31	3,8	3,05	7.56E-4	PGC
Smad1		GSMG0038636	1,36	0,091652	1,34	1,46	1,28	3.11E-3	PGC

B Global gene expression analysis in FACS-isolated motor neuron subsets



Supplemental Figure S4. Gene expression profiles of LMC/PGC motor neurons and MMC motor neurons on microarrays.

A. Gene expression profile of marker genes in LMC/PGC-MN and MMC-MN. Fold changes (mean, sd, sample pairs #1, #2, #3) and p-values by student's t-test are indicated.

B. Global gene expression analysis in FACS-isolated motor neuron subsets. Scatter plots and correlation coefficients of gene expression (R) between independent biological replicates #1, #2 and #3 of LMC/PGC-MN and MMC-MN show high standardization. Expression values are on log2 scale.

Supplemental Fig. S5
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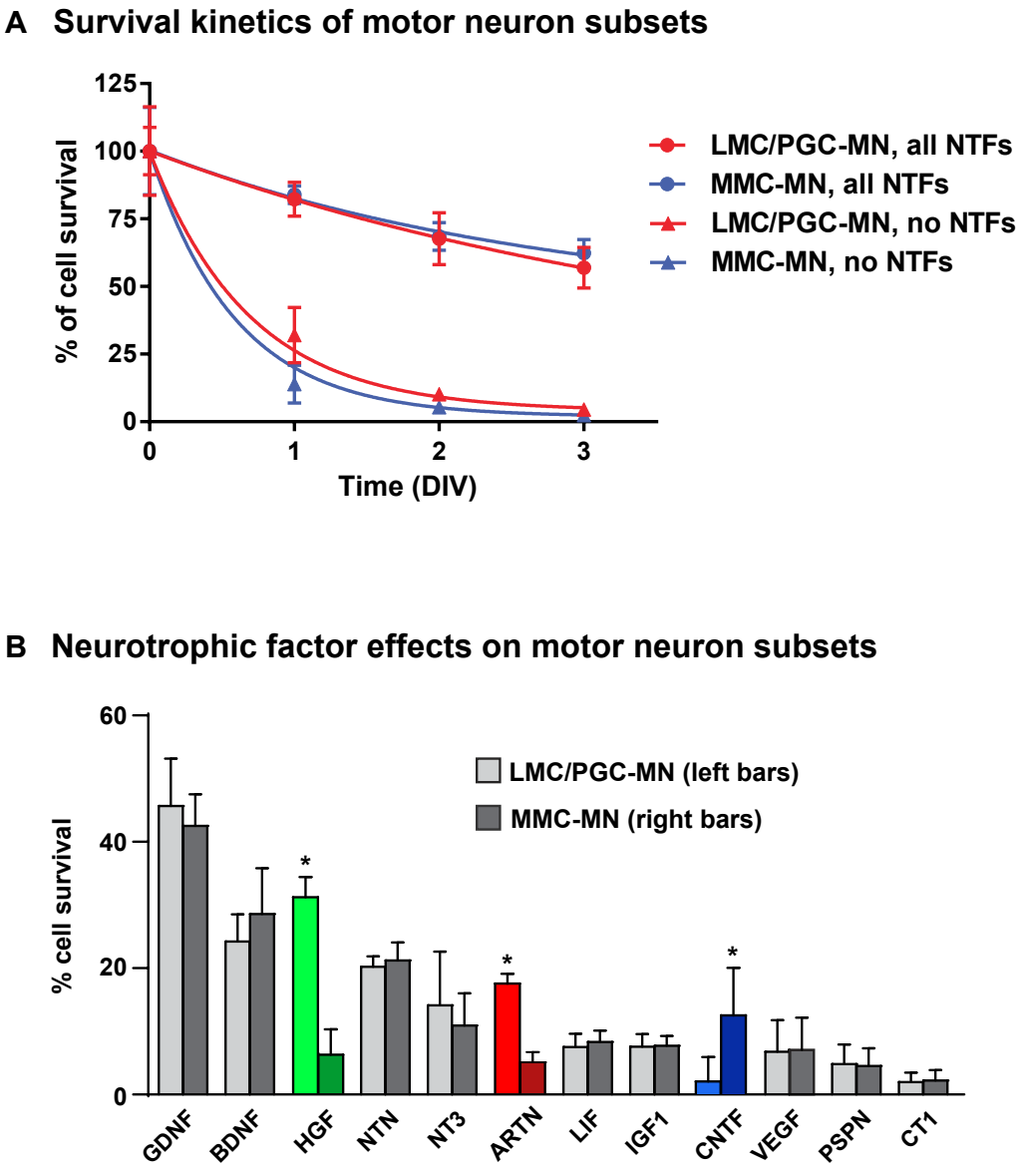

Supplemental Figure S5. Gene expression profile and NTF survival responses of LMC/PGC and MMC motor neurons.

a. Similar survival kinetics (mean ± sd) of FACS-isolated LMC/PGC-MN and MMC-MN from 0 to 3 DIV. There are no significant survival differences between LMC/PGC-MN and MMC-MN cultured in the presence or absence of NTFs. The kinetic data were fitted by a non-linear least-squares method (GraphPad Prism) using a one phase exponential decay equation.

b. Similar survival responses of LMC/PGC-MN (light shaded bars) and MMC-MN (dark shaded bars) to the neurotrophic factors CNTF, BDNF, NTN, LIF, IGF1, VEGF, PSPN and CT1. Distinct survival responses are seen for HGF, ARTN and CNTF (mean ± sd, * $p < 0.05$, Mann & Whitney test). Survival values are expressed relative to the value obtained in the presence of all NTFs (100 %) or no NTF (0 %).

Supplemental Fig. S6
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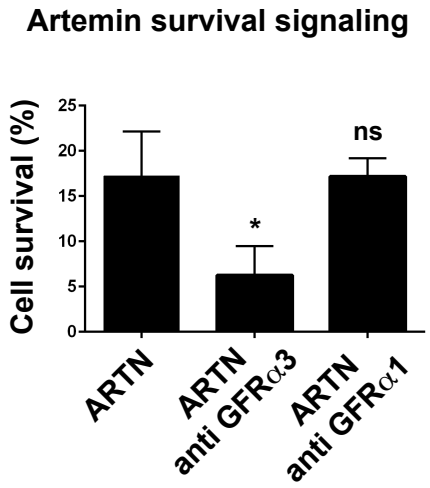

Supplemental Figure S6. GFRα3-mediated Artemin survival signaling.
Cell survival of motor neurons cultured in the presence of Artemin (ARTN) is significantly inhibited by pre-incubation with antibodies against the extracellular domain of GFRα3 (2 μg/ml, 1 h) but not with antibodies against GFRα1 (10 μg/ml, 1 h). *, p = 3.28 E-7 by student's t-test; ns: not significant.

Supplemental Materials and Methods

Reagents. Antibodies, neurotrophic factors, pharmacological inhibitors and antisense probes are listed in **Tables 1-6**. Other reagents (references, suppliers) were as follows: DIG-labeling kits, yeast RNA and RNase (Roche), bovine serum albumin (BSA), DNase I (DN25), trypsin, bovine hemoglobin (H2625-25G), poly-DL-ornithin (P8638) (Sigma), L15 (11415-064), B27 (17504-044), Neurobasal without riboflavin (041-96399M) (Thermofisher), laminin (354232, Corning), calcein (FP-855422, Interchim), Triton X-100 (Euromedex), Facsflow (342003), Facsrinse (340346) (Becton Dickinson), 24-well plates (142475, Nunc), 96-well plates (655090, Greiner or 353219, Falcon) and 384-well plates (781091, Greiner).

Gene expression profiling. FACS-isolated motor neurons were collected at a minimum yield of 60.000 cells per subtype in each of three replicate experiments. RIN values were between 8.1 and 9.5 (mean 8.9). Gene expression profiling was performed using Mouse Exon 1.0 ST arrays (Affymetrix, Hygh Wycombe, UK) according to the supplier's recommendations. Raw data were controlled with Expression console (Affymetrix) and analyzed by GenoSplice using EASANA software and FAST DB annotations (2).

Immunoblot analyses. FACS-isolated motor neurons were collected in ice-cold Laemmli buffer, protein extracts corresponding to 20.000 motor neurons separated by SDS-PAGE and blotted on membranes. Membranes were reacted with antibodies (**Table 3**) and revealed with a ChemiDoc XRS+ imager (Biorad).

Immunohistochemistry. Transverse sections of E12 mouse embryos were incubated with primary antibodies and biotinylated or fluorochrome-conjugated secondary reagents (**Table 1**). Slides were mounted with Vectashield/DAPI and images acquired with an inverted Leica DMI 4000B microscope or a Zeiss LSM510 confocal microscope.

Retrograde labeling. Urogenital organs including bladder (3) of E12 Hb9:GFP embryos were identified using a fluorescence dissection microscope (Leica FZ III). Injection of Dextrans conjugated to AlexaFluor or Tetramethylrhodamine were performed using a microcapillary. Embryos were incubated for 3 to 4 hours at 30°C in Hibernate medium containing 1% penicillin-streptomycin under continuous CO_2/O_2 bubbling. Embryos were then either processed for immunohistochemistry or their spinal cords were dissected out. Preparation of single cell suspensions and flow cytometry analysis were done as described above.

Signaling studies. Pharmacological inhibitors are listed in **Table 5**. The monovalent antibody against mouse c-Met (OA-118) was designed, produced and tested for its biological activity as described (4). Unless otherwise stated, FACS-isolated motor neurons were pre-incubated with the inhibitors for 1 h before NTF addition. Optimal concentrations of each inhibitor were empirically determined.

Whole mount labelings. E12 spinal cords were reacted with digoxigenin (DIG)-labelled RNA probes (**Table 6**) or anti-GFRα3 antibodies. In situ hybridization and in situ immunohistochemistry were performed by standard techniques (5) and images acquired with a Leica MZ 12 microscope.

Tables

Table 1. Antibodies used in immunohistochemistry

Primary Antibodies	Dilution	Supplier	Reference
Choline Acetyl Transferase (goat)	1:100	Chemicon	Ab144P
Engrailed 1 (EN1) (mouse)	1:20	DSHB	4G11
CHX10 (sheep)	1:50	Exalpha	X1179P
GFRα3 (goat)	1:500	R&D Systems	AF2645
Neurofilament M (rabbit)	1:1000	Merck Millipore	AB1987
Neurofilament H non phosphorylated SMI32 (mouse)	1:500	Sternberger	SMI-32R
nNOS C-ter (rabbit)	1:300	Immunostar	24287
Fox P1 (rabbit)	1:500	Abcam	ab16645

Secondary antibodies and tracers	Dilution	Supplier	Reference
anti-goat Biotin (donkey)	1:500	Jackson Lab.	705-066-147
Streptavidin Alexa Fluor 594	1:500	Invitrogen	S32356
anti-rabbit Cy5 (donkey)	1:300	Jackson Lab.	711-175-152
anti-mouse Cy5 (donkey)	1:200	Jackson Lab.	711-175-151
anti-sheep Cy3 (donkey)	1:200	Jackson Lab.	713-165-147
Dextran Alexa 647	50 mg/ml	Invitrogen	D22914
Dextran Tetramethylrodamine	50 mg/ml	Invitrogen	D1817

Table 2. Antibodies used in flow cytometry

Primary Antibodies	Dilution	Supplier	Reference
Hb9 (mouse)	1:100	DSHB	81.5C10
Islet-1 / Islet-2 (mouse)	1:100	DSHB	40.2D6 / 39.4D5
Lhx 1/2 (mouse)	1:100	DSHB	4F2
Lhx 3 (mouse)	1:100	DSHB	67.4E12
Foxp1 (mouse)	1:2000	Abcam	ab32010
Oct6 (mouse)	1:2000	S. Driegen	(6)

Secondary Antibodies	Dilution	Supplier	Reference
anti-mouse IgG Alexa Fluor 568 (donkey)	1:1000	Invitrogen	A10037
anti-rabbit IgG Alexa Fluor 568 (donkey)	1:1000	Invitrogen	A10042
anti-mouse IgG Alexa Fluor 633 (goat)	1:2000	Invitrogen	A21052
anti-rabbit IgG Alexa Fluor 633 (goat)	1:2000	Invitrogen	A21070
anti-sheep IgG (donkey)	1:2000	Invitrogen	A21099

Table 3. Antibodies used in immunoblots

Primary Antibodies	Dilution	Supplier	Reference
c-Met (mouse)	1:1000	Cell Signaling	3127
c-Ret (C-19) (rabbit)	1:200	Santa Cruz	sc-167

GFRα3 (goat)	1:100	R&D Systems	AF2645
Gp130 (C-20) (rabbit)	1:100	Santa Cruz	sc-655
H3 (mouse)	1:200	Merck Millipore	05-499
LIF Rβ (H-220) (rabbit)	1:100	Santa Cruz	sc-20752
Syndecan-3 (rabbit)	1:500	Abcam	Ab63932

Secondary Antibodies	**Dilution**	**Supplier**	**Reference**
HRP - anti-rabbit (goat)	1:40.000	Invitrogen	62-6120
HRP - anti-goat (donkey)	1:40.000	Jackson Lab.	705-036-147
HRP - anti-mouse (goat)	1:40.000	Jackson Lab.	115-036-062
HRP - anti-rabbit (goat)	1:40.000	Jackson Lab.	111-036-045
HRP - anti-rabbit (goat)	1:40.000	Jackson Lab.	111-035-144

Table 4. Neurotrophic factors

Neurotrophic factor	**Final concentration (ng/ml)**	**Supplier**	**Reference**
ARTN	10	R&D Systems	1085-AR
BDNF	1	R&D Systems	248-BD
CNTF	10	R&D Systems	557-NT
CT1	10	R&D Systems	438-CT
GDNF	1	Life Technologies	PHC7041
HGF	2	R&D Systems	2207-HG
IGF1	100	R&D Systems	791-MG
LIF	10	Life Technologies	PMC4054
NT3	10	R&D Systems	267-N3
NTN	10	R&D Systems	477-MN
PSPN	10	R&D Systems	2479-PS
VEGF	100	R&D Systems	493-MV

Table 5. Pharmacological inhibitors

Inhibitor	**Conc. / Dilution**	**Supplier**	**Reference**
EMD 1204831	100 nM	Merck	(7)
OA-118	5 μg/ml	Genentech	(4)
Heparinase III	1:10.000	R&D Systems	6145-GH
PI-PLC	2 μg/ml	Sigma-Aldrich	P8804
anti-GFRα1	10 μg/ml	R&D Systems	AF560
anti-GFRα3	2 μg/ml	R&D Systems	AF2645

Table 6. Antisense probes used for in situ hybridization

Probe	Restriction Enzyme/ RNA Polymerase	Origin	Reference
Met 5'	BamHI/T3	Y. Yamamoto	(8)
Met 3'	NotI/T3	Y. Yamamoto	(8)
Ret	NotI/T7	V. Pachnis	(9)
Gfra3	BamHI/T3	P. Ernfors	(10)
Hb9	EcoRV/T7	J. Livet	unpublished
Lifrb	EcoRI/T3	H. Nishimune	(11)
Raldh2	XbaI/T7	T. Jessell	(12)

Supplemental References

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