



Article – Supplementary information

Overexpression of miR-124 in Motor Neurons Plays a Key Role in ALS Pathological Processes

Supplementary Figures

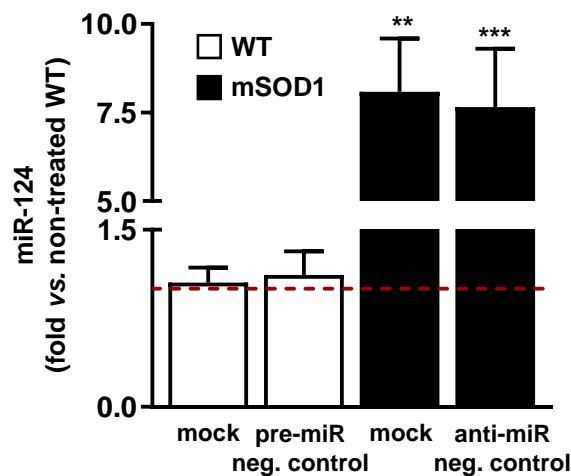


Figure S1. MiR-124 expression is not affected by the transfection method either in WT and mSOD1 MNs. White bars represent WT MNs treated with XtremeGENE™ HP DNA Transfection Reagent (mock control) or modulated with Pre-miR™ Negative Control. Black bars represent mSOD1 MNs treated with XtremeGENE™ HP DNA Transfection Reagent (mock control) or modulated with Anti-miR™ Negative Control. Results are expressed as fold change relatively to WT MNs incubated with Optimem (dashed red line). ** $p<0.01$ vs. WT MNs. WT, wild type; MN, motor neurons (NSC-34 cell line); mSOD1, MNs overexpressing G93A mutation in superoxide dismutase 1.

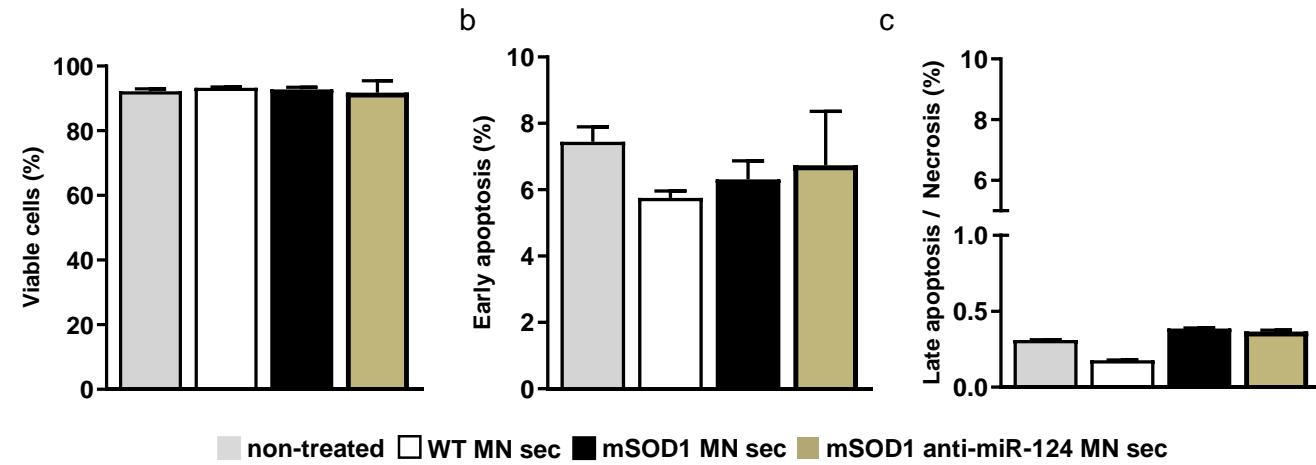


Figure S2. Microglia viability is not affected by the presence of neuronal secretome, derived from either WT, mSOD1 or anti-miR-124 mSOD1 MNs. Two days in vitro primary spinal microglia were incubated with WT MN, mSOD1 MN secretome or with the secretome from anti-miR-124 mSOD1 MNs for 4 h. Non-treated cells were used as controls. After incubation, the percentage of viable, early apoptotic, and late-apoptotic/necrotic cells were determined by flow cytometry with phycoerythrin-conjugated annexin V (annexinV-PE) and 7-amino-actinomycin D (7-AAD). The three populations were distinguished as follows: (a) viable cells (annexin V-PE and 7-AAD negative), (b) early apoptotic cells (annexinV-PE positive and 7-AAD negative), and (c) cells in late stages of apoptosis or necrosis (annexinV-PE and 7-AAD positive). Results are mean \pm SEM from at least 3 independent experiments performed in duplicate. WT, wild type; MN, motor neurons (NSC-34 cell line); mSOD1, MNs overexpressing G93A mutation in superoxide dismutase 1.

Supplementary Tables**Table S1.** List of primer sequences used in RT-qPCR to amplify miRNA.

miRNA	Target sequence (5'-3')
hsa-miR-124-3p	UAAGGCACGCCGUGAAUGC
hsa -miR-125b-5p	UCCCUGAGACCUAACUUGUGA
hsa miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
hsa miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
SNORD110	Reference gene
RNU1A1	Reference gene

RT-qPCR, real-time quantitative polymerase chain reaction; miRNA, microRNA; hsa, homo sapiens.

Table S2. List of primer sequences used in RT-qPCR to amplify protein-coding genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Synaptophysin	GACGTTGGTAGTGCCTGTGA	GCACAGGAAAGTAGGGGTC
DLG4	GAGGCTGGCGGCCAGTACACCAG	ACAGAGCAGGCGGTCA
Kif5b	GGTCCTACAGTTGCCACCTA	ATTGAAATACGCCAGGCCA
Dynein	GCCTCAGTCTCTGTCCCAC	AAGTCCTGGGTAAGGTGCT
IL-1 β	CAGGCTCCGAGATGAACAAAC	GGTGGAGAGCTTCAGCTCAT
IL-18	TGGTCCATGCTTCTGGACTCCT	TTCCTGGCCAAGAGGAAGTG
HMGB1	CTCAGAGAGGTGGAAGACCATGT	GGGATGTAGGTTTCATTCTCTTTC
iNOS	ACCCACATCTGGCAGAATGAG	AGCCATGACCTTCGCATTAG
IL-10	ATGCTGCTTGCTCTTACTGA	GCAGCTCTAGGAGCATGTGG
CX3CL1	CTCACGAATCCCAGTGGCTT	TTTCTCCTCGGGTCAGCAC
S100B	GAGAGAGGGTGACAAGCACAA	GGCCATAAACCTCTGGAAGTC
GFAP	CAAACCTGGCTGATGTCTACC	GCTTCATCTGCCTCCTGTCTA
CX3CR1	ATGGGGTCTCTGTCTGCTCT	TACTGGCAATGGGTGGCATT
MFG-E8	AGCCTGAATGGTAGGGTTGG	GAGACTGCATCCTGCAACCA
β -actin	GCTCCGGCATGTGCAA	AGGATCTTCATGAGGTAGT

DLG4, discs large MAGUK scaffold protein 4 (that encodes for postsynaptic density protein 95, PSD-95); Kif5b encodes for the protein kinesin -1 heavy chain; IL, interleukin; HMGB1, high mobility group box 1; iNOS, inducible nitric oxide synthase; MFG-E8, milk fat globule-EGF factor 8 protein, CX3CL1, C-X3-C motif chemokine ligand 1/fractalkine; S100B, S100 calcium-binding protein B; GFAP, glial fibrillary acidic protein; CX3CR1, C-X3-C motif chemokine receptor 1; β -actin, beta-actin.

Table S3. List of antibodies used for immunocytochemistry (ICC) or immunohistochemistry (IHC).

	Antibodies	Source	Species	Dilution	Assay
Primary	Anti-β-III tubulin	Merck Millipore, MAB1637	Mouse	1:500	ICC
	Anti- mitofusin 2	AbCam, ab50838	Rabbit	1:50	ICC
	Anti-Drp1	AbCam, ab140494	Mouse	1:150	ICC
	Anti-iNOS	BDBiosciences, 610329	Mouse	1:100	ICC
	Anti-Arg 1	Santa Cruz, sc18355	Goat	1:50	ICC
	Anti-GFAP	NovoCastra, GFAP-GA5-6035278	Mouse	1:100	IHC
	Anti-Iba1	Wako, 019-19741	Rabbit	1:250	IHC
Secondary	AlexaFluor 488 anti-mouse	Invitrogen, A-10680	Goat	1:1000	IHC
	AlexaFluor 488 anti-rabbit	Invitrogen, A-11008	Goat	1:1000	IHC
	AlexaFluor 594 anti-mouse	Invitrogen, A-11005	Goat	1:1000	IHC
	AlexaFluor 594 anti-goat	Invitrogen, A-21468	Chicken	1:1000	IHC
	AlexaFluor 647 anti-mouse	Invitrogen, A-21236	Goat	1:500	IHC
	AlexaFluor 594 anti-rabbit	Invitrogen, A-11012	Goat	1:500	IHC

Arg1, arginase 1; Drp 1, dynamin-related protein 1; GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide synthase; Iba-1, ionized calcium-binding adapter molecule 1.

Table S4. Absolute values of the heatmap indicated in Figure 6g for mSOD1 and mSOD1+anti-miR-124 MN secretome versus WT spinal cord slices.

	mSOD1 (fold change vs. WT) Mean ± SEM	mSOD1 + anti-miR-124 MN secretome (fold change vs. WT) Mean ± SEM
Genes		
iNOS	3.71 ± 1.32	1.26 ± 0.41
IL-1 β	2.99 ± 0.44	0.70 ± 0.18
IL-10	2.72 ± 0.42	0.51 ± 0.08
HMGB1	0.54 ± 0.11	0.82 ± 0.07
CX3CL1	0.71 ± 0.07	0.85 ± 0.08
PSD-95	0.42 ± 0.06	0.81 ± 0.10
Synaptophysin	1.61 ± 0.18	0.83 ± 0.12
S100B	1.09 ± 0.06	0.84 ± 0.05
GFAP	0.42 ± 0.08	1.28 ± 0.28
MFG-E8	0.75 ± 0.07	0.79 ± 0.33
CX3CR1	1.94 ± 0.36	0.83 ± 0.09
Inflamma-miRs		
hsa-miR-124-3p	1.57 ± 0.14	0.74 ± 0.02
hsa-miR-125b-5p	0.94 ± 0.13	0.95 ± 0.21
hsa-miR-146a-5p	1.85 ± 0.09	1.75 ± 0.25
hsa-miR-21-5p	2.16 ± 0.40	2.38 ± 0.39
PI ⁺ cells	1.65 ± 0.14	0.81 ± 0.13

Organotypic cultures were obtained from the spinal cord of 10–12-weeks-old mice, as described in methods. Slices were collected for necrosis evaluation and mRNA expression of iNOS, IL-1 β , IL-10, HMGB1, CX3CL1, PSD-95, Synaptophysin, S100B, GFAP, MFG-E8, and CX3CR1 by reverse transcriptase quantitative real-time PCR (RT-qPCR), using β -actin as reference gene. Results are expressed as fold change versus WT organotypic slices incubated with MN culture media (control) and are mean \pm SEM. The values considered statically significant are in bold. iNOS, inducible nitric oxide synthase; IL-1 β , interleukin-1 beta; IL-10, interleukin-10; HMGB1, high mobility group protein 1; CX3CL1, C-X3-C motif chemokine ligand 1; PSD-95, postsynaptic density protein 95 (gene Dlg4); S100B, S100 calcium-binding protein B; GFAP, glial fibrillary acidic protein; MFG-E8, milk fat globule-EGF factor 8; CX3CR1, C-X3-C motif chemokine receptor 1; mSOD1, MNs overexpressing G93A mutation in superoxide dismutase 1; PI, propidium iodide.