3 **Dysregulated balance of D- and L-amino acids modulating glutamatergic neurotransmission**
4 **in severe spinal muscular atrophy**

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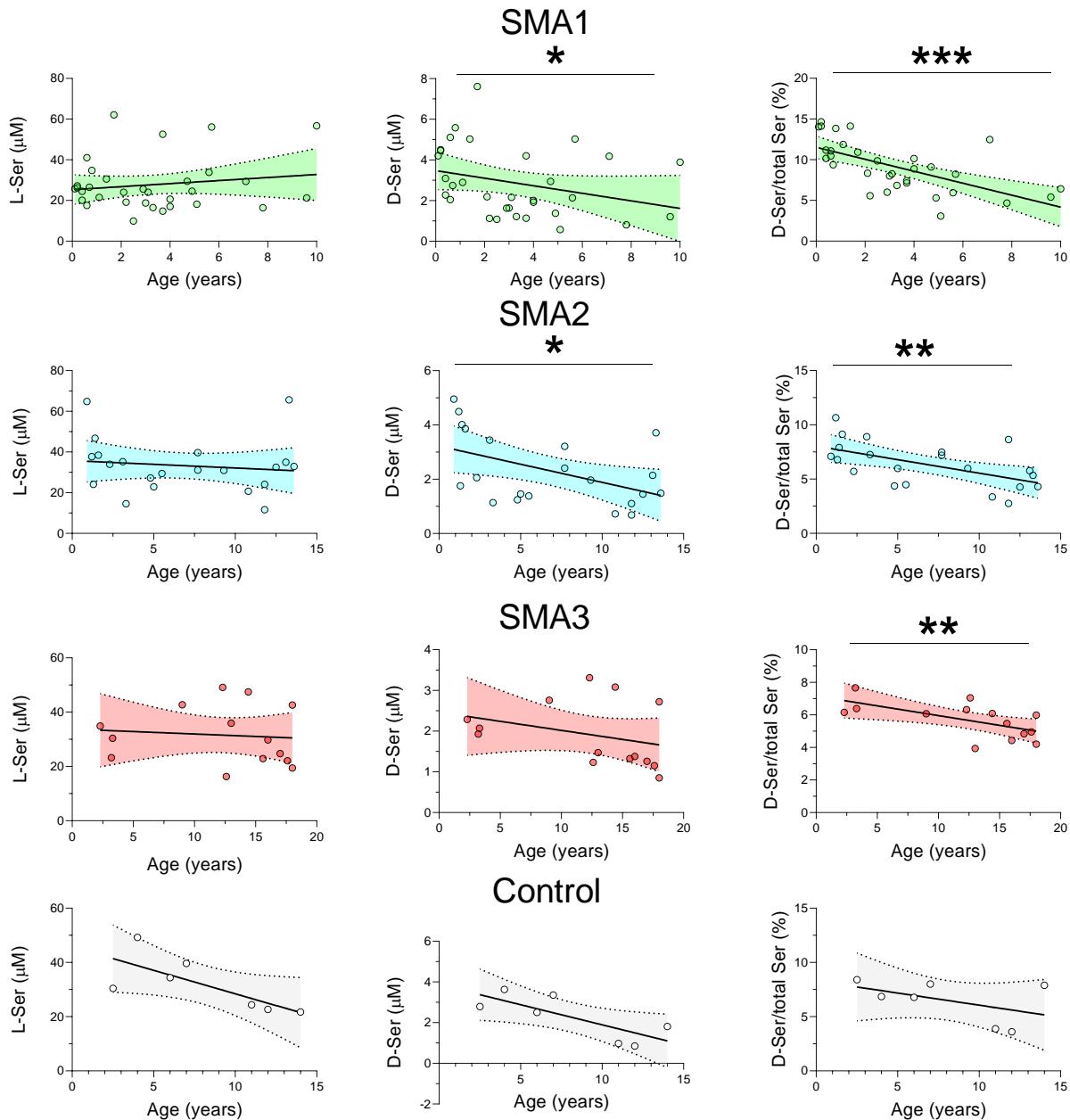
Suppl Fig 1: Correlation between age and amino acid concentrations in naive cohorts of SMA1, SMA2 and SMA3 patients as well as normal controls. Association between L-Ser, D-Ser, or D-Ser/tot Ser ratio with age of SMA1, SMA2, SMA3, and control individuals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, Spearman correlation.

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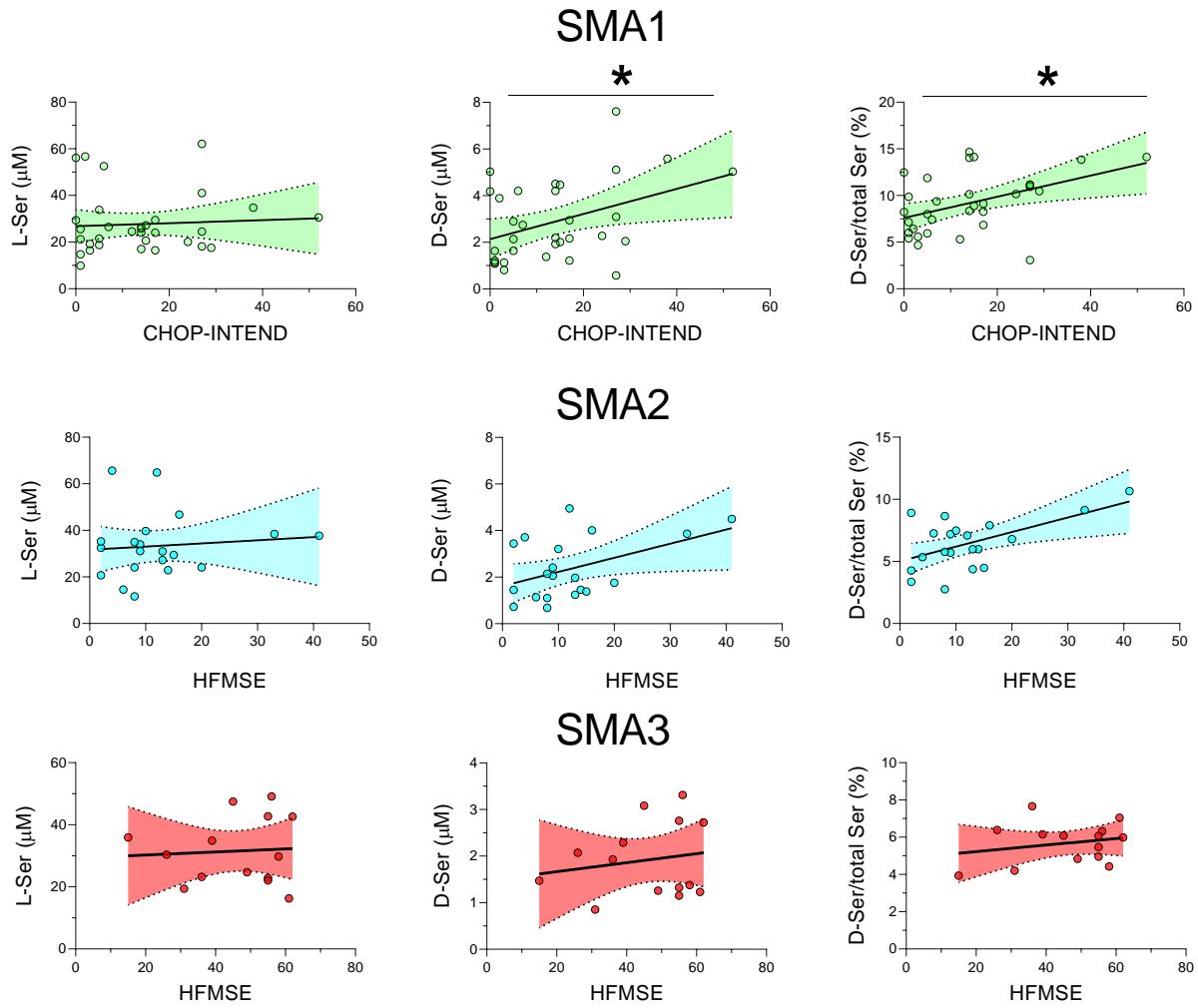
Suppl Fig 2: Correlation between amino acid concentrations and CHOP-INTEND or HFMSE in naive cohort of SMA1, SMA2 and SMA3 patients. Association between L-Ser, D-Ser, or D-Ser/tot Ser ratio with CHOP-INTEND (SMA1) or HFMSE (SMA2 and SMA 3). *P < 0.05, Spearman correlation.

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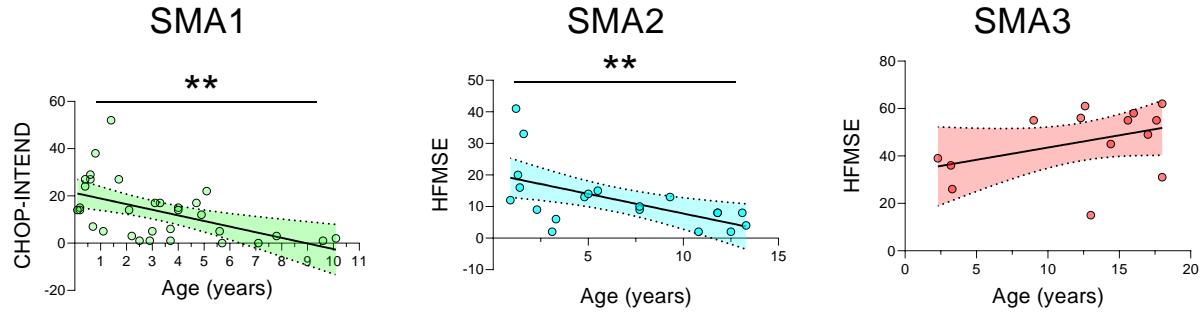
SMA1 SMA2 SMA3

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68 **Suppl Fig 3:** Correlation of CHOP-INTEND or HFMSE with age of naive SMA1, SMA2 and SMA3
69 patients. **P* < 0.01, Spearman correlation.
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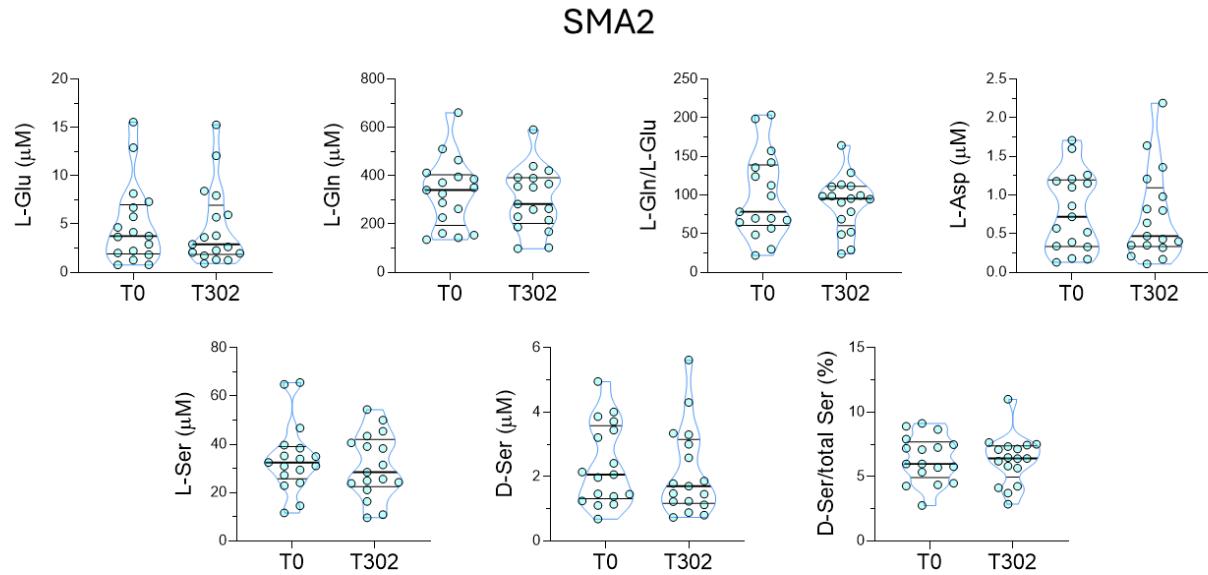
SMA2

Suppl Fig 4: Effect of Nusinersen on amino acids levels in the CSF of SMA2 patients. Levels of L-glutamate (L-Glu), L-glutamine (L-Gln), L-glutamine/L-glutamate ratio (L-Gln/L-Glu), L-aspartate (L-Asp), L-serine (L-Ser), D-serine (D-Ser) and D-serine/total serine (L-Ser/total Ser) percentage in the CSF of SMA2 patients prior to treatment (T0, $n = 17$) and at the time of the sixth (T302, $n = 17$) injection of Nusinersen. Data are shown as violin plots representing median with interquartile range (IQR). Dots represent individual patients' values.

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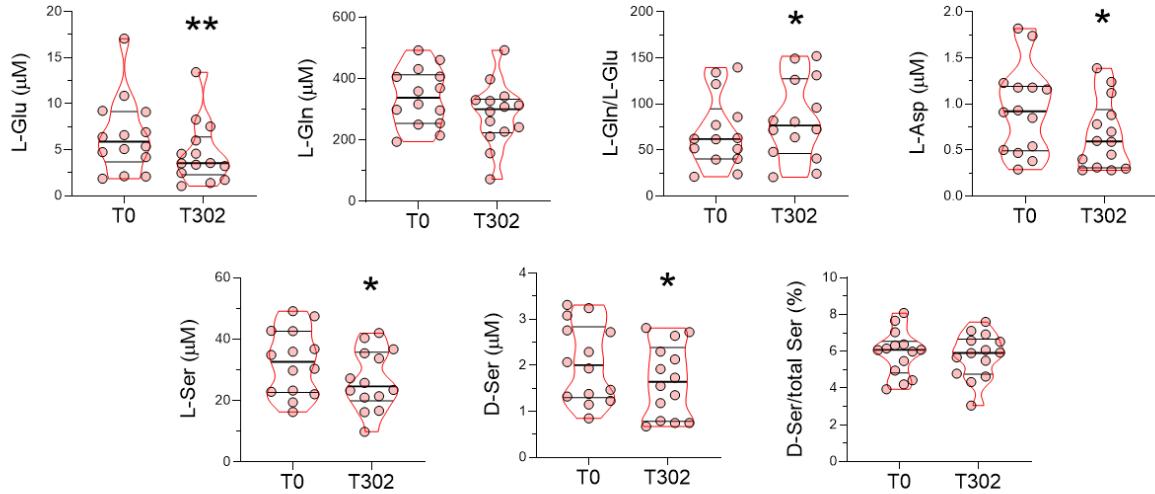
Suppl Fig 5: Effect of Nusinersen on amino acids levels in CSF of SMA3 patients. Levels of of L-glutamate (L-Glu), L-glutamine (L-Gln), L-glutamine/L-glutamate (L-Gln/L-Glu) ratio, L-aspartate (L-Asp), L-serine (L-Ser), D-serine (D-Ser) and D-serine/total serine (L-Ser/total Ser) percentage ratio in the CSF of SMA3 patients prior to treatment (T0, $n = 14$) and at the time of the sixth (T302, $n = 14$) injection of Nusinersen. $*P < 0.05$, $**P < 0.01$, compared to T0 (Wilcoxon matched-pairs signed ranks test). Data are shown as violin plots representing median with interquartile range (IQR). Dots represent individual patients' values.

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SMA1 (T302)
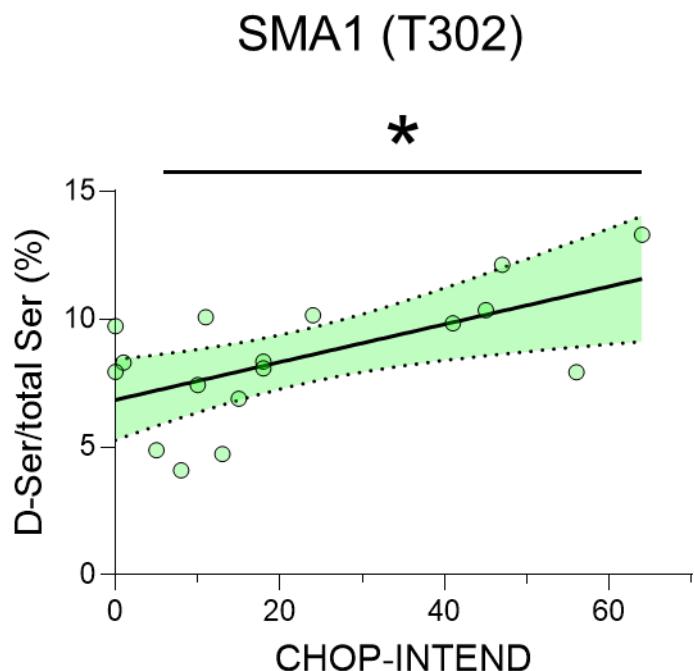
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124 **Suppl Fig 6**: Effect of Nusinersen on the association between D-serine/total serine (L-Ser/total Ser)
125 percentage and CHOP-INTEND of SMA1 patients. *$P < 0.05$, Spearman correlation.
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SMA2 (T302)
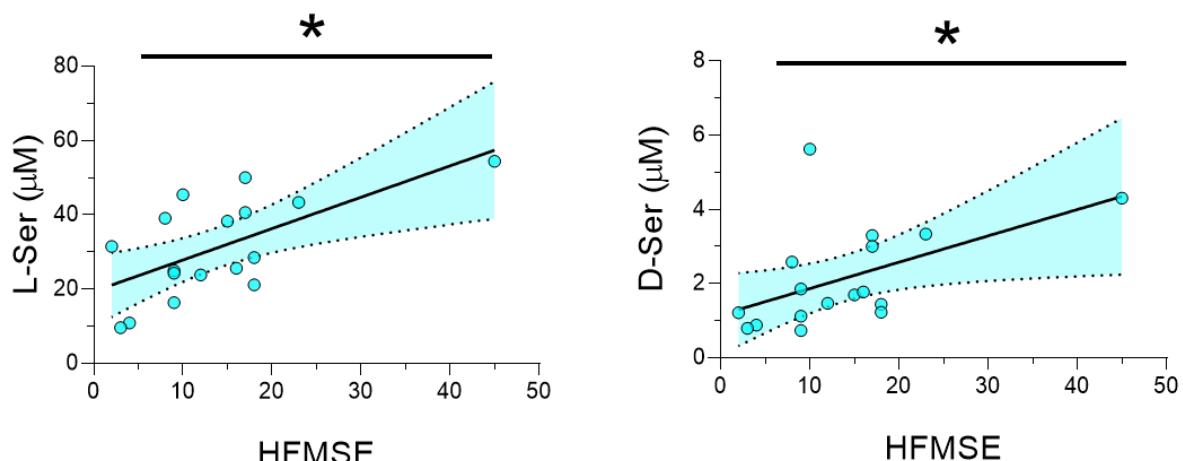
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133 **Suppl Fig 7:** Effect of Nusinersen on the association between L-Ser or D-Ser and HFMSE of SMA2
134 patients. *$P < 0.05$, Spearman correlation.

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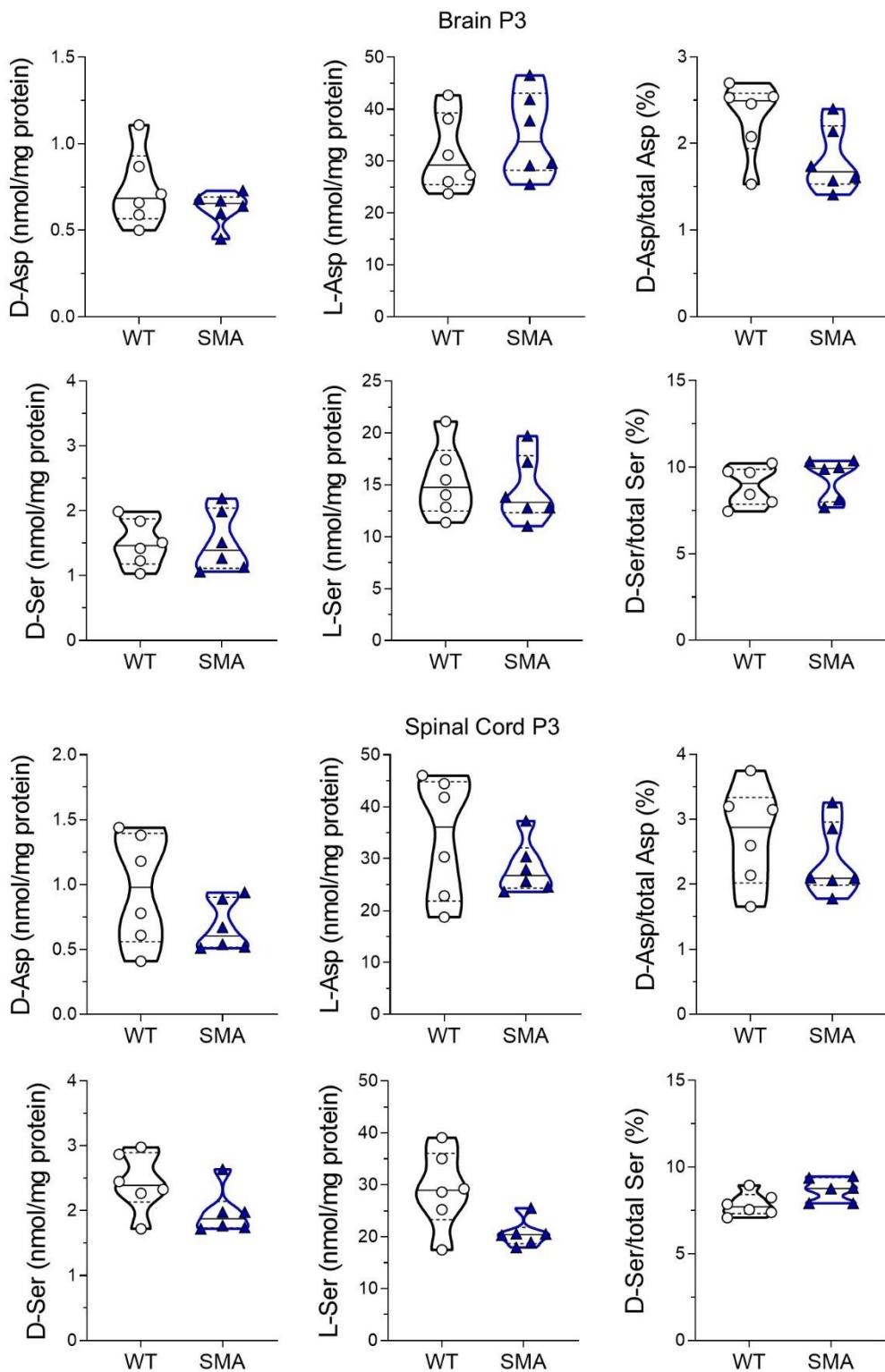
Suppl Fig 8: Analysis of D-aspartate (D-Asp), L-aspartate (L-Asp), D-serine (D-Ser) and L-serine (L-Ser) and their relative ratios between WT and SMA mice in the brain and spinal cord at P3. The average amounts of amino acids detected were normalized for mg of proteins. Dots represent values from individual mice. Amino acids levels are expressed as violin plots representing median with interquartile range (IQR) and analyzed by Mann-Whitney.
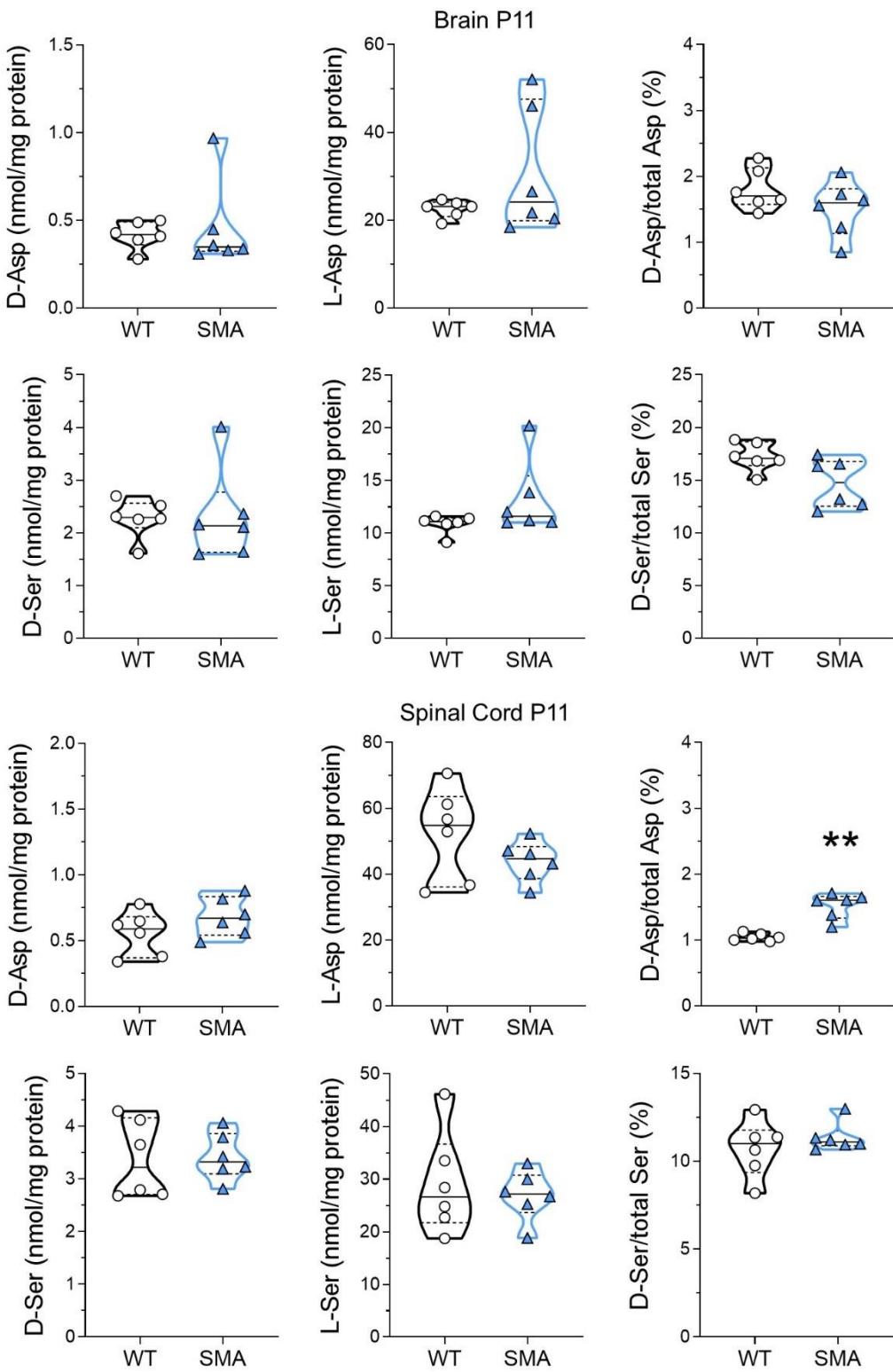
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149 **Suppl Fig 9:** Analysis of D-aspartate (D-Asp), L-aspartate (L-Asp), D-serine (D-Ser) and L-serine
150 (L-Ser) and their relative ratios between WT and SMA mice in the brain and spinal cord at P11. The
151 average amounts of amino acids detected were normalized for mg of proteins. Dots represent values
152 from individual mice. Amino acids levels are expressed as violin plots representing median with

153   interquartile range (IQR) and analyzed by Mann-Whitney. **$P < 0.01$, compared to age-matched WT
154   mice.
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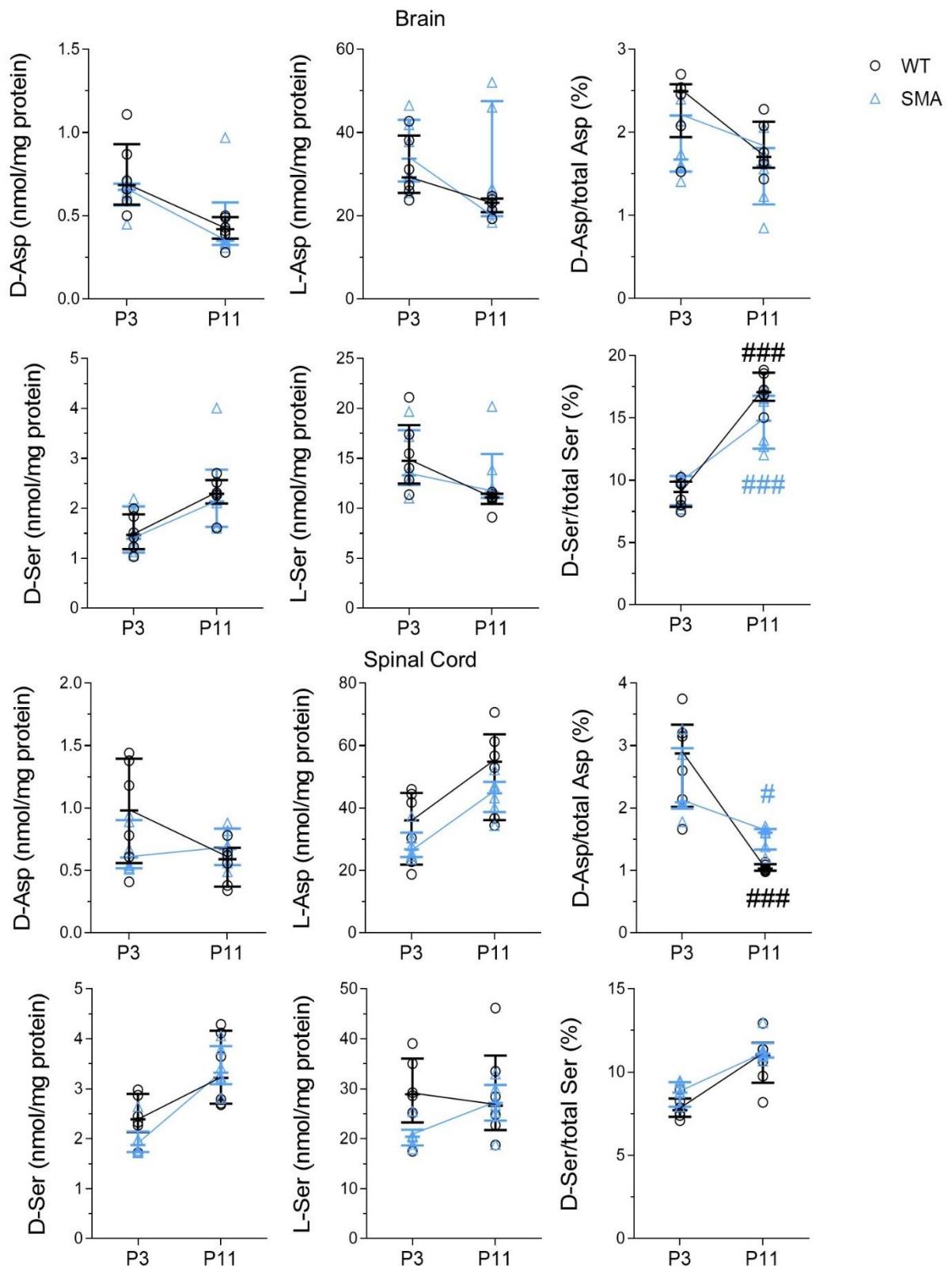
Suppl Fig 10: Analysis of D-aspartate (D-Asp), L-aspartate (L-Asp), D-serine (D-Ser) and L-serine (L-Ser) and their relative ratios between WT and SMA mice at P3 and P11 analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. #$P < 0.05$, ##$P < 0.01$, P11 compared to genotype-matched P3 mice. Amino acids levels were shown as scatter dot plots representing median with interquartile range (IQR) while dots represent individual animals' values.

Supplementary Table 1. Amino acids levels in controls and SMA patients.

Amino acids	Controls ($n = 7$)	SMA1 ($n = 34$)	SMA2 ($n = 22$)	SMA3 ($n = 17$)	Kruskal-Wallis (P-value)[a]	Post-hoc analysis (P-value)[b]
L-Gln (μM)	257 (206-315)	256 (174-288)	307 (211-400)	312 (259-406)	**0.023**	SMA1 vs SMA3: **0.024**
L-Glu (μM)	8.96 (5.28-9.34)	2.50 (1.11-5.04)	3.96 (2.15-6.86)	5.36 (2.29-9.16)	**0.007**	Controls vs SMA1: **0.024**
L-Gln/L-Glu	27.5 (21.7-59.7)	120 (53.4-188)	74.2 (54.9-129)	62.3 (40.1-119)	**0.005**	Controls vs SMA1: **0.006** Controls vs SMA2: **0.036**
L-Asp (μM)	0.83 (0.40-0.87)	0.39 (0.23-0.67)	0.63 (0.34-1.16)	0.85 (0.43-1.18)	**0.037**	
L-Ser (μM)	30.4 (22.7-39.6)	25.0 (19.1-34.1)	32.7 (24.1-38.7)	29.8 (23.1-39.7)	0.166	
D-Ser (μM)	2.50 (0.98-3.36)	2.47 (1.57-4.20)	2.01 (1.35-3.54)	1.93 (1.26-2.74)	0.374	
D-Ser/total Ser (%)	6.86 (3.88-8.01)	8.61 (6.74-11.1)	6.39 (4.45-7.73)	6.07 (4.63-6.71)	**<0.001**	SMA1 vs SMA2: **0.012** SMA1 vs SMA3: **<0.001**

Data are expressed as median (IQR). Analysis by non-parametric Kruskal-Wallis[a], post-hoc analysis by Mann-Whitney with Bonferroni's correction[b]. L-Gln, L-Glu, L-Gln/L-Glu ratio, L-Asp, D-Ser/total Ser (%) levels were natural log transformed and ANCOVA analysis was performed, considering sex and age as possible confounders. At ANCOVA, only L-Glu ($P = 0.006$) and L-Glu/L-Gln ratio ($P = 0.003$) remain significantly associated. Abbreviation: L-glutamine (L-Gln), L-glutamate (L-Glu), L-glutamine/L-glutamate (L-Gln/L-Glu), L-aspartate (L-Asp), L-serine (L-Ser), D-serine (D-Ser), D-serine/total serine (D-Ser/total Ser).

Supplementary Table 2. Variations of amino acids at T0 vs T302 among SMA patients.

Amino acids	SMA1 T0 vs T302	SMA2 T0 vs T302	SMA3 T0 vs T302
L-Gln (μM)	237 vs 285 (***P* = 0.025**) (*n* = 18)	341 vs 283 (*P* = 0.758) (*n* = 17)	338 vs 300 (*P* = 0.064) (*n* = 14)
L-Glu (μM)	2.80 vs 3.70 (***P* = 0.035**) (*n* = 18)	3.76 vs 2.91 (*P* = 0.831) (*n* = 17)	5.87 vs 3.54 (***P* = 0.006**) (*n* = 14)
L-Gln/L-Glu	92.1 vs 102 (*P* = 0.777) (*n* = 18)	78.4 vs 95.5 (*P* = 0.492) (*n* = 17)	62.0 vs 76.7 (***P* = 0.019**) (*n* = 14)
L-Asp (μM)	0.37 vs 0.57 (*P* = 0.287) (*n* = 18)	0.72 vs 0.47 (*P* = 0.379) (*n* = 17)	0.92 vs 0.59 (***P* = 0.038**) (*n* = 14)
L-Ser (μM)	24.5 vs 35.4 (***P* = 0.004**) (*n* = 18)	32.5 vs 28.5 (*P* = 0.586) (*n* = 17)	32.6 vs 24.6 (***P* = 0.019**) (*n* = 14)
D-Ser (μM)	2.82 vs 2.83 (*P* = 0.248) (*n* = 18)	2.06 vs 1.70 (*P* = 0.492) (*n* = 17)	2.00 vs 1.64 (***P* = 0.016**) (*n* = 14)
D-Ser/total Ser (%)	9.24 vs 8.19 (***P* = 0.007**) (*n* = 18)	5.98 vs 6.41 (*P* = 0.653) (*n* = 17)	6.08 vs 5.91 (*P* = 0.221) (*n* = 14)

Data are expressed as median. *P*-values are related to non-parametric Wilcoxon test. The number (*n*) of patients is also indicated.

Supplementary Table 3. Levels of amino acids in the brain and spinal cord of wild type (WT) and SMNΔ7 mice at P3 and P11.

		BRAIN				SPINAL CORD			
		P3		P11		P3		P11	
		WT	SMA	WT	SMA	WT	SMA	WT	SMA
L-Glu	Median	69.33	71.51	71.92	67.07	107.0	77.42	133.5	107.5
	(IQR)	(59.58-88.42)	(54.71-86.47)	(64.26-73.90)	(53.54-85.03)	(78.22-129.5)	(72.51-87.12)	(103.7-151.8)	(94.23-125.8)
	P-value	0.5887		0.5887		0.0931		0.1797	
L-Gln	Median	48.58	48.61	37.21	50.09	274.3	209.8	152.6	220.3
	(IQR)	(45.17-70.35)	(40.17-54.23)	(32.06-41.73)	(46.70-62.42)	(205.4-324.2)	(194.7-216.0)	(126.1-185.6)	(192.5-255.8)
	P-value	0.5887		**0.0043**		0.0649		**0.0152**	
L-Gln/L-Glu	Median	0.750	0.620	0.560	0.740	2.590	2.585	1.230	1.995
	(IQR)	(0.663-0.875)	(0.608-0.753)	(0.468-0.633)	(0.728-0.893)	(2.500-2.678)	(2.500-2.750)	(1.070-1.313)	(1.763-2.295)
	P-value	0.2900		**0.0022**		0.9697		**0.0022**	
D-Ser	Median	1.465	1.390	2.290	2.135	2.390	1.875	3.220	3.325
	(IQR)	(1.180-1.878)	(1.113-2.040)	(2.098-2.565)	(1.630-2.773)	(2.133-2.898)	(1.735-2.145)	(2.703-4.163)	(3.095-3.858)
	P-value	0.9091		0.4848		0.1429		0.8182	
L-Ser	Median	14.76	13.31	11.10	11.61	28.93	20.40	26.63	27.18
	(IQR)	(12.48-18.34)	(12.33-17.82)	(10.44-11.45)	(11.04-15.44)	(23.28-36.04)	(18.67-21.81)	(21.73-36.67)	(23.65-90.76)
	P-value	0.4848		0.1797		0.0931		>0.9999	
D-Ser/total Ser	Median	9.065	9.935	17.07	14.78	7.715	8.770	11.01	11.11
	(IQR)	(7.865-9.880)	(8.003-10.34)	(16.38-18.64)	(12.54-16.77)	(7.323-7.920)	(8.425-9.398)	(9.37-11.77)	(10.88-11.75)
	P-value	0.3095		0.0649		0.0584		0.6991	
D-Asp	Median	0.685	0.655	0.420	0.350	0.980	0.605	0.590	0.670
	(IQR)	(0.567-0.930)	(0.563-0.693)	(0.363-0.493)	(0.325-0.580)	(0.560-1.395)	(0.518-0.903)	(0.370-0.683)	(0.543-0.835)
	P-value	0.5887		0.5887		0.3095		0.2576	
L-Asp	Median	29.25	33.73	23.13	24.16	36.08	26.76	54.85	44.71
	(IQR)	(25.47-39.28)	(28.24-43.07)	(20.89-24.15)	(19.94-47.58)	(21.83-44.83)	(24.33-32.09)	(36.15-63.62)	(38.68-44.71)
	P-value	0.5887		0.6667		0.5887		0.2403	
D-Asp/total Asp	Median	2.495	1.675	1.705	1.600	2.875	2.095	1.030	1.605
	(IQR)	(1.943-2.580)	(1.530-2.205)	(1.575-2.130)	(1.135-1.813)	(2.020-3.338)	(1.990-2.960)	(0.995-1.100)	(1.335-1.665)
	P-value	0.0931		0.2403		0.3939		**0.0022**	

Data are expressed as median. *P*-values are related to non-parametric Mann-Whitney test, $n = 6$ animals per group.

Supplementary Table 4. Age effect on amino acids levels in the brain and spinal cord of wild type (WT) and SMA mice at P3 and P11.

		BRAIN			SPINAL CORD		
		F (DFn, DFd)	*P*-value	Tukey's multiple comparisons test	F (DFn, DFd)	*P*-value	Tukey's multiple comparisons test
L-Glu	Interaction	F (1, 20) = 0.1622	0.6914		F (1, 20) = 0.029	0.8657	
	Age	F (1, 20) = 0.1019	0.7529		F (1, 20) = 10.82	0.0037	
	Genotype	F (1, 20) = 0.02570	0.8742		F (1, 20) = 6.201	0.0217	
L-Gln	Interaction	F (1, 20) = 6.632	**0.0181**	P3:SMA vs. P3:WT *P* = 0.6519 P3:SMA vs. P11:SMA *P* = 0.6444 P3:WT vs. P11:WT *P* = 0.0978 P11:SMA vs. P11:WT *P* = 0.0955	F (1, 20) = 13.45	**0.0015**	P3:SMA vs. P3:WT *P* = 0.0970 P3:SMA vs. P11:SMA *P* = 0.8975 P3:WT vs. P11:WT *P* = **0.0012** P11:SMA vs. P11:WT *P* = 0.0583
	Age	F (1, 20) = 0.8153	0.3773		F (1, 20) = 7.202	0.0143	
	Genotype	F (1, 20) = 0.8474	0.3682		F (1, 20) = 0.03362	0.8564	
L-Gln/L-Glu	Interaction	F (1, 20) = 14.71	**0.001**	P3:SMA vs. P3:WT *P* = 0.4531 P3:SMA vs. P11:SMA *P* = 0.2055 P3:WT vs. P11:WT *P* = **0.0145** P11:SMA vs. P11:WT *P* = **0.0043**	F (1, 20) = 20.21	**0.0002**	P3:SMA vs. P3:WT *P* > 0.9999 P3:SMA vs. P11:SMA *P* = **0.0028** P3:WT vs. P11:WT *P* < **0.0001** P11:SMA vs. P11:WT *P* < **0.0001**
	Age	F (1, 20) = 0.8907	0.3566		F (1, 20) = 106.2	<0.0001	
	Genotype	F (1, 20) = 2.913	0.1033		F (1, 20) = 19.89	0.0002	
D-Ser	Interaction	F (1, 20) = 8.407E-4	0.9772		F (1, 20) = 1.440	0.2442	
	Age	F (1, 20) = 11.56	0.0028		F (1, 20) = 31.61	<0.0001	
	Genotype	F (1, 20) = 0.01518	0.9032		F (1, 20) = 0.9909	0.3314	
L-Ser	Interaction	F (1, 20) = 1.670	0.211		F (1, 20) = 1.317	0.2647	
	Age	F (1, 20) = 5.610	0.028		F (1, 20) = 1.284	0.2706	
	Genotype	F (1, 20) = 0.3861	0.5414		F (1, 20) = 3.733	0.0676	
D-Ser/total Ser	Interaction	F (1, 20) = 5.412	**0.0306**	P3:SMA vs. P3:WT *P* = 0.9557 P3:SMA vs. P11:SMA *P* < **0.0001** P3:WT vs. P11:WT *P* < **0.0001** P11:SMA vs. P11:WT *P* = 0.052	F (1, 20) = 0.06080	0.8077	
	Age	F (1, 20) = 112.4	<0.0001		F (1, 20) = 43.27	<0.0001	
	Genotype	F (1, 20) = 2.576	0.1242		F (1, 20) = 3.189	0.0893	
D-Asp	Interaction	F (1, 20) = 1.118	0.3029		F (1, 20) = 3.824	0.0646	
	Age	F (1, 20) = 11.25	0.0032		F (1, 20) = 3,702	0.0687	
	Genotype	F (1, 20) = 0.2173	0.6461		F (1, 20) = 0,580	0.4551	
L-Asp	Interaction	F (1, 20) = 0.3941	0.5372		F (1, 20) = 0.089	0.7685	
	Age	F (1, 20) = 3.065	0.0953		F (1, 20) = 17.09	0.0005	
	Genotype	F (1, 20) = 2.503	0.1293		F (1, 20) = 2.962	0.1007	
D-Asp/total Asp	Interaction	F (1, 20) = 0.4019	0.5333		F (1, 20) = 4.789	**0.0407**	P3:SMA vs. P3:WT *P* = 0.5209 P3:SMA vs. P11:SMA *P* = **0.0363** P3:WT vs. P11:WT *P* < **0.0001** P11:SMA vs. P11:WT *P* = 0.3463
	Age	F (1, 20) = 6.351	0.0203		F (1, 20) = 40.51	<0.0001	
	Genotype	F (1, 20) = 6.142	0.0222		F (1, 20) = 0.05086	0.8239	

Data were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test.