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Supplementary Information for

Adult spinal motoneurons change their neurotransmitter phenotype to control locomotion

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This PDF file includes:

Figs. S1 to S7
Tables S1 to S2

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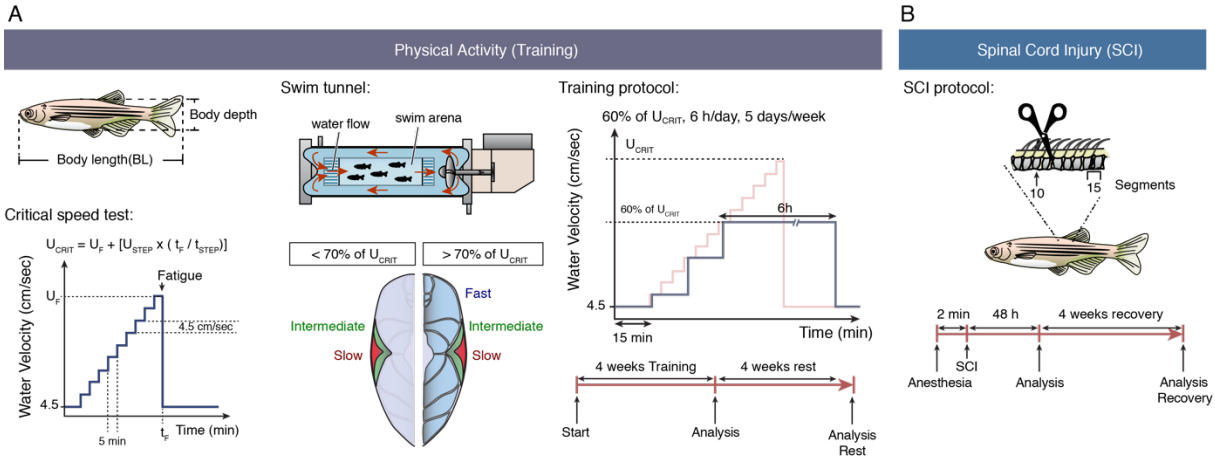

Fig. S1. Experimental design to induce spinal cord plasticity. (*A*) Graphical presentation of the critical speed test protocol to determine the U_{CRIT} (maximum speed of locomotion) of the experimental animals using the swim tunnel. 70% of the U_{CRIT} represents the threshold of engagement of fast motor units. Below the 70% threshold the slow and intermediate motor units are responsible for the animal's locomotion. The animals were trained 6 h per day, 5 days per week for 4 weeks at 60% of the critical speed (U_{CRIT}). Another group of animals were allowed to survive for 4 more weeks to analyze how spinal motoneurons respond to post-training. (*B*) Protocol to induce adaptive phenomena studied here (segment 15) after total lesion of the rostral spinal cord segment 10. Group of experimental animals were allowed to recover for 4 weeks post-injury.

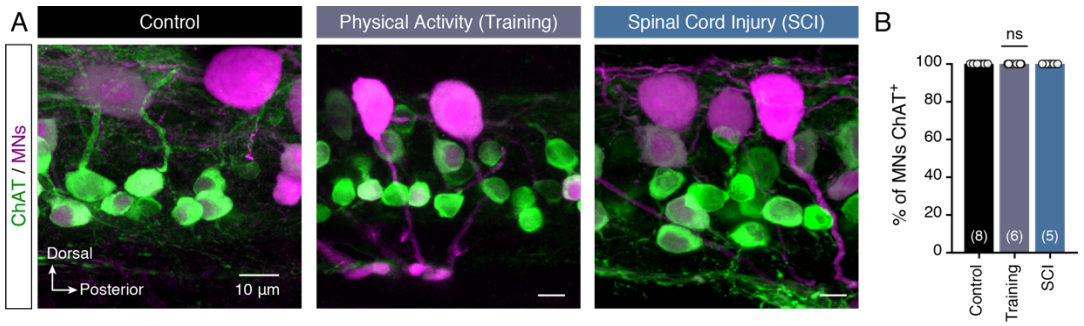

Fig. S2. Motoneurons always remain cholinergic. (*A*) Representative whole mount confocal images showing ChAT immunoreactivity of retrograde labelled spinal motoneurons in control, after training and following spinal cord injury (SCI). (*B*) Quantification of the spinal motoneurons positive for ChAT immunolabeling. Data are presented as mean ± SEM; ns, non-significant.

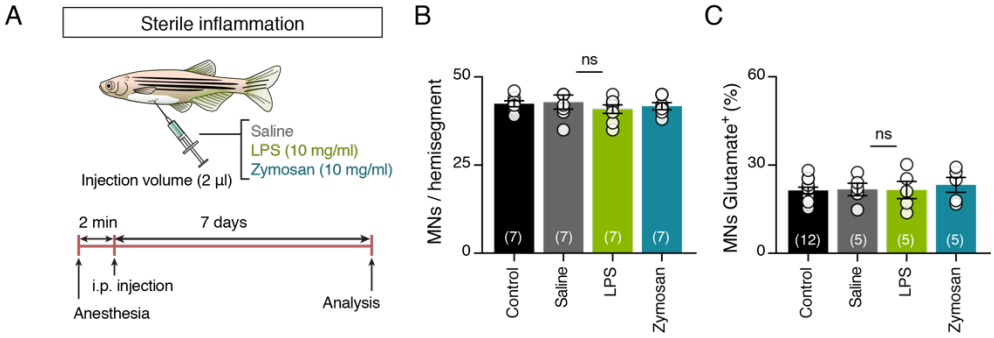

Fig. S3. Induction of sterile inflammatory response does not alter the motoneuron glutamatergic phenotype. (*A*) Experimental design for the induction of acute inflammatory response by single intraperitoneal injection of immunogenic particles LPS or Zymosan. (*B*) Quantification of the axial motoneuron number per spinal hemisegment. (*C*) Quantification of the motoneurons that also co-express glutamate. Data are presented as mean ± SEM; ns, non-significant. For detailed statistics see *SI Appendix*, Table S1.

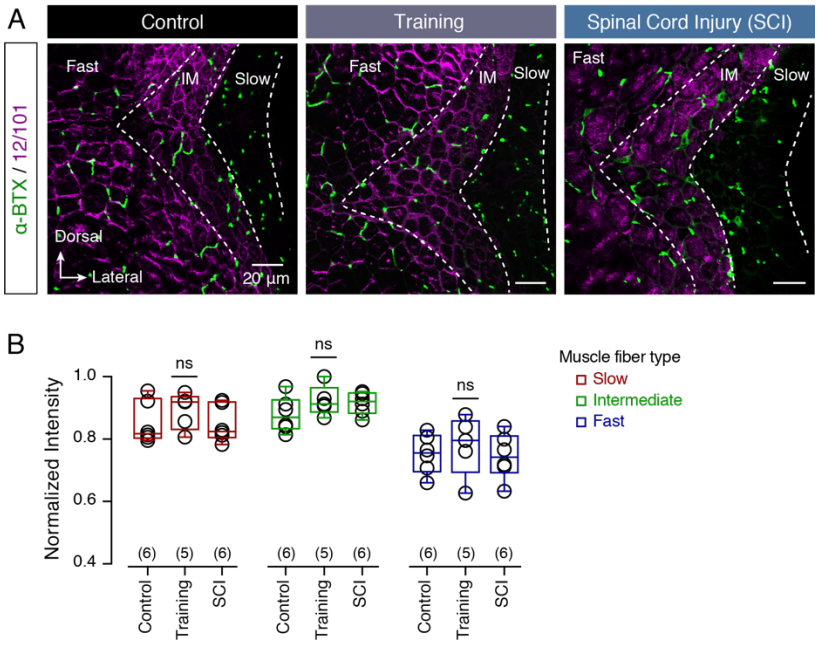

Fig. S4. Neuromuscular junctions remain the same after training and spinal cord injury. (*A*) Representative transverse confocal images from adult zebrafish myotome (purple) showing the location and the number of the neuromuscular junctions (α-BTX; green) in control and after physical exercise and spinal cord injury. (*B*) Quantification of the normalized intensity of the α-BTX staining in slow, intermediate and fast muscle fibers show no significant changes following training and spinal cord injury. Data are presented as boxplots showing the median, 25th and 75th percentile (box and line), minimal and maximal values (whiskers), mean ± SEM; ns, non-significant; IM, intermediate. For detailed statistics see *SI Appendix*, Table S1.

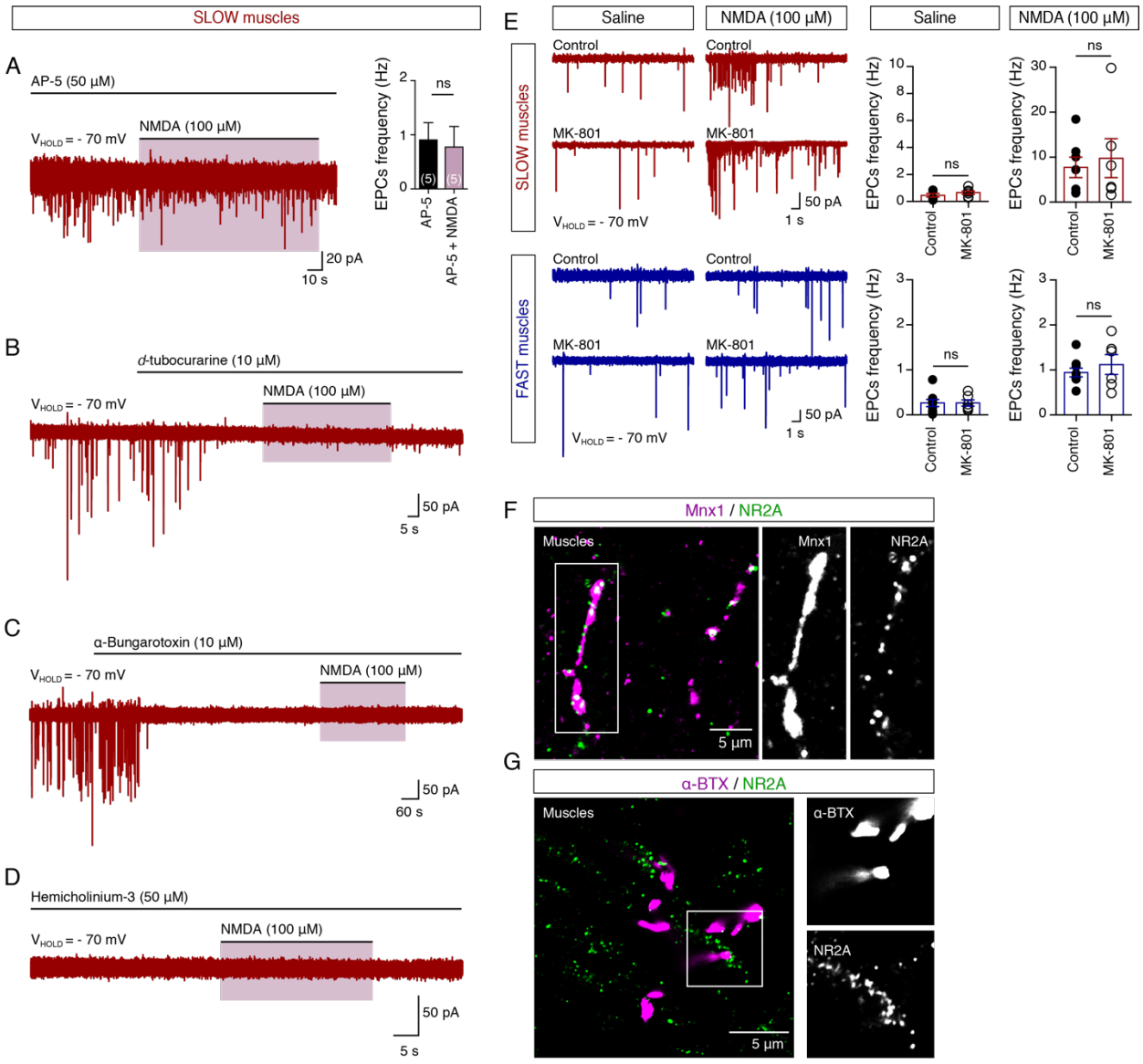

Fig. S5. Presynaptic NMDA receptors induce muscle activity. (*A*) Representative trace of whole cell voltage-clamp recording from slow muscle fiber showing that in a presence of 50 µM AP-5 the NMDA-induced ACh release is blocked. (*B-D*) Representative traces of whole cell voltage-clamp recordings showing that in presence of nicotinic acetylcholine receptor blockers (*d*-tubocurarine and α-Bungarotoxin, 10 µM) and the selective blocker of the choline uptake (Hemicholinium-3, 50 µM) NMDA cannot induce any EPCs, suggesting that the NMDA receptors are located at the presynaptic terminals, and their activation from glutamate enhance the release of acetylcholine. (*E*) EPC recordings from both slow and fast muscle fibers in presence of the non-competitive NMDA receptor antagonist (MK-801; 1 mM) in the intracellular solution show no significant changes in the frequency of post-synaptic events. (*F*) Close apposition of motoneuron axon NR2A expression (green) to the post-synaptic muscle nicotinic receptors (α-BTX; magenta). (*G*) NMDA receptor subunit 2B (NR2B) expression (green) on the motoneuron presynaptic terminals at the neuromuscular junctions (α-BTX; magenta). Whole cell voltage-clamp recordings were obtained at a holding potential of -70 mV in all recordings. Data are presented as mean ± SEM; ns, non-significant. For detailed statistics see *SI Appendix*, Table S1.

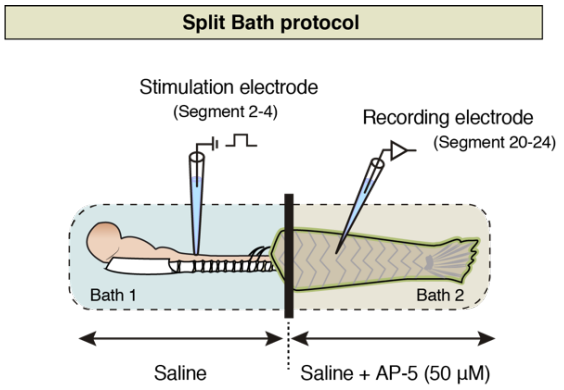

Fig. S6. Experimental strategy to study the direct release of glutamate in the axial neuromuscular junctions. Split bath experiment in the adult zebrafish *ex-vivo* preparation. Electrical stimulation of the initial segments (2-4) of spinal cord depolarized supra-threshold the spinal motoneurons to produce muscle twitch. Recordings of EPCs performed from caudal muscle fibers (segment 20-24) in a presence of the NMDA receptor selective blocker AP-5 (50 µM).

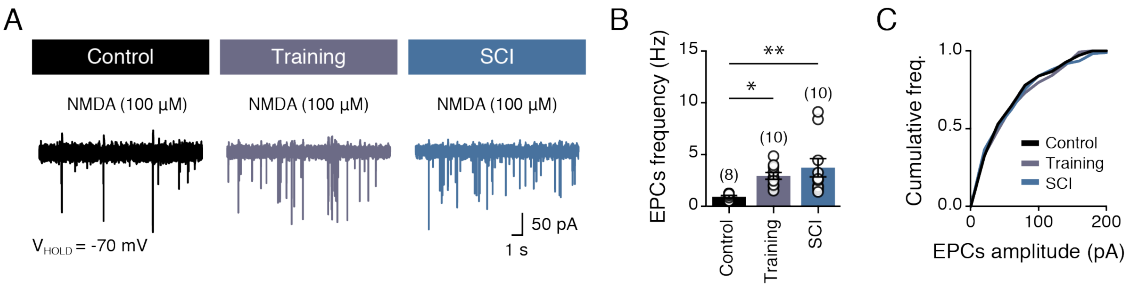

Fig. S7. Neuromuscular changes after training and SCI. (*A-C*) Fast muscle fibers displayed a significant increase in EPCs frequency after bath application of NMDA (100 µM) following training and SCI (P = 0.01, one-way ANOVA). Whole cell voltage-clamp recordings were obtained at a holding potential of -70 mV in all recordings. Data are presented as mean ± SEM. *P < 0.05. For detailed statistics see *SI Appendix*, Table S1.

Table S1. Detailed statistics.

Figure	Statistics	Result	Post-hoc Test	comparison	Significance	*P* value
1*B*	Descriptive	Mean ± SEM: 21.41 ± 1.091				
1*E*	Descriptive	Mean ± SEM $_{slow}$: 42.67 ± 2.245% Mean ± SEM $_{intermediate}$: 26.32 ± 1.911% Mean ± SEM $_{fast}$: 18.07 ± 1.036%				
	One-way ANOVA	$F_{(3, 49)}$ = 124.9 *P* < 0.0001	Tukey's test	Slow Intermediate	****	*P* < 0.0001
				Slow Fast	****	*P* < 0.0001
				Intermediate Fast	**	*P* = 0.0012
1*F* Soma size	Unpaired *t*-test	t = 5.16, df = 108 (Two-tailed)		Glutamate+ Glutamate-	****	*P* < 0.0001
1*F* Position	Unpaired *t*-test	t = 2.364, df = 72 (Two-tailed)		Glutamate+ Glutamate-	*	*P* = 0.0208
2*B*	One-way ANOVA	$F_{(2, 14)}$ = 0.00034			ns	*P* = 0.999
2*C*	Descriptive	Mean ± SEM $_{Control}$: 21.41 ± 1.091% Mean ± SEM $_{Training}$: 39.54 ± 3.014% Mean ± SEM $_{SCI}$: 33.21 ± 1.884%				
	One-way ANOVA	$F_{(2,23)}$ = 26.55 *P* < 0.0001	Dunnett's test	Control Training	****	*P* = 0.0001
				Control SCI	***	*P* = 0.0003
2*D* Slow MNs	One-way ANOVA	$F_{(2,19)}$ = 0.1243			ns	*P* = 0.8839
2*D* Intermediate MNs	One-way ANOVA	$F_{(2,21)}$ = 1.069			ns	*P* = 0.3614
2*E*	One-way ANOVA	$F_{(4, 33)}$ = 17.65 *P* < 0.0001	Dunnett's test	Control Training	****	*P* = 0.0001
				Control SCI	****	*P* = 0.0001
				Control Training-rest	ns	*P* = 0.8012
				Control SCI-recovery	ns	*P* = 0.6921
2*E* Soma size	One-way ANOVA	$F_{(2,174)}$ = 4.738 *P* = 0.0099	Dunnett's test	Control Training	**	*P* = 0.0088
				Control SCI	*	*P* = 0.0113
2*E* Position	One-way ANOVA	$F_{(2,117)}$ = 4.917 *P* = 0.0089	Dunnett's test	Control Training	**	*P* = 0.0087
				Control SCI	**	*P* = 0.0094
3*C* Total distance	Unpaired *t*-test	t = 3.284, df = 10 (Two-tailed)		Control Training	*	*P* = 0.0106
3*C* Traveled area	Unpaired *t*-test	t = 1.701 , df = 10 (Two-tailed)		Control Training	ns	*P* = 0.1198
3*C* Immobility	Unpaired *t*-test	t = 1.628, df = 10 (Two-tailed)		Control Training	ns	*P* = 0.1345

3*C* Average velocity	Unpaired *t*-test	t = 2.708, df = 10 (Two-tailed)		Control Training	*	*P* = 0.022
3*C* Max velocity	Unpaired *t*-test	t = 4.361, df = 10 (Two-tailed)		Control Training	**	*P* = 0.0014
3*D*	Unpaired *t*-test	t = 4.847, df = 17(Two-tailed)		Control Training	***	*P* = 0.0002
4*B*	Unpaired *t*-test	t = 3.377, df = 28 (Two-tailed)		Control Training	**	*P* = 0.0022
4*D* Slow muscles	Unpaired *t*-test	t = 6.611, df = 496 (Two-tailed)		Control Training	****	*P* < 0.0001
4*D* Intermediate muscles	Unpaired *t*-test	t = 4.823, df = 549 (Two-tailed)		Control Training	****	*P* < 0.0001
4*D* Fast muscles	Unpaired *t*-test	t = 0.3116, df = 803 (Two-tailed)		Control Training	ns	*P* = 0.7554
4*F*	One-way ANOVA	$F_{(2, 32)}$ = 240.5, *P* < 0.0001	Tukey's test	Slow Intermediate	****	*P* < 0.0001
				Slow Fast	****	*P* < 0.0001
				Intermediate Fast	****	*P* < 0.0001
5*B*	Repeated Measures One-way ANOVA	$F_{(1.216, 6.079)}$ = 39.07 *P* = 0.0006	Tukey's test	Slow Intermediate	**	*P* = 0.0069
				Slow Fast	**	*P* = 0.0027
				Intermediate Fast	**	*P* = 0.0038
5*C* Slow muscles	One-way ANOVA	$F_{(2, 18)}$ = 0.3972			ns	*P* = 0.678
5*C* Intermediate muscles	One-way ANOVA	$F_{(2, 18)}$ = 2.935			ns	*P* = 0.0789
5*C* Fast muscles	One-way ANOVA	$F_{(2, 18)}$ = 8.775 *P* = 0.0022	Dunnett's test	Control Training	**	*P* = 0.0019
				Control SCI	**	*P* = 0.0060
5*E* Slow muscles	One-way ANOVA	$F_{(2, 18)}$ = 11 *P* = 0.0008	Dunnett's test	Saline NMDA	**	*P* = 0.0013
				Saline Washout	ns	*P* = 0.9974
5*E* Fast muscles	One-way ANOVA	$F_{(2, 21)}$ = 10.82 *P* = 0.0006	Dunnett's test	Saline NMDA	***	*P* = 0.0003
				Saline Washout	ns	*P* = 0.059
5*F*	Repeated Measures One-way ANOVA	$F_{(1.557, 6.228)}$ = 7.172 *P* = 0.0279	Dunnett's test	Saline AP-5	*	*P* = 0.0107
				Saline Washout	ns	*P* = 0.3555
5*I*	One-way ANOVA	$F_{(2, 30)}$ = 4.531 *P* = 0.0191	Dunnett's test	Control Training	*	*P* = 0.0215
				Control SCI	*	*P* = 0.0326
S3*B*	One-way ANOVA	$F_{(3, 25)}$ = 0.4496			ns	*P* = 0.7198

S3*C*	One-way ANOVA	$F_{(3, 23)} = 0.1856$			ns	$P = 0.9051$
S4*B* Slow muscles	One-way ANOVA	$F_{(2, 14)} = 0.7632$			ns	$P = 0.4846$
S4*B* Intermediate muscles	One-way ANOVA	$F_{(2, 14)} = 1.418$			ns	$P = 0.2749$
S4*B* Fast muscles	One-way ANOVA	$F_{(2, 14)} = 0.3081$			ns	$P = 0.7397$
S5*A*	Wilcoxon matched-pairs signed rank test			AP-5 AP-5+NMDA	ns	$P = 0.3125$
S5*E*	Unpaired *t*-test	t = 1.023, df = 11 (Two-tailed)		*Slow (Saline)* Control MK-801	ns	$P = 0.3285$
	Unpaired *t*-test	t = 0.437, df = 11 (Two-tailed)		*Slow (NMDA)* Control MK-801	ns	$P = 0.6706$
	Unpaired *t*-test	t = 0.9936, df = 11 (Two-tailed)		*Fast (Saline)* Control MK-801	ns	$P = 0.9936$
	Unpaired *t*-test	t = 0.8328, df = 11 (Two-tailed)		*Fast (NMDA)* Control MK-801	ns	$P = 0.4200$
S7*B*	One-way ANOVA	$F_{(2, 25)} = 5.549$ $P = 0.0101$	Dunnett's test	Control Training	*	$P = 0.0496$
				Control SCI	**	$P = 0.006$

Table S2. Antibodies Used[1]

Antigen	Host	Source	Code	Dilution
Primary				
ChAT	Goat	Millipore	AB144P; RRID: AB_2079751	1:150
Glutamate	Rabbit	Sigma	G6642; RRID: AB_259946	1:6000
Mef2	Rabbit	Santa Cruz	SC313; RRID: AB_631920	1:50-1:80
Mnx1	Mouse	DSHB	81.5C10; RRID: AB_2145209	1:50
NR2A	Rabbit	Millipore	07-632; RRID: AB_310837	1:500
NR2B	Mouse	BD Biosciences	610416; RRID: AB_397796	1:500
S58	Mouse	DSHB	S58; RRID: AB_528377	1:10-1:20
VAChT	Goat	Millipore	ABN100; RRID: AB_2630394	1:500
VGluT1	Guinea Pig	Millipore	AB5905; RRID: AB_2301751	1:800
VGluT2	Guinea Pig	Millipore	AB5907; RRID: AB_2301731	1:300
12/101	Mouse	DSHB	12/101; RRID: AB_531892	1:50
Secondary				
Goat IgG-568	Donkey	ThermoFisher	A-11057; RRID: AB_2534104	1:500
Goat IgG-488	Donkey	ThermoFisher	A-11055; RRID: AB_2534102	1:500
Guinea Pig IgG-568	Goat	ThermoFisher	A-11075; RRID: AB_2534119	1:500
Mouse IgG-647	Donkey	ThermoFisher	A-31571; RRID: AB_162542	1:500
Mouse IgG-568	Goat	ThermoFisher	A-11004; RRID: AB_2534072	1:500
Mouse IgG-488	Donkey	ThermoFisher	A-21202; RRID: AB_141607	1:500
Rabbit IgG-488	Donkey	ThermoFisher	A-21206; RRID: AB_2535792	1:500
Rabbit IgG-647	Donkey	ThermoFisher	A-31573; RRID: AB_2536183	1:500
Rabbit IgG-568	Donkey	ThermoFisher	A-10042; RRID: AB_2534017	1:500

[1]ChAT, choline-acetyltransferase; mef2, myocyte enhancer factor-2; Mnx1, motoneuron homebox 1; NR2A, NMDA receptor subunit 2A; NR2B, NMDA receptor subunit 2B; S58, Myosin heavy chain, slow contracting muscle; VAChT, vesicular acetylcholine transporter; VGluT1, vesicular glutamate transporter 1; VGluT2, vesicular glutamate transporter 2; 12/101, skeletal muscle marker, 102 kDa.