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Supplemental Information

Synaptogenesis Stimulates a Proteasome-Mediated Ribosome Reduction in Axons

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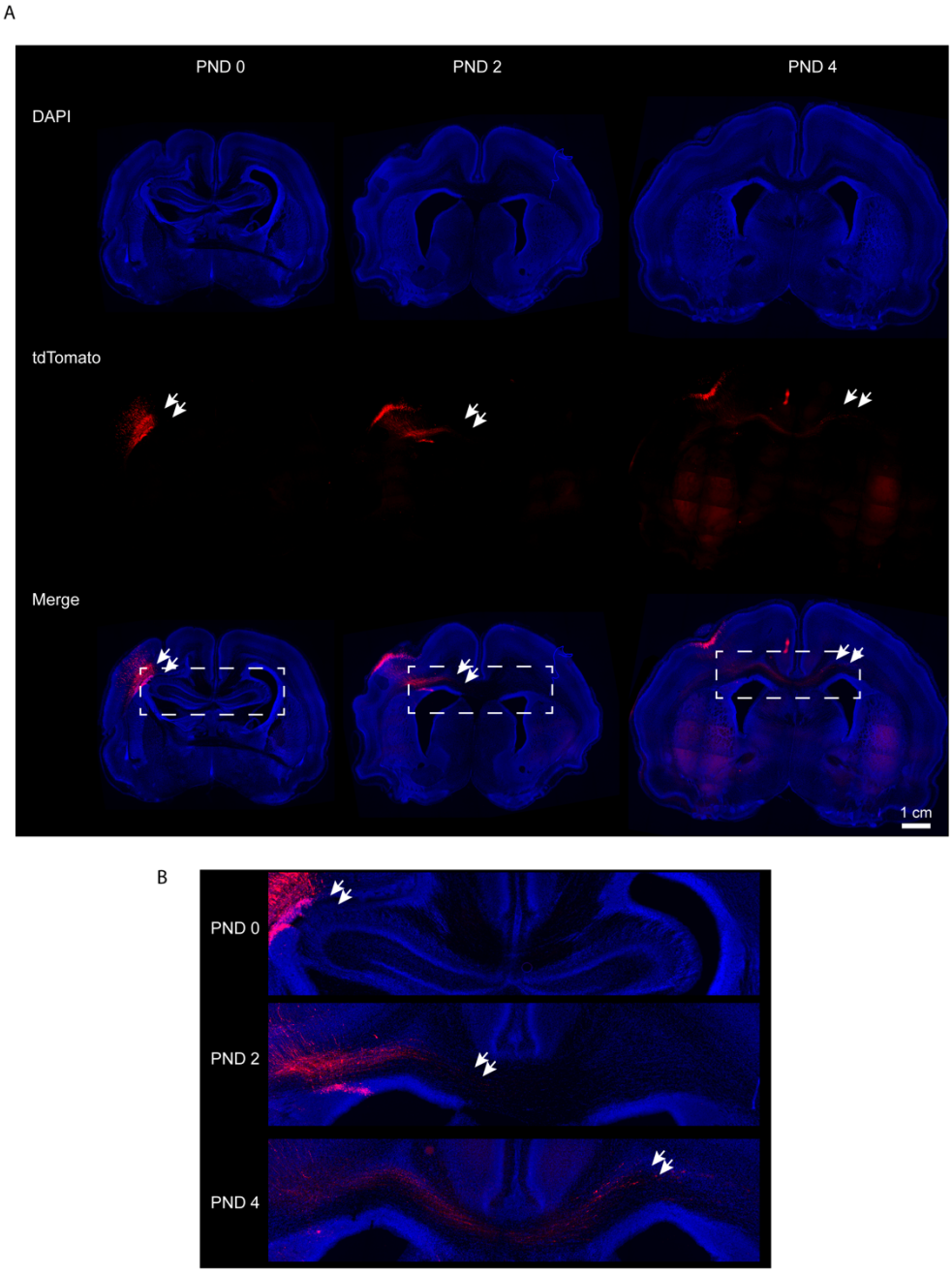

Figure S1. Study of *corpus callosum* axonal development through *in utero* somatosensory cortex electroporation, related to Figure 1.

(A) Development of callosal projection neurons (CPN) axons in vivo. CPN, present at somatosensory cortex, were electroporated in utero with pCAGGS-tdTomato expressing vector, as described in Figure 1. At post-natal days (PND) 0, 2 and 4, brains were perfused/fixed and then sliced. Imaging of brain slices (80 μm thick) show the CPN axonal development towards the contralateral side. DAPI (blue) was used to label the nucleus, while tdTomato (red) allowed the visualization of CPN axonal development. Results show that electroporated CPN neurons project axons that pass the *corpus callosum* midline area (border between both hemispheres) between PND 2 and PND 4. Before this period, no signal is visible in corpus callosum midline area, demonstrating the specificity of the somatosensory cortex electroporation. Scale bar is 1 cm.

(B) Higher magnification of dashed white rectangles indicated in A). CPN axons are filled with tdTomato (red) and the cell bodies labelled with DAPI (blue). Results show the development of CPN axons towards the contralateral hemisphere. White arrows in (A) and (B) depict the area already reached by CPN axons.

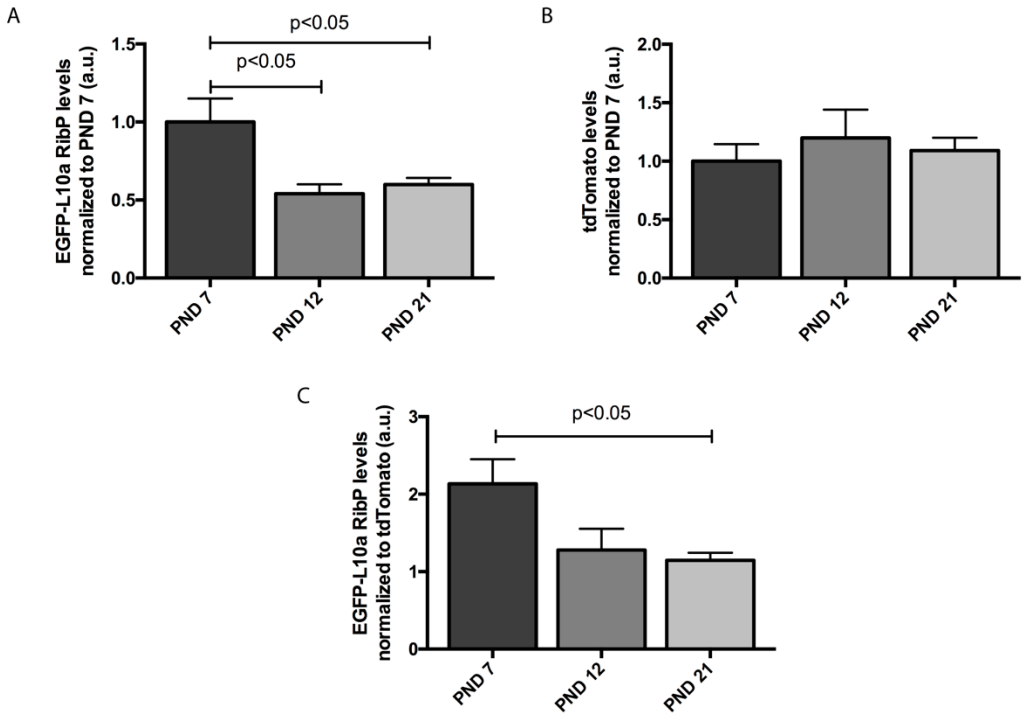

Figure S2. Synaptogenesis triggers a specific reduction of intra-axonal ribosomal levels *in vivo*, related to Figure 1.

(A-C) Ribosomal protein (RibP) L10a and tdTomato analysis by western blot (raw data, used in figure 1 G elaboration). Electroporated brains were dissected at post-natal days (PND) 7, 12 and 21 and proteins contained in *corpus callosum* midline area extracted and prepared for western blot, as explained in figure 1. Results show that RibP L10a levels are reduced in callosal projecting neurons (CPN) axons as they reach the postsynaptic target in the contralateral hemisphere, when compared to PND 7 (A), while tdTomato levels remain unaltered during the experimental period (B). To evaluate the ratio of tagged ribosomes in electroporated neurons EGFP-10a levels were normalized for tdTomato and similar results were obtained (C). Bars represent the mean ± SEM of at least 4 independent experiments. At least 9 animals were used per condition (PND 7, n=9; PND 12, n=8; PND 21, n=9). Statistical significance by one-way ANOVA followed by multiple-comparison Tukey's post-hoc test.

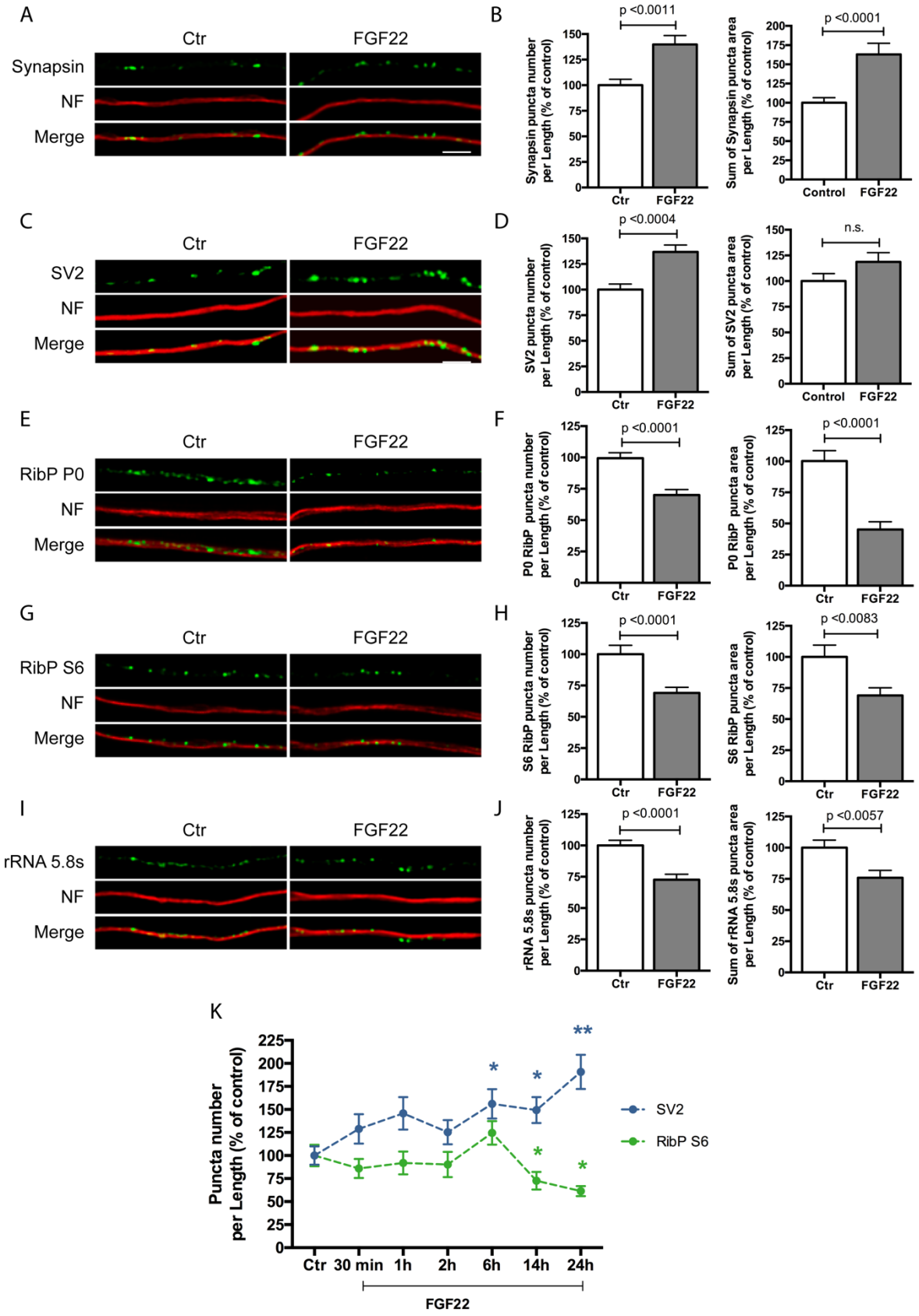

Figure S3. FGF22 induces presynaptic differentiation and ribosomal proteins and rRNA 5.8s decrease in axons, related to Figure 2.

(A, C) Presynaptic clusters increase upon FGF22 treatment. Spinal motor neurons, cultured in normal coverslips, were globally stimulated (both cell bodies and neurites), at DIV 3/4, with either BSA (Ctr) or FGF22 (2 nM). At DIV 4, neurons were immunostained against synapsin (A) or SV2 (C) (green) and neurofilament (NF; red). Scale bar is 2.5 µm.

(B, D) Quantitative data of the number, and area of synapsin (B), SV2 (D) clusters per axonal length. The number of synapsin and

SV2 puncta, puncta area per axon length increased significantly after FGF22 stimulation when compared to control. Results are expressed as % of control. Bars represent the mean ± SEM of at least 3 independent experiments. In each independent experiment, at least 12 fields of view (FOV) were analysed per condition (n=36). Statistical significance by unpaired student's t-test.

(E, G, I) Ribosomal proteins and rRNA 5.8s decrease upon FGF22 treatment. Spinal motor neurons, cultured in normal coverslips, were globally stimulated (both cell bodies and neurites), at DIV 3/4, with either BSA (Ctr) or FGF22 (2 nM). At DIV 4, neurons were immunostained against RibP P0 (E), RibP S6 (G) or rRNA 5.8s (I) (green) and neurofilament (NF; red). The number of RibP P0, RibP S6 or rRNA 5.8s puncta, and puncta area per axon length decreased significantly after FGF22 stimulation when compared to control. Scale bar is 2.5 µm. These three endogenous ribosomal proteins belong to different categories.; 1) RibP P0, is part of the same family of ribosomal proteins as RibP L10, and part of the 60S ribosomal subunit (Uchiumi and Kominami, 1997); 2) RibP S6, belongs to the small 40S ribosomal subunit (Hutchinson et al., 2011) and 3) ribosomal RNA 5.8S (rRNA 5.8s or Y10B), is part of the large subunit of the ribosome (Abou Elela and Nazar, 1997). Respectively, these proteins are essential for the translation initiation step of protein synthesis (1), for the recruitment of translation factors to the ribosome (2), and for the protein synthesis elongation step (3).

(F, H, J) Quantitative data of the number and area of RibP P0 (F), RibP S6 (H) and rRNA 5.8s (J) clusters per axonal length. The number of ribosomes decreased significantly after FGF22 stimulation when compared to control. Results are expressed as % of control. Bars represent the mean ± SEM of at least 3 independent experiments. In each independent experiment, at least 12 fields of view (FOV) were analysed per condition (n=36). Statistical significance by unpaired student's t-test.

(K) Ribosomal protein decrease in axons correlates with FGF22 induced presynaptic differentiation. Spinal motor neurons, cultured in normal coverslips, were stimulated (global application) for different periods of time, at DIV 3/4, with either BSA (Ctr) or FGF22 (2 nM). At DIV 4/5, neurons were immunostained against SV2 or S6 and neurofilament. Quantitative data shows that FGF22-induced increase in synaptic vesicle 2 (SV2) puncta clusters precedes ribosomal protein S6 (RibP S6) decrease. The number of SV2 puncta per axon length (blue line) increases gradually, reaching its maximum value at 24h (190.76%, $p<0.01$). In contrast, the number of ribosomal protein S6 puncta per axon length (green line) decreases approximately 27% after 14h (72.63%, $p<0.05$), with a minimal value reached after 24h (61.47%, $p<0.05$). For each independent experiment, results were normalized to the mean of control. Values are the mean ± SEM of at least two independent experiments. In each independent experiment, at least 12 fields of view (FOV) were analysed per condition (n=24 or n=36). Statistical significance by one-way ANOVA followed by multiple-comparison Dunnett's post-hoc test. *$p < 0.05$; **$p < 0.01$; significantly different when compared to control.

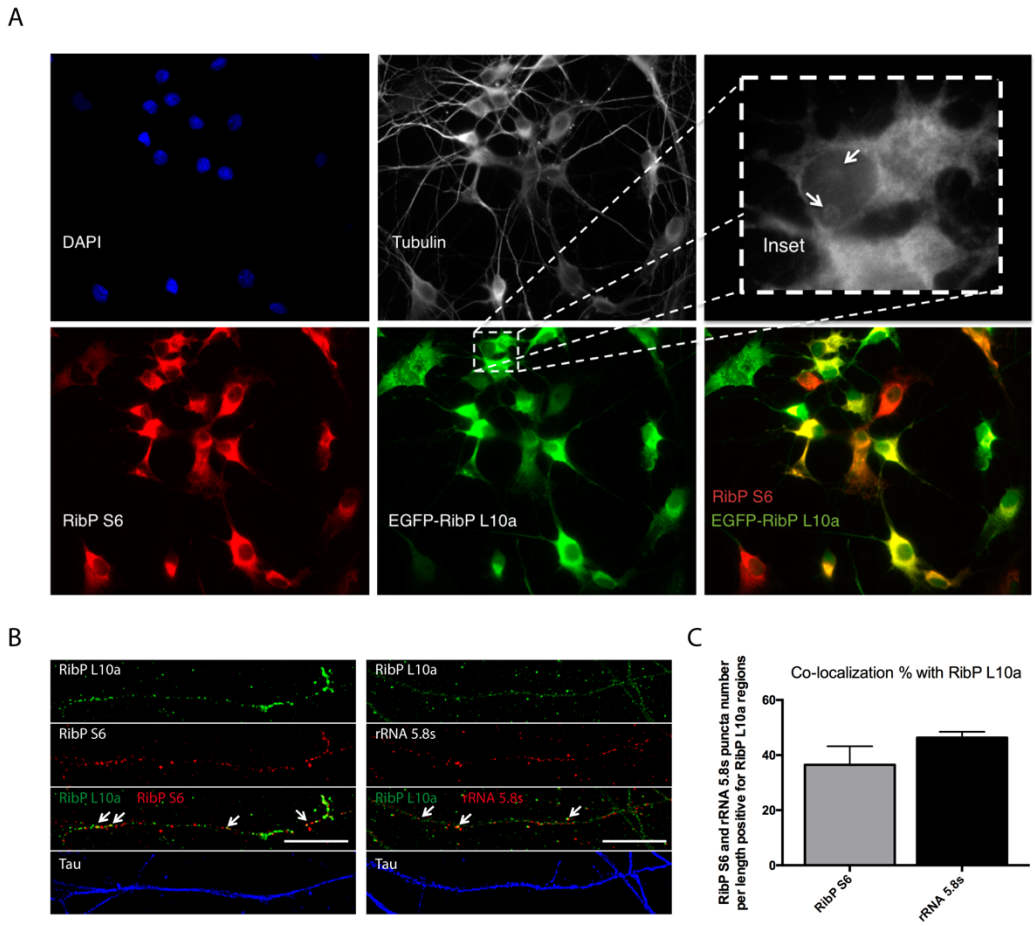

Figure S4. Validation of a ribosomal reporter (EGFP-L10a) in spinal motor neuron cultures, related to Figure 4.

(A) Exogenously expressed EGFP-L10a co-localizes with endogenous RibP S6. Rat spinal motor neurons were transduced at DIV 1 with a lentivirus containing EGFP fused to the 60s RibP L10a. At DIV 4, neurons were labelled with DAPI (blue; nucleus), and immunostained against GFP (green; EGFP-L10a), synapsin (red) and tubulin (grey). Merge image show the co-localization of both exogenous RibP L10a and endogenous RibP S6. The inset image shows the RibP L10a presence on the nucleolus (white arrows), the ribosomal assembly site.

(B) EGFP-L10a co-localizes with endogenous RibP S6 and rRNA 5.8s in axons. Rat spinal motor neurons were infected at DIV 1 with a lentivirus containing EGFP fused to the 60s RibP L10a. At DIV 4, neurons were immunostained against GFP (green; EGFP-L10a), RibP S6 or rRNA 5.8s (red) and tau (blue). Co-localization is observed between RibP L10a/S6 and RibP L10a/rRNA 5.8s (yellow, white arrows). Scale bar is 10 µm.

(C) Quantitative data of the number of RibP S6 and rRNA 5.8s puncta that co-localize with Rib L10a clusters per axonal length. Results are expressed as % of the total RibP S6 or rRNA 5.8s puncta number per length that co-localize with RibP L10a puncta. Bars represent the mean ± SEM of 1 independent experiment where at least 12 fields of view (FOV) were analysed per condition.

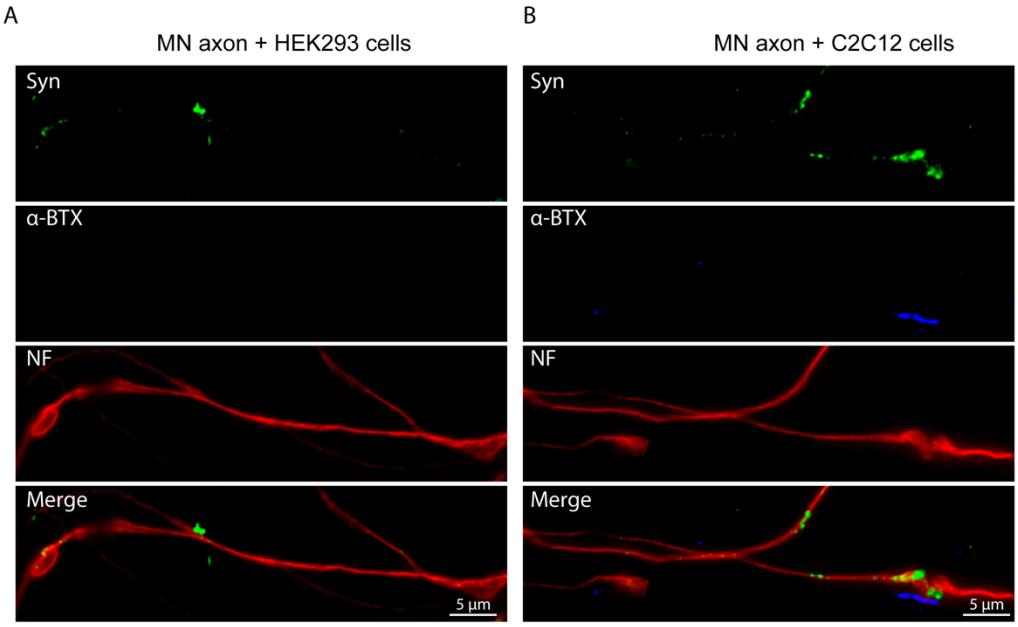

Figure S5. Neuromuscular junction formation between spinal motor neurons and C2C12 myofibers, related to Figure 4.
(A-B) Spinal motor neurons (MN) were plated in the somal compartment of microfluidic chambers as described in figure 4A and at DIV 1, HEK293 cells (control) or C2C12 muscle fibbers were added to axonal compartment. At DIV 4/5, MN-HEK293 (A) and MN-muscle (B) co-cultures were labelled with α-bungarotoxin (α-BTX, post-synaptic marker; blue) and immunostained against synapsin (Syn, pre-synaptic marker; green;), and neurofilament (NF; axonal marker; red). Results show an accumulation of synaptic vesicles in a juxtaposed region to AChRs in MN-C2C12 co-cultures indicating neuromuscular junction formation (B). No synapse formation is observed in MN-HEK293 co-cultures (A). Scale bar is 5 µm.