Supporting Information

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Spinal cord neuronal network formation in a 3D printed reinforced matrix – a model system to study disease mechanisms

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Figure S1: Schematic overview of an inhibitory synapse

top - spinal cord depicted with an enlarged view of the inhibitory synapse (circle). Pre- and postsynapse are shown by opposing membranes. Typical structural proteins for inhibitory synapse formation that are monitored in the present study are labelled: synapsin – localized at synaptic vesicles. Synaptic vesicles contain chemical neurotransmitters: e.g. glycine. Glycine is loaded into synaptic vesicles by the vesicular-GABA transporter (VGAT). At the postsynapse, ligand-gated glycine receptors (GlyR) are anchored to the post-synaptic membrane via the scaffold protein gephyrin.
**Figure S2:** Laser Cutting of PCL reinforcement frames. The laser power was set to 2.1W on the software Minijamager 2.4.1 and the cutting was varied through the laser speed percentage and number of passes in the same software. Stereomicroscopy of samples in this study that were laser cut with A-B) 6 cm.s⁻¹, C) 4.5 cm.s⁻¹ and D-E) 3 cm.s⁻¹ indicate only partially laser cut samples, when the cutting speed was too rapid or overly melted edges when the speed was too low. F) shows the ideal cutting outcome with a more rapid speed of 6 cm.s⁻¹ but with four circular passes to produce the scaffolds used in this study.
Figure S3: Scanning electron microscopy image of the melt electrowritten PCL reinforcing frames used in this study.
### Table S1: Cyclic compression tests of MEW-frame, Matrigel and MEW-frame + Matrigel

|                      | MEW-frame | Matrigel | MEW-frame + Matrigel |
|----------------------|-----------|----------|----------------------|
| **Maximum values**   | n         | 1st cycle| 2nd cycle | 3rd cycle | 1st cycle | 2nd cycle | 3rd cycle | 1st cycle | 2nd cycle | 3rd cycle | 1st cycle | 2nd cycle | 3rd cycle | 1st cycle | 2nd cycle | 3rd cycle |
| **of Cyclic**        |           | -1.373 ± 0.086 | -0.4209 ± 0.119 | -0.5796 ± 0.315 | -1.3492 ± 0.064 | -0.2159 ± 0.045 | -0.3824 ± 0.151 | -1.3484 ± 0.044 | -0.2249 ± 0.051 | -0.3091 ± 0.106 |
| **Compression tests**|           | 0.8929 ns | 0.0319 * | 0.0655 | 0.9284 ns | 0.0394 * | * | 0.0106 |
| **1st cycle vs 2nd cycle** |           | - | <0.0000 *** | <0.0000 *** | - | <0.0000 *** | <0.0000 *** | - | <0.0000 *** | <0.0000 *** |
| **1st cycle vs 3rd cycle** |           | - | <0.0000 *** | <0.0000 *** | - | <0.0000 *** | <0.0000 *** | - | <0.0000 *** | <0.0000 *** |
| **x vs MEW-frame**   |           | - | <0.0000 *** | <0.0000 *** | - | <0.0000 *** | <0.0000 *** | - | <0.0000 *** | <0.0000 *** |

Significance values: *p<0.05; ****p<0.0001, ns = not significant; n = number of measured constructs.
|                  | DIV3 | DIV7 | DIV10 | DIV14 | DIV17 | DIV21 |
|------------------|------|------|-------|-------|-------|-------|
| **Whole cell**   |      |      |       |       |       |       |
| 2D n             | 100  | 100  | 81    | 100   | 80    | 79    |
| 3D n             | 17   | 30   | 39    | 40    | 40    | 40    |
| VGAT 2D mean     | 53.2±3.1 | 60±2 | 47±2.1 | 46.7±2.2 | 45.2±2.1 | 47.3±2.1 |
| ± SEM            | 17   | 30   | 39    | 40    | 40    | 40    |
| 3D mean          | 47.1±4.1 | 45.9±4.9 | 48.3±7 | 48.2±3.7 | 49.2±3.8 | 53.8±3.7 |
| ± SEM            | 17   | 30   | 39    | 40    | 40    | 40    |
| 2D vs 3D p       | 0.4322 | 0.0037 | 0.7764 | 0.7237 | 0.3159 | 0.1031 |
| DIVx vs DIV21    |      |      |       |       |       |       |
| 2D p             | 0.2670 | 0.0021 | 0.9998 | <0.0001 | 0.9585 | - |
| 3D p             | 0.7809 | 0.4386 | 0.6916 | 0.7224 | 0.8488 | - |
| Whole cell 2D n  | 180  | 200  | 181   | 200   | 160   | 158   |
| 3D n             | 47   | 50   | 79    | 80    | 80    | 80    |
| gephrin 2D mean  | 63.5±2.1 | 65.6±1.4 | 62.8±1.2 | 62±1.6 | 60.6±1.5 | 63.6±1.8 |
| ± SEM            | 17   | 30   | 39    | 40    | 40    | 40    |
| 3D mean          | 39.5±3.7 | 56±4.2 | 57±2.4 | 58.3±2.5 | 55.6±3.2 | 63.2±2.5 |
| ± SEM            | 17   | 30   | 39    | 40    | 40    | 40    |
| 2D vs 3D p       | <0.0001 | 0.0057 | 0.0217 | 0.2070 | 0.1111 | 0.9137 |
| DIVx vs DIV21    |      |      |       |       |       |       |
| 2D p             | >0.9999 | 0.8228 | 0.9981 | 0.9457 | 0.6023 | - |
| 3D p             | <0.0001 | 0.3594 | 0.3743 | 0.6142 | 0.1942 | - |
| Whole cell 2D n  | 100  | 100  | 81    | 100   | 80    | 80    |
| 3D n             | 17   | 30   | 39    | 40    | 40    | 40    |
| GlyR 2D mean     | 42.17±3.4 | 60±3.1 | 49±3  | 39.6±2.6 | 61.2±4.1 | 69.2±3.8 |
| ± SEM            | 17   | 30   | 39    | 40    | 40    | 40    |
| 3D mean          | 4.2±1.3 | 25.5±5.5 | 50±4  | 55.4±3.6 | 55.2±2.9 | 52.8±4.1 |
| ± SEM            | 17   | 30   | 39    | 40    | 40    | 40    |
| 2D vs 3D p       | <0.0001 | <0.0000 | 0.8310 | 0.0011 | 0.3272 | 0.0080 |
| DIVx vs DIV21    |      |      |       |       |       |       |
| 2D p             | <0.0001 | 0.1580 | 0.0003 | <0.0001 | 0.3462 | - |
| 3D p             | <0.0001 | 0.9810 | 0.9857 | 0.9905 | - |

Significance values: *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001, ns = not significant; n = number of counted dendrites
### Table S3: Intracellular colocalization of VGAT versus GlyR and gephyrin versus GlyR

|                | DIV3 | DIV7 | DIV10 | DIV14 | DIV17 | DIV21 |
|----------------|------|------|-------|-------|-------|-------|
| **Whole Cell Co-localization** |      |      |       |       |       |       |
| VGAT vs GlyR   |      |      |       |       |       |       |
| 2D n           | 100  | 30   | 39    | 100   | 80    | 80    |
| 3D n           | 17   | 30   | 39    | 40    | 40    | 40    |
| 2D mean ± SEM  | 14.24±1.9 | 28.9±2.2 | 24.5±2.3 | 20.2±1.9 | 31.7±2.8 | 36.3±2.5 |
| 3D mean ± SEM  | 1.3±0.7  | 16.1±3.9  | 29.6±2.8  | 32.6±3.8  | 31.9±2.7  | 35.1±3.1  |
| 2D vs 3D       | p    | <0.0069 | 0.0056 | 0.1902 | 0.0016 | 0.9625 | 0.7741 |
|                 | **   | **    | ns    | ns    | ns    | ns    |
| DIVx vs DIV21  |      |      |       |       |       |       |
| 2D p           | <0.0001 | 0.0882 | 0.0025 | <0.0001 | 0.5129 | -     |
| 3D p           | <0.0001 | 0.003  | 0.6011 | 0.9728 | 0.9275 | -     |
|                | ****  | ns    | **    | ****  | ns    | ns    |
|                | ****  | ***   | ns    | ns    | ns    | ns    |
| **Whole cell Co-localization** |      |      |       |       |       |       |
| gephyrin vs GlyR|      |      |       |       |       |       |
| 2D n           | 100  | 100  | 81    | 100   | 80    | 80    |
| 3D n           | 17   | 30   | 39    | 40    | 40    | 40    |
| 2D mean ± SEM  | 22.2±2.3 | 31.6±2.4 | 28.6±2.1 | 24.6±1.9 | 30.9±2.9 | 35.3±2.7 |
| 3D mean ± SEM  | 0.25±0.2  | 15±3.2  | 31.6±3.3  | 39.3±3.3  | 35.7±3.5  | 37.9±4.1  |
| 2D vs 3D       | p    | <0.0001 | 0.0006 | 0.4328 | <0.0001 | 0.3226 | 0.5959 |
|                 | ****  | ***   | ns    | ****  | ns    | ns    |
| DIVx vs DIV21  |      |      |       |       |       |       |
| 2D p           | 0.0005 | 0.6875 | 0.1948 | 0.0066 | 0.5885 | -     |
| 3D p           | <0.0001 | <0.0001 | 0.5515 | 0.9981 | 0.9898 | -     |
|                | ****  | ****  | ns    | ns    | ns    | ns    |

Significance values: **p<0.01, ***p<0.001; ****p<0.0001, ns = not significant; n = number of counted dendrites.
|                  | DIV3   | DIV7   | DIV10  | DIV14  | DIV17  | DIV21  |
|------------------|--------|--------|--------|--------|--------|--------|
| **Surface GlyR** |        |        |        |        |        |        |
| 2D n             | 80     | 100    | 100    | 100    | 80     | 78     |
| 3D n             | 30     | 30     | 40     | 40     | 40     | 40     |
| 2D mean ± SEM    | 14.5±2.4 | 18.6±2.3 | 24.1±1.9 | 39.3±2.8 | 56.2±3.3 | 50.7±2.9 |
| 3D mean ± SEM    | 14±3.5  | 14±3.5 | 43.1±4.1 | 60.8±3.2 | 61.7±3.2 | 62.3±3.8 |
| **2D vs 3D**     |        |        |        |        |        |        |
| p                | 0.9148 | 0.3089 | <0.0001 | <0.0001 | 0.2857 | 0.0207 |
| ns               | ns     | ns     | ****    | ****    | ns     | ns     |
| **DIVx vs DIV21**|        |        |        |        |        |        |
| 2D p             | <0.0001 | <0.0001 | <0.0001 | 0.0092 | 0.482 | - |
| ****            | **** | **** | **** | ns | ns |
| 3D p             | <0.0001 | <0.0001 | 0.0005 | 0.9978 | 0.9998 | - |
| ****            | **** | **** | *** | ns | ns |
| **Surface Co-localization** |        |        |        |        |        |        |
| gephrin vs GlyR  |        |        |        |        |        |        |
| 2D n             | 80     | 100    | 99     | 100    | 80     | 78     |
| 3D n             | 30     | 20     | 40     | 40     | 40     | 40     |
| 2D mean ± SEM    | 7.6±1.5 | 9.7±1.3 | 10.5±1.1 | 23.8±2.1 | 34.8±2.5 | 29.5±2.4 |
| 3D mean ± SEM    | 8.3±2.8 | 17.6±2.5 | 28.4±3.2 | 45.2±3.2 | 45.2±4.6 | 52.5±3.9 |
| **2D vs 3D**     |        |        |        |        |        |        |
| p                | 0.8043 | 0.0125 | <0.0001 | <0.0001 | 0.0324 | <0.0001 |
| ns               | *      | ****   | ****    | ****    | *      | ****   |
| **DIVx vs DIV21**|        |        |        |        |        |        |
| 2D p             | <0.0001 | p<0.0001 | <0.0001 | 0.1149 | 0.1867 | - |
| ****            | **** | **** | **** | ns | ns |
| 3D p             | <0.0001 | <0.0001 | 0.4497 | 0.4548 | - |
| ****            | **** | **** | **** | ns | ns |

Significance values: *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001, ns = not significant; n = number of counted dendrites.
Table S5: Cyclic compression tests of spinal cord-MEW-frame-Matrigel constructs at DIVs 0, 3, 7, 10, and 14

|                      | DIV0      | DIV3      | DIV7      | DIV10     | DIV14     |
|----------------------|-----------|-----------|-----------|-----------|-----------|
| Maximum values       | n         |           |           |           |           |
| of Cyclic            |           |           |           |           |           |
| Compression tests    |           |           |           |           |           |
| 1st cycle            | -0.6790 ± 0.236 | -0.7679 ± 0.3 | -1.039 ± 0.281 | -0.8318 ± 0.291 | -0.8328 ± 0.136 |
| DIVx vs DIV0         | p         |           |           |           |           |
|                      | -         | 0.9384    | 0.0258    | 0.6801    | 0.6967    |
|                      | -         | ns        | *         | ns        | ns        |

Significance values: *p<0.05; ns = not significant; n = number of measured constructs.
Supporting Video 1 Caption: High resolution SEM image of the PCL MEW reinforcement frames, with 9.05 ± 0.2µm diameter individual fibers and 200µm spacing in a grid pattern. Distortions at the edges of the scaffold at low magnification are due to electron microscope aberrations.

Supporting Video 2 Caption: 3D reconstruction of immunocytochemical z-stack of a DIV14 composite shown in Figure 7a. Proteins marked are VGAT (cyan), GlyR(magenta), Nuclei (DAPI), MEW-scaffold shows an autofluorescence in the DAPI-channel.