SUPPLEMENTARY INFORMATION

Dual-function injectable angiogenic biomaterial for the repair of brain tissue following stroke

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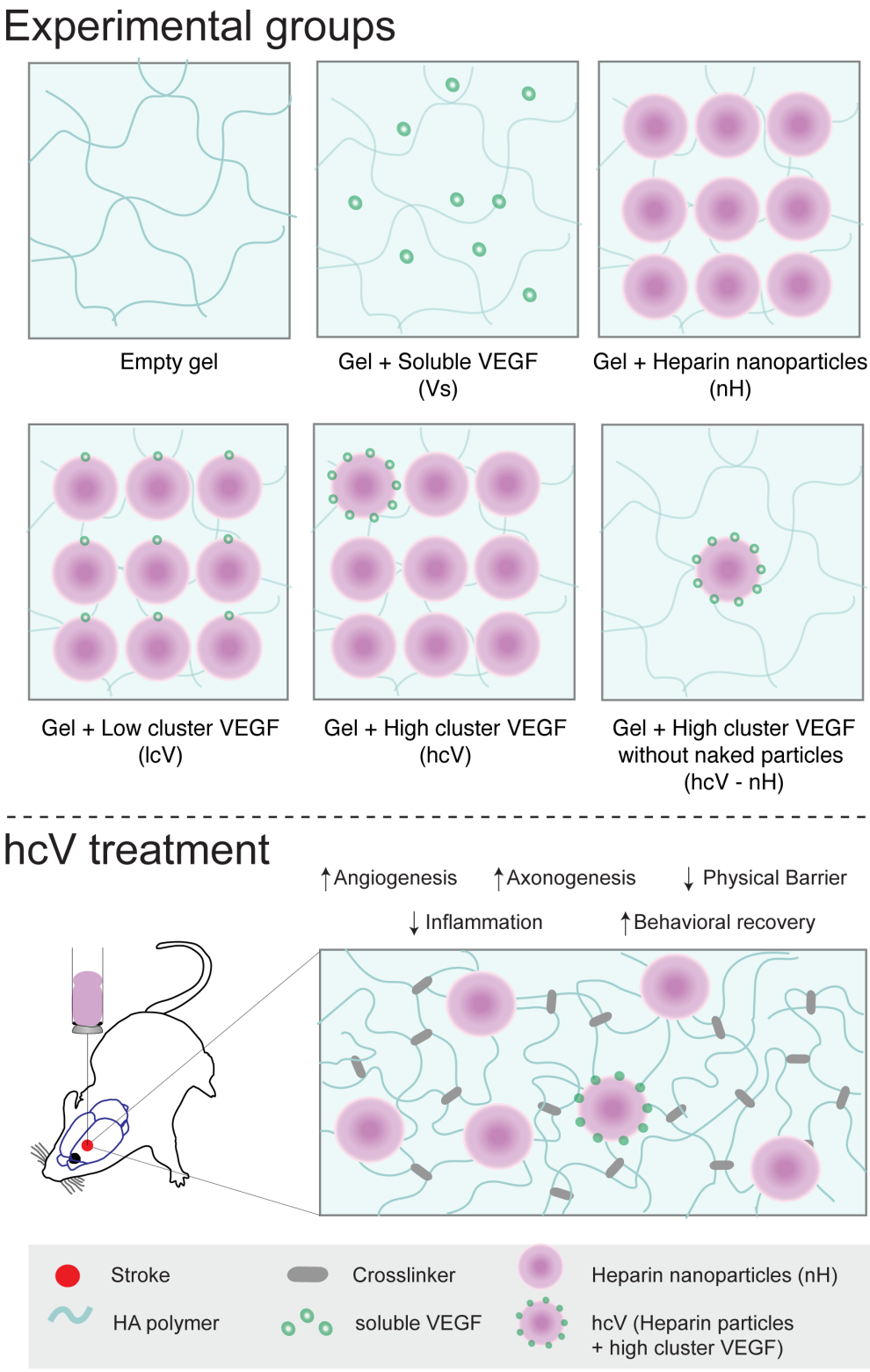
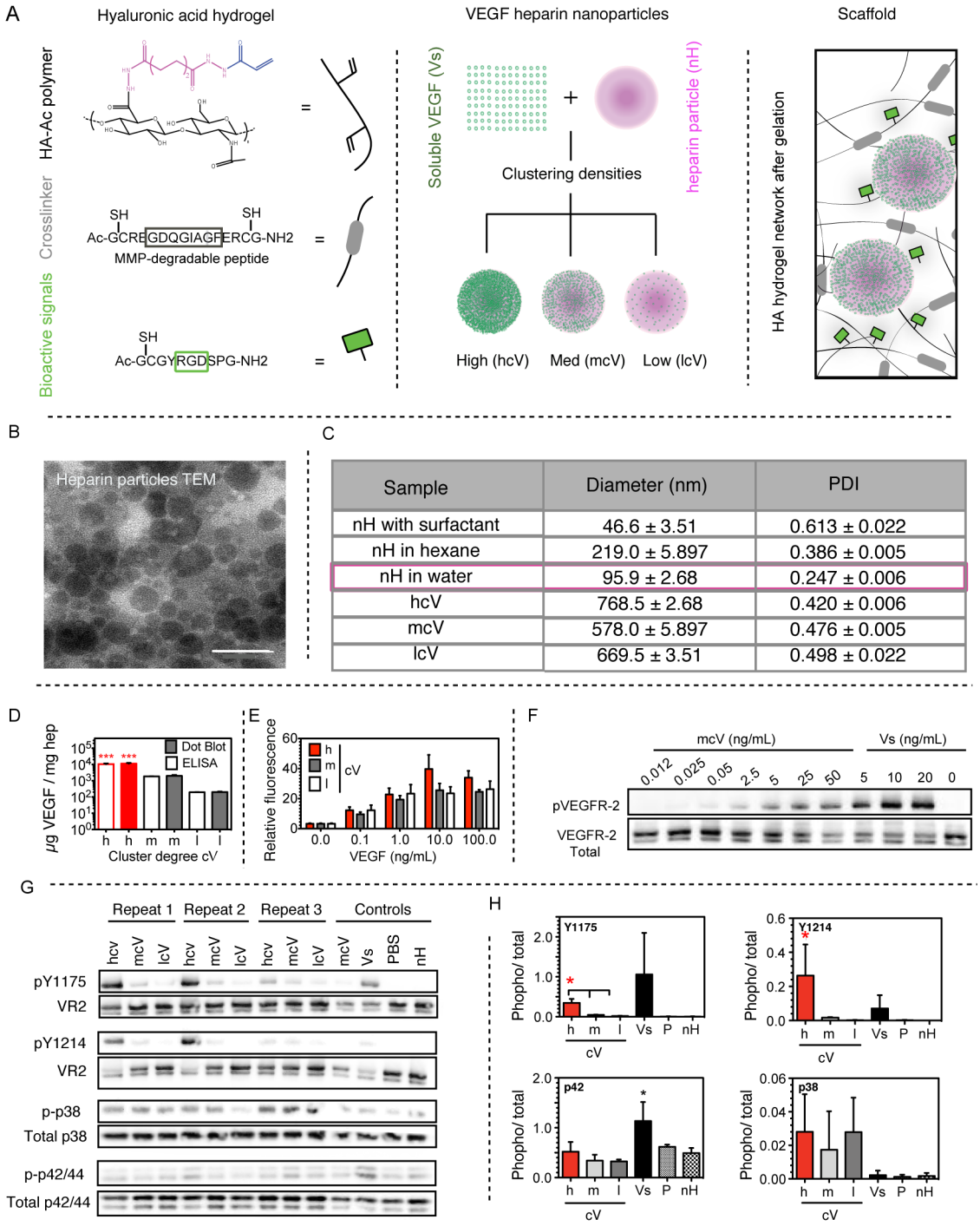
Supplementary figures and captions



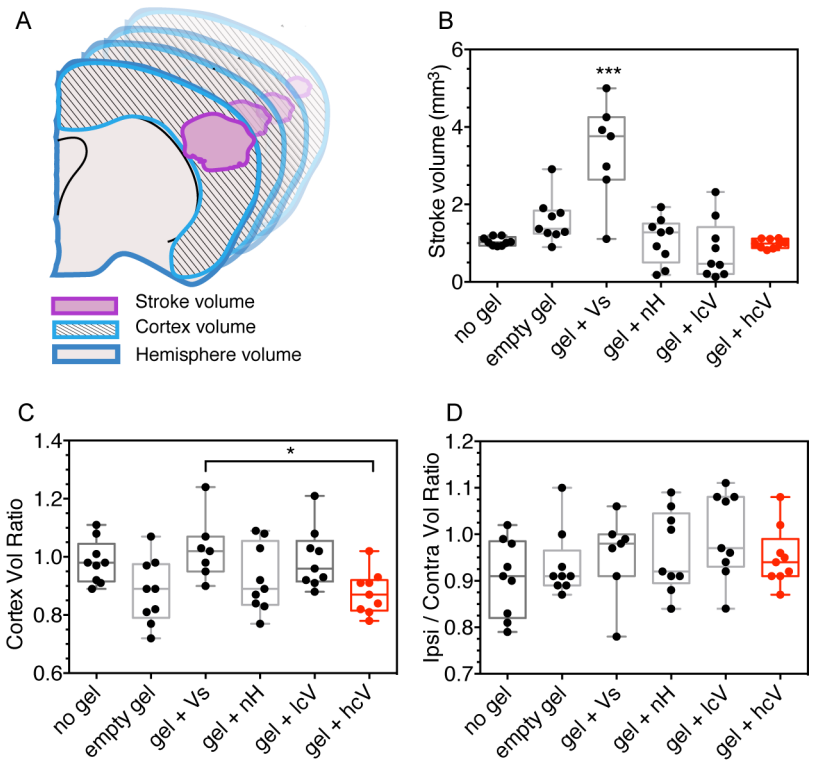
Sup Fig 1: Therapeutic angiogenic hydrogel concept

This study aims at designing and testing a pro-angiogenic therapy for stroke through a tissue engineering approach where the precise 3D presentation of VEGF to its receptor is controlled through its binding onto a nanoparticle's surface and its packing density onto the particle. For this, we designed an injectable hyaluronic acid (HA) hydrogel encapsulating highly clustered VEGF (hcV) onto nanoparticles of heparin and compared it directly to a condition of a low packing density, or low cluster of VEGF (lcV) where the total amount of heparin and VEGF was kept equal. In order to determine the effect of soluble VEGF (Vs), heparin nanoparticles only (nH), or the hydrogel (Empty gel) components of the hcV treatment, each of these conditions was tested, along with a stroke only (no treatment) as a negative control (For, the composition of each treatment, see Supplementary note 1).

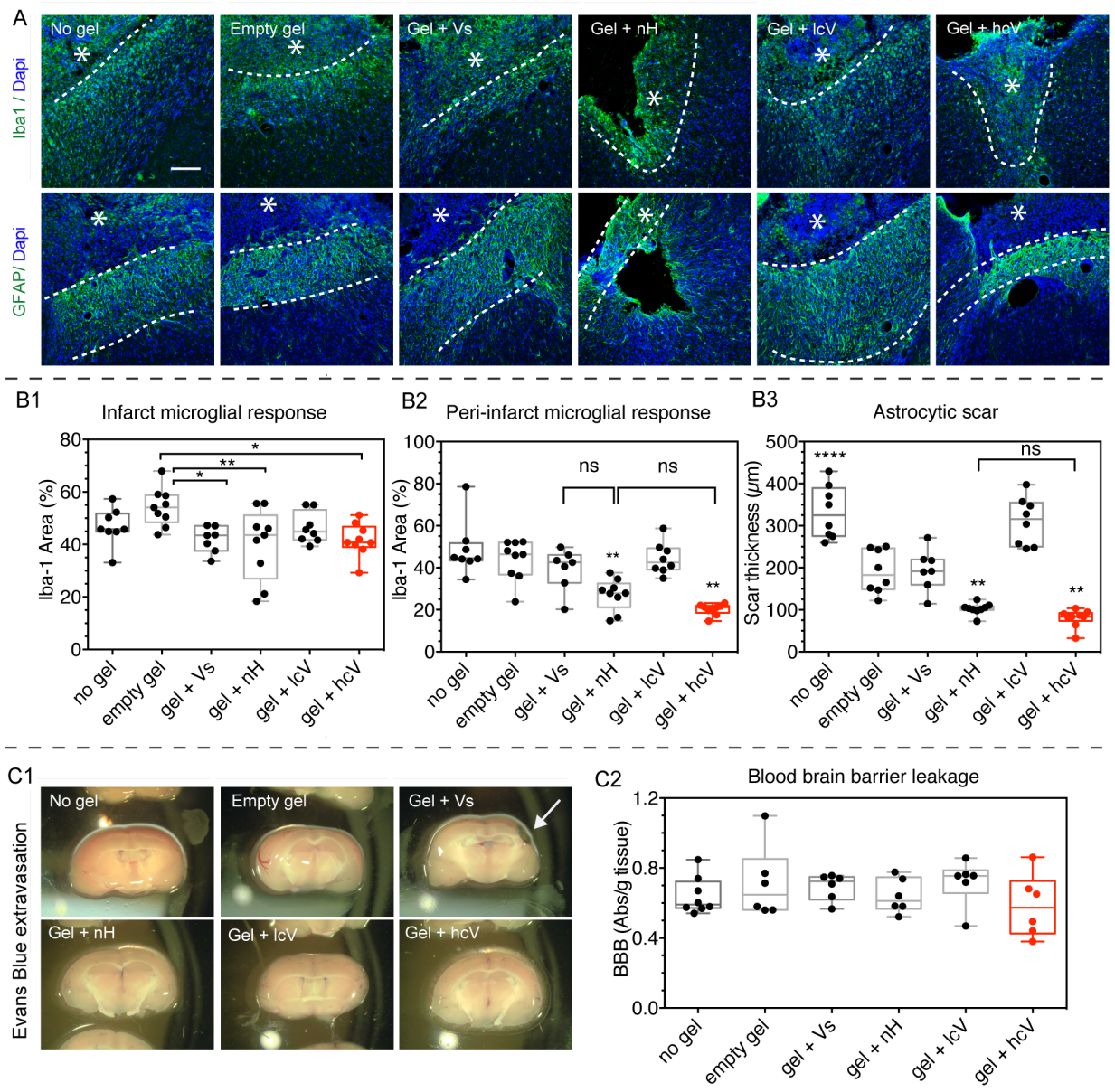


Sample	Diameter (nm)	PDI
nH with surfactant	46.6 ± 3.51	0.613 ± 0.022
nH in hexane	219.0 ± 5.897	0.386 ± 0.005
nH in water	95.9 ± 2.68	0.247 ± 0.006
hcV	768.5 ± 2.68	0.420 ± 0.006
mcV	578.0 ± 5.897	0.476 ± 0.005
lcV	669.5 ± 3.51	0.498 ± 0.022

Sup Fig 2: Engineered hydrogel and heparin particles. (A) Schematic illustration of the hydrogel scaffold made of injectable acrylated hyaluronic acid (HA), MMP degradable motifs and adhesion peptides, as well as heparin nanoparticles and the different VEGF clustering densities. Different clustering densities of VEGF were created by mixing the same amount of VEGF with different amounts of heparin particles, leading to a low (lcV), medium (mcV) and high cluster density (hcV) of the growth factor onto the particle's surface. (B) Characterization of the particles by Transmission electron microscopy (TEM) and (C) Dynamic light scattering (DLS) (95.9 nm, and a PDI of 0.268). The size of nanoparticles was measured after each step of the formation (surfactants, hexane then water) showing an increase in diameter after attachment with VEGF in the 3 cluster conditions. Values represent average values obtained with 3 independent measurements, with standard deviation. (D) Two methods were used to determine the VEGF content in the heparin particles, a direct method that measures the amount of VEGF on the nanoparticle surface using dot blots and an indirect method that measures the amount of VEGF not bound to nH during synthesis using ELISA. The two methods showed the same VEGF concentration per weight of heparin for each of the clustering densities: 20 mg, 2 mg and 200 µg VEGF/mg heparin for high, medium and low cluster, respectively. (E) To ensure that the process of VEGF clustering did not impact the activity of VEGF, the ability of VEGF clusters to enhance endothelial cell (EC) proliferation and (F,G,H) to induce VEGF-receptor-2 (VEGFR-2) phosphorylation on the Y1175 and Y1114 sites and the MAP-Kinase protein p38 and p-42/44 were tested. Data represent the average ± SEM obtained from three independent experiments, and P values were determined by One-way Anova, Tukey's post-hoc test, with *** indicating $P < 0.001$.

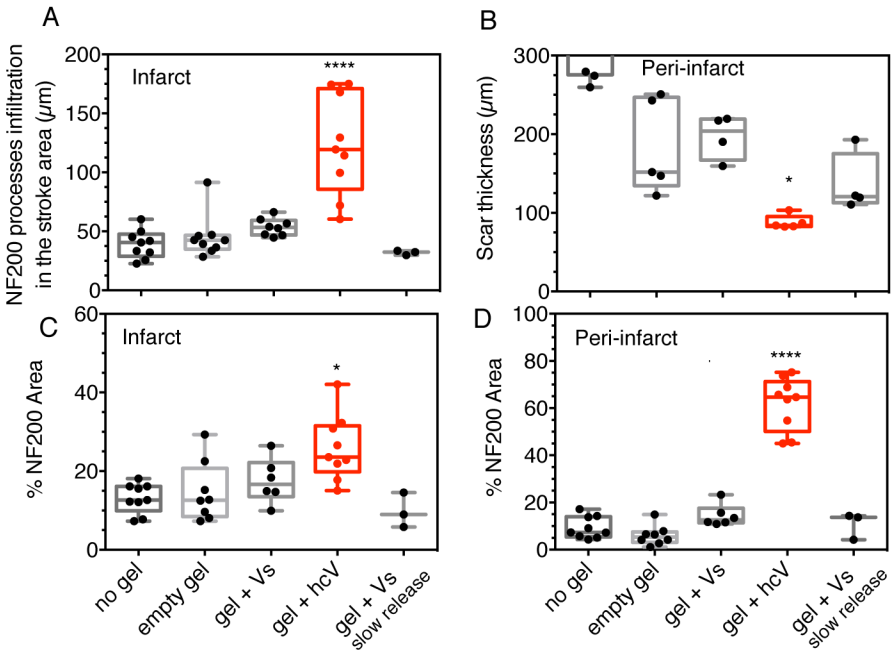


Sup Fig 3: Evaluation of infarct size. (A) Schematic illustration of the ipsilateral hemisphere of a mouse brain. (B) Quantification of the stroke, (C) Cortex and (D) hemisphere at day 10 after gel injection. No gel = stroke only condition, empty gel = HA hydrogel, gel + Vs = HA hydrogel loaded with 200ng of soluble VEGF, gel + nH = HA hydrogel with 1µg heparin nanoparticles (nH), gel + lcV = HA hydrogel with 1µg nH loaded with 200 ng VEGF, gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH. Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test with * and *** indicating $P < 0.05$ and $P < 0.001$, respectively.

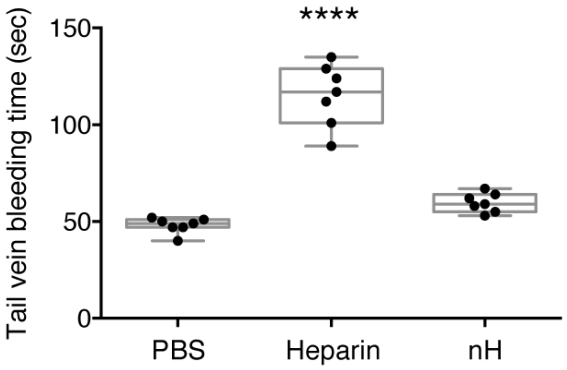


Sup Fig 4: Post-stroke inflammatory response. (A) Fluorescent images of microglial cells (Iba-1) and astrocytes (GFAP) at the stroke site (*) at day 10 after gel transplantation. (B1) Quantification of the infarct and (B2) peri-infarct positive area for Iba-1 and (B3) the GFAP-labeled astrocytic scar thickness. (C1) Images of brain sections showing Evans blue extravasation in the stroke site (white arrow) after intravenous injection of the dye, and (C2) quantification by spectrophotometry of the amount of Evans blue measured per g of brain tissue. No gel = stroke only condition, empty gel = HA hydrogel, gel + Vs = HA hydrogel loaded with 200ng of soluble VEGF, gel + nH = HA hydrogel with 1µg heparin nanoparticles (nH), gel + lcV = HA hydrogel with 1µg nH loaded with 200 ng VEGF, gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH. Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way

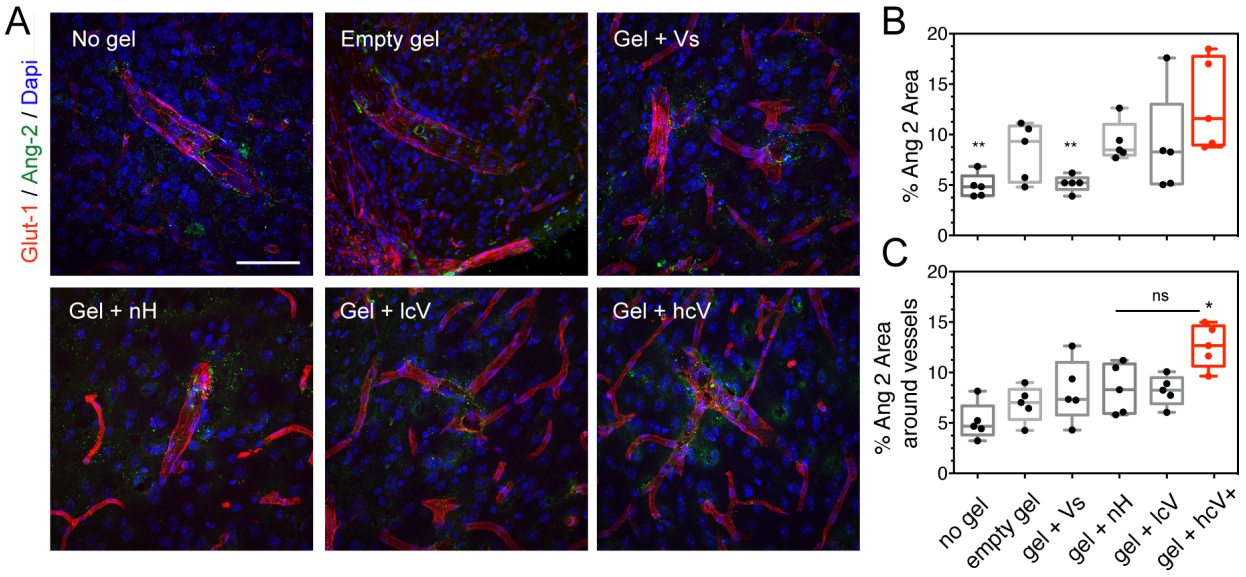
ANOVA with a Tukey's post-hoc test, with * and ** indicating $P < 0.05$ and $P < 0.01$, respectively. Scale bar: 100 µm.



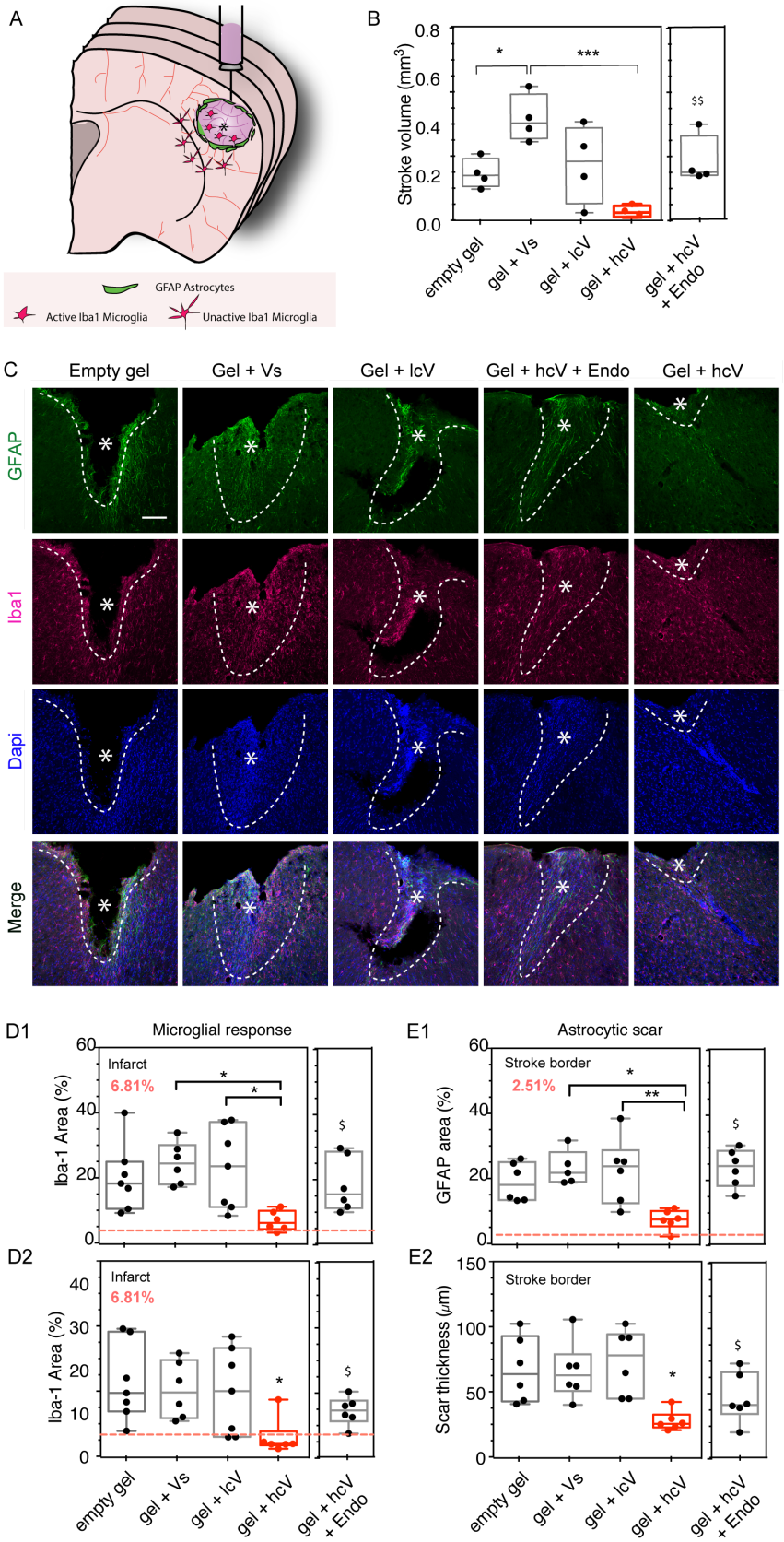
Sup Fig 5: VEGF slow release. (A) Quantification of the NF200 processes infiltration distance and the positive area for NF200 in the stroke site 2 weeks after gel injection. (B) Quantification of the peri-infarct scar thickness and positive area for NF200 around the stroke site. In this set of data, a supplemental group of mice were injected a slow delivery of soluble VEGF and compared with the other VEGF-injected conditions. No gel = stroke only condition, empty gel = HA hydrogel, gel + Vs = HA hydrogel loaded with 200ng of soluble VEGF, gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH, gel + Vs slow release = HA hydrogel loaded with 200ng of soluble VEGF slowly released from the gel. Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with * and **** indicating $P < 0.05$ and $P < 0.0001$, respectively.



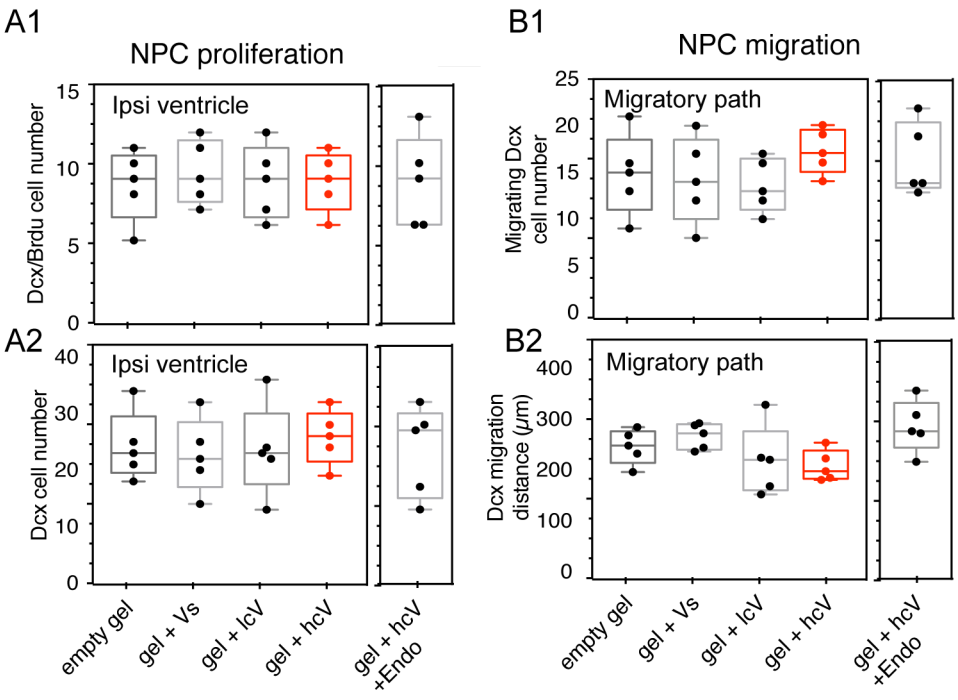
Sup Fig 6: Tail vein bleeding assay. Measurement of the tail vein bleeding time (sec) in mice injected intravenously with saline (PBS), 2μg of heparin or heparin nanoparticles (nH). Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with **** indicating $P < 0.0001$.



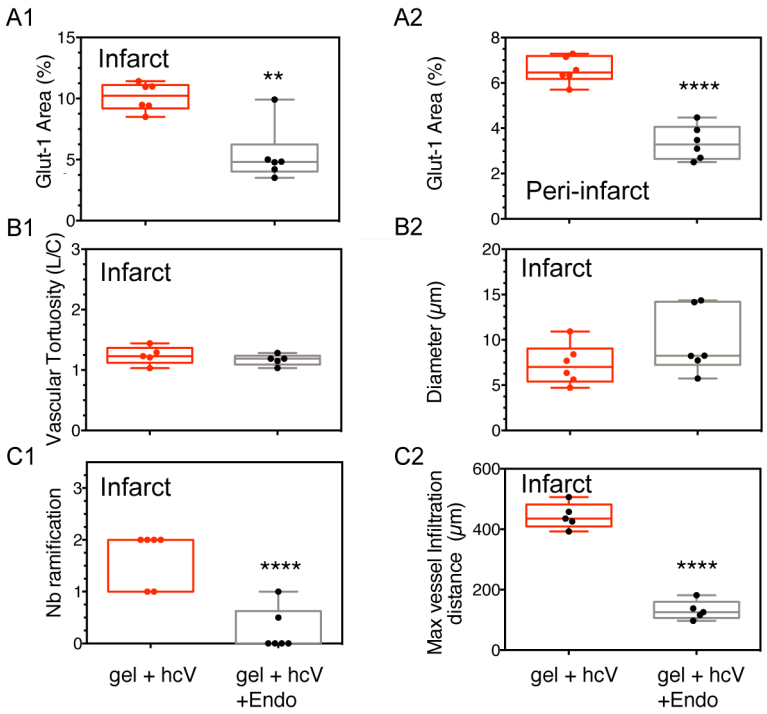
Sup Fig 7: Angiopoietin-2 secretion. (A) Fluorescent images of vessels (Glut-1, red), Angiopoietin-2 (green) and Dapi (Blue) in the peri-infarct area in the different conditions 2 weeks after stroke. (B) Quantification of the positive area for Angiopoetin-2 in the peri-infarct area and (C) around vessels (10 µm distance from vessels). No gel = stroke only condition, empty gel = HA hydrogel, gel + Vs = HA hydrogel loaded with 200ng of soluble VEGF, gel + nH = HA hydrogel with 1 µg unloaded nH, gel + lcV = HA hydrogel with 1µg nH loaded with 200 ng VEGF, gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH, Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with * and ** indicating $P < 0.05$ and $P < 0.01$, respectively. Scale bar: 50 µm.


A
GFAP Astrocytes
Active Iba1 Microglia
Unactive Iba1 Microglia
B
Stroke volume (mm3)
0.8
0.6
0.4
0.2
0.0
*
***
$$
empty gel
gel + Vs
gel + lcV
gel + hcV
gel + hcV + Endo
C
Empty gel
Gel + Vs
Gel + lcV
Gel + hcV + Endo
Gel + hcV
GFAP
Iba1
Dapi
Merge
D1
Microglial response
Iba-1 Area (%)
Infarct
6.81%
60
40
20
0
*
*
$
D2
Iba-1 Area (%)
Infarct
6.81%
40
30
20
10
0
*
$
E1
Astrocytic scar
GFAP area (%)
Stroke border
2.51%
60
40
20
0
*
**
$
E2
Scar thickness (μm)
Stroke border
150
100
50
0
*
$
empty gel
gel + Vs
gel + lcV
gel + hcV
gel + hcV + Endo

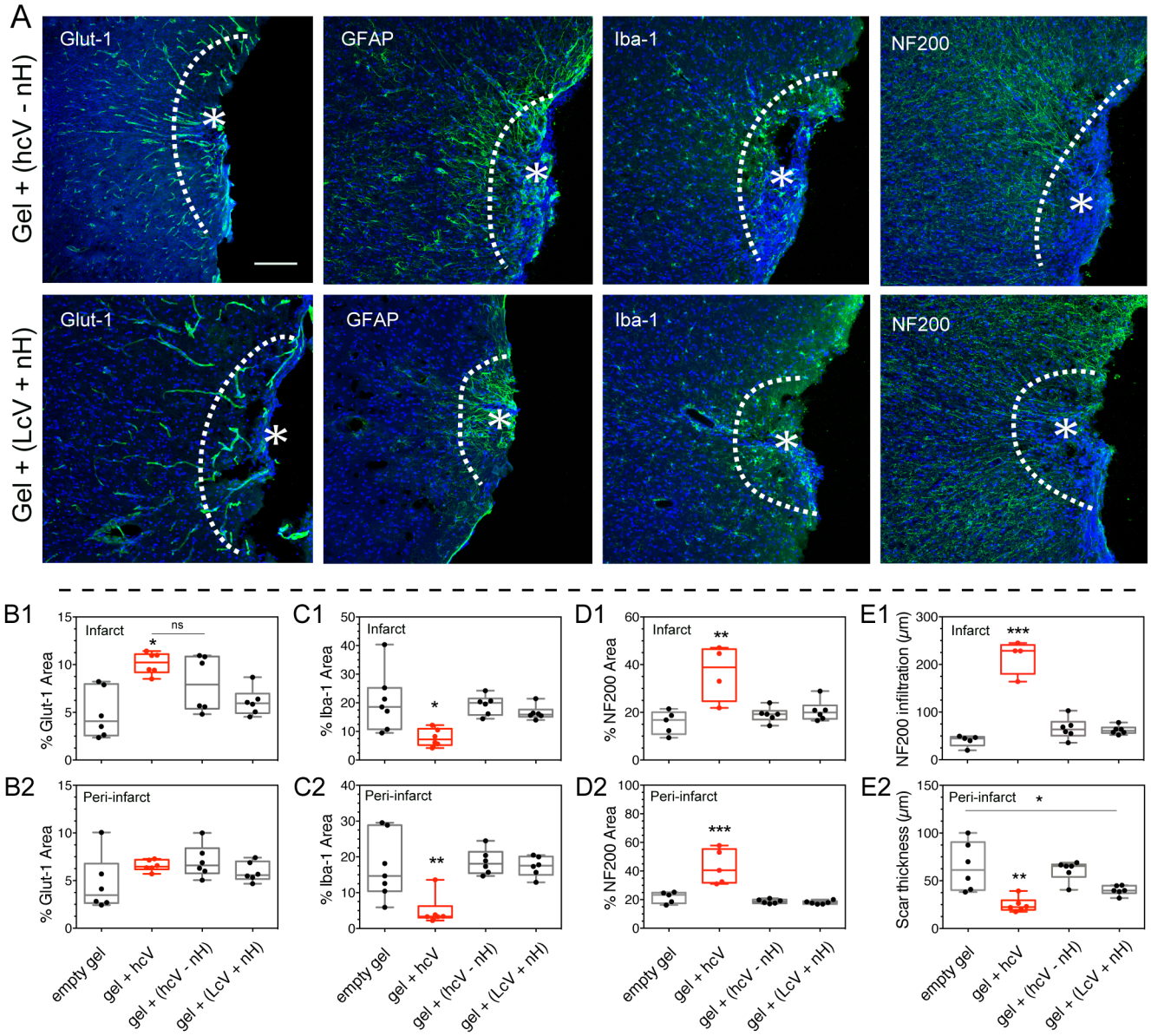
Sup Fig 8: Long-term post-stroke inflammatory response. (A) Schematic illustration of the ipsilateral hemisphere of a mouse brain, showing the injection of the hydrogel through a needle directly into the stroke site (*) where both astrocytes and microglial cells in and around the lesion are represented. (B) Quantification of the stroke volume 16 weeks after gel transplantation. (C) Fluorescent images of astrocytes (GFAP, green) and microglial cells (Iba1, red) in and around the stroke site (*) in the different conditions. (D1) Quantification of the infarct and (D2) peri-infarct positive area for Iba-1. (E1) Quantification of the astrocytic area and (E2) thickness. The average value obtained for the contralateral side of the same mice was added as a positive control (average shown on the left upper corner and the dotted line). Empty gel = HA hydrogel, gel + Vs = HA hydrogel loaded with 200ng of soluble VEGF, gel + lcV = HA hydrogel with 1µg nH loaded with 200 ng VEGF, gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH, gel + hcV + Endo = Gel + hcV + i.p administration of endostatin from day 5 to 15 after stroke. Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with **** indicating $P < 0.0001$, respectively. Scale bar: 100 µm. P values between gel + hcV and gel + hcV + endostatin were determined by t test, with $ indicating $P < 0.05$.



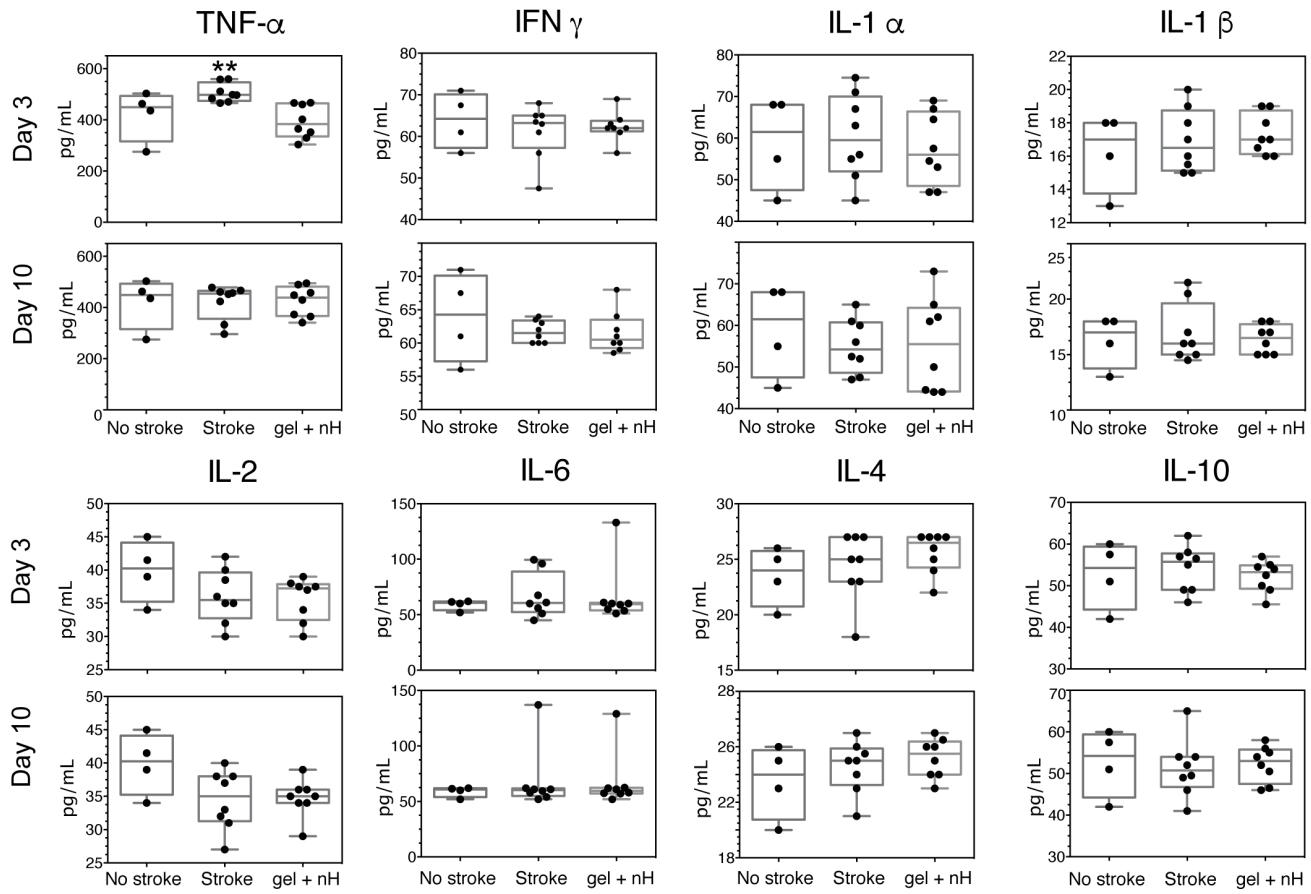
Sup Fig 9: Long-term neurogenesis. (A1) Quantification of the total number of proliferating neuroblasts and (A2) total number of neuroblasts on the ipsilateral ventricle, and (B1) number of cells migrating an (B2) distance of migration at 16 weeks after stroke. Empty gel = HA hydrogel, gel + Vs = HA hydrogel loaded with 200ng of soluble VEGF, gel + lcV = HA hydrogel with 1µg nH loaded with 200 ng VEGF, gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH. Data is presented using a min to max box plot, gel + hcV + Endo = gel + hcv + i.p administration ef endostatin from day 5 to 15 after stroke. Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test.



Sup Fig 10: Long-term effect of endostatin on vessel post-stroke vessel formation. (A1) Quantification of the vascular area in the infarct and (A2) peri-infarct area, Quantification of the vessel morphology: vessel tortuosity (B1) and diameter (B2), Quantification of vessel growth with number of ramifications (C1) and maximum infiltration distance of the vessels into the stroke site (C2) 16 weeks after stroke. Gel + hcV = HA hydrogel with 0.01 µg nH loaded with VEGF and 0.99 µg unloaded nH, Gel + hcV + Endo = Gel + hcV + i.p administration of endostatin from day 5 to 15 after stroke. Data is presented using a min to max box plot. Each dot in the plots represent one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with ** and **** indicating $P < 0.01$ and $P < 0.0001$, respectively.


A
Gel + (hcV - nH)
Gel + (LcV + nH)
Glut-1
GFAP
Iba-1
NF200
B1
C1
D1
E1
B2
C2
D2
E2
Infarct
Peri-infarct
% Glut-1 Area
% Iba-1 Area
% NF200 Area
NF200 infiltration (μm)
Scar thickness (μm)
ns
*
**
***
empty gel
gel + hcV
gel + (hcV - nH)
gel + (LcV + nH)

Sup Fig 11: Role of naked heparin particles in the hcV treatment at 16 weeks post-stroke. Since the injected high and low cluster treatments (respectively hcV and lcV) were designed to contain equal amounts of heparin and VEGF, the VEGF clusterization in the hcV treatment was obtained on a low amount of heparin, leaving a high amount of naked particles. In order to understand the contribution of these naked particles in the pro-repair effect of the hcV, two supplemental group were studied: the hcV – nH where the naked particles from the hcV were removed, and the LcV + nH where additional naked particles were added to the low VEGF cluster condition. (A) Fluorescent images of vessels (Glut-1), astrocytic scar (GFAP), microglia (Iba-1) and axonal neurofilaments (NF200) in and around the stroke site (*) of gel + (hcV – nH) and LcV + nH) conditions, 16 weeks post-stroke. Quantitative assessment of the vascular area in the infarct (B1) and the peri-infarct area (B2), microglial area in the infarct (C1) and the peri-infarct area (C2), axonal area in the infarct (D1) and the peri-infarct area (D2), axonal infiltration distance (E1) and astrocytic scar (E2) at 16 weeks post-stroke. hcV – nH = 0.01 µg nH loaded with 200 ng VEGF, lcV = 1µg nH loaded with 200 ng VEGF and 0.99 µg unloaded nH. Data is presented using a min to max box plot. Each dot in the plots represents one animal and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with *, **, and *** indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Data represent the average. Scale bar: 100 µm.



Sup Fig 12: Brain levels of inflammatory cytokines 3 and 10 days after gel transplantation in the no stroke, no gel and gel + nH conditions. Gel + nH = HA hydrogel with 1μg heparin nanoparticles (nH). Data is presented using a min to max box plot. Each dot in the plots represents one brain sample (concentrations in pg/mL) and p values were determined by One-way ANOVA with a Tukey's post-hoc test, with **, indicating $p < 0.01$.

Supplementary Notes 1

The composition of each injected treatment condition is as follows:

- No gel = stroke only condition
- Empty gel = 6 HA μL hydrogel,
- Gel + Vs = 6 HA μL HA hydrogel + 200ng Vs
- Gel + nH = 6 HA μL HA hydrogel + 1μg nH
- Gel + lcV = 6 HA μL HA hydrogel + 1μg nH bound to 200 ng VEGF,
- Gel + hcV = 6 HA μL HA hydrogel + 0.01 μg nH bound to 200 ng VEGF + 0.99 μg naked nH.

The brain injection of hcV in stroke mice promoted the formation of a reparative niche with the generation of a vascularized network of regenerated functional neuronal connections in the stroke lesion. These results were also associated with a reduction of the post-stroke glial inflammation and an improved behavioral recovery compared with all the other conditions. It is important to note that the injected high and low cluster treatments (respectively hcV and lcV) were designed to contain equal amounts of heparin and VEGF. Therefore, the VEGF distribution in the hcV treatment is reduced to a low amount of heparin in the hcV, leaving a high amount of naked particles. We showed that the injection of heparin particles only (nH) reduced inflammation in the stroke brain at both short and long term. In order to understand the contribution of these naked particles in the pro-repair effect of the hcV, an additional hcV group where the naked particles were removed (hcV-nH) was tested. The results showed that the hcV - nH treatment did improve vessel formation in the stroke site; however, this angiogenesis was not followed by the formation of axons, and was not associated with a reduced glial inflammation.

Supplementary Notes 2

The other cytokines tested showed no significant difference between groups, however. The technical challenges of dissecting the injected gel without the surrounding peri-infarct tissue may have hindered the quality of the harvested samples. In addition, the presence of the hydrogel itself may have increased the background in the injected brains, reducing the sensitivity of cytokines detection. A perfected and customized protocol may be able detect significant differences of the other inflammatory cytokines. Nevertheless, our data is consistent with previous reports. Qiu et al reported a protective effect of low molecular weight heparin in a rat model of pancreatic encephalopathy by reducing significantly serum levels of TNF-α brain water content, neuronal, apoptosis and edema[46]. Similarly, Babazada et al reported that self-assembling glycol-split heparin nanoparticles blocks the production of TNF- α from lipopolysaccharide (LPS)-stimulated macrophages and dendritic cells in an experimental arthritis model in vitro[47]. They suggest that the anti-inflammatory effect is mediated through a selective inhibition of TLR4-mediated NFκB signaling pathway. Recently, Yazeji et al described a similar effect of low molecular weight heparin –based nanoparticles in an experimental ulcerative colitis model[48].