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Endothelial cells promote excitatory synaptogenesis and improve ischemia-induced motor deficits in neonatal mice

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ABSTRACT

Brain microvascular endothelial cells (BMEC) are highly complex regulatory cells that communicate with other cells in the neurovascular unit. Cerebral ischemic injury is known to produce detectable synaptic dysfunction. This study aims to investigate whether endothelial cells in the brain regulate postnatal synaptic development and to elucidate their role in functional recovery after ischemia. Here, we found that *in vivo* engraftment of endothelial cells increased synaptic puncta and excitatory postsynaptic currents in layers 2/3 of the motor cortex. This pro-synaptogenic effect was blocked by the depletion of VEGF in the grafted BMEC. The *in vitro* results showed that BMEC conditioned medium enhanced spine and synapse formation but conditioned medium without VEGF had no such effects. Moreover, under pathological conditions, transplanted endothelial cells were capable of enhancing angiogenesis and synaptogenesis and improved motor function in the ischemic injury model. Collectively, our findings suggest that endothelial cells promote excitatory synaptogenesis *via* the paracrine factor VEGF during postnatal development and exert repair functions in hypoxia-ischemic neonatal mice. This study highlights the importance of the endothelium-neuron interaction not only in regulating neuronal development but also in maintaining healthy brain function.

1. Introduction

As the brain develops, neurons elaborate axons and dendrites that connect at synapses to form a functional network. The maintenance of normal brain function depends on this precise neural network. Synaptic connections between neurons actively change in structure and function throughout life (Fu and Zuo, 2011). Understanding the molecular and cellular mechanisms underlying the regulation of synaptic connectivity could help identify novel therapeutic targets for the promotion of functional synaptic recovery in neurological diseases.

In recent years, cell-cell interactions in the brain have been extensively investigated. It has been obvious that other brain cells can influence synapse formation besides neurons. Accumulating evidence demonstrates that neuronal-glial cell interactions may regulate the formation and remodeling of synapses and circuits (Kettenmann et al., 2013; Pfrieger and Barres, 1997; Stogsdill and Eroglu, 2017; Ullian et al., 2001). Like glial cells, brain microvascular endothelial cells (BMEC) are an indispensable part of nervous system networks. The interaction between BMEC and neurons has also received great attention. Endothelial cells have been reported to stimulate the proliferation of neuronal precursors and potentiate the differentiation towards neuronal lineage (Gama Sosa et al., 2007; Shen et al., 2004). Furthermore, endothelial cells can enhance migration of neurons in subependymal zone explants (Leventhal et al., 1999) and accelerate neurite outgrowth of spinal motor neurons (Dugas et al., 2008). However, much less is known about the vascular endothelial regulation of ongoing developmental processes, synapse formation and maturation. We recently found that neuron-endothelial cocultures promoted the formation of synaptic connectivity *in vitro* (Wu et al., 2017). Therefore, it is necessary to investigate the neuron-endothelial interaction in the postnatal brain to determine whether endothelial cells regulate synaptic formation and plasticity during postnatal development.

Cell-based therapy shows great potential for the treatment of ischemic stroke (Zhang and Chopp, 2013). Vascular endothelial cells were previously shown to have neuroprotective and regenerative effects in experimental models of cerebral ischemia. Transplantation of endothelia into ischemic brains reduced the infarct volume and promoted behavioral recovery (Iijima et al., 2015; Moon et al., 2016; Puentes

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et al., 2012). However, the underlying therapeutic mechanisms are unknown. Because synaptic dysfunction and motor function deficits are hallmarks of cerebral ischemic injury, here we tested whether grafted endothelial cells contributed to motor recovery by stimulating synapse formation.

In this study, we established *in vivo* and *in vitro* experimental models to investigate the interaction between neurons and the endothelium. Using immunostaining and whole-cell patch-clamp technique, we analyzed the effects of endothelial cells on angiogenesis, synaptogenesis and neurotransmission. We also established a neonatal ischemic injury model to study the regulatory capacity of endothelial cells on angiogenesis and synaptogenesis as well as motor repair after hypoxia-ischemic (HI) injury. Our results indicate that endothelial cells promote synaptic connectivity and also have important implications for functional recovery after HI injury.

2. Materials and methods

2.1. Animal

All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Advisory Committee at Fudan University. All mice were housed at the Animal Facility of Fudan University under a 12 h light/dark cycle and had access to food and water. All efforts were made to minimize animal suffering and reduce the number of animals used. A total of eighty-two C57BL/6 mice were used in the following experiments. Animals were randomly assigned to the experimental groups, which consisted of animals belonging to both sexes.

2.2. Cell culture

Mouse cortical neurons were prepared as previously described (Wu et al., 2016; Wu et al., 2017). Briefly, cortical neurons were prepared from C57BL/6 E16 mouse embryos. Cerebral cortices were dissected and digested with trypsin. Following digestion, the precipitate was re-suspended and the isolated cells were plated on poly-L-lysine-coated dishes. Neurons were grown in Neurobasal supplemented with 2% B27.

BMEC were obtained from 2-day-old neonatal C57BL/6 mice as described previously (Wu et al., 2016; Wu et al., 2017). In brief, after removal of brainstems, cerebella and thalami, the isolated forebrain was digested with type-2 collagenase. After digestion, the precipitate was re-suspended in 20% BSA and centrifuged. After centrifugation, the bottom cells were re-suspended, carefully added on the top of the 50% Percoll gradient and centrifuged again. The microvessel endothelial cells were collected and plated on gelatin-coated dishes. Cells were grown in EGM-2 medium.

2.3. Cell supernatant collection (conditioned medium)

After the endothelial cells grew to 80% confluency, the cultured medium was replaced with Neurobasal supplemented with 2% B27 and harvested after 6 h as BMEC-conditioned medium (B-CM). The conditioned medium was passed through a 0.22 μm filter before use. For B-CM treatment, half the volume of the medium in the neuron cultures was changed with mixed medium of Neurobasal containing 2% B27 supplement and B-CM (1:1).

For VEGF immunodepletion, we incubated 5 μg of a monoclonal anti-VEGF antibody (catalog sc-152; Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 1 ml of B-CM for 60 min at 4 °C and then incubated the mixture with a protein A/agarose suspension for 3 h. Agarose beads with the immunoprecipitate were cleared by centrifugation and the supernatant removed.

2.4. Brain microvascular endothelial cells transplantation

The C57BL/6 mice were subjected to anesthesia, and the skull was exposed by skin incision. Stereotaxically, holes were bored above both hemispheres (lateral 1.5 mm and anterior 1.0 mm from bregma). BMEC in 0.5 μl of phosphate-buffered saline (PBS; 1×10^5 cells/μl) were injected into the left hemisphere (depth 2.0 mm from surface of the skull) using Syringe Pumps (KD Scientific, Holliston, MA, USA) with 33-gauge needle at a rate of 0.1 μl/min. After injection, the needle was held steady for 1 min, and then gradually withdrawn for 5 min. In the same manner, PBS (0.5 μl) was injected into the right hemisphere as a control.

2.5. Cerebral blood flow measurements

Transcranial laser Doppler flowmetry (MP150; Biopac Systems, Santa Barbara, CA, USA) was used to monitor regional cerebral blood flow (CBF) at 7 days after transplantation. The previous transplanted point was thinned with a drill for positioning of the fiber optic probe. CBF in each hemisphere was measured for 5 min. The data are presented as a percentage of right hemisphere values.

2.6. Electrophysiological recordings

Whole-cell patch-clamp analysis of cortical pyramidal neurons from Layers 2/3 (L2/3) were used to record spontaneous miniature excitatory postsynaptic currents (mEPSC). For further details, please see Supplementary Materials.

2.7. Immunofluorescence

For the vessel formation evaluation, the coronal sections at the levels of the motor cortex (0.5 to 1.5 mm rostral to bregma) were stained with a mouse anti-CD31 antibody (a marker of endothelial cells, 1:100; catalog ab24590; Abcam, Cambridge, UK). Alexa-conjugated secondary antibodies (anti-mouse IgG Alexa Fluor 488, 1:1000; catalog A21202 Invitrogen, Carlsbad, CA, USA) were used for detection. To detect the proliferating endothelial cells, the coronal sections were co-stained with mouse anti-CD31 and rabbit anti-Ki67 (a proliferation marker, 1:100; catalog ab16667; Abcam) antibodies. Alexa-conjugated secondary antibodies (anti-rabbit IgG Alexa Fluor 594, 1:1000; catalog A21207 and anti-mouse IgG Alexa Fluor 488, 1:1000; catalog A21202; Invitrogen) were used for detection.

For evaluation of the synapse formation, coronal sections containing motor cortex (0.5 to 1.5 mm rostral to bregma) or cultured neurons were co-stained for presynaptic markers VGlut-1 (rabbit anti-VGlut-1, 1:200; catalog 48-2400; Invitrogen) or VGlut-2 (rabbit anti-VGlut-2, 1:200; catalog 25261-1-AP; Proteintech, Chicago, IL, USA) with post-synaptic marker PSD95 (mouse anti-PSD95, 1:200; catalog MAB1596; Millipore). Alexa-conjugated secondary antibodies (anti-rabbit IgG Alexa Fluor 594, 1:1000; catalog A21207 and anti-mouse IgG Alexa Fluor 488, 1:1000; catalog A21202; Invitrogen) were used for detection. The fluorescent images were captured using a confocal microscope (SP8, Leica, Wetzlar, Germany).

The length and area of vessels were evaluated in the motor cortex using Image-Pro Plus6.0 software (Media Cybernetics, Rockville, MD, USA). Synaptic puncta densities were determined by counting the number of VGlut-1^+-PSD95^+ (L2/3) or VGlut-2^+-PSD95^+ (L4) puncta in the primary motor cortex (M1). Layers were identified using DAPI staining. At least 3 sections from each mouse in which measurements were collected from a minimum of 4 areas close to the injection sites were analyzed per mouse. Spine and synapse analysis in the cultured neurons were performed as previously described (Wu et al., 2017).

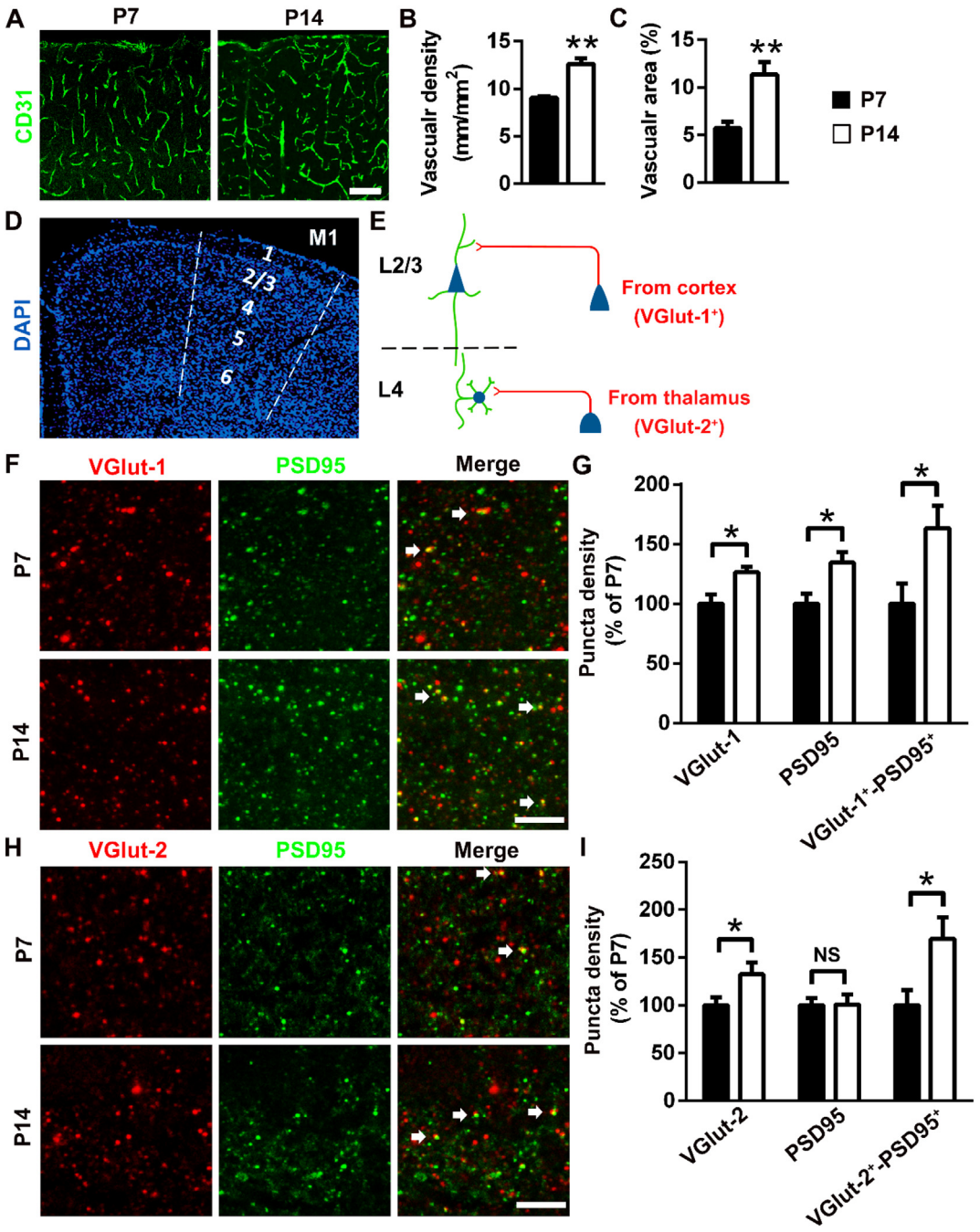

Fig. 1. Angiogenesis is concurrent with excitatory synapse formation during postnatal development. (A) Representative images of CD31-positive microvessels in mouse cerebral cortex at 7 days and 14 days after birth. Scale Bar = 100 μm. (B, C) Quantification of CD31-positive microvascular density (B) and percent area occupied by vascular structures (C). (**p < 0.01; n = 4 for each group). (D) A DAPI-stained section with M1 and cortical layers 1–6 indicated. (E) Schematic of synaptic input to area M1. The intracortical connections are VGlut-1$^+$ in L2/3. The thalamus sends VGlut-2$^+$ projections to L4. (F) Representative images show individual channels for VGlut-1 (red) and PSD95 (green) staining, as well as the merged image in L2/3 of M1. Scale Bar = 5 μm. (G) Quantification of VGlut-1$^+$, PSD95$^+$ and VGlut-1$^+$-PSD95$^+$ puncta (arrows). Co-localization of VGlut-1 (red) and PSD95 (green) revealed an increase in intracortical synapses in the second postnatal week. (*p < 0.05; n = 7 for each group). (H) Representative images show individual channels for VGlut-2 (red) and PSD95 (green) staining, as well as the merged image in L4 of M1. (I) Quantification of VGlut-2$^+$, PSD95$^+$ and VGlut-2$^+$-PSD95$^+$ puncta (arrows). VGlut-2 (red) and PSD95 (green) co-localization, representing thalamocortical synapses was significantly increased in the second postnatal week. Scale Bar = 5 μm. (*p < 0.05; n = 7 for each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.8. Immunohistochemistry

Coronal sections were incubated with mouse anti-microtubule-associated protein (MAP2, 1:200; catalog M4403; Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-SMI32 antibody (1:200; catalog ab27375; Abcam) respectively, and immunoreactivity was visualized with a Vectastain ABC kit (Vector Laboratories, Burliname, CA, USA).

Brain damage was analyzed at a location equivalent to 1.0 mm rostral to bregma in mice by outlining staining area on full section images using ImageJ. Ipsilateral MAP2 or SMI32 area loss was calculated as follows: (1 − (ipsilateral positive staining area/contralateral positive staining area)) × 100% (van Velthoven et al., 2010; van Velthoven et al., 2013).

2.9. Neonatal hypoxia-ischemic brain injury

Hypoxia-ischemic brain injury was induced in P7 pups as previously described (Rice 3rd et al., 1981; Yang et al., 2013). Briefly, animals were anesthetized by inhaled isoflurane. The left common carotid artery was exposed and ligated using an electrocoagulation device. Following the surgery, pups were returned to the cages for 60 min. After 60 min of recovery, pups were placed in a sealed tank aerated with 8% O_2 / 92% N_2 gas mixture for 60 min. After 60 min of hypoxia, the pups were returned to the cages. Sham animals were anesthetized, and their left common carotid artery was exposed and separated without ligation or hypoxia.

3. Behavioral test

3.1. Negative geotaxis reflex

We assessed vestibular and proprioceptive function using a negative geotaxis reflex task at 7 days after HI insult according to a previously reported method (Huang et al., 2017a,b). The mice were placed head down on a 45° inclined wooden board, and the time taken to rotate 180° and climb up the board was measured. For each trial, the maximum scored time was 20 s.

3.2. Cliff avoidance reflex

We assessed the integration of exteroceptive input and locomotor output using a cliff avoidance reflex task at 7 days after HI insult according to a previously reported method (Huang et al., 2017a,b). Mouse pups were placed on the wooden board so that their front paws hung over the edge of the board. The time taken to avoid the “cliff” by turning the body 90° to either side was measured. For each trial, the maximum scored time was 20 s.

3.3. Cylinder Test

We assessed the impairment of forelimb function using the cylinder test at 7 days after HI insult according to a previously reported method with minor modifications (Bae et al., 2012). We placed each mouse in a transparent cylinder (inner diameter: 15 cm; height 30 cm), and quantified the number of times that the mouse touched the wall with either forepaw. A total of 20 contacts were recorded for each animal. The percentage of impaired forelimb use was calculated as right (impaired) side/(left + right) × 100%.

3.4. Statistical analysis

All data were analyzed using Stata, version 12 (StataCorp, College Station, TX, USA) and statistical significance was determined using Kolmogorov–Smirnov test for cumulative probabilities distribution, Student's *t*-test for comparison of two groups, or ANOVA and Tukey *post hoc* test for comparison of multiple groups. Statistical differences were defined as $p < 0.05$.

4. Results

4.1. Angiogenesis is concurrent with excitatory synapse formation during postnatal development

To investigate the effects of vascular endothelial cells on neuronal development *in vivo*, we first detected the relationship between vessel and synapse formation in the neonatal mouse brain during development. By immunochemical staining with an antibody against CD31, a marker of endothelial cells, we found that the length and area of blood vessels were significantly increased in coronal sections at the level of the motor cortex in mice in the second postnatal week (Fig. 1A–C).

Neurons in M1 receive two major sources of excitatory inputs based on the layers of the cortex. In L2/3, the inputs are from cortex neurons and highly express VGlut-1. In L4, the inputs are from the thalamus neurons and express VGlut-2 (Bopp et al., 2017; Kaneko and Fujiyama, 2002) (Fig. 1D,E). Therefore, in the present study, we performed double immunostaining with antibodies against PSD95, a postsynaptic marker, and VGlut-1 or VGlut-2 to indicate intracortical synapses (VGlut-1$^+$-PSD95$^+$) and thalamocortical synapses (VGlut-2$^+$-PSD95$^+$), respectively. We quantified the number of VGlut-1$^+$ and PSD95$^+$ puncta in individual channels and found that both markers were increased in L2/3 of motor cortex in the second postnatal week compared to the first postnatal week (Fig. 1F,G). In L4, the number of VGlut-2$^+$ puncta was increased significantly in the second postnatal week (Fig. 1H,I). In the merged image, we observed that the number of VGlut-1$^+$-PSD95$^+$ puncta and VGlut-2$^+$-PSD95$^+$ puncta were significantly increased in the corresponding regions in the second postnatal week compared to the first postnatal week (Fig. 1F–I), suggesting intracortical synapses and thalamocortical synapses are simultaneously formed in the cortex during postnatal development. Taken together, these results show that synapse formation correlates with the proliferation of cerebral endothelial cells, indicating that endothelial cells might play a role in regulating synaptogenesis during postnatal development.

4.2. Grafted endothelial cells promote intercortical synaptic formation and transmission

To determine the role of BMEC in the formation of cortical synapses, we stereotactically injected mouse BMEC or vehicle into the M1 of P7 mice and quantified the number of excitatory synapses as the co-localization of the presynaptic VGlut-1 or VGlut-2 puncta with postsynaptic PSD95 puncta at 7 days after BMEC injection. We observed that BMEC transplantation significantly increased the number of VGlut-1$^+$, PSD95$^+$ and VGlut-1$^+$-PSD95$^+$ puncta in L2/3 of M1 (Fig. 2A,B). However, this treatment did not change the number of VGlut-2$^+$, PSD95$^+$ and VGlut-2$^+$-PSD95$^+$ puncta in L4 of M1 (Fig. 2C,D).

Next, we used immunochemical staining with an anti-CD31 antibody to investigate the length and area of blood vessels in the motor cortex in coronal brain sections and measured regional CBF in each cerebral hemisphere by transcranial laser doppler flowmetry at 7 days after vehicle or BMEC injection. As shown in Fig. 2E–H, BMEC injection had no effect on the density of vessels or blood flow in the cortical regions compared with vehicle injection (Fig. 2E–H). These results indicate that promotion of synapse formation by endothelial cells is not dependent on vessel formation.

To determine whether these morphological changes reflected alterations in synaptic function, we recorded mEPSC in cortical pyramidal neurons from L2/3 of M1. We found that grafted BMEC significantly increased the amplitude of postsynaptic currents demonstrating that endothelial cells strengthen synaptic activity (Fig. 3A–C). Injection of BMEC also led to a significant increase in the frequency of the synaptic events (Fig. 3D,E), which is consistent with the robust increase we observed in the number of synaptic puncta (Fig. 2A,B). Together, these results indicate that endothelial cells induce excitatory synapse formation and enhance synaptic function.

4.3. Brain microvascular endothelial cells promote excitatory synapse formation and transmission via endothelial VEGF

Previous study demonstrated endothelium-secreted factors promoted excitatory synapse formation through VEGF (Wu et al., 2017). Therefore, in this study, to identify the source of VEGF involved in prosynaptogenesis, we deleted VEGF from B-CM by immunodepletion with VEGF antibody (B-CM VEGF IP). Consistent with previous study (Wu et al., 2017), B-CM treatment in cultured neurons increased VGlut-1$^+$ puncta by 43.4%, PSD95$^+$ puncta by 27.3% and VGlut-1$^+$-PSD95$^+$ puncta by 47.8% compared to the control. Cultured neurons treated

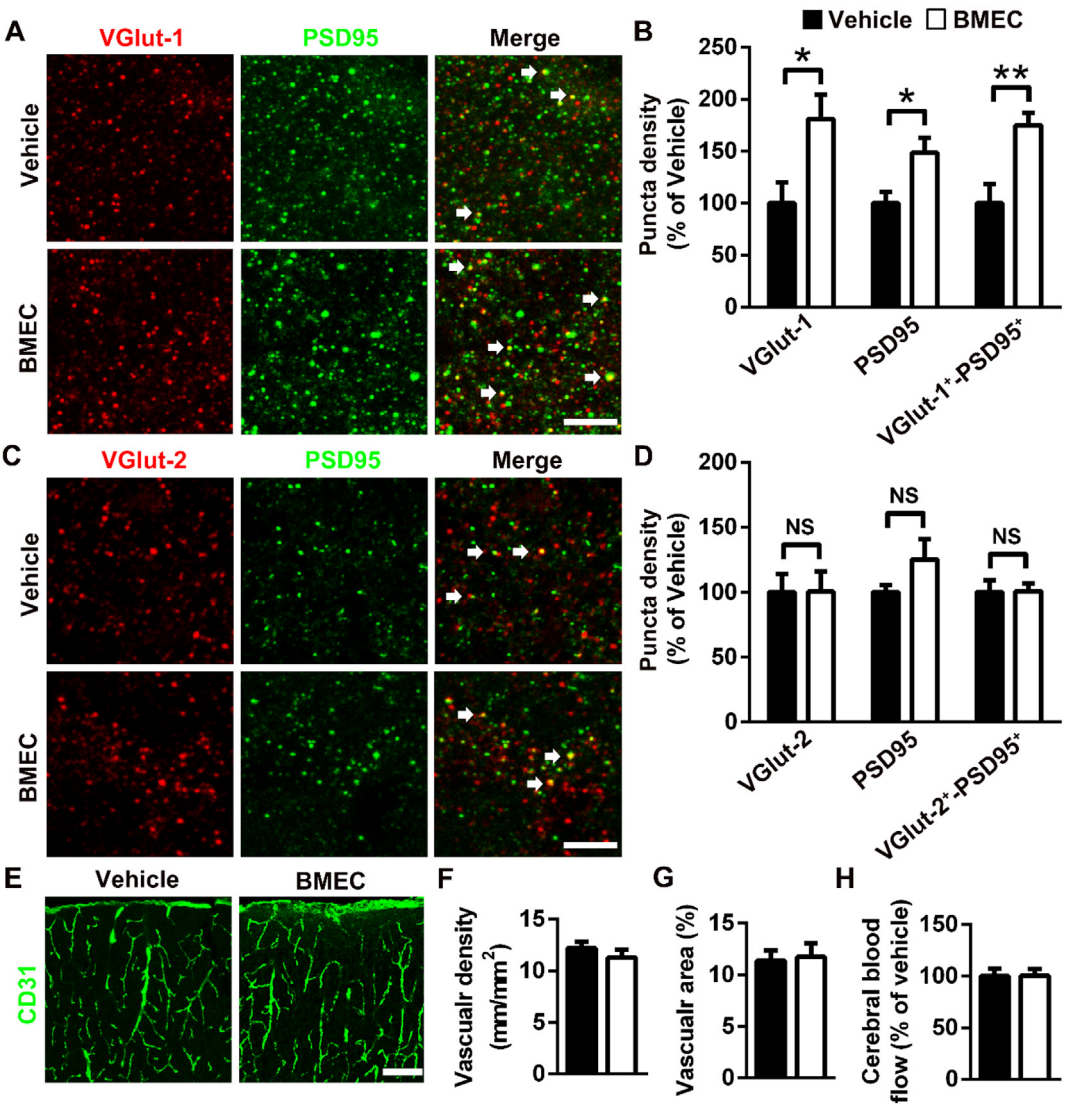

Fig. 2. Grafted endothelial cells promote intercortical synaptic formation. (A) Representative images show individual channels for VGlut-1 (red) and PSD95 (green) staining, as well as the merged image in L2/3 of M1. Scale Bar = 5 μm. (B) Quantification of VGlut-1, PSD95 and VGlut-1$^+$-PSD95$^+$ puncta (arrows). Puncta density were normalized to Vehicle. Grafted endothelial cells increased the number of VGlut-1$^+$, PSD95$^+$ and VGlut-1$^+$-PSD95$^+$ puncta. (*$p < 0.05$, **$p < 0.01$; n = 5 for each group). (C) Representative images show individual channels for VGlut-2 (red) and PSD95 (green) staining, as well as the merged image in L4 of M1. Scale Bar = 5 μm. (D) Quantification of VGlut-2$^+$, PSD95$^+$ and VGlut-2$^+$-PSD95$^+$ puncta (arrows). Puncta density were normalized to Vehicle. Grafted endothelial cells had no effect on the number of VGlut-2$^+$, PSD95$^+$ and VGlut-2$^+$-PSD95$^+$ puncta. (n = 5 for each group). (E) Representative images of CD31-positive microvessels in the brain sections from hemispheres injected with vehicle or BMEC. Scale Bar = 100 μm. (F, G) Quantification of CD31-positive microvascular density (F) and percent area occupied by vascular structures (G). (n = 5 for each group). (H) Quantification of cerebral blood flow in BMEC or Vehicle-transplanted hemisphere. Data were normalized to Vehicle. (n = 5 for each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with B-CM VEGF IP showed no B-CM-induced increases in puncta (Fig. 4A,B), indicating that endothelial VEGF promotes excitatory synaptogenesis.

During early postnatal development, postsynaptic dendritic spines receive multiple excitatory inputs from presynaptic terminals. To evaluate the ability of endothelial VEGF to modulate postsynaptic partner *in vitro*, we assessed dendritic spine formation. We observed a noticeable increase in spine density in B-CM-treated neurons. However, treatment with B-CM VEGF IP led to a noticeable blockade of B-CM-induced increase in spine formation (Fig. 4C,D), demonstrating that endothelial VEGF is necessary to promote postsynaptic spine formation.

To further confirm the effects of endothelial VEGF on excitatory synapse formation *in vivo*, we downregulated VEGF expression by VEGF siRNA in cultured BMEC and then grafted the cells into motor cortex. As shown in Fig. 5B, VEGF siRNA transfection decreased the level of VEGF protein by 63.9% in the BMEC (Fig.5B). This resulted in fewer PSD95$^+$ and VGlut-1$^+$-PSD95$^+$ puncta in the VEGF siRNA-treated BMEC group than those in the control BMEC group, (Fig. 5C,D) demonstrating that the ability of the grafted BMEC to promote synaptic formation depends on the presence of endothelial VEGF. Next, we recorded mEPSC in cortical pyramidal neurons from L2/3 of M1 in brain slices. We found that the mEPSC amplitude, but not the frequency was reduced in the VEGF siRNA-treated BMEC group compared to those measured in the control BMEC group (Fig. 6).

4.4. Grafted endothelial cells promote vessel and synapse formation and motor recovery in hypoxia-ischemic neonatal mice

To investigate whether endothelial cells contributed to brain repair after injury, we induced HI injury in P7 mice, then stereotactically

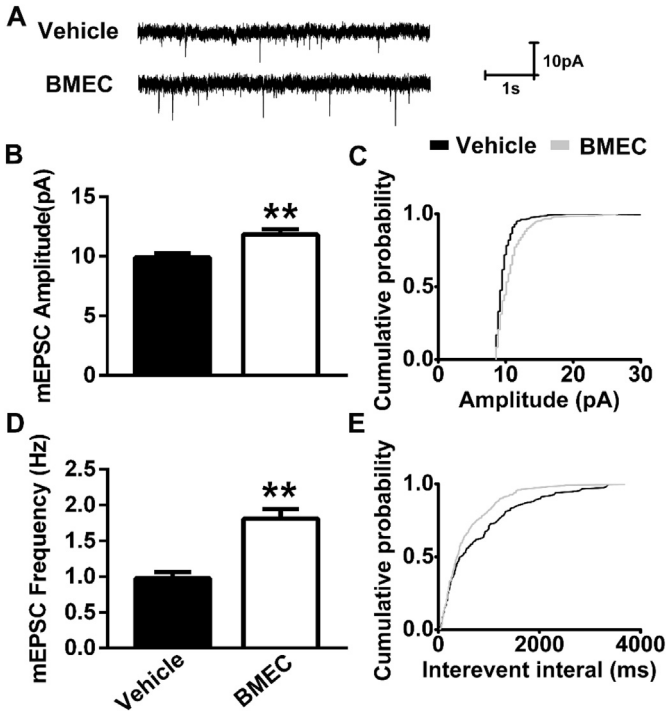

Fig. 3. Grafted endothelial cells promote excitatory synaptic transmission. (A) Example traces from whole-cell patch-clamp recordings showing mEPSC in cortical pyramidal neurons of L2/3 from hemispheres injected with vehicle or BMEC. (B-E) Quantification of the mean values of mEPSC amplitudes (B) and the frequency (D) of synaptic events. Cumulative probability of mEPSC amplitude and interevent interval were indicated in C and E. (**p < 0.01; n = 7 for each group).

injected mouse BMEC or vehicle into cerebral motor cortex of mouse brains at 24 h after HI injury. By immunostaining with antibodies against MAP2 and SMI32, we detected neurons (MAP2$^+$) and neuronal axons (SMI32$^+$) in the brain slices of mice at 7 days after HI injury. Our results showed that HI injury significantly induced the loss of MAP2$^+$ neurons and SMI32$^+$ neuronal axons in the ipsilateral hemisphere (Fig. 7B–D). However, BMEC injection did not change HI injury-induced loss of MAP2$^+$ neurons or SMI32$^+$ neuronal axons compared to vehicle injection (Fig. 7B–D). We further used the geotaxis reflex test, the cliff avoidance reflex test and the cylinder test to assess neurobehavioral outcomes in the mice with different treatments at 7 days after HI injury. As we expected, HI injury caused longer latency in geotaxis reflex test (Fig. 7E) and cliff avoidance test (Fig. 7F), and reduced use of impaired forelimb in the cylinder test (Fig. 7G), indicating that HI injury induces brain function deficits. Our results indicate that BMEC transplantation significantly improves recovery from neural function deficits induced by HI injury compared with the vehicle treatment.

To test whether grafted endothelial cells promoted angiogenesis, which could contribute to brain function recovery (Ishikawa et al., 2013), we stained with CD31 antibody to evaluate vessel formation in the cortex. We found that ischemic injury decreased vascular length and area in the cortex and engraftment of endothelial cells increased them, compared to vehicle treatment (Fig. 8A–C). Furthermore, engraftment of endothelial cells increased the number of proliferating endothelial cells (CD31$^+$-Ki67$^+$), compared to vehicle treatment after ischemia (Fig. 8D,E). It has been reported that ischemic injury also impairs the integrity of neural connectivity and this interrupts synaptic transmission, leading to motor function deficits (Curristin et al., 2002; Soleman et al., 2010). To investigate whether endothelial pro-synaptogenesis was associated with the motor recovery of HI injured mice, we further detected VGlut-1$^+$-PSD95$^+$ puncta in mice brain sections and quantified the number of excitatory synapses. We found that HI injury decreased the number of VGlut-1$^+$-PSD95$^+$ puncta in L2/3 of M1.

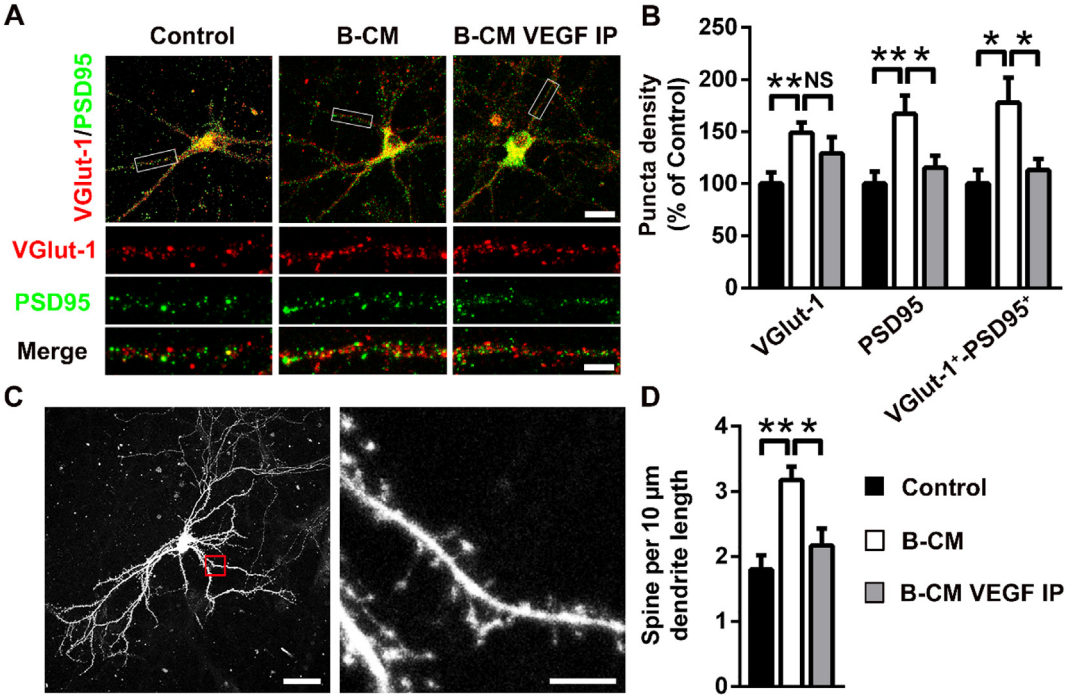

Fig. 4. Endothelial cell-secreted VEGF promotes synapse and spine formation in cortical neurons. (A) Representative images of immunostaining of VGlut-1 (red) and PSD95 (green) in cultured neurons. Scale Bar = 20 μm; (magnification) 5 μm. (B) Quantification of VGlut-1$^+$, PSD95$^+$ and VGlut-1$^+$-PSD95$^+$ puncta in neurons treated with B-CM or VEGF immunodepleted B-CM (B-CM VEGF IP) and age-matched control neurons. Puncta density were normalized to Control. (*p < 0.05, **p < 0.01; n = 7 for each group) (C) The representative image of a dendritic segment from neurons treated with B-CM. Scale Bar = 20 μm; (magnification) 5 μm. (D) Quantification of spine density along dendrites from cells, as in B. (*p < 0.05, **p < 0.01; n = 7 for each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

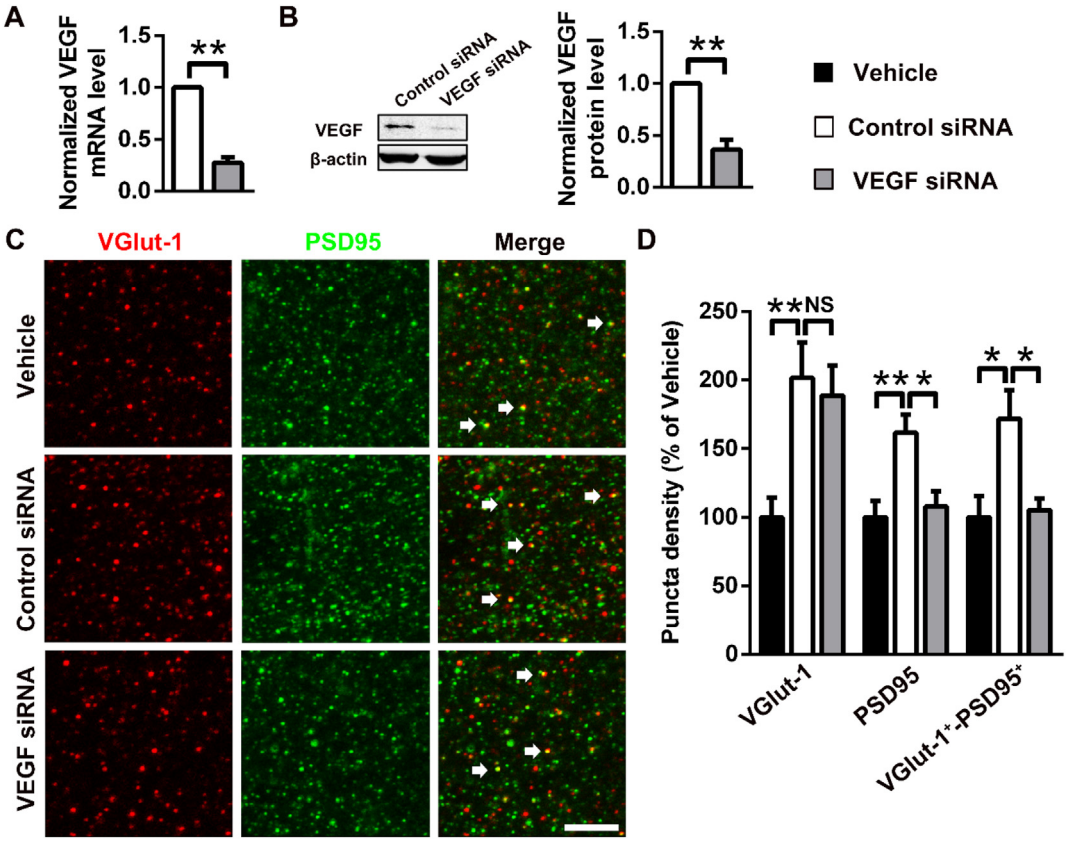

Fig. 5. Endothelial cell-secreted VEGF is necessary for the pro-synaptogenesis of BMEC *in vivo*. (A) RT-PCR from cultured BMEC transfected with VEGF siRNA or a negative control siRNA (Control siRNA). Data from four independent experiments. **p < 0.01. (B) Representative western blots of VEGF from cultured BMEC and quantification of VEGF expression showing VEGF siRNA transfection significantly decreased VEGF protein level in BMEC. Data from three independent experiments. **p < 0.01. (C) Representative images show individual channels for VGlut-1 (red) and PSD95 (green) staining, as well as the merged image in L2/3 of M1 from mice injected with vehicle or BMEC with control siRNA or VEGF siRNA. Scale Bar = 5 μm. (D) Quantification of VGlut-1$^+$, PSD95$^+$ and VGlut-1$^+$-PSD95$^+$ puncta (arrows). Puncta density were normalized to Vehicle. (*p < 0.05; n = 5 for each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Interestingly, BMEC injection significantly increased the number of VGlut-1$^+$-PSD95$^+$ puncta compared with vehicle injection (Fig. 8F,G).

5. Discussion

In the present study, we reported that endothelial cells promoted excitatory synapse formation and transmission *via* endothelium-secreted VEGF. The synaptic regulatory effects of endothelial VEGF were essentially due to changes in the postsynaptic terminal. BMEC transplantation also enhanced synapse formation and motor function recovery after HI injury in neonatal mice. These results indicate endothelial cells contribute to appropriate neuronal wiring during brain development and have important implications for motor function recovery from ischemic injury.

In the central nervous system, neural and vascular cells form a functionally integrated network, whereby neuronal and endothelial signals are tightly coupled (Iadecola, 2004; Quaegebeur et al., 2011). It has been reported that endothelial cells provide metabolic support for neurogenesis (Gama Sosa et al., 2007) and neuronal migration (Kamat et al., 1995; Plane et al., 2010). In this study, we found endothelial cells populated the cerebral cortex in the second postnatal week, concurrently with the synaptogenic period (Fig. 1). Synaptic formation and maturation are hallmarks of neuron maturation and are critical for the establishment of functional neuronal circuitry. These phenomena raise the possibility that endothelial cells might regulate synapse development and neural network construction. To test this possibility, we grafted endothelial cells into cortex and found increases in the number of VGlut-1$^+$ intracortical synapses but not VGlut-2$^+$ thalamocortical synapses (Fig. 2). The data provide novel evidence that neurovascular cells are trophically and metabolically coupled, confirming the pro-synaptogenic effects of endothelial cells. These results also reveal that the formation of VGlut-1$^+$ intracortical and VGlut-2$^+$ thalamocortical synapses are differentially regulated by vascular endothelial cells during brain development. In addition, we found engraftment of endothelial cells for 1 week had no effect on vessel formation and CBF, indicating that the promotion of synapse formation by endothelial cells is dependent on endothelial neurotrophy but not vessel formation.

Understanding the neurotrophic factors that regulate synaptic maturation contributes to our understanding of plasticity and function of the brain. VEGF, as an important neurotrophic factor (Pan et al., 2017; Sun and Guo, 2005), could enhance neurogenesis and prolong neurite outgrowth of developing neurons (Jin et al., 2002; Shen et al., 2016; Wang et al., 2009; Wang et al., 2007). During brain development, neural cells express and secret VEGF (Yang et al., 2017), which acts as a signaling protein to simulate angiogenesis (Ogunshola et al., 2002; Ogunshola et al., 2000). Recently, VEGF expression in endothelial cells has also been reported (Virgintino et al., 2003). As an autocrine factor, VEGF could promote endothelial survival postnatally (Domigan et al., 2015; Lee et al., 2007). As a paracrine factor from endothelial cells, VEGF could affect corticogenesis and promote migration of interneurons in the embryonic forebrain (Barber et al., 2018; Li et al., 2013). We investigated its role in synaptic formation and maturation in this study. We found that depletion of VEGF by genetic modification or immunodepletion in cultured endothelia abolished the endothelial pro-

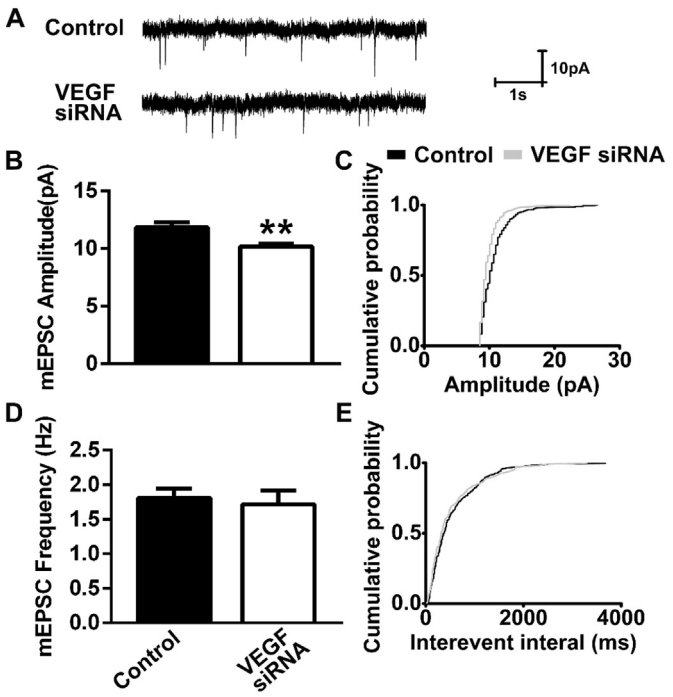

Fig. 6. Depletion of VEGF from BMEC by siRNA-mediated knockdown in cultured BMEC inhibits synaptic transmission *in vivo*. (A) Example traces from whole-cell patch-clamp recordings showing mEPSCs in cortical pyramidal neurons of L2/3 from mice injected with control BMEC or VEGF siRNA-treated BMEC (VEGF siRNA). (B-E) Quantification of the mean values of mEPSC amplitudes (B) and the frequency (D) of synaptic events. Cumulative probability of mEPSC amplitude and interevent interval were indicated in C and E. (**p < 0.01; n = 7 for each group).

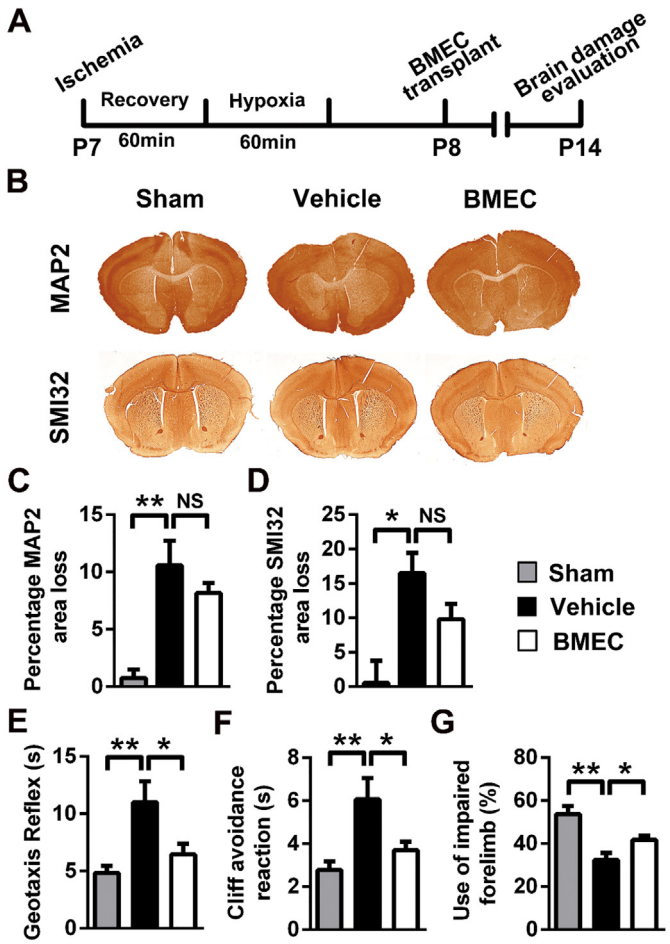

Fig. 7. Effects of BMEC transplant at 1 day after HI on lesion size and functional outcome. (A) Experimental protocol. Mice underwent HI insult at P7, and received cell transplant at P8. The behavioral tests and brain damage evaluation were performed at P14. (B) Representative MAP2 or SMI32-stained coronal brain sections. (C, D) Quantification of MAP2 (C) and SMI32 (D) positive area loss calculated as follows: (1 - (ipsilateral positive staining area / contralateral positive staining area)) × 100%. (n = 4 for each group). (E) Geotaxis reflex test. (n = 9 for each group). (F) Cliff avoidance test. (n = 9 for each group). (G) Cylinder test. (sham group, n = 8; vehicle group, n = 7; BMEC group, n = 7). *p < 0.05, **p < 0.01.

synaptogenic effects. Our findings also provided functional electrophysiological evidence as well as morphological evidence that endothelium-secreted VEGF promoted the formation of functional excitatory synapses (Fig. 6). The results enrich the diversity of biological function of this cell-specific molecule. Moreover, the electrophysiological data showed that knockdown of VEGF in cultured endothelium blocked the increased amplitude but not the frequency of mEPSC (Fig. 6). Combined with the evidence for endothelial regulation on postsynaptic PSD95 and spines, these results indicate that the regulatory effects of endothelial VEGF are essentially due to changes in the postsynaptic terminal.

To investigate the therapeutic significance of grafted endothelium for neurological disorders during early postnatal development, we induced HI injury in neonatal mice. We found engraftment of endothelial cells into the motor cortex, starting at 24 h after injury, resulted in a significant improvement in motor function recovery even though cell transplantation did not affect the lesion size (Fig. 7). Our results are in accordance with several studies on neonatal HI injury that have reported that cell therapies induce behavioral benefit without any morphological improvement (Bae et al., 2012; Huang et al., 2017a,b; Ohshima et al., 2016), indicating functional recovery is independent of morphological improvement. Consistent with previous study (Ishikawa et al., 2013), we found that engraftment of endothelial cells promoted angiogenesis in the ischemic brain, in contrast to the intact brain as mentioned above. A possible explanation is that the ischemic microenvironment induced more pro-angiogenesis factors from the endothelium. Under pathological conditions, endothelial cells not only promoted angiogenesis but also enhanced neuronal survival (Dugas et al., 2008; Guo et al., 2008; Ishikawa et al., 2013; Wu et al., 2016), and both processes contributed to brain function recovery after injury. Remodeling of synaptic connectivity and neural circuitry are also implicated in the recovery (Gherardini et al., 2015; Soleman et al., 2010).

In this study, we evaluated synapse formation after HI injury and found BMEC injection induced the formation of more synapses than vehicle injection (Fig. 8). These results indicate that the functional recovery might be attributed to the promotion of angiogenesis and establishment of synaptic connections after BMEC transplantation in neonatal HI injured mice.

In summary, we demonstrated that grafted endothelial cells promoted synaptic formation and transmission *via* the paracrine factor VEGF by targeting postsynaptic partners during development; this was accompanied by an improved behavioral recovery after ischemic insult. These results identify a novel role for endothelial VEGF in postnatal development and lead to a better understanding of neurovascular interaction in the regulation of neural plasticity and network function. Our data also indicate that endothelial cells should be considered as a potential source of trophic support for neuronal maturation and survival.

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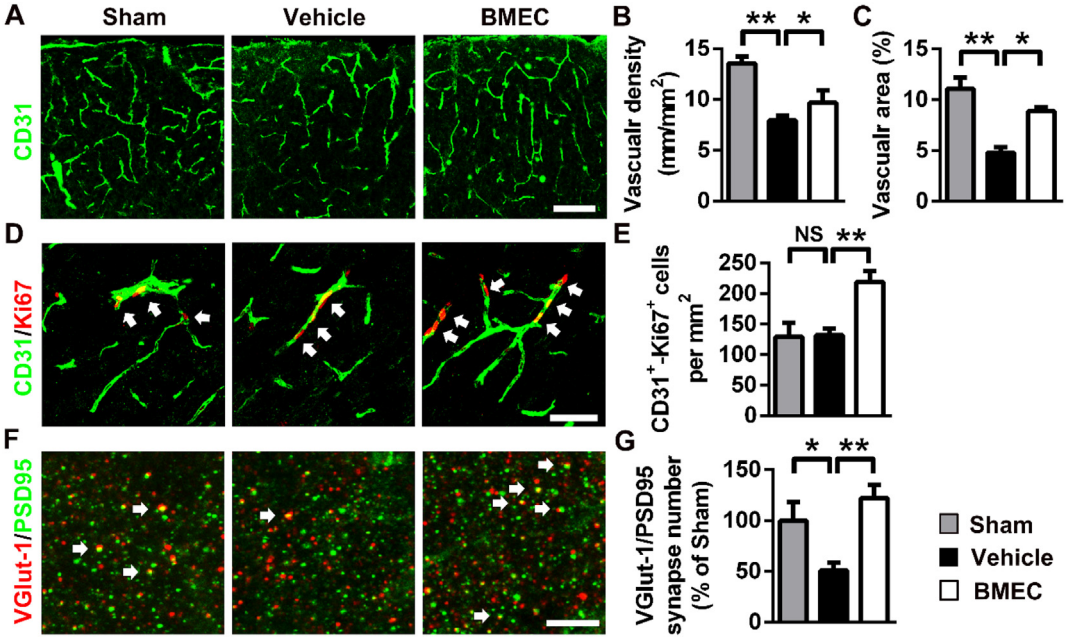

Fig. 8. Grafted endothelial cells at 1 day after HI injury promote vessel and synapse formation in motor cortex. (A) Representative images of CD31-positive microvessels in motor cortex. Quantification of CD31-positive microvascular density (B) and percent area occupied by vascular structures (C). Scale Bar = 100 μm. (D, E) Representative images of vascular branches and $CD31^+$-$Ki67^+$ proliferating endothelial cells (arrows) in motor cortex and quantification of the data. Scale Bar = 50 μm. (F) Representative images show VGlut-1 (red) and PSD95 (green) co-stained image in L2/3 of M1. Scale Bar = 5 μm. (G) Quantification of VGlut-1^+-$PSD95^+$ puncta (arrows). Puncta density were normalized to Sham. (*$p < 0.05$, **$p < 0.01$; n = 4 for each group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2018.10.006.

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