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Manuscript Draft

Manuscript Number: BRES-D-15-00999R1

Title: A human neural stem cell line provides neuroprotection and improves neurological performance by early intervention of neuroinflammatory system

Article Type: Research Report

Section/Category: Cell Biology, Signaling and Synaptic Transmission

Keywords: Neural stem cell; middle cerebral artery occlusion; transplantation; neuroinflammation; neuroprotection

Corresponding Author: Prof. Atsushi Nagai, MD, PhD

Corresponding Author's Institution: Shimane University Faculty of Medicine

First Author: Tatsuzo Watanabe

Order of Authors: Tatsuzo Watanabe; Atsushi Nagai, MD, PhD; Abdullah M Sheikh; Shingo Mitaki; Kiryo Wakabayashi; Seung U Kim; Shotai Kobayashi; Shuhei Yamaguchi

Manuscript Region of Origin: JAPAN

Abstract: A human neural stem cell line, HB1.F3, demonstrated neuroprotective properties in cerebral ischemia animal models. In this study, we have investigated about the mechanisms of such neuroprotection, mainly focusing on the neuroinflammatory system at an earlier time point of the pathology. Cerebral ischemia model was generated by middle cerebral artery occlusion (MCAO) in adult male Wister rats. HB1.F3 cells were transplanted through jugular vein 6 h after MCAO. Forty eight hours after MCAO, transplanted rats showed better neurological performance and decreased TUNEL positive apoptotic cell number in the penumbra. However, haematoxylin and eosin staining and immunostaining showed that, HB1.F3 cells did not affect the necrotic cell death. Twenty four hours after MCAO (18 h after HB1.F3 transplantation), infiltrated granulocytes and macrophage/microglia number in the core regions were decreased compared to PBS-treated controls. Immunohistochemical analysis further demonstrated that the transplantation decreased inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expressing cell number in the core and penumbra, respectively. Double immunofluorescence results revealed that iNOS was mainly expressed in granulocytes and macrophage/microglia in the core region, and COX-2 mainly expressed in neurons, endothelial cells and granulocytes in penumbra. Further analysis showed that although the percentage of iNOS expressing granulocytes and macrophage/microglia was not decreased, COX-2 expressing neurons and vessel number was decreased by the transplantation. In vitro mRNA analysis showed that brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (βFGF) and bone morphogenic protein (BMP)-4 expression was high in cultured HB1.F3 cells. Thus, our results demonstrated that HB1.F3 cell transplantation provide neuroprotection

possibly through the regulation of early inflammatory events in the cerebral ischemia condition.

To,

Prof. DR. Irwin B. Levitan Date: November 09, 2015

Editor-in-Chief:

The Brain Research

Subject: Submission of revised manuscript **BRES-D-15-00999**

Dear Sir,

Thank you for considering our research report titled "**A human neural stem cell line provides neuroprotection and improves neurological performance by early intervention of neuroinflammatory system" Ms. No.: BRES-D-15-00999**, for the Brain Research. We also thank the Reviewers for their constructive review of the manuscript. According to the reviewer's suggestion, we have done some additional experiments. The experiments produced some interesting data, and the results are included in the revised manuscript. Now, we think the quality of the manuscript has improved significantly.

Therefore, I hope that you will consider our manuscript on a positive way for publication in your reputed journal

Sincerely yours'

Atsushi Nagai,

Department of Laboratory Medicine,

Shimane University School of Medicine,

89-1 Enya Cho, Izumo 693-8501

Japan

BRES-D-15-00999

Response to reviewer’s comments:

We thank the reviewers for the constructive review. According to their comments, we have done additional experiments. The results are included in the revised manuscript. We have changed some parts of the manuscript according to their suggestions, tried to clarify the ambiguity. Also we tried to improve the figures to publication quality. The specific responses to the comments are described below:

Reviewer Comments

Reviewer #2: This is a review of manuscript BRES-D-15-00999 "A human neural stem cell line provides neuroprotection and improves neurological performance by early intervention of neuroinflammatory system" by Watanabe et al.

This study demonstrated that HB1.F3 cell transplantation provide neuroprotection through the modulation of early inflammatory events in the focal cerebral ischemia. This is an interesting study to evaluating a neuroprotective effects of neural stem cells after stroke. This study demonstrated several lines of evidence to prove their hypothesis. However, this reviewer found this study is premature to be published in this content.

First, although they tried to understand the mechanism of the neuroprotective effects of stem cell after ischemia, this study showed **no clear evidence** for that

mechanism. This study feels a kind of comparative study with NS-398 and HBF3. Thus, this reviewer feels it's not a novel study in this field. Authors should demonstrate somewhat new findings for this manuscript.

Response: NS-398 was used as a control anti-inflammatory agent. And in this study, as a possible mechanism of neuroprotection, we found that HB1.F3 cell transplantation inhibits granulocyte accumulation, and inhibit proinflammatory gene expression including COX-2. However, in granulocytes or in macrophage/microglia, HB1.F3 transplantation did not affect iNOS expression, as did by NS-398. Thus, we think that regulation of inflammatory cell accumulation might be the main feature of the beneficial effects of HB1.F3 transplantation at an earlier time point. In this respect, the mechanism of immune regulation by HB1.F3 is different from the conventional anti-inflammatory agent, such as NS-398. To our knowledge, such regulation of early inflammatory phenomenon in MCAO condition by NSC is a novel finding and helps us to understand the overall mechanism of neuroprotection by NSC transplantation. This point has been discussed in the discussion section of revised manuscript (Page 12, line 20 to page 13, line 2). We are currently investigating how NSC transplantation affects granulocytes and other inflammatory cell accumulation.

Second, authors claimed that **less TUNEL (+) cells in the penumbra represents less injury after ischemia.** However, authors should consider **less TUNEL (+)** cells in the penumbra has **more severe brain injury** after focal

cerebral ischemia **since necrotic cells showed no TUNEL (+) cells**. So, authors **should demonstrate** the **total infarct volume** after vehicle or stem cell treatment.

Response: We have checked the infarct volume by MRI. But at this earlier time point (only 48 h after MCAO), we did not find any difference among the groups. This information has been given in the revised manuscript in the result section (Page 7, line 7 to page 7, line 8). Moreover, a previous report also showed that HB1.F3 did not decreased infarct size (International Journal of Neuroscience, 121, 457–461, 2011).

In the penumbra area, apoptosis is the main mechanism of cells death than necrosis. H.E. staining showed that the bodies of some cells were increased; however, the number of such cell type was similar among the groups. Also tissue vacuolation was similar. We also showed the data of RIPK-1 staining (a necroptosis related protein). Here also we found no difference among the groups. This result has been described in the abstract (page 2, line 11 to page 2, line 12), Result (page 7, line 9 to 16), discussion (page 12, line 15 to 16), Experimental Procedure (page 16, line 21 to 23, page 17, line 10 to 16) and figure legends (page 22, line 11 to 18)

Third, authors should **describe why they consider the BMP-4** is important for **neuroprotection** after stem cell treatment.

Response: According to reviewer's suggestion, we have discussed about the importance of BMP-4 in neuroprotection in the discussion section (page 12, line

1 to 14)

<u>Fourth,</u> the images in the **Figure 4, 5 are not acceptable for publication**.

Response: According to the reviewer's suggestion, we have changed the images of figure 4 and 5. Granulocytes are identified by immunostaining. And double immunofluorescence staining showed that a few granulocytes are also positive for COX-2. This finding is included in the revised manuscript. Also, the quality of other staining pictures is improved. Due to the changes of granulocytes detection method (granulocyte detection by immunostaining), some changes have been made in the manuscript: page 9, line 1 to 2, page 9, line 20, page 10, line 1 to 3 (result section), page 14, line 11 to 14 (discussion section), page 17, line 6 to 7, page 17, line 24 to page 18, line 1 (Experimental Procedures), page 23, line 3 to 4, page 24, line 2, page 24, line 19 to 20 (figure legends).

Research Highlights

1. HB1.F3 cell transplantation improved neurological performance in MCAO rats
2. HB1.F3 cell transplantation decreased granulocytes infiltration in MCAO rats
3. HB1.F3 cell transplantation decreased macrophage infiltration in MCAO rats
4. HB1.F3 cell transplantation decreased COX-2 expression in MCAO rats
5. HB1.F3 cell transplantation did not decreased iNOS expression in MCAO rats

***Suggested Reviewers**

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Masao Nagayama, nagay001@iuhw.ac.jp, international university of health and welfare

Naohumi Hosoki, nhosomi@hiroshima-u.ac.jp, Department of Clinical Neuroscience and Therapeutics, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima

Tomohide Adachi, adachi3@saichu.jp, Saiseikai Cent Hosp, Tokyo

Title: A human neural stem cell line provides neuroprotection and improves neurological performance by early intervention of neuroinflammatory system

Tatsuzo Watanabe[1], Atsushi Nagai[2*], Abdullah Md. Sheikh[2], Shingo Mitaki[1], Kiryo Wakabayashi[1], Seung U. Kim[3, 4], Shuhei Yamaguchi[1]

[1]Department of Internal Medicine III, Shimane University School of Medicine, 89-1 Enya Cho, Izumo 693-8501, Japan
[2]Department of Laboratory Medicine, Shimane University School of Medicine, 89-1 Enya Cho, Izumo 693-8501, Japan
[3]Department of Neurology, UBC Hospital, University of British Columbia, Vancouver, Canada
[4]Medical Research Institute, Chung-Ang University College of Medicine, Seoul, Republic of Korea

*Address correspondence to:
Atsushi Nagai
Department of Laboratory Medicine
Shimane University School of Medicine
89-1 Enya Cho, Izumo 693-8501, Japan
Tel.: +81-0853-20 2312
Fax: +81-0853-20 2312
E mail anagai@med.shimane-u.ac.jp

Abbreviations: iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenese-2, BDNF: brain derived neurotrophic factor, FGF: fibroblast growth factor, BMP-4: bone morphogenic protein-4. MCAO: middle cerebral artery occlusion

Abstract:

A human neural stem cell line, HB1.F3, demonstrated neuroprotective properties in cerebral ischemia animal models. In this study, we have investigated about the mechanisms of such neuroprotection, mainly focusing on the neuroinflammatory system at an earlier time point of the pathology. Cerebral ischemia model was generated by middle cerebral artery occlusion (MCAO) in adult male Wister rats. HB1.F3 cells were transplanted through jugular vein 6 h after MCAO. Forty eight hours after MCAO, transplanted rats showed better neurological performance and decreased TUNEL positive apoptotic cell number in the penumbra. However, haematoxylin and eosin staining and immunostaining showed that, HB1.F3 cells did not affect the necrotic cell death. Twenty four hours after MCAO (18 h after HB1.F3 transplantation), infiltrated granulocytes and macrophage/microglia number in the core regions were decreased compared to PBS-treated controls. Immunohistochemical analysis further demonstrated that the transplantation decreased inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expressing cell number in the core and penumbra, respectively. Double immunofluorescence results revealed that iNOS was mainly expressed in granulocytes and macrophage/microglia in the core region, and COX-2 mainly expressed in neurons, endothelial cells and granulocytes in penumbra. Further analysis showed that although the percentage of iNOS expressing granulocytes and macrophage/microglia was not decreased, COX-2 expressing neurons and vessel number was decreased by the transplantation. In vitro mRNA analysis showed that brain-derived

neurotrophic factor (BDNF), basic fibroblast growth factor (βFGF) and bone morphogenic protein (BMP)-4 expression was high in cultured HB1.F3 cells. Thus, our results demonstrated that HB1.F3 cell transplantation provide neuroprotection possibly through the regulation of early inflammatory events in the cerebral ischemia condition.
Key words: Neural stem cell, middle cerebral artery occlusion, neuroinflammation, neuroprotection, HB1.F3 cells.

1. Introduction

Cerebral ischemia or stroke is a leading cause of death and long term disability worldwide. It results from transient or permanent disruption of cerebral blood flow, leading to necrotic death of the brain tissue supplied by the affected artery (Kumar et al., 2010). Such event activates an inflammatory condition in the affected area, marked by infiltration of inflammatory cells (Hallenbeck, 1996; Wang et al., 2007; Zheng and Yenari, 2004). Infiltration of granulocytes starts at very early time point, within hours of the initiation of the process, and progressively increases up to 48 h (Barone and Feuerstein, 1999; Gronberg et al., 2013; Kumar et al., 2010; Wang et al., 2007). Phagocytic cells including resident and circulating macrophage/microglia infiltration and accumulation follow the granulocytes, which are usually evident after 48 h, and become prominent inflammatory cell type during following 2 to 3 weeks (Gronberg et al., 2013; Kumar et al., 2010). These accumulated inflammatory cells produce various type inflammatory factors including cytokines, chemokines and enzymes (del Zoppo et al., 2000; Doll et al., 2014), and also clear up the dead tissues (Woo et al., 2012). Such processes are vital for the reparative process that ensue the ischemic insult. On the other hand, inflammation can induce an apoptotic cell death in the transition region between necrotic and normal tissue, so called penumbra, for a fairly prolonged period of time (Villa et al., 2003). Hence, the mature infarct size is usually much bigger than necrotic brain tissue of affected artery supply area. Such brain tissue of penumbra that is ‘at risk’ of apoptotic cell death, is salvageable by proper interventions of apoptotic and

inflammation processes (Barone, 2009).

In recent years, the management and treatment protocols for stroke have been evaluated and improved (Grossman and Broderick, 2013), yet that fall far behind with respect to the disease modifying and restorative capability. However, based on the remarkable advances about the understanding of stroke pathology, several potential targets have been identified and accordingly strategies are being developed and tested. Strategies such as control of neuroinflammation, regeneration of neural tissue by exogenous stem cell transplantation or stimulation of endogenous neurogenesis show promises regarding this matter (Chang et al., 2013; Sheikh et al., 2011; Taguchi et al., 2004). Interestingly, exogenous stem cell based studies not only demonstrated the homing ability of these cells to the lesion area, but also showed neuroprotective and neuroinflammation modulatory functions, along with being differentiated into neural tissue (Chang et al., 2013; Sheikh et al., 2011; Wakabayashi et al., 2010). Hence, stem cell transplantation is suggested to modulate most of the potential targets of stroke pathology. Several stem cell types including mesenchymal stem cells, neural stem cells (NSC), embryonic stem cells and induced pluripotent cells are being tested (Chen et al., 2010; Takahashi et al., 2008; Wakabayashi et al., 2010; Yanagisawa et al., 2006). Among these cell types, NSC-based therapy could be important because of its neuronal differentiation capability, along with the ability to enhance angiogenesis and endogenous neurogenesis, and modulation of neuroinflammatory system (Kim et al., 2008; Kim et al., 2009; Sheikh et al., 2011; Tang et al., 2014). Indeed, NSC transplantation has been found to improve functional neurological recovery

in cerebral ischemia animal models (Kim et al., 2008; Takahashi et al., 2008; Tang et al., 2014). Although, NSC is shown to be differentiated into mature neurons in the lesion area of cerebral ischemia animal models, it is difficult for such neurons to integrate into the neural circuitry. Hence, immune modulation might be an important aspect for such beneficial effects of NSC transplantation.

Several cell transplantation studies demonstrated that the transplantation during subacute phase, about 24 h after middle cerebral artery occlusion (MCAO), provide better result (Hao et al., 2014; Song et al., 2011). Accordingly, immune modulatory effects of cell transplantation during subacute phase are being investigated extensively (Sheikh et al., 2011; Wang et al., 2013). However, intervention during very early phase and understanding the modulatory effects at that time might also be important because during this time the events of neuroinflammation and other pathological aspects of stroke are different than that of sub-acute phase (Gronberg et al., 2013; Kumar et al., 2010). Therefore, in this study we aimed to investigate the effects of a neural stem cell line (HB1.F3) transplantation during early phage on the pathological changes in a cerebral ischemia condition. We found that HB1.F3 cell line transplantation at an earlier time point affects the initial events of neuroinflammation at the level of cell infiltration and pro-inflammatory gene expression.

2. Results:

2.1 Effects of HB1.F3 transplantation on neurological performances, tissue damage and cellular apoptosis in MCAO rat brains. The rats included in the study showed no neurological deficit prior to MCAO. Six hours after MCAO, animals having neurological deficit with NSS score between 10 and 12 were

randomly divided into 3 groups. There was no significant difference in NSS score among the groups at this time point. Forty eight hours after MCAO (42 h after HB1.F3 transplantation), NSS assessment was done again. The results showed that compared to PBS-treated group, the neurological performance did not improved in NS-398 (a COX-2 selective Inhibitor)-treated group. However, HB1.F3 transplanted group showed a slight, but significant improvement (Figure 1B). Nevertheless, infarct size was not changed either by HB1.F3 transplantation or by NS-398 treatment, as revealed by MRI (data not shown). Twenty four h after MCAO, haematoxylin and eosin staining revealed that the tissue damage, such as vacuolation and necrotic cells in the core area was not different among the groups (Figure 1C). In the penumbra, the bodies of some cells were found to be irregular in shape, and larger than the cells of contralateral cortices; however, the number of such cell type was similar among the groups (Figure 1C). Moreover, an immunostaining result showed that RIPK1, a necroptosis related protein, expressing cell number was similar among the groups, both in core and penumbra cortices (Figure 1D and 1E).

Next, apoptotic cells were evaluated by TUNEL assay. We found that TUNEL positive apoptotic cells were present only in the ischemic boundary zone (IBZ) of MCAO brains after 48 h. Hence, apoptotic cells were counted in that area. The results demonstrated that TUNEL positive apoptotic cell number was significantly decreased in HB1.F3 transplanted group compared to PBS group (Figure 1F and 1G). Apoptotic cell were also decreased in NS-398 group, however that reduction was not statistically significant (Figure 1F and 1G).

2.2 Effects of HB1.F3 transplantation on inflammatory cell infiltration.

Previously, it has been shown that the state inflammatory condition affects apoptotic neuronal death in MCAO (Elango and Devaraj, 2010). As HB1.F3 transplantation decreased apoptosis cell number after 48 h, we checked its immunomodulatory capability before that time point. After analyzing the distribution of inflammatory cells including granulocytes and macrophage/microglia in the rat brains 24 h after MCAO, we found that the former cell type were predominant in the lesion area than the latter. Interestingly, HB1.F3 transplantation significantly decreased both granulocytes and macrophage/microglia cell number compared to PBS- or NS 398-treated group (Figure 2). Conversely, NS 398-treated group did not show significant reduction of inflammatory cell infiltration (Figure 2).

2.3 Effects of HB1.F3 transplantation on iNOS expression in MCAO rats.

Next, we investigated the effects of HB1.F3 transplantation on the expression of pro-inflammatory factors including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, which have been shown to be regulated in MCAO condition. Our immunostaining results demonstrated that 24 h after MCAO, iNOS was expressed mainly in the cells of ischemic core region near the penumbra. A few cells in the penumbra region were also positive for iNOS. Hence, we counted iNOS positive cells in those areas. The results showed that both HB1.F3 transplantation and NS-398 treatment decreased iNOS positive cells in MCAO rat brains after 24 h (Figure 3A and 3B).

To identify the cells that expressed iNOS in MCAO condition, we employed double immunofluorescence staining with cell type specific markers including NeuN (neuron marker), ED-1 (macrophage/microglia marker) and vWF

(endothelial cell marker). Granulocytes were identified using an anti-granulocyte antibody. The results showed that iNOS was expressed mainly in ED-1 positive macrophage/microglia and granulocytes (Figure 4A). Counting iNOS positive granulocytes or macrophage/microglia revealed that both HB1.F3 transplantation and NS-398 treatment decreased the number of iNOS positive granulocytes and macrophage/microglia (Figure 4B). As NS-398 treatment did not decrease the accumulation (see Figure 2), then we analyzed whether it affected the percentage of iNOS positive granulocytes or macrophage/microglia. The results showed that indeed NS-398 treatment decreased the percentage of iNOS expressing granulocytes and macrophage/microglia; whereas, HB1.F3 transplantation did not affect that percentage (Figure 4C).

2.4 Effects of HB1.F3 transplantation on COX-2 expression in MCAO rats.

Immunofluorescence staining using COX-2 specific antibody revealed that it was expressed in the penumbra area and in the ischemic core near the penumbra; hence the positive cells were counted in those areas. The staining results demonstrated that both HB1.F3 transplantation and NS-398 treatment decreased COX-2 positive cell number in the examined area (Figure 5A). To identify the cells that expressed COX-2 in MCAO condition, we employed double immunofluorescence staining with cell type specific markers including NeuN, ED-1 and vWF. Granulocytes were identified using an anti-granulocyte antibody. Double immunofluorescence results showed that COX-2 was mainly expressed in NeuN positive neurons and vWF positive endothelial cells. Counting COX-2 positive neurons and vessels revealed that both HB1.F3 transplantation and NS-398 treatment decreased the number of COX-2 positive neurons and

vessels (Figure 5C and 5D). A few granulocytes were also positive for COX-2. Again, both HB1.F3 transplantation and NS-398 decreased COX-2 positive granulocytes number.

2.5 Basal expression levels of growth factors and cytokines in HB1.F3 in culture condition. To understand the possible effects of transplantation on MCAO condition, we have investigated the basal expressional levels of several growth factors and cytokines in HB1.F3 that was prepared to be used for transplantation. HB1.F3 cells in normal culture condition expressed mRNA for several neurotrophic factors and cytokines (Figure 6). Among the neurotrophic factors we have checked, the mRNA of bone morphogenic protein (BMP)-4, basic fibroblast growth factor (βFGF), brain derived neurotrophic factor (BDNF) and BMP-6 were high, whereas epidermal growth factor (EGF), hepatocyte growth factor (HGF), neurotrophin (NT)-3 and vascular endothelial growth factor (VEGF) mRNA were showed relatively low levels of expression (Figure 6). In the case of cytokines, only the mRNA level of interleukin (IL)-5 was found to be considerably high.

3. Discussion:

In this study, we have found that the transplantation of a neural stem cell line, HB1.F3, during early phase of cerebral ischemia, provided neuroprotection and improved functional neurological recovery in cerebral ischemia animal model. We also demonstrated that the transplantation modulates inflammatory cell infiltrations and the expression of proinflammatory factors including COX-2. As neuroinflammation plays a vital role to determine the pathological course of cerebral ischemia (Barone and Feuerstein, 1999; Gronberg et al., 2013;

Hallenbeck, 1996; Wang et al., 2007; Zheng and Yenari, 2004), our results suggest that the modulation of such system might be one of the major factor that mediates HB1.F3-induced beneficial effects.

In a previous study, HB1.F3 cells have been found to differentiate into neuronal and astroglial cells in a cerebral ischemia model (Kim et al., 2008). In this study, the improvement of neurological performance in HB1.F3 transplanted rats was observed at very early time point, within 42 h after transplantation. During such a short period of time, differentiation of transplanted cells and integration into the neural circuitry might not be the principal feature. Hence, replacement and restoration of damaged tissue by transplantation could be a very minor aspect during the early events. Rather neuroprotective function of HB1.F3 transplantation might have a bigger role at this stage. Indeed, we have found the reduction of apoptotic cell death in the penumbral area, pointing the neuroprotective function of HB1.F3 transplantation. Previously it has been demonstrated that the culture supernatant of HB1.F3 cells have anti-apoptotic properties on SH-SY5Y cells and fetal rat ventral mesencephalic dopaminergic neurons through increasing Bcl-2 levels (Yasuhara et al., 2006), suggesting HB1.F3-secreted soluble factor(s) is responsible for such neuroprotective activity. In this study, we have found that in culture condition, βFGF and BDNF expression are high in the cells. These growth factors are demonstrated to regulate Bcl-2 expression and provide anti-apoptotic neuroprotection (Allsopp et al., 1995; Ay et al., 2001). Although we did not examine the role of Bcl-2 in neuroprotective effects in MCAO condition, there is a possibility that transplanted cells secreted factors including βFGF and BDNF might protect neuronal cells

from apoptosis by regulating Bcl-2. We also found that the basal expression of BMP-4 was high in HB1.F3 culture. In a previous report, it was shown that BMP-4 promotes differentiation of neural progenitor cells to astrocytes without decreasing neuronal cell type in a neurosphere culture (Xin et al., 2006). Astrocytes can modulate cerebral edema and neuroinflammation, and can provide neuroprotection through production of neurotrophic factors (Trendelenburg and Dirnagl, 2005). Also, in cerebral ischemia animal model, BMP-4 expression in astrocytes is decreased, and restoration of that expression by bone marrow mesenchymal stem cell transplantation increased Connexin-43 and Synaptophysin expression, along with functional recovery (Zhang et al., 2006). Taken together, BMP-4 might have an important role in HB1.F3-mediated regulation of cerebral ischemia pathology. It will be interesting to investigate the effects of HB1.F3 cell transplantation on endogenous neural cell migration, differentiation, and expression of BMP-4 in cerebral ischemic condition.

We found in this study that HB1.F3 transplantation specifically decreased apoptotic cell death without affecting necrosis in MCAO condition. Apoptotic neuronal death in cerebral ischemia is influenced by local inflammatory condition, which can be altered by modulation of that inflammatory condition (Barone and Feuerstein, 1999; Hallenbeck, 1996; Wang et al., 2007; Zheng and Yenari, 2004). In our results, we have demonstrated that leukocytes including granulocytes and macrophage/ microglia accumulation in the lesion area were specifically inhibited by HB1.F3 transplantation without affecting iNOS expression. Conversely, in NS-398 treated animals, iNOS expression was decreased without affecting infiltration of inflammatory cells. Hence, regulation of

inflammatory cell infiltration might be one of the main features of HB1.F3 transplantation-induced modulation of neuroinflammation in this condition. Neutrophils are shown to play a great role in determining the size of the lesion and disease outcome of stroke. For example, studies have demonstrated that depletion of systemic neutrophils improve the stroke condition in various animal models (Bednar et al., 1991; Matsuo et al., 1994). The increased production of reactive oxygen species (ROS) is suggested to be the possible mechanism of neutrophil-induced neurotoxicity in cerebral ischemia condition (Matsuo et al., 1995). Although we did not investigate about the role of HB1.F3 on MCAO-induced ROS formation, the correlation of decreased apoptotic cells, decreased inflammatory cell infiltrations and improved neurological performance might suggest similar underlying mechanism in this case.

To further investigate about the regulation of inflammatory condition by HB1.F3 transplantation, we have analyzed proinflammatory factors including COX-2 and iNOS in MCAO brains. Previous studies have shown that COX-2 and iNOS contribute to increase lesion formation through production of neurotoxic prostanoids, superoxide and reactive nitrogen species (del Zoppo et al., 2000; Nogawa et al., 1997; Sairanen et al., 1998; Zhu et al., 2002). In this study, we have found that HB1.F3 transplantation decreased iNOS expressing cell number. iNOS was found to be expressed in leukocytes, in both granulocytes and macrophage/microglia. As these cell populations were decreased by transplantation, there is a possibility that reduction of iNOS expressing cells number was due to decreased accumulation of iNOS expressing cells, not due to inhibition of iNOS gene expression. To clarify that matter, we checked whether

the percent of iNOS expressing granulocytes or macrophage/microglia were decreased. As the percentage did not decreased, HB1.F3 cell transplantation did not affect the expression of iNOS at protein level. On the other hand, COX-2 was found to express mostly in neurons and endothelial cells in the penumbral region after 24 h of MCAO. Moreover, the number of COX-2 expressing neurons and vessels were decreased, suggesting that the expression of COX-2 at protein level was regulated by HB1.F3. As prostaglandin E2, produced by COX-2 activity, is suggested to contribute to ischemic cell damage by disrupting Ca^{2+} homeostasis in neurons (Shimamura et al., 2013), reduction of its expression in neurons might provide protection by maintaining Ca^{2+} homeostasis. We also found a few granulocytes that expressed COX-2 in that area. As COX-2 positive granulocytes number is very few, such expression might not be as important as the expression in neurons or endothelial cells; nevertheless HB1.F3 transplantation decreased such positive cell number.

In conclusion, this study provides evidence that early transplantation of a neural stem cell line in cerebral ischemia condition provide neuroprotection through regulation of leukocyte infiltration and inhibition of proinflammatory gene expression. Such early intervention might be a good strategy for the therapy of cerebral ischemia condition.

4. Experimental Procedure:

4.1 HBF3 cell culture:

HB1.F3 cells were generated from primary cell culture of human fetal telencephalon of 14 weeks gestation (Cho et al., 2002). The primary telencephalon cells were infected with an amphotropic, replication incompetent

retroviral vector-containing v-myc. One selected clone, HB1.F3, was demonstrated to express nestin and vimentin, the cell specific markers for NSC. The cells were grown in T25 flasks in Dulbecco's modified Eagle medium (DMEM, Gibco, Life technologies) with high glucose, supplemented with 5% horse serum, 20 mg/ml gentamicin (Wako pure chemicals, Richmond, VA, USA), and 2.5 mg/ml amphotericin B (Sigma, St. Louis, MO. USA).

4.2 Focal cerebral ischemia animal model

Focal cerebral ischemia animal model was generated by transiently occluding the middle cerebral artery (MCA). Adult male Wistar rats (Charles River, Yokohama, Japan), weighing 250–300 g, were used to prepare transient middle cerebral artery occlusion (MCAO) model, and the procedure was done following a previously described method (Wakabayashi et al., 2010). In a brief, the rat was initially anesthetized with 4% halothane. Rectal temperature was maintained at 37°C throughout the surgical procedure by means of a feedback-regulated water heating system. Common carotid artery, external carotid artery, and internal carotid artery of left side was exposed. A length of 4-0 monofilament nylon suture (20 mm), with its tip rounded, was advanced from external carotid artery into the lumen of internal carotid artery until it blocked the origin of middle cerebral artery. Then the rat was allowed to recover from anesthesia. Sixty minutes after MCAO, the animal was re-anesthetized with halothane, and reperfusion of ischemic area of brain was allowed by withdrawal of the suture. The experimental protocol and procedures were approved by the Ethical Committee of the Shimane University School of Medicine.

4.3 Behavioral test

All animals underwent behavioral tests before, and 6 h and 48 h after MCAO. A neurological severity score (NSS) system was used to grade the various aspects of neurological functions, which was adopted from a previous report (Wakabayashi et al., 2010), with some modification. NSS (Supplemental Table 1) system is a composite of motor, sensory, beam balance and reflex tests, in which meticulous sensory examination for vision, touch and proprioceptive sensation was performed. The total score for the test was 22 points. Increasing score indicates the severity of injury.

4.4 Intravenous injection of HB1.F3 human NSC

Six hours after MCAO, the rat was anesthetized with halothane, the jugular vein on the right side was exposed, and 3×10^6 HB1.F3 cells in 100 µl phosphate-buffered saline (PBS) were injected into the jugular vein. Then the rat was allowed to recover from anesthesia and returned to the cage. Immunosuppressants were not used in the present study.

4.5 Histological and immunohistochemical analysis

Twenty four or 48 h after MCAO, rats were deeply anesthetized and transcardial perfused sequentially with normal saline and 4% paraformaldehyde (PFA) in 0.1mol/L phosphate buffer (PB, pH 7.4). Then the rat brains were immersed in 20% sucrose for 48 hours, embedded in TissueTek OCT compound and frozen on dry ice. The brain tissues were cut into equally spaced (thickness 2 mm) coronal blocks, then sectioned into 10 µm slices using a cryostat. To investigate the histological changes in the brain tissue, haematoxylin and eosin (H.E.) staining was performed.

To investigate about the cellular infiltration, macrophage/microglia and granulocytes were identified immunohistochemically using anti-rat CD68 (ED-1) and anti-granulocytes IgG, respectively. In a brief, the sections were incubated in blocking solution containing 5% normal goat or horse serum and 0.2% Triton X-100 in PBS. Then the sections were incubated with primary antibody against microglia/macrophage-specific ED-1 (mouse 1:100, Serotech, Oxford, UK), or anti-granulocyte antibody (mouse 1:50, Abcam, Cambridge, UK). Following incubation with primary antibody, the sections were incubated in FITC-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To identify the nuclei, Hoechst staining was done. RIPK1 expressing cells in the MCAO brains were identified by immunostaining using anti-RIPK1 IgG (rabbit 1:100, Sigma-Aldrich, Saint Lois, MO, USA). After incubation with primary antibody, the sections were then incubated in biotin-conjugated anti-rabbit IgG (1:100, Vector, Ingold Road, CA) and avidin–biotin–peroxidase complex (ABC, Vector). The immune reaction products were visualized with 3, 30-diaminobenzidine (DAB, Sigma).

Analysis of cyclooxygenese-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressing cells in MCAO rat brains were done by immunofluorescence staining using specific antibodies (anti-COX-2 IgG, rabbit 1:100, Santa Cruz; anti-iNOS IgG, rabbit 1:100, Santa Cruz). To determine COX-2 and iNOS expressing cells in the infracted brains, double immunofluorescence staining of COX-2 or iNOS, and cell type specific markers (ED-1 for macrophage/microglia, Serotech; vWF for endothelial cells, Santa Cruz; NeuN for neurons, Millipore, Billerica, MA, USA) were done. Granulocytes

were identified using an anti-granulocyte antibody (Abcam). Stained sections were examined under a fluorescent microscope (NIKON, ECLIPSE E600), and cells were counted in a blinded manner.

4.6 Terminal deoxynucleotidyl transferase dUTP-biotin nick-end-labeling (TUNEL) assay

The TUNEL assay was performed using a kit (In Situ Cell Death Detection Kit, POD, Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the tissue sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Then the apoptotic cells were detected by labeling the DNA nicks with fluorescein-conjugated nucleotides using label solution and enzyme solution provided by the manufacturer. For identification of cells, nuclei were stained with Hoechst. The cells were then examined under a fluorescent microscope (NIKON, ECLIPSE E600) and counted at 400X magnification.

4.7 Total RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated from HB1.F3 cell culture using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To prepare first-strand cDNA, 2 μg of total RNA was reverse transcribed with reverse transcriptase enzyme (RiverTraAce, Toyobo, Osaka, Japan) in 20 μl reaction mixture. To analyze mRNA level, real-time PCR was performed with an ABI Prism 7000 Sequence Detector system (Applied Biosystems, Foster City, CA, USA). For quantification, a relative quantification method was employed where the mRNA level of a target gene was

normalized by GAPDH mRNA, and expressed as mRNA copy number relative to 10^6 GAPDH mRNA copy.

4.8 Statistical analysis

Data are presented as mean values ± SD. The counted cell numbers and behavior scores (NSS) were statistically analyzed. Statistical analysis was done by one-way ANOVA, followed by Scheffe's post hoc test or paired *t*-test. The statistical significance level was set at $p < 0.05$.

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Figure legends

Figure 1. Effects of HB1.F3 transplantation on neurological performance, tissue damage and cellular apoptosis in a MCAO rat model. Six hours after generation of MCAO, PBS (control) or HB1.F3 cells were transplanted intravenously. Another group of rats were treated with a selective COX-2 inhibitor (NS-398), as shown in the study protocol (A). (B) Forty eight hours after MCAO, the neurological performance of the rats were evaluated using a neurological scoring system, as described in the Experimental Procedure. To evaluate the necrotic cells and the extent of tissue damages 24 h after MCAO, haematoxylin and eosin staining was done, and representative photomicrographs of ischemic core, penumbra and contralateral cortices are shown in (C). A necroptosis related protein, RIPK1 level in MCAO rat brains were evaluated 24 h after MCAO by immunohistochemistry. Representative photomicrographs of ischemic core, penumbra and contralateral cortices after RIPK1 immunohistochemistry are shown in (D), and average number of positive cells in (E). Apoptotic cell number in the penumbra was evaluated 48 h after MCAO by TUNEL assay. Representative photomicrographs of TUNEL positive cells are shown in (F), and the average number of positive cells is presented in (G).. Numerical data are presented here as average ± SD of 5 rats in a group. Statistical significance are denoted as follows; *p< 0.05 vs PBS (control) rats.

Figure 2. Effects of HB1.F3 transplantation on cellular infiltration in ischemic area of MCAO rats. To evaluate the effect of HB1.F3 transplantation on infiltration of inflammatory cells, granulocytes and macrophage/microglia accumulation in the ischemic core region was analyzed 24 h after MCAO. Granulocytes and macrophage/microglia was identified by immunostaining using

anti-granulocyte and anti-rat CD68 (ED-1) IgG, respectively, as described in the Experimental procedure. The cells were counted at 5 random microscopic fields of 400X magnification in the core region of 3 brain tissue sections at a distance of 2 mm, and the average number of a total 15 fields represented the cell number of that animal. The cell numbers are presented here as average ± SD of 5 rats in a group. Statistical significance are denoted as follows; *p< 0.05 vs PBS (control) rats, $^{\#}p$< 0.05 vs NS-398 group.

Figure 3. Effects of HB1.F3 transplantation on iNOS expression in the ischemic core region of MCAO rat brains. To determine the expression of iNOS in the ischemic core areas, immunostaining technique was employed. Twenty four hours after MCAO, iNOS positive cells were evaluated by immunofluorescence staining using iNOS-specific antibody and Texas Red conjugated species specific IgG (A). The cells were counted at 5 random microscopic fields of 400X magnification in the core region of 3 brain tissue sections at a distance of 2 mm, and the average number of a total 15 fields represented as the cell number of that animal. The cell numbers are presented here as average ± SD of 5 rats in a group, and shown in (B). Statistical significance are denoted as follows; *p< 0.05 vs PBS (control) rats.

Figure 4. Identification of iNOS expressing cells in MCAO rat brains. To determine iNOS expressing cell in rat brains 24 h after MCAO, double immunofluorescence staining with cell specific markers including NeuN (neurons), ED-1 (macrophage/microglia) and vWF (endothelial cells) were done. To identify granulocytes, an anti-granulocyte antibody was used. Representative photomicrographs of cell specific markers, iNOS and their merged image are

shown in (A). To determine the cell number, the cells were counted at 5 random microscopic fields of 400X magnification in the core region of 3 brain tissue sections at a distance of 2 mm, and the average number of a total 15 fields represented as the cell number of that animal. The number of iNOS expressing granulocytes and macrophage/microglia are presented here as average ± SD of 5 rats in a group, and shown in (B). In (C), % of granulocytes or macrophage/microglia expressing iNOS in MCAO rat brains are shown. Statistical significance are denoted as follows; *p< 0.05 vs PBS (control) rats.

Figure 5. Effects of HB1.F3 transplantation on COX-2 expression in MCAO rat brains. To determine COX-2 expression in the penumbra of MCAO rat brains, immunostaining technique was employed. Twenty four hours after MCAO, COX-2 positive cells were evaluated by immunofluorescence staining using COX-2-specific antibody and Texas Red conjugated species specific IgG (A). To identify the cells that were expressing COX-2, double immunofluorescence staining with cell specific markers including NeuN (neurons), ED-1 (macrophage/microglia) and vWF (endothelial cells) were done. To identify granulocytes, an anti-granulocyte antibody was used. Representative photomicrographs of cell specific markers, COX-2 and their merged image are shown in (B). To determine the cell number, the cells were counted at 5 random microscopic fields of 400X magnification in the penumbra region of 3 brain tissue sections at a distance of 2 mm, and the average number of a total 15 fields represented as the cell number of that animal. The number of COX-2 expressing neurons (C), vessels (D) and granulocytes (E) are presented here as average ± SD of 5 rats in a group. Statistical significance are denoted as follows; *p< 0.05

vs PBS (control) rats.

Figure 6. Basal expression levels of growth factors and cytokines mRNA in HB1.F3 cells. Total RNA was isolated from cultured HB1.F3 cells, and real-time PCR was performed using gene-specific primers, as described in the Experimental Procedure. The results were calculated relatively as specific gene mRNA copy number per 10^6 GAPDH mRNA copy, and are expressed as average ± SD of three separate experiments.

Figure
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Figure 1

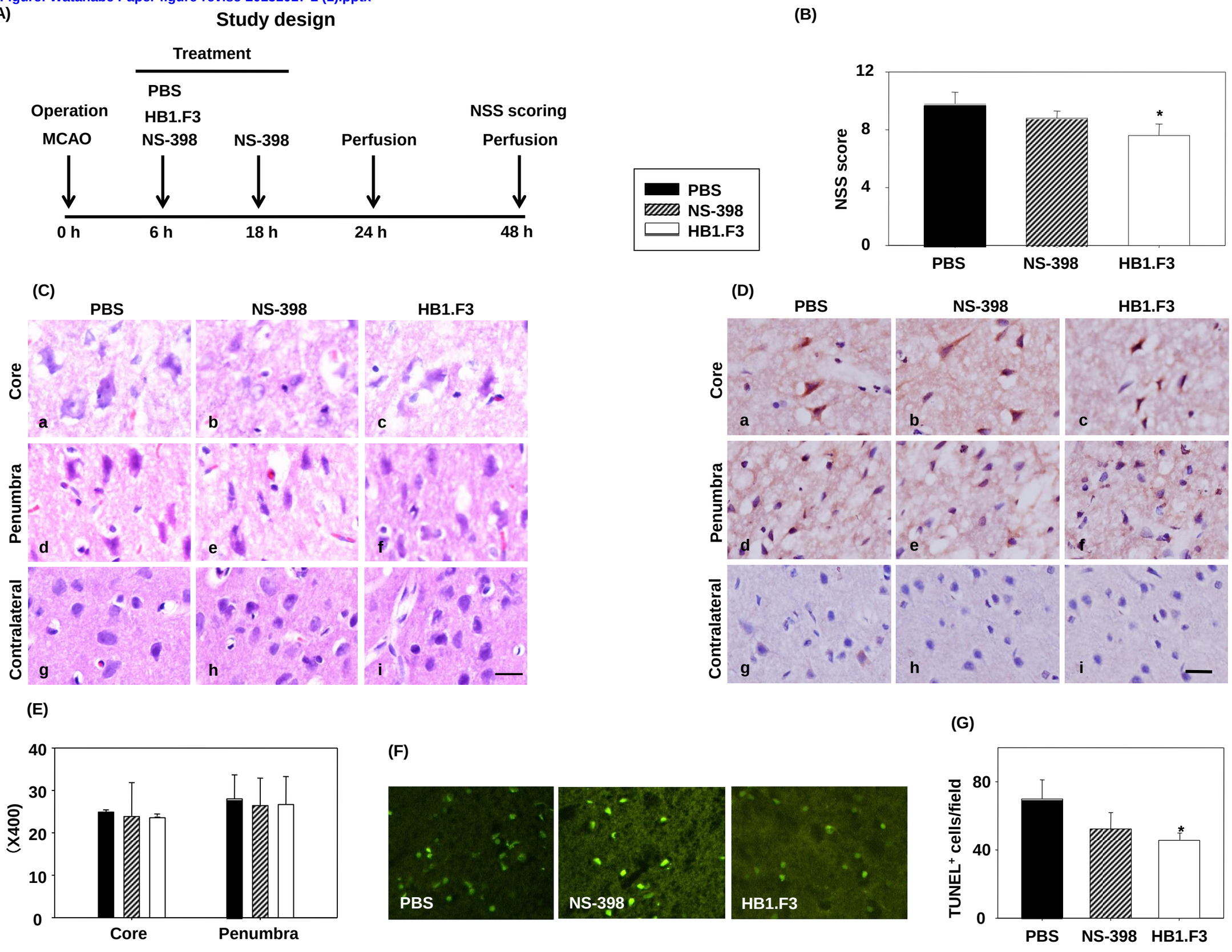
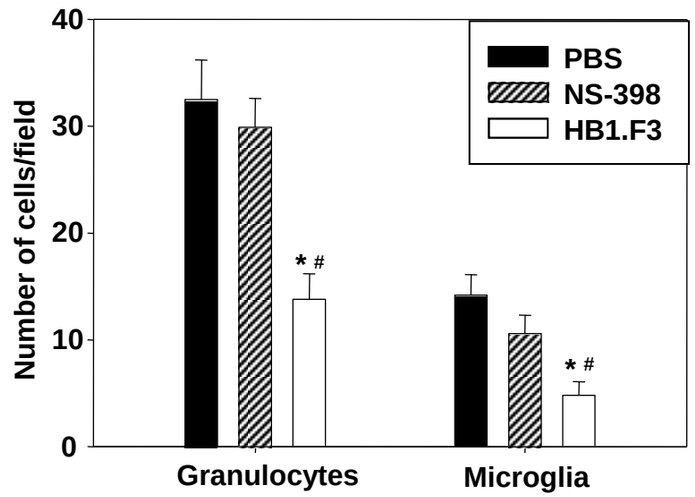


Figure 2


40
30
20
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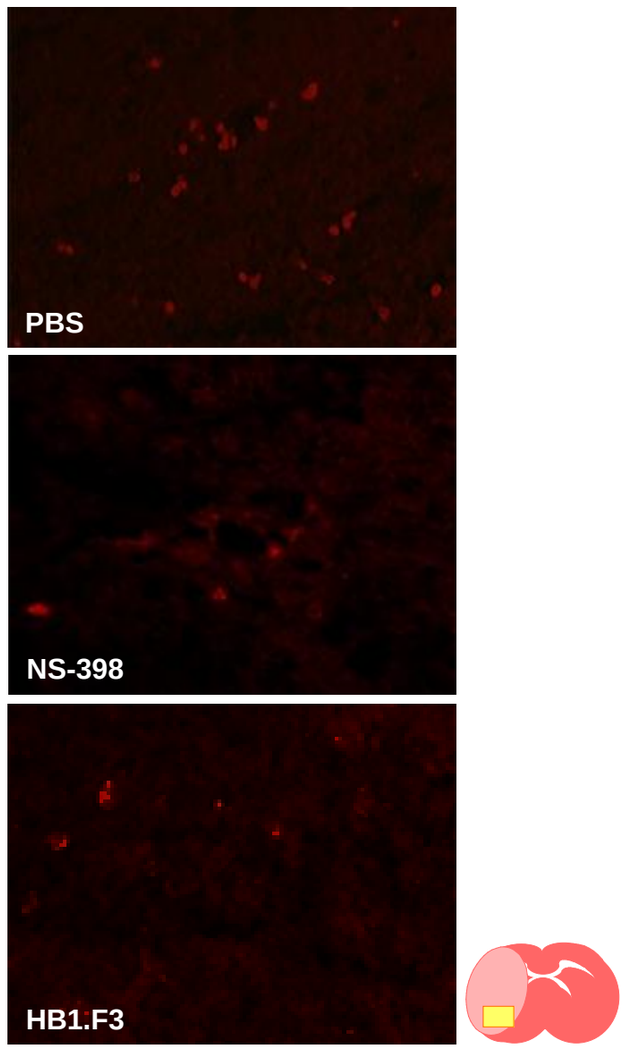
Number of cells/field
PBS
NS-398
HB1.F3
* #
* #
Granulocytes
Microglia

Figure 3

(A)



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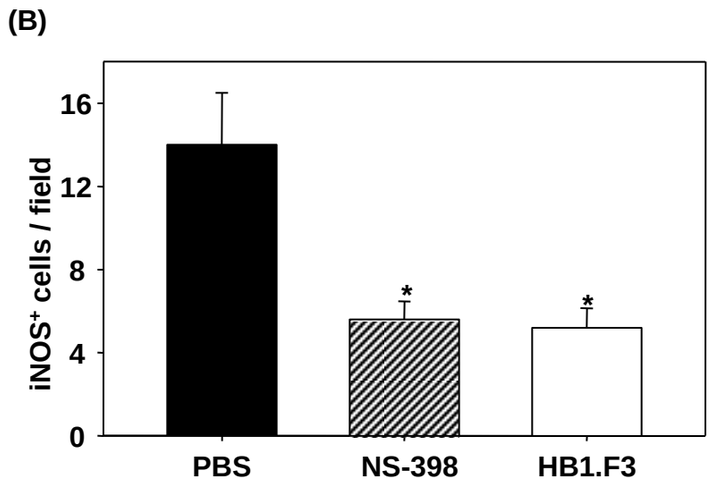


Figure 4

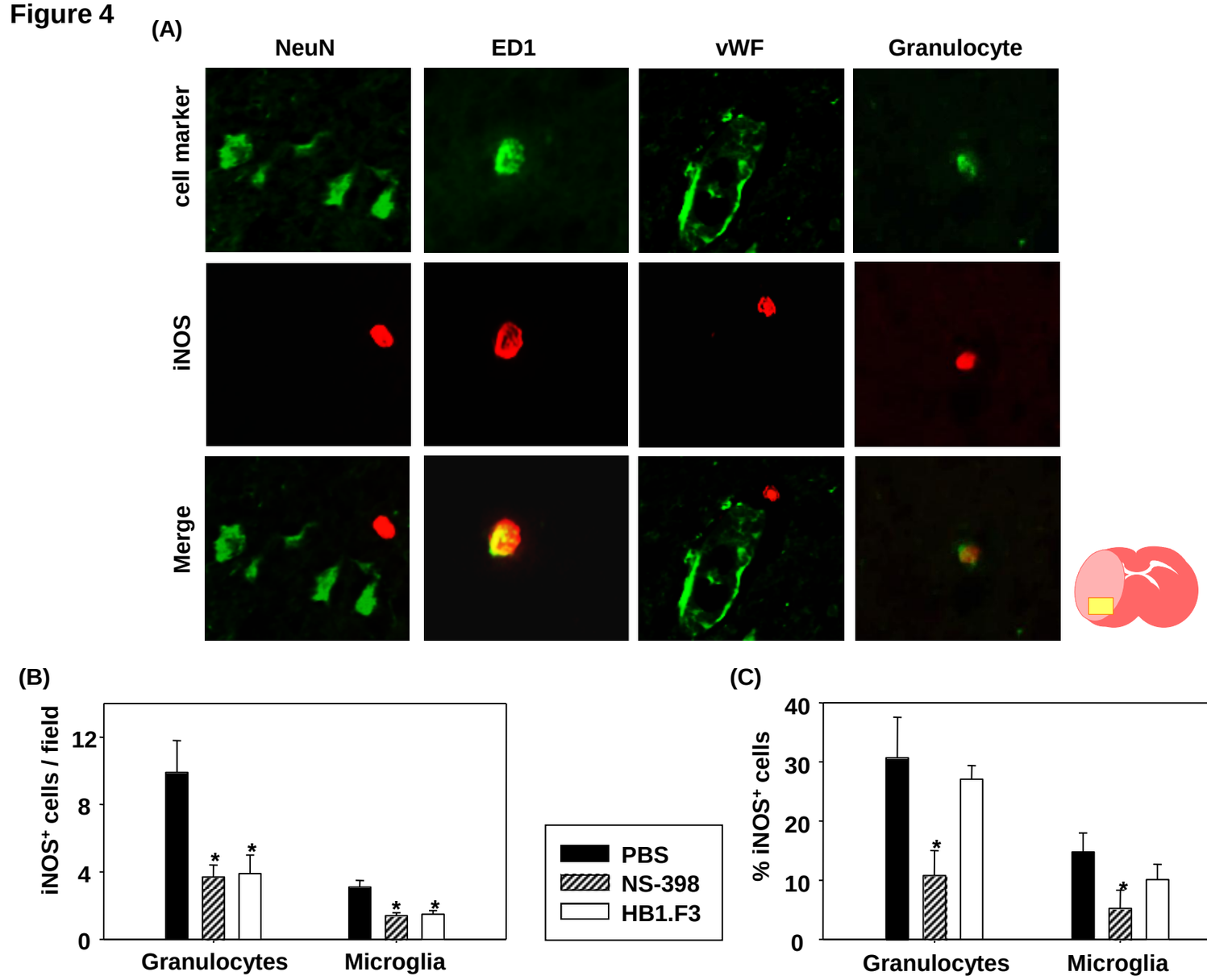

(A)
NeuN
ED1
vWF
Granulocyte
cell marker
iNOS
Merge
(B)
iNOS+ cells / field
12
8
4
0
*
Granulocytes
Microglia
PBS
NS-398
HB1.F3
(C)
% iNOS+ cells
40
30
20
10
0
*
Granulocytes
Microglia

Figure 5

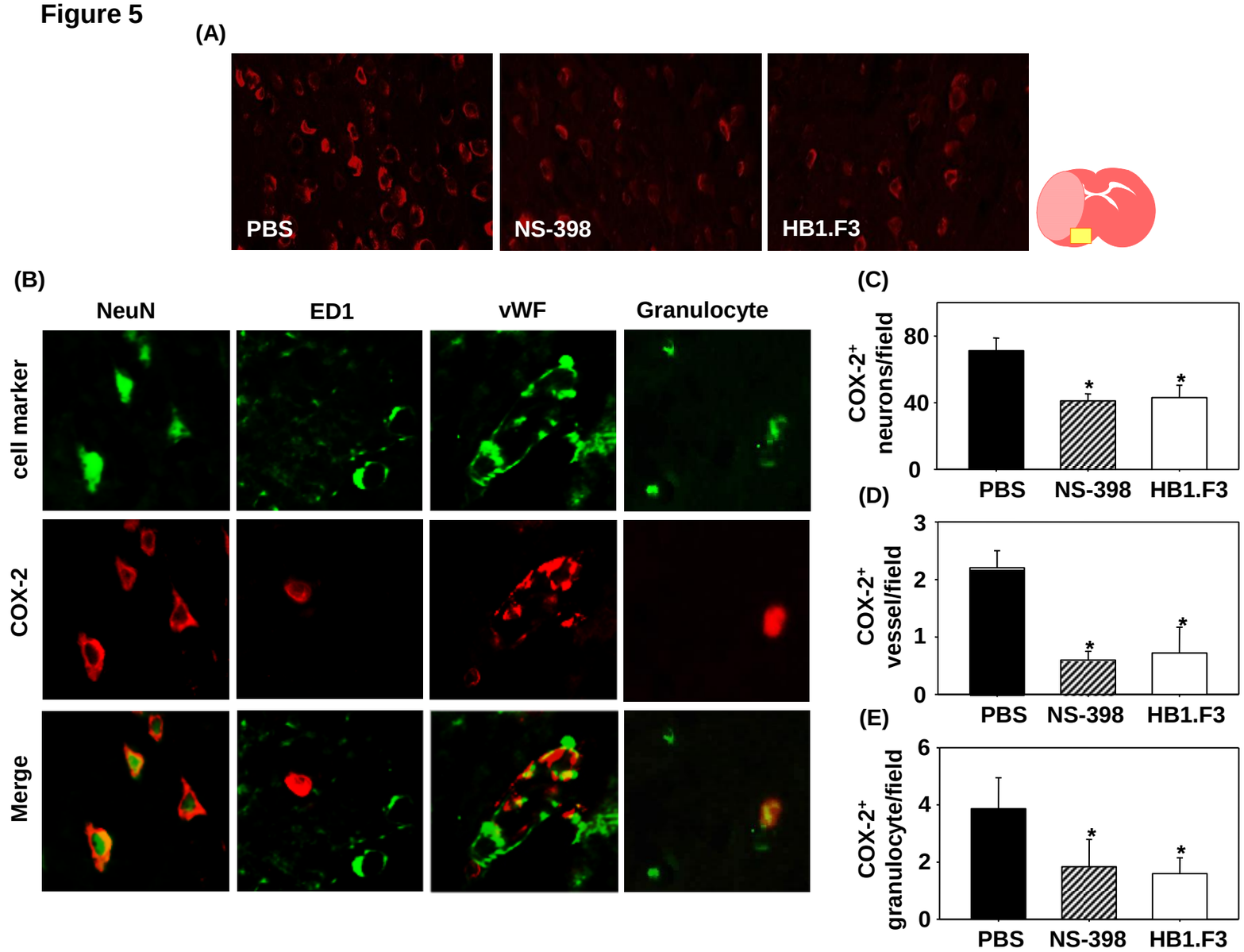
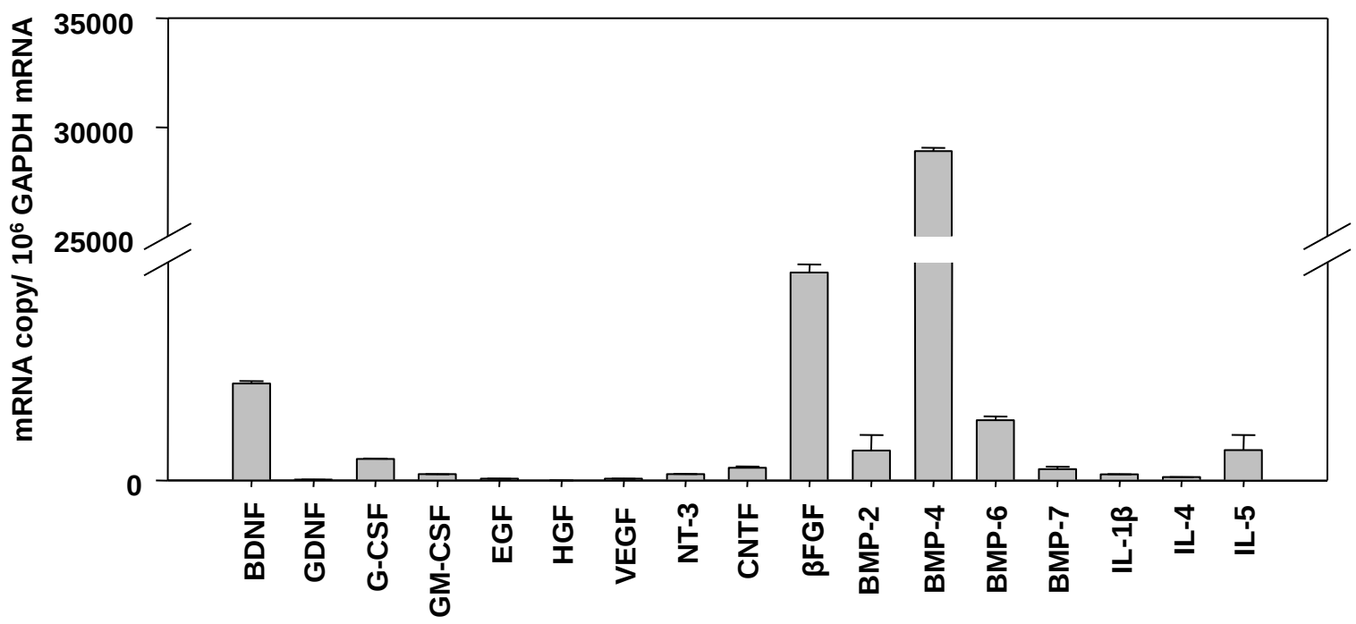

(A)
PBS
NS-398
HB1.F3
(B)
NeuN
ED1
vWF
Granulocyte
cell marker
COX-2
Merge
(C)
COX-2+ neurons/field
80
40
0
PBS
NS-398
HB1.F3
(D)
COX-2+ vessel/field
3
2
1
0
PBS
NS-398
HB1.F3
(E)
COX-2+ granulocyte/field
6
4
2
0
PBS
NS-398
HB1.F3

Figure 6


mRNA copy/ 10⁶ GAPDH mRNA
35000
30000
25000
0
BDNF
GDNF
G-CSF
GM-CSF
EGF
HGF
VEGF
NT-3
CNTF
βFGF
BMP-2
BMP-4
BMP-6
BMP-7
IL-1β
IL-4
IL-5

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