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Title	Timing and cell dose determine therapeutic effects of bone marrow stromal cell transplantation in rat model of cerebral infarct
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Citation	Neuropathology, 33(2), 140-148 https://doi.org/10.1111/j.1440-1789.2012.01335.x
Issue Date	2013-04
Doc URL	https://hdl.handle.net/2115/70801
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Type	journal article
File Information	Neuropathology33_140.pdf



ABSTRACT

Objective – Stereotactic transplantation of bone marrow stromal cells (BMSCs) enables efficient delivery to the infarct brain. This study was aimed to assess its optimal timing and cell dose for ischemic stroke.

Methods – The BMSCs were harvested from the GFP-transgenic rats and were labeled with quantum dots. The BMSCs (1×10^5 or 1×10^6) were stereotactically transplanted into the ipsilateral striatum of the rats subjected to permanent middle cerebral artery occlusion at 1 or 4 weeks post-ischemia. Motor function was serially assessed. Using *in vivo* near infrared (NIR) fluorescence imaging, the engrafted BMSCs were visualized at 3 weeks post-transplantation. Immunohistochemistry was performed to evaluate their fate.

Results – Functional recovery was significantly enhanced when both low and high doses of BMSCs were transplanted at 1 week post-ischemia, but such therapeutic effects was observed only when the high-dose BMSCs were transplanted at 4 weeks post-ischemia. Both optical imaging and immunohistochemistry revealed their better engraftment in the peri-infarct area when the high-dose BMSCs were transplanted at 1 or 4 weeks post-ischemia.

Conclusion – These findings strongly suggest the importance of timing and cell dose to yield therapeutic effects of BMSC transplantation for ischemic stroke. Earlier transplantation requires a smaller number of donor cells for beneficial effects.

(203 words)

Running title:

Optimal timing/dose of cell therapy (31 characters)

Key words:

Bone marrow stromal cell, cerebral infarct, timing, dose, and transplantation

There is increasing evidence that bone marrow stromal cells (BMSCs) may be the most appropriate source to enhance functional recovery after ischemic stroke, because they can be harvested from the patients themselves without ethical issue and can be transplanted without immunological problems. Recent studies have shown that the engrafted BMSCs survive, migrate toward injured tissue, express the proteins specific for the neural cells, and improve neurological function when transplanted into various kinds of animal models of central nervous system (CNS) disorders, including cerebral infarct^{1, 2}. Alternatively, the BMSCs are known to rescue the injured neurons by releasing neuroprotective or neurotrophic factors^{3, 4}. In fact, preliminary clinical trials of BMSC transplantation for CNS disorders have been already started⁵⁻⁷.

As recently pointed out by several researchers, however, it should be reminded that several problems to be solved still exist to launch its clinical application for CNS disorders. The issues include the optimal route, timing, and dose of donor cell delivery. Although all of them are critical to yield its therapeutic significance, there are few studies that scientifically determine the most favorable protocol even in animal experiments^{2, 8-12}. Very recently, the authors have demonstrated that intracerebral transplantation of 1×10^6 of the BMSCs at one week after the onset of permanent middle cerebral artery (MCA) occlusion significantly enhances functional recovery of rats, whereas intravenous administration of 3×10^6 of the BMSCs at the same timing does not promote it. *In vivo* near infrared (NIR) fluorescence imaging could clearly visualize their migration towards cerebral infarct during 4 weeks after transplantation in intracerebral group, but not in intravenous group. The BMSCs were widely distributed in the ischemic brain and a certain subgroup of them expresses neural cell markers in the intracerebral group, but not in the intravenous group¹³.

Based on these considerations, this study was aimed to clarify the optimal timing and dose of BMSC transplantation for cerebral infarct in rats. For this purpose, 1×10^5 or 1×10^6 of BMSC were stereotactically transplanted at 1 or 4 weeks after the onset of permanent MCA occlusion. In addition to behavioral test, *in vivo* NIR fluorescence imaging was employed to track the engrafted cells. Finally, the distribution and phenotypic fate of engrafted cells were evaluated with immunohistochemistry.

MATERIALS AND METHODS

Preparation of BMSC

All animal experiments were approved by the Animal Studies Ethical Committee of the Hokkaido University Graduate School of Medicine. Green fluorescence protein (GFP)-transgenic rats were purchased from Japan SLC, Inc., and Sprague-Dawley (SD) rats were purchased from CLEA Japan, Inc. (n=40). The BMSCs were isolated from 8-week-old GFP rat as described previously^{14, 15}. The cells were passed 3 times for subsequent experiments.

The cultured BMSCs were labeled with QD800 Q-tracker cell labeling kits (CdSeTe/ZnS:

-10 nm core-shell type, 5-10 nm polymer coating; Invitrogen, Hayward, CA, USA), as described before^{13, 15}. The cells were incubated at 37°C for 15 hr and were washed 3 times in phosphate-buffered saline (PBS) before transplantation.

Permanent MCA Occlusion Model

A focal cerebral infarct was induced by permanent occlusion of the right middle cerebral artery (MCA) with 1-hr occlusion of the bilateral common carotid arteries (CCAs), as described before¹³⁻¹⁶. Briefly, the 8-week-old male SD rats were anesthetized, and the bilateral CCA were exposed through a ventral midline incision of the cervical portion. Then, a 1.5-cm vertical skin incision was performed between the right eye and ear. The temporal muscle was scraped from temporal bone, and a 7 x 7 mm temporal craniotomy was performed, using a small dental drill. The dura mater was carefully kept intact, and the right MCA was ligated using 10-0 nylon thread through the dura mater. Then, the craniotomy was closed with the temporal bone flap. The temporal muscle and skin were sutured with 4-0 nylon threads. Subsequently, the bilateral CCAs were occluded by surgical microclips for 1 hr. Only animals that circled towards the paretic side after surgery were included in this study¹⁷.

BMSC Transplantation

The QD800-labeled BMSCs or PBS were transplanted into the ipsilateral striatum at 1 or 4 weeks after the onset of ischemia, as described previously¹³⁻¹⁶. The number of transplanted cells was 1×10^5 or 1×10^6 (n=8 in each group). Briefly, the animals were anesthetized and were fixed to a stereotactic apparatus. The skull was exposed and a burr hole was made 3 mm right to the bregma, using a small dental drill. A Hamilton syringe was inserted 6 mm into the brain parenchyma from the surface of the dura mater, and 10 μ l of cell suspension or vehicle was introduced into the striatum over a period of 5 min, using an automatic microinjection pump.

Assessment of Motor Function

Motor function of the animals was semi-quantitatively assessed before and after the onset of ischemia, using a rotarod treadmill (Model MK-630; Muromachi Kikai Co.). The rotarod was set to the acceleration mode of 4 to 40 rpm for 3 min, as described previously. The two best values of four trials in each animal were used to represent performance. The data were expressed as the percentages of those before the onset of ischemia¹³⁻¹⁶.

In vivo NIR Fluorescence Imaging

The engrafted QD800-labeled cells were visualized at 3 weeks post-transplantation, using *in vivo* NIR fluorescence imaging, as described previously^{13, 15, 18}. Briefly, the animals were anesthetized

and were transferred to the chamber of the IVIS 200 Imaging System (Xenogen Co., Alameda, CA, USA). The fluorescence emitted from QD800 was detected through the scalp and the skull, using an 800-nm emission filter and a 710-nm excitation filter. Exposure time was set to 5 seconds. On captured images, the regions of interest (ROIs) were placed on the right parietal area (ROI-1) and on the contralateral parietal area as the reference (ROI-2). Signal intensity in each ROI was expressed in the units of total efficiency (TE) defined as total efficiency (%) = total emission light (photons/seconds) / total excitation light (photons/seconds). The target-to-normal ratio (TNR) was determined by calculating the ratio of TE_{ROI-1}/TE_{ROI-2} .

Histological Analysis

At 8 weeks after the onset of ischemia, the animals were deeply anesthetized and transcardially perfused, as described previously¹³⁻¹⁶. The brain was removed, immersed in 4 % paraformaldehyde for two days and embedded in paraffin. The 4- μ m-thick coronal sections were prepared for subsequent analysis. To determine the distribution and phenotypic fate of engrafted BMSC, double fluorescence immunohistochemistry was performed, as described previously¹⁹⁻²². Each section was treated with the mouse monoclonal antibody against GFAP (dilution 1:500; BD Bioscience, Franklin Lakes, NJ, USA) or NeuN (dilution 1:100; Chemicon, Temecula, CA, USA) at 4 °C overnight and with rhodamine- conjugated goat anti-mouse antibody (dilution 1:200; Chemicon) at room temperature for 1 hr. Subsequently, the sections were treated with mouse monoclonal anti-GFP antibody (dilution 1:100, Santa Cruz) and Zenon Alexa Fluor 488 Mouse IgG Labeling Kits (dilution 1:100, Molecular Probes Inc, Eugene, OR, USA) at 4°C overnight. The fluorescence emitted was observed through appropriate filter under a fluorescence microscope (BS51; Olympus) and digitally photographed using a CCD camera equipped to the microscope (Model VB-6000/6010; Keyence Co.). To semi-quantitatively determine the distribution of engrafted cells, totally 10 ROIs (100 μ m x 100 μ m) were placed in the ipsilateral peri-infarct area, corpus callosum, and striatum. Furthermore, to analyze their capacity neural differentiation, the percentages of the cells doubly positive for GFP and NeuN or GFAP were calculated as described previously^{15, 23}.

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation. Continuous data were compared, using one- or two-factor ANOVA followed by Scheffe's post-hoc test among more than 3 groups. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Timing and Dose of BMSC Transplantation Affect Functional Recovery

All animals survived throughout the experiment and were used for subsequent analysis. As shown in Fig. 1, all animals showed severe neurological deficit during 1 week after the onset of ischemia. There was no significant difference in motor deficits among the groups at 7 days post-ischemia. When the BMSCs were stereotactically transplanted at 1 week post-ischemia, both 1×10^5 ($P < 0.05$) and 1×10^6 of them ($P < 0.05$) significantly enhanced the recovery of motor function at 4 weeks post-transplantation (Fig. 1A). On the other hands, there were no significant differences in motor deficits during 4 weeks post-ischemia among the animals that were treated with BMSCs or vehicle at 4 weeks post-ischemia. However, only the animals treated with 1×10^6 of BMSCs showed significant recovery of motor function after transplantation ($P < 0.05$, Fig. 1B).

In vivo NIR Fluorescence Imaging

In vivo NIR fluorescence imaging was performed to track the QD800-labeled BMSCs at 3 weeks post-transplantation. The fluorescence emitted from QD800 could clearly be observed in the right parietal region, *i.e.*, peri-infarct area. Representative images are shown in Fig. 2. The TNR value was 1.10 ± 0.06 in the vehicle-transplanted animals. When 1×10^5 or 1×10^6 of BMSCs were transplanted at 1 week post-ischemia, the TNR value was 1.33 ± 0.20 ($P < 0.05$) and 2.01 ± 0.49 ($P < 0.01$), respectively. On the other hands, when 1×10^5 or 1×10^6 of BMSCs were transplanted at 4 weeks post-ischemia, the TNR value was 1.33 ± 0.40 ($P > 0.05$) and 1.73 ± 0.31 ($P < 0.05$), respectively. Therefore, the intensity of NIR fluorescence was the most prominent in the peri-infarct area when 1×10^6 of BMSCs were transplanted at 1 week post-ischemia (Fig. 2).

Engraftment and Differentiation of BMSCs

The distribution of GFP-positive cells was evaluated at 8 weeks post-ischemia. Fig. 3 shows their representative distribution in the peri-infarct area. The GFP-positive cells were densely engrafted in the peri-infarct area, correlating with the findings on *in vivo* NIR fluorescence imaging. Their number was 196 ± 28 and $736 \pm 97/\text{mm}^2$ in the animals that were treated with 1×10^5 or 1×10^6 of BMSC at 1 week post-ischemia, respectively. On the other hands, their number was 190 ± 19 and $594 \pm 17/\text{mm}^2$ in the animals that were treated with 1×10^5 or 1×10^6 of BMSC at 4 weeks post-ischemia, respectively. Therefore, the number of engrafted cells in the peri-infarct area was proportional to the cell dose at each time point. Smaller number of GFP-positive cells was also identified in the ipsilateral corpus callosum and striatum.

Double fluorescence immunohistochemistry revealed that a certain subgroup of GFP-positive cells were also positive for NeuN (Fig. 4) or GFAP (Fig. 5). The percentages of the cells doubly positive for GFP and NeuN were $51.3 \pm 2.7\%$ and $66.7 \pm 5.8\%$ in the animals receiving

1×10^5 and 1×10^6 of BMSC at 7 days post-ischemia, respectively. The values were $66.7 \pm 5.8\%$ and $66.7 \pm 15.3\%$ in the animals receiving 1×10^5 and 1×10^6 of BMSC at 4 weeks post-ischemia, respectively. The percentages of the cells doubly positive for GFP and GFAP were $20.7 \pm 10.1\%$ and $6.7 \pm 5.8\%$ in the animals receiving 1×10^5 and 1×10^6 of BMSC at 7 days post-ischemia, respectively. The values were $16.7 \pm 15.3\%$ and $13.3 \pm 11.5\%$ in the animals receiving 1×10^5 and 1×10^6 of BMSC at 4 weeks post-ischemia, respectively. Thus, there were no significant differences in their neural differentiation among experimental groups.

DISCUSSION

The present study clearly shows that the timing and dose of stereotactic BMSC transplantation are critical factors to determine the therapeutic effects for ischemic stroke. Thus, there is no significant difference in functional recovery between 1×10^5 group and 1×10^6 group when the BMSCs are transplanted at one week after the onset, although *in vitro* optical imaging and histological analysis demonstrate that their distribution in the peri-infarct area differs between two groups. On the other hands, 1×10^6 , but not 1×10^5 , of BMSC significantly promote functional recovery when transplanted at 4 weeks after the onset. *In vitro* optical imaging and histological analysis also reveal that their distribution in the peri-infarct area is proportional to their therapeutic effects. Therefore, these findings strongly suggest that optimal timing and dose should precisely be evaluated when establishing the treatment protocol of BMSC transplantation for ischemic stroke.

Unresolved Issues in Cell Therapy for Ischemic Stroke

As aforementioned, some investigators have already started preliminary clinical trials of BMSC transplantation for ischemic stroke. Thus, Bang et al. intravenously injected the autologous BMSC into 5 patients with severe neurological deficits due to ischemic stroke at 5 to 9 weeks after the onset, and concluded that autologous BMSC infusion is a feasible and safe therapy that may improve functional recovery⁵. Honmou et al. intravenously transplanted the BMSC into 12 patients with ischemic stroke 36 to 133 days post-stroke⁶. Very recently, Lee et al. performed an open-label, observer-blinded clinical trial of 52 patients with ischemic stroke, and followed up them for up to 5 years. As the results, they concluded that intravenous transplantation of autologous BMSC could be safe and effective strategy for ischemic stroke⁷. These studies indicate that BMSC transplantation may be at least safe and feasible for patients with ischemic stroke. However, there are many variables that may affect the efficacy of BMSC transplantation in clinical setting. They include donor cell factors (safety, autologous or allogeneic, *ex vivo* cell expansion), patient factors (age, stroke type), treatment factors (interval since onset, delivery route, cell dose), and validation factors (neurological assessment, imaging)^{2, 8-12}.

Optimal Timing and Dose of Cell Therapy for Ischemic Stroke

In the majority of animal studies, the BMSCs are transplanted *within 24 hours or 7 days* after the onset of cerebral ischemia, whereas they are usually transplanted several weeks (or even several months) after the onset in clinical situation⁵⁻⁷. Therefore, a considerable gap of treatment protocol exists between animal experiments and clinical trials, which may correspond to “*inadequate preclinical testing*” on neuroprotective agents²⁴. The difficulty to rapidly expand the autologous BMSCs may largely explain this “*delay*” of cell therapy for patients with ischemic stroke. Especially, this discrepancy between animal experiments and clinical testing would be more distinct when considering clinical application of BMSC transplantation for the elder patients with ischemic stroke, because their BMSCs are known to have the limited capacity of *in vitro* proliferation²⁵. Therefore, it is quite important to examine the therapeutic time window of stereotactic BMSC transplantation for ischemic stroke. However, there are a limited number of studies that aimed to clarify the optimal timing and dose to promote functional recovery, using clinically relevant protocol.

Thus, Saporta et al. stereotactically transplanted 5 to 160×10^3 of the human neuroteratocarcinoma (hNT) neurons at 1 month after the onset of 1-hr MCA occlusion. They found a dose-dependent manner in functional recovery and their engraftment in the ischemic brain²⁶. Chen et al. intravenously infused 1×10^6 or 3×10^6 of BMSCs at 1 or 7 days after the onset of 2-hr MCA occlusion, and found that intravenous administration of 3×10^6 of BMSCs at 1 or 7 days post-ischemia yielded significant functional recovery²⁷. Borlongan et al. stereotactically transplanted 1 to 20×10^4 of the BMSC immediately after 1-hr MCA occlusion. They reported that cerebral blood flow and blood-brain barrier function were restored in a dose-dependent manner. However, they did not refer to the effects on functional recovery²⁸. Vendrame et al. intravenously injected 1×10^4 to 5×10^7 of the human umbilical cord blood cells at 24 hr after the onset of permanent MCA occlusion. They demonstrated a dose-dependent therapeutic effect on motor function and infarct volume²⁹. Omori et al. intravenously infused the human BMSC at a single 6 hr time point (low and high cell doses) and various multiple time points after cerebral infarct, and obtained the greatest therapeutic benefit in the animals receiving a single high cell dose injection at 6 hr post-ischemia³⁰. Stroemer et al. stereotactically transplanted 4500, 45 000, or 450 000 of the human neural stem cells into the ipsilateral striatum of rats subjected to 1-hr MCA occlusion at one month post-ischemia. As the results, they found a dose-dependent recovery of sensorimotor function³¹. Darsalia et al. stereotactically transplanted different numbers of neural stem cells into the ipsilateral striatum of the rats subjected to 30-min MCA occlusion at 48 hr or 6 weeks after the insult. They quantified the engrafted cells and concluded that better cell survival was achieved by transplantation of the cells shortly after the stroke (48 hr) as compared with a later time point (6 weeks). They further found that increasing the number of grafted cells beyond a certain number

did not result in a greater number of surviving cells. However, they did not assess the therapeutic effects on functional recovery³². Therefore, direct transplantation of the hNT neurons or human neural stem cells at one month post-ischemia would yield significant functional recovery in a dose-dependent manner.

Considering these observations, this study is the first direct evaluation of how number of transplanted BMSCs and the timing of stereotactic transplantation after ischemic stroke affect the therapeutic benefits. Thus, stereotactic transplantation therapy may require a smaller number of BMSCs to yield significant beneficial effects in early phase (*within 7 days*) of ischemia. However, a larger number of BMSCs would be crucial to achieve same therapeutic effects in late phase (*around 4 weeks*) of ischemia.

As aforementioned, the BMSCs are considered to promote functional recovery after ischemic stroke through multiple mechanisms³. A certain subpopulation of BMSCs may directly differentiate into the neural cells and replace the lost neural tissue^{33,34}. They can alter their gene expression profile in response to the surrounding microenvironment and differentiate into the neurons without evidence of cell fusion^{3,35}. Alternatively, the BMSCs may support the survival of host neurons by releasing the neuroprotective or neurotrophic factors, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). This speculation is quite natural because the BMSC *per se* support the homing and proliferation of the hematopoietic cells in the bone marrow by producing a variety of cytokines³⁶. When the BMSCs are co-cultured with the glutamate-exposed neurons under three-dimensional co-culture paradigm, the BMSCs increase their release of NGF and BDNF and significantly ameliorate neuronal damage³. The BMSCs also promote the neurite extension from the neurons in the organotypic slice of the brain and spinal cord^{4,37}. These observations may explain the reason why a smaller number of BMSCs (1×10^5) can yield therapeutic benefits similar to a larger number of them (1×10^6) in spite of worse cell survival, only when transplanted at 7 days after the insult. Thus, their neuroprotective effects may largely work and support the survival of host neural cells in early phase (*within 7 days*) of cerebral ischemia. As the results, therapeutic effects may be comparable between low-dose group and high-dose group. However, their neuroprotective actions may exhibit less pronounced effects on infarct brain in later phase (*around 4 weeks*), because tissue damages are already established. Instead, their ability of neural differentiation may mainly contribute to restore neurological function in this period, requiring a high-dose transplantation.

Cell Tracking Technique in Regenerative Medicine

Interestingly, this study clearly demonstrates that *in vivo* NIR fluorescence imaging can non-invasively provide information on the engraftment of BMSCs in the brain. The NIR-emitting quantum dots (QDs) are known to emit fluorescence with long wavelengths (>700 nm) that allow

easy penetration of the tissue, including the bone and skin. In fact, the authors have recently shown that *in vivo* NIR fluorescence imaging can serially track the QD800-labeled BMSC over 8 weeks after transplantation, when they are stereotactically transplanted into the ipsilateral striatum of the rats subjected to permanent MCA occlusion at 7 days after the insult. The intensity of NIR fluorescence gradually increases in the peri-infarct area, reaches the peak at 3 to 4 weeks after transplantation, and gradually decreases thereafter due to the denaturation of QDs. The findings on *in vivo* NIR fluorescence imaging correlate very well with those on *ex vivo* NIR fluorescence imaging and histological analysis¹⁵. Furthermore, this technique is proven valuable to distinguish the therapeutic benefits between stereotactic and intravenous BMSC transplantation for ischemic stroke¹³.

In this study, there are significant correlations between the TNR values and the number of engrafted cells in the peri-infarct neocortex, suggesting the utility of non-invasive optical imaging to quantify the cell engraftment in the living animals. These findings would provide important information on cell kinetics in the brain, when considering clinical application of BMSC transplantation for ischemic stroke. Finally, it would be essential to establish *in vivo* imaging technique to objectively evaluate the fate of donor cells in the human brain when applying cell transplantation therapy into clinical situations³⁸.

CONCLUSIONS

The present findings strongly suggest the importance of optimal timing and cell dose to yield therapeutic effects of BMSC transplantation for ischemic stroke. First, a therapeutic time window for stereotactic BMSC transplantation at least ranges from one to four weeks after the onset of ischemic stroke. Second, optimal number of BMSCs may differ in the treatment period. Earlier transplantation requires a smaller number of donor cells for beneficial effects.

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Figure Legends

Fig. 1

Rotarod treadmill performance. A) Line graph shows the temporal profiles of functional recovery in animals that were treated with vehicle (black), 1×10^5 of bone marrow stromal cells (BMSCs) (blue), and 1×10^6 of BMSCs at 1 week after the onset of permanent middle cerebral artery occlusion (MCAO). B) Line graph shows the temporal profiles of functional recovery in animals that were treated with vehicle (black), 1×10^5 of bone marrow stromal cells (BMSCs) (blue), and 1×10^5 of BMSCs at 4 week after the onset of permanent MCAO. * $P < 0.05$ and ** $P < 0.01$ compared with the vehicle-treated animals.

Fig. 2

Representative *in vivo* near infrared (NIR) fluorescence images of the animals at 3 weeks after stereotactic transplantation of vehicle (A) and 1×10^5 (B, D) or 1×10^6 of BMSCs (C, E) at 1 week (A-C) and 4 weeks after the onset of MCAO (D, E). F) Column graph shows the target to normal ratio (TNR) in each group. * $P < 0.05$ and ** $P < 0.01$ compared with the vehicle-treated animals.

Fig. 3

Photomicrographs of fluorescence immunohistochemistry at 8 weeks post-ischemia show that the GFP-positive cells (green) are distributed in the peri-infarct area in each group. Scale bar = 100 μm .

Fig. 4

Photomicrographs of double fluorescence immunohistochemistry at 8 weeks post-ischemia of the animals that were treated with 1×10^6 of BMSCs at 1 week post-ischemia, showing that a certain subgroup of the GFP-positive cells is also positive for NeuN in the peri infarct neocortex (A-C), corpus callosum (D-E), and ipsilateral striatum (G-I). Scale bar = 20 μm .

Fig. 5:

Photomicrographs of double fluorescence immunohistochemistry at 8 weeks post-ischemia of the animals that were treated with 1×10^6 of BMSCs at 1 week post-ischemia, showing that a certain subgroup of the GFP-positive cells is also positive for GFAP in the peri infarct neocortex (A-C), corpus callosum (D-E), and ipsilateral striatum (G-I). Scale bar = 20 μm .

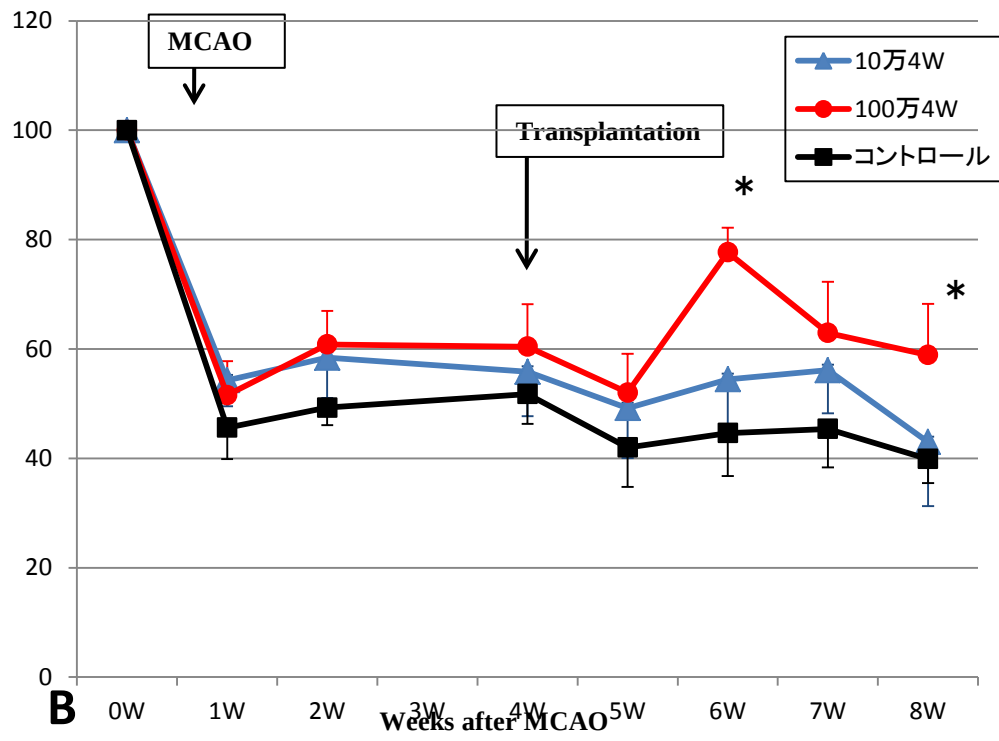
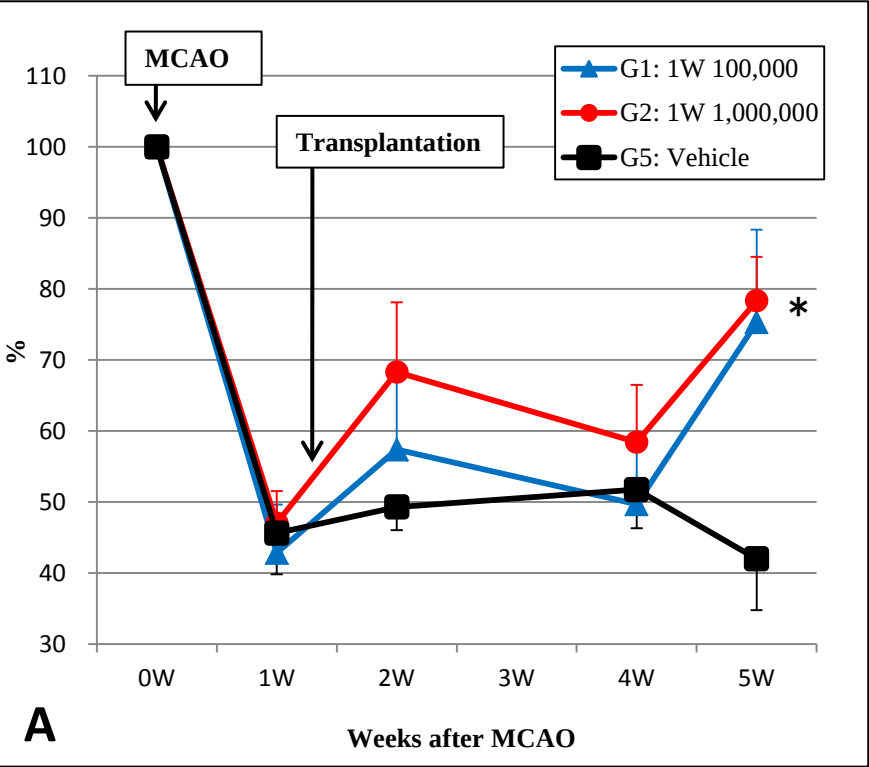


Fig. 1

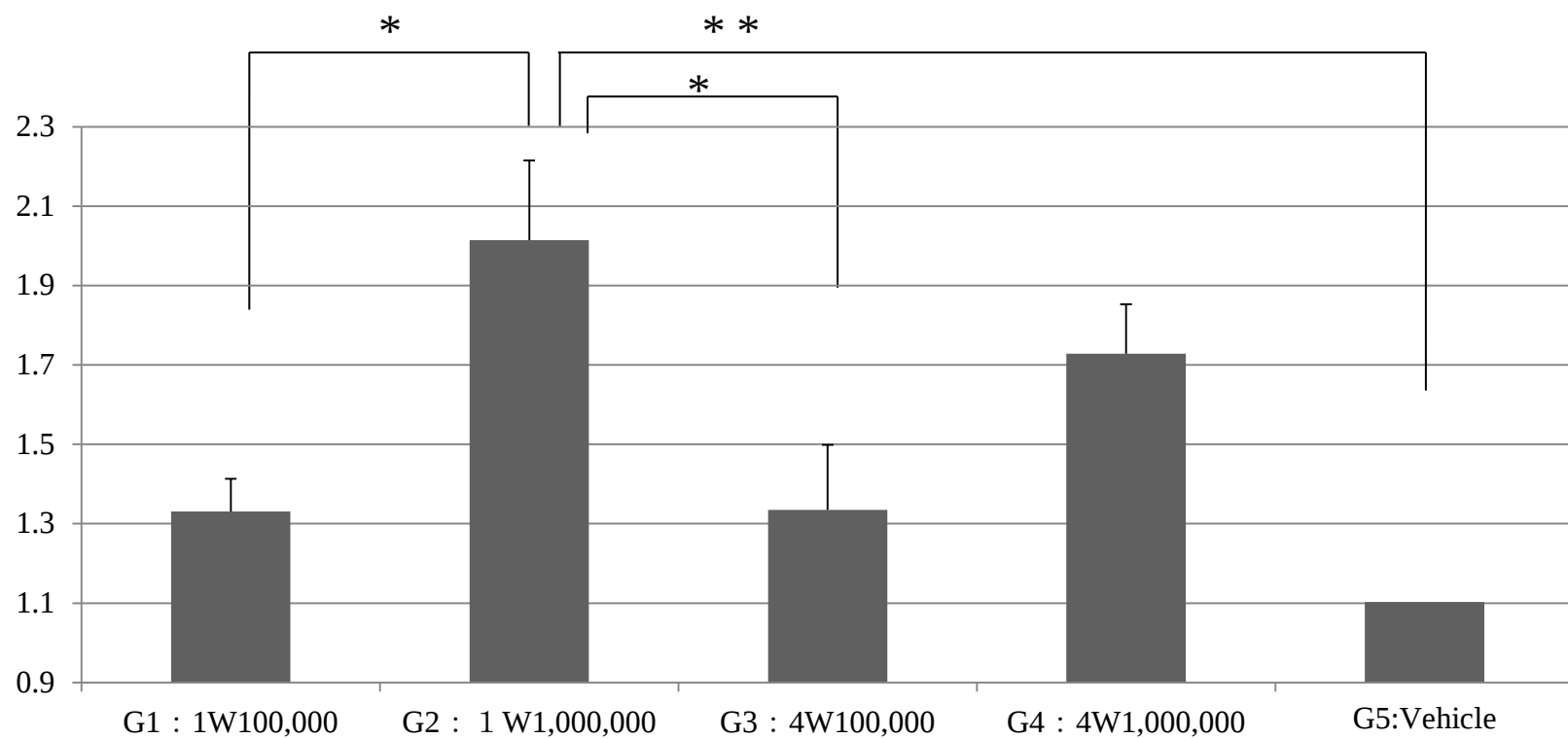
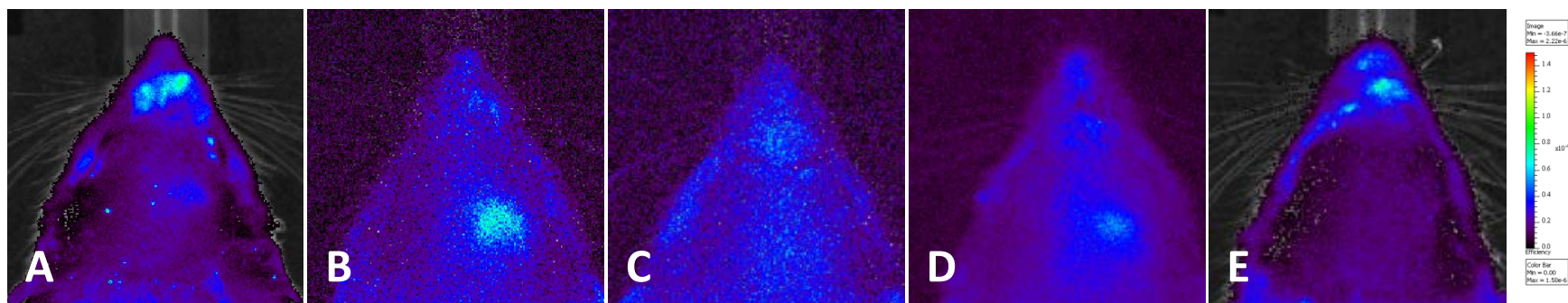


Fig. 2

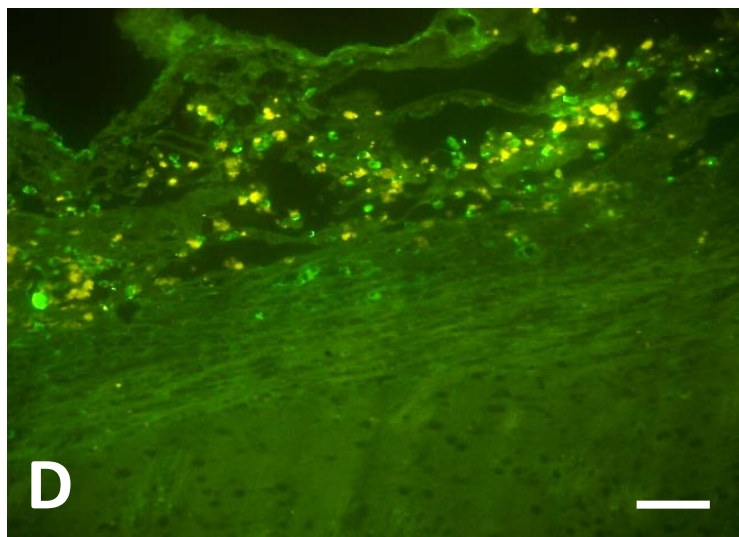
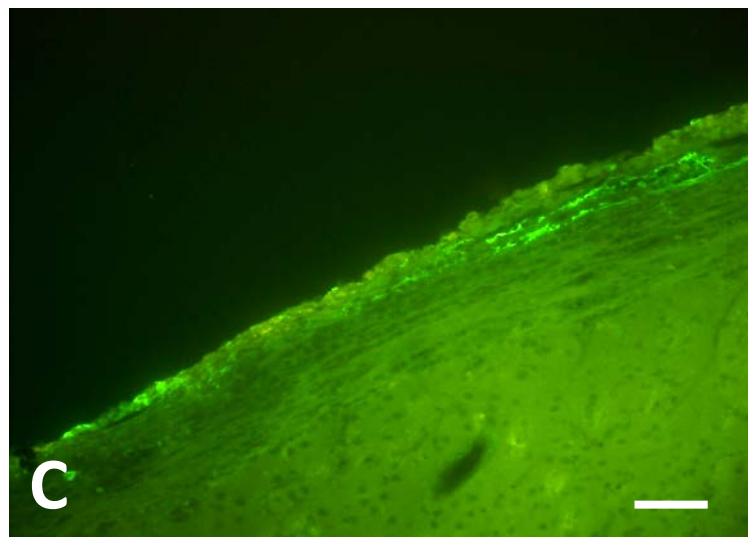
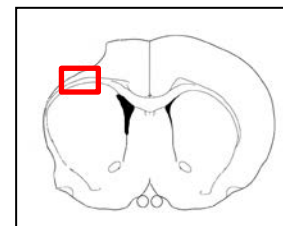
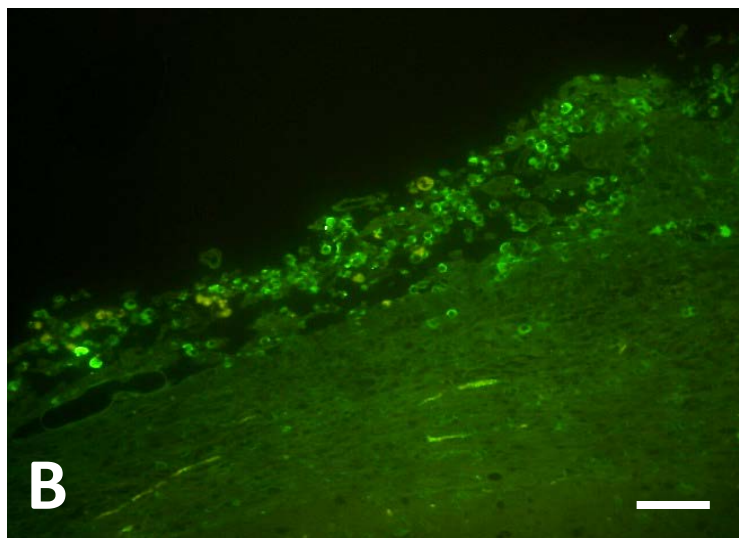
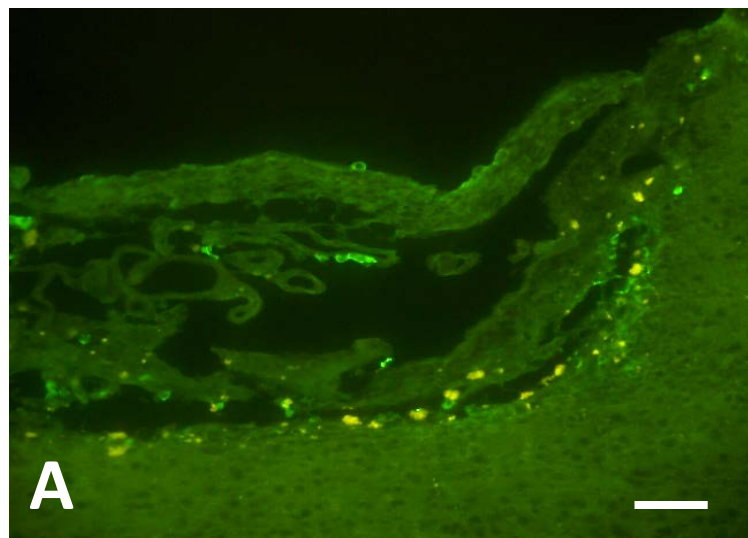


Fig. 3

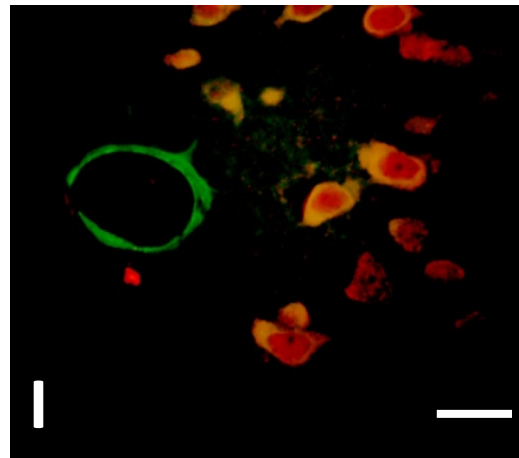
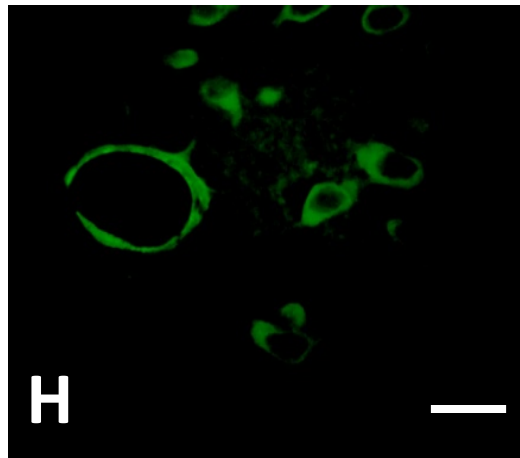
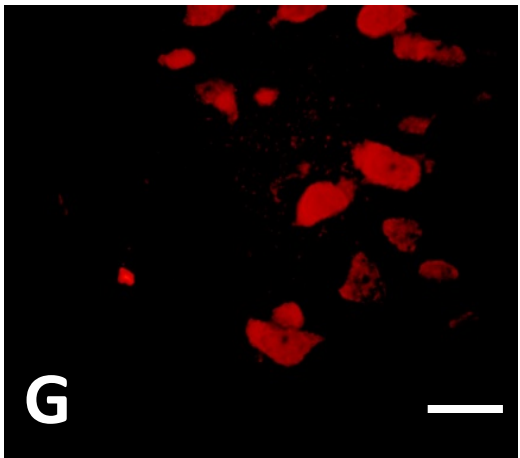
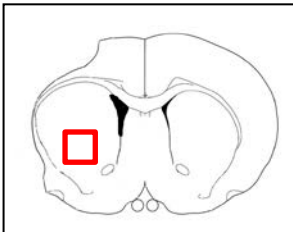
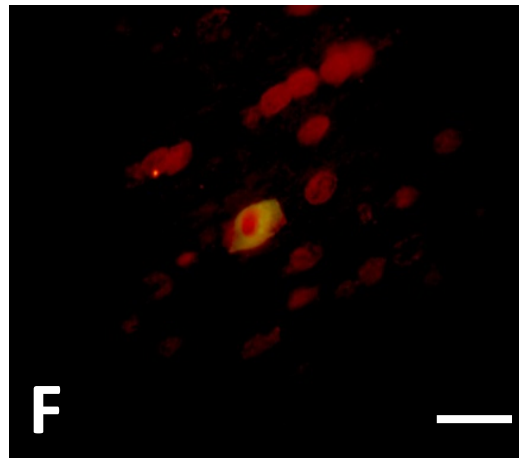
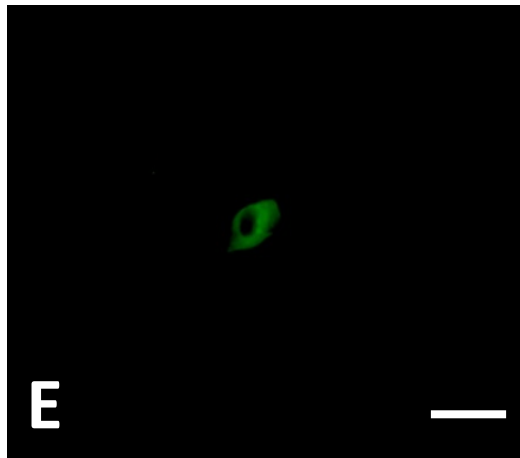
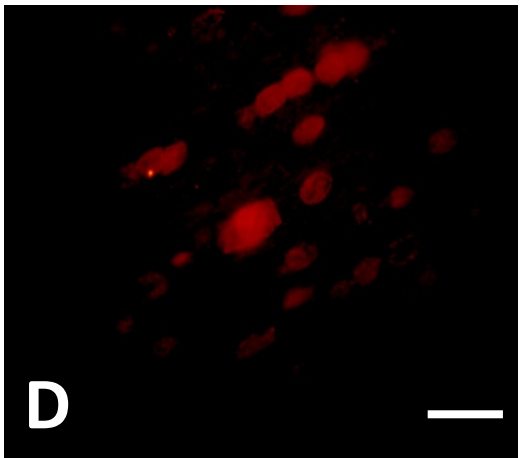
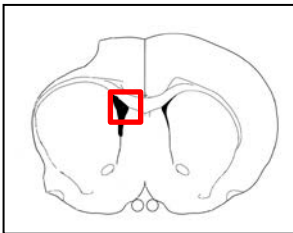
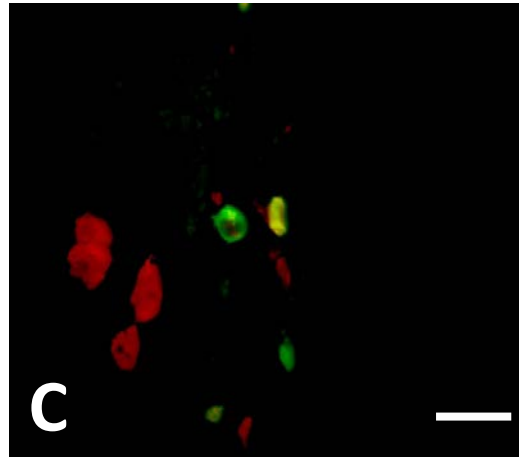
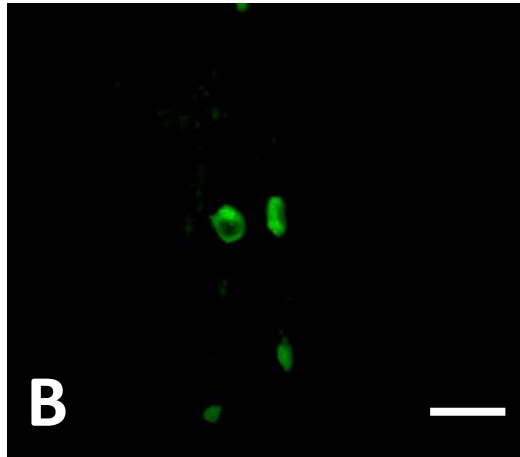
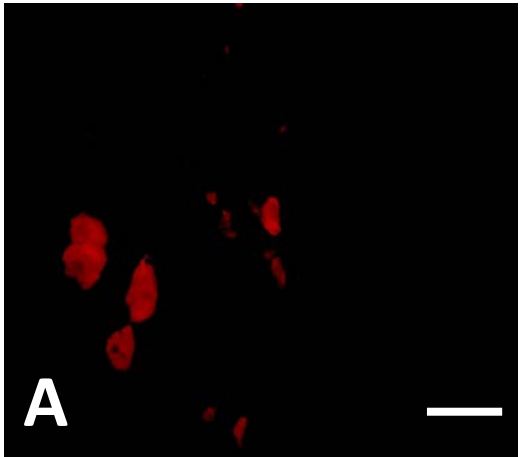
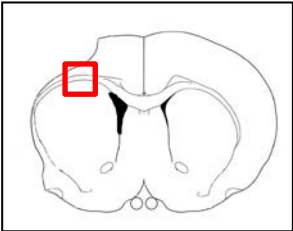


Fig. 4

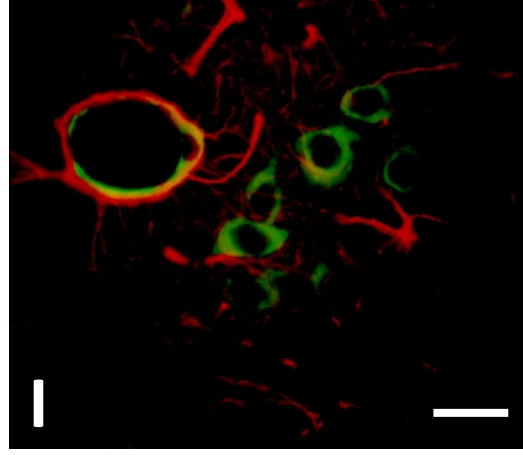
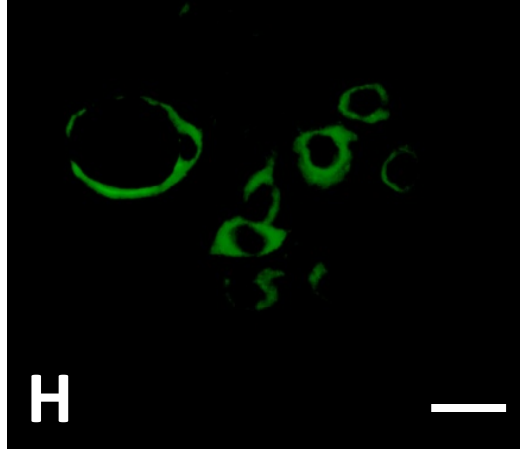
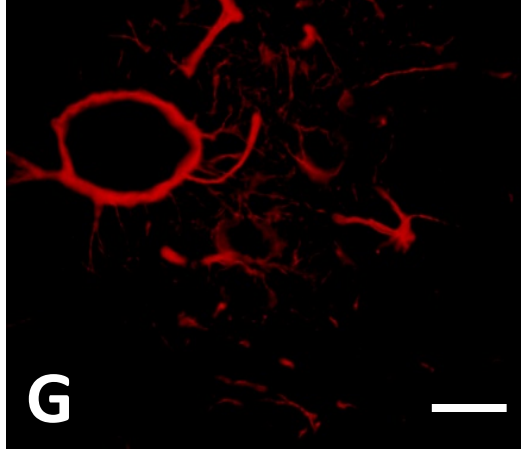
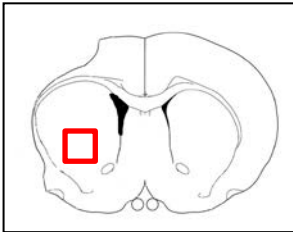
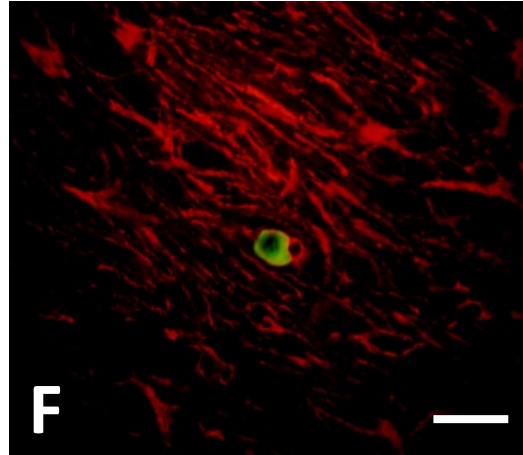
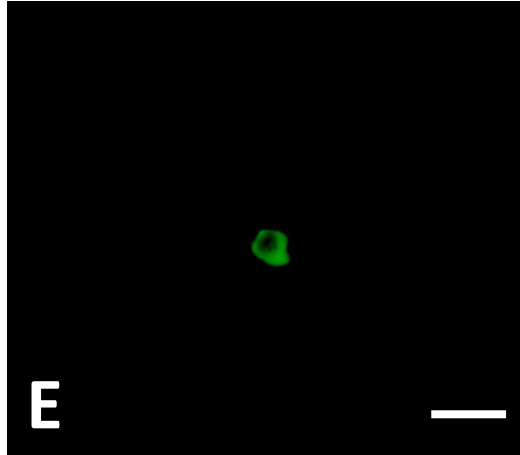
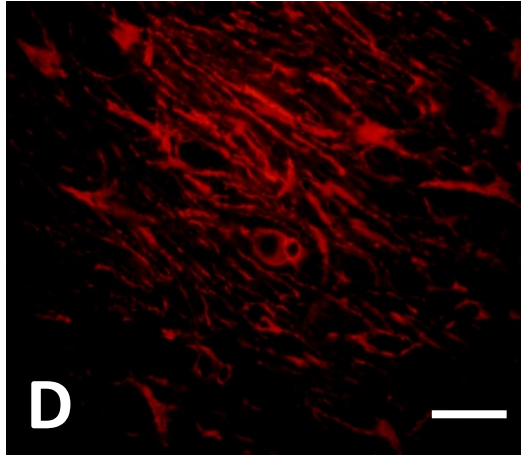
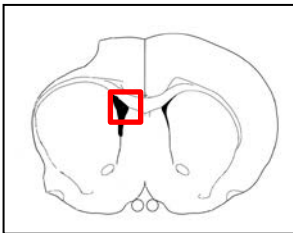
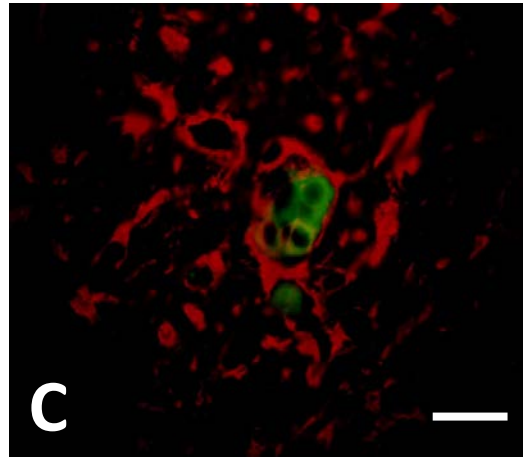
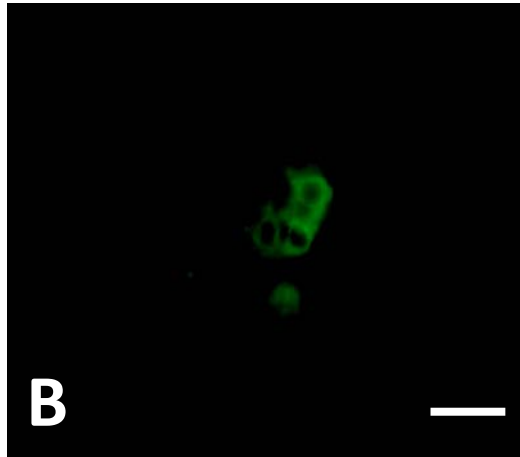
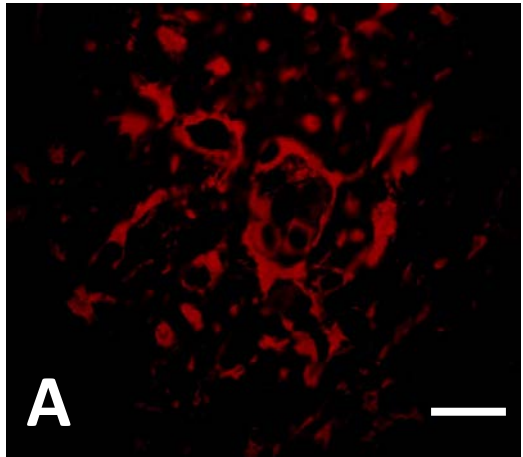
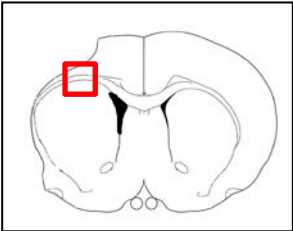


Fig. 5