



Title	Triggering Receptor Expressed on Myeloid Cells-2 Correlates to Hypothermic Neuroprotection in Ischemic Stroke
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Triggering receptor expressed on Myeloid Cells-2 (TREM-2) correlates to hypothermic neuroprotection in ischemic stroke

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Running title: TREM-2 in hypothermia

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Abstract

Hypothermia is neuroprotective against many acute neurological insults including ischemic stroke. We and others have previously shown that protection by hypothermia is partially associated with the suppression of the inflammatory. Phagocytes are thought to play an important role in the clearance of necrotic debris, paving the way for endogenous repair mechanisms to commence, but the effect of cooling and phagocytosis has not been extensively studied. Triggering receptor expressed on myeloid cells-2 (TREM2) is a newly identified surface receptor shown to be involved in phagocytosis. In this study, we examined the effect of therapeutic hypothermia on TREM2 expression. Mice underwent permanent middle cerebral artery occlusion (MCAO) and were treated with one of 2 cooling paradigms: one where cooling (30 C) began at the onset of MCAO (early hypothermia) and another where cooling began 1h later (delayed hypothermia). In both groups, cooling was maintained for 2h. A 3rd group was maintained at normothermia as a control (37C). Mice from the normothermia and delayed hypothermia groups had similar ischemic lesions sizes and neurological performance, but early hypothermia group showed marked protection as evidenced by smaller lesion size and less neurological deficits up to 30 days after the insult.

Microglia and macrophages increased after MCAO as early as 3 days, peaked at 7 days, and decreased by 14 days. Both hypothermia paradigms were associated with decreased numbers of microglia and macrophages at 3 and 7 days, with greater decreases in the early paradigm. However, the proportion of the TREM2 positive microglia/macrophages was actually increased among the early hypothermia group at day 7. Early hypothermia showed long term neurological benefit, but neuroprotection did not correlate to immune suppression. However, hypothermic neuroprotection was associated with relative increased in TREM2 expression, and suggests that TREM2 may serve a beneficial role in brain ischemia. (296/300 words)

Introduction

Hypothermia is recognized as one of the most potent neuroprotectants studied in the laboratory and clinical settings(Busto, et al., 1989, Feuerstein, et al., 1997). Recent clinical studies have established a role for therapeutic cooling in neuroprotection in some clinical conditions, including anoxic brain injury due to cardiac arrest and hypoxic ischemic neonatal encephalopathy(Yenari and Han, 2012). In contrast to many other neuroprotective treatment strategies, hypothermia influences multiple aspects of brain pathophysiology in the acute, subacute, and even in chronic stages of ischemia. It affects pathways leading to excitotoxicity, apoptosis, inflammation and free radical production, as well as blood flow, metabolism and blood-brain barrier integrity(Yenari and Han, 2012). Thus, hypothermia could be viewed as model of neuroprotection, whereby potential therapeutic targets may be identified. Post-ischemic phagocytosis is thought to be involved in the clearance of necrotic debris, paving the way for endogenous repair mechanisms to commence. Triggering receptor expressed by myeloid cells-2 (TREM2) is a newly indentified molecule described on activated microglia and macrophage(Daws, et al., 2001, Sessa, et al., 2004, Takahashi, et al., 2005), involved in innate immunity and is considered to play an important role in the phagocytosis microbes(Daws, Lanier, Seaman and Ryan, 2001). Its ligand has also been

detected in the brain(Daws, et al., 2003), and has been postulated to mediate phagocytosis of injured neurons(Daws, Sullam, Niemi, Chen, Tchao and Seaman, 2003, Hsieh, et al., 2009, Stefano, et al., 2009, Takahashi, Rochford and Neumann, 2005). In this study, we compared paradigms where hypothermia was protective and where hypothermia was not protected in a mouse stroke model to characterize the inflammatory response and TREM2 expression.

Methods

All animal experiments were approved by the San Francisco VA Medical Center's Institutional Animal Care and Use Committee (IACUC), and were in accordance with NIH guidelines.

Stroke model

A focal cerebral infarct was induced by permanent occlusion of the left middle cerebral artery (MCA) as described previously with slight modifications.(Kawabori, et al., 2012, Kawabori, et al., 2012) All surgical techniques were performed under aseptic conditions. Male C57BL/6 mice (Simonson Labs, Gilroy), weighing 20-25g, and 8-12 weeks old were used. Mice were allowed free access to food and water and housed in a

climate-controlled environment (25°C). Anesthesia was induced by inhalation of 4.0% isoflurane in N₂/O₂ (80:20%); a surgical procedure was performed under spontaneous ventilation in 1.5%-2.0% isoflurane in N₂/O₂ (80:20%).

The animals were placed on the heating pad and a 1 cm skin incision was created between the left margin of the orbit and the tragus, and the temporalis muscle was incised. A small craniotomy was made above the proximal segment of MCA, and the MCA was exposed after the dura was opened and retracted. The MCA was occluded by short coagulation with a bipolar at the MCA segment just proximal to the olfactory branch, which was consistently present. Rectal temperature was maintained between 36.5-37.5°C during the procedures, by using a thermometer connected to a heating pad (Harvard Apparatus, MA). A total of 75 mice were subjected to distal MCA occlusion (dMCAO). Mice were randomized into three groups: early hypothermia (eHT), delayed hypothermia (dHT), and normothermia (NT). In the eHT group, cooling began at the time of dMCAO and maintained for 2 hours, with a rectal temperature of 29.5-30.5°C, followed by rewarming. In the dHT group, cooling began 1 hour after dMCAO and continued for 2 hours, with a rectal temperature of 29.5-30.5°C. In NT group, rectal temperature was maintained in the normal range (36.5-37.5 °C) throughout the experiment. We previously showed that rectal temperature of 37°C and 30°C correspond

to brain temperatures of 38°C and 33°C, respectively.(Yenari, et al., 2000) The duration of anesthesia was maintained the same for all three groups (Fig. 1). Animals with no observable deficits at the time of recovery from anesthesia were removed from the experiment. The animals were sacrificed at 1, 3, 7, 14, and 30 days after ischemia for the subsequent studies (n=5/group).

Behavior studies

Focal neurological deficits were evaluated at above mentioned time points; 1, 3, 7, 14, and 30 days after dMCAO using a neurological score based on that developed by Bederson et al, and modified for use in mice as previously described(Tang, et al., 2008, Tang, et al., , Zheng, et al., 2008): grade 0 = no observable neurological deficits, grade 1 = fails to extend right forepaw, grade 2 = circles to the right, grade 3 = falls to the right, and grade 4 = cannot walk spontaneously.

In addition, the elevated body swing test (EBST) was conducted to evaluate asymmetrical motor behavior at each time-point as previously described.(Borlongan, et al., 1995) Briefly, animals were held by the tail and elevated approximately 10 cm above the bench top. The direction of the body swing, defined as an upper body turn of >10 degrees to either side, was recorded for each 30 trials. The numbers of left and

right turns were counted, and the percentage of turns made to the side contralateral to the ischemic hemisphere (right side bias) was determined.

Histological examination

After animals were perfused with the ice cold phosphate buffered saline (PBS), the brain was harvested. The brains were then sunk in 20% sucrose for overnight, and frozen at -80°C stored until use. Fresh frozen sections were cryosectioned in the coronal plane (25- μ m-thick).

Infarct Size

A series of 25- μ m-thick coronal sections from 6 brain regions (between 1.5 mm anterior and 1.5 mm posterior to the bregma) were collected at 500 μ m intervals throughout the ischemic lesion and stained with cresyl violet staining. The infarct areas in each section were measured using an image analysis system (Image J), and calculated by subtracting the normal ipsilateral area from that of the contralateral hemisphere to reduce errors due to cerebral swelling as described previously.(Kawabori, Kuroda, Ito, Shichinohe, Houkin, Kuge and Tamaki, 2012, Kawabori, Kuroda, Sugiyama, Ito, Shichinohe, Houkin, Kuge and Tamaki, 2012)

Histochemistry and Fluorescent Microscopy

Microglia were identified using lectin histochemistry. Brain sections were treated for endogenous peroxidases with 1.0 % hydrogen peroxidase. Sections were then incubated with peroxidase labeled *Griffonia simplicifolia* isolectin-B4 (IB4) (10µg/ml; Sigma-Aldrich, St. Louis; catalogue # L5391) overnight at 4°C, followed by diaminobenzidine (Vector laboratories, Burlingame), then counterstained with hematoxylin. Positive cells were countered as previously described.(Tang, Cairns, Cairns and Yenari, 2008, Tang, et al., 2007, Tang, Zheng, Giffard and Yenari, 2011). Briefly, 1-mm anterior to the bregma section was used to quantify the cell numbers, and the positive cells were optically counted from its five random non-overlapping x 400 high-power fields within the cortex adjacent to the outer boundary of the infarct as delineated by hematoxylin in four mice for each groups.

Adjacent sections were stained for CD68 to identify phagocytes. CD68 rat anti-mouse monoclonal antibody (1:200; Abcam, Cambridge, MA; catalogue # ab53444) was used as a primary antibody, followed by biotin conjugated goat anti-rat (1:1000, Santa Cruz, CA; catalogue # sc-2041). Elite Vectastain ABC kit (Vector Laboratories) and diaminobenzidine was done to visualize the proteins and hematoxylin staining was

underwent as a counterstain. In order to visualize TREM2, sections were incubated with biotinylated rat monoclonal anti mouse TREM2 (20 µg/ml; R&D systems, Minneapolis, MN; catalogue # BAF1729) and the antigen detected using an Elite Vecstain ABC kit, followed by diaminobenzidine, then counterstained with hematoxylin. In order to elucidate the proportion of TREM2 positive phagocytic cells, tissues were double labeled for CD68 and TREM2. Brain sections were blocked with normal goat serum and Streptavidin blocking solution (Vector Laboratories, Burlingame). The section was incubated in antibodies against TREM2 (20ug/ml; R&D systems, Minneapolis, MN; catalogue # BAF1729) overnight, followed by Streptavidin Alexa 594 (1:1000; Molecular Probe, catalogue # S-11227). After the sections were rinsed twice for 5 min with PBS, the sections were then incubated with rat monoclonal anti-mouse CD68 (1:1000; Abcam; catalogue # ab53444) at room temperature for 1 hour, followed by Donkey anti-rat Alexa Fluor 488 at room temperature for 1 hour. The sections were mounted on glass slides using Vector-Shield mounting medium. Slides analysis was conducted by microscopy (Zeiss Aviovert 40 CFL, Germany) and the numbers of the each positive cells were counted as described above.

Since the 60 kD heat shock protein (HSP60) has been implicated as potential ligand of TREM2,(Stefano, Racchetti, Bianco, Passini, Gupta, Panina Bordignon and

Meldolesi, 2009) brains were stained for this protein. Since this was previously described on neurons and astrocytes, brains were double labeled for HSP60, the proposed ligand for TREM2, plus MAP-2 to identify neurons and GFAP to identify astrocytes. Brain sections were reacted with mouse antibodies against HSP60 (1:50, Stressgen, catalogue # SPA-807), and rabbit polyclonal anti MAP2 (1:100, Millipore, catalogue # NG1723932,) or rabbit polyclonal anti GFAP (1:1000, abcam, catalogue # ab63366) at room temperature for 1 hour. After the sections were rinsed twice for 5 min with PBS, Alexa 594-conjugated goat anti-mouse IgG (H+L) (1:200, Invitrogen, catalogue # 835723) and Alexa 488-conjugated goat anti-rabbit IgG (H+L) (1:200, Invitrogen, catalogue # 792513) was added and incubated at room temperature for 1 hours, then mounted on glass slides using Vector-Shield mounting medium. Slides analysis was conducted by microscopy (Zeiss Aviovert 40 CFL, Germany). Positive cells were countered as described above.

Statistical Analysis

All experiments were carried out in a random fashion, and ratings were carried out by investigators blinded to the experimental manipulations. All statistical analyses were performed using Sigma Stat 3.1 (Systat Software, San Jose CA). Quantitative data

were presented as mean \pm S.E. Multiple comparisons were performed using one-way ANOVA followed by Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

Results

Early Hypothermia but Not Delayed Hypothermia Ameliorated Neurological Deficits

Neurological deficits were significantly reduced only in the eHT group in both Modified Bederson Score (Fig. 2A) and Elevated body swing test (Fig. 2B) compared with the NT group and dHT hypothermia group. eHT group showed better neurological recover compared with the NT group between Day 1 and Day 14 according to the Modified Bederson Score and between Day 1 and Day 30 in the elevated body swing test. eHT also showed better neurological recovery compare to dHT groups between Day 1 and Day7 checked by Modified Bederson Score and between Day 1 and Day 30 by elevated body swing test. However, dHT group did not show any significant neurological recovery compared with other groups.

Early Hypothermia but Not Delayed Hypothermia Decreased Infarct Size

The eHT group showed reduced infarct size by about 30% (Fig. 3) compared with NT and dHT groups. The infarct size seems to be expand through the time course

between 1D and 30D, however, eHT group showed consistent reduction of the infarct brain compared with the other groups. Representative photos of cresyl violet stained brain sections at Day 7 are shown in Fig. 3A-C. Notably early hypothermia protected peri-infarct regions.

Hypothermia Decreased Microglial Activation

Hypothermia significantly decreased numbers of activated microglia at Day 3 and Day 7 (Fig. 4). Both the eHT and dHT groups showed significant reduction of the activated microglia at Day 3 by about 30-40% (normothermia; 88.8 ± 22.8 cells/hpf, delayed hypothermia; 35.5 ± 4.2 cells/hpf early hypothermia; 25.3 ± 14.4 cells/hpf, $P < 0.01$), while only the eHT group showed significant reduction of activated microglia in Day 7 by 40% (normothermia; 188.3 ± 91.5 cells/hpf, delayed hypothermia; 147.3 ± 33.6 cells/hpf, early hypothermia; 72.9 ± 13.3 cells/hpf, $P < 0.01$) compare to the NT group. There was no difference of IB4 positive cell counts after day 14. Representative images of NT, dHT, and eHT brains and IB4 staining at Day 7 can be seen in Fig. 4 A-C, respectively.

Early Hypothermia decreased Phagocytic Activation of Myeloid Cells

Similar to that observed in IB4 positive microglia, early hypothermia significantly decreased the expression of CD68 positive phagocytes at Day 3 and at Day 7 by 4 % (normothermia; 50.6 ± 6.5 cells/hpf, delayed hypothermia; 29.6 ± 5.9 cells/hpf, early hypothermia; 2.4 ± 0.1 cells/hpf, $P < 0.01$) and 64 % (normothermia; 94.4 ± 4.9 cells/hpf, delayed hypothermia; 112.0 ± 6.0 cells/hpf, early hypothermia; 60.4 ± 7.6 cells/hpf, $P < 0.01$) compared to the NT group, respectively (Fig. 5). Representative images of CD68 stained brain sections from NT, dHT, and eHT at Day 3 are shown in Fig. 5 A-C.

Proportionately higher TREM2 expression when hypothermia is protective

TREM2 is located on the surface of the microglia and macrophages and its activation has been shown to lead to phagocytosis. Recent work indicates that TREM2 may be involved in the phagocytosis of injured or apoptotic brain cells.(Hsieh, Koike, Spusta, Niemi, Yenari, Nakamura and Seaman, 2009, Takahashi, Rochford and Neumann, 2005) We first characterized the temporal expression of the TREM2 after MCAO, and the effect of cooling. TREM2 expression amongst the normothermic controls showed little to no staining at 1 day, but markedly increased by day 3, peaking at 7 days, with a few positive cells remaining by day 30 (Fig. 6A-D). Hypothermia,

whether early or delayed decreased overall numbers of TREM2 positive cells at 3 and 14 days, but little difference was noticed at 7 and 30 days. Since TREM2 is only seen on a fraction of myeloid cells, and overall microglial/ macrophage numbers were decreased by cooling, we then normalized TREM2 counts to those of total IB4 counts, and found that the proportion of myeloid cells that expressed TREM2 were actually increased in the eHT group compared to the other groups at day 7, a time when IB4 positive cells were at a peak (Fig. 6E). However, this pattern did not persist at days 14 and 30, but interestingly, the proportion of TREM2 positive microglia reached over 80%.

To validate these observations, we then double labeled for CD68 and TREM2 at day 7. The proportion of double labeled cells to all CD68 positive phagocytes were increased for the eHT group, but not for dHT compared to NT (Fig. 7).

Hypothermia failed to influence HSP60 expression

While the precise ligand of TREM2 in the brain has defied investigators in the field for quite some time, one study implicated the 60 kD heat shock protein (HSP60).(Stefano, Racchetti, Bianco, Passini, Gupta, Panina Bordignon and Meldolesi, 2009) Thus, we carried out immunostains to determine the extent of HSP60 staining in our model. Consistent with the prior report, HSP60 was present in both neurons and

astrocytes (Fig. 8 A-F). Numbers of HSP60 positive cells increased 1-7 days post ischemia, but decreased by day 30. However, neither cooling paradigm affected the numbers of positive cells compared to NT. (Fig. 8G))

Discussion

Studies of the inflammatory response to brain infarction contribute to the development of effective neuroprotective therapies. One approach to such investigations is to delineate mechanisms rendering the effects of the already established neuroprotective strategies, such as hypothermia. In this study, we first established a model of hypothermic neuroprotection (early hypothermia) following permanent MCAO where the effects persisted out to 30 days. We then compared this to a hypothermic paradigm that was not protective (delayed hypothermia). We found that cooling, whether protective or not, led to an attenuated inflammatory response as detected by microglial staining. However, by using a marker specific to macrophages and/or phagocytic microglia (CD68), we found decreased CD68 positive cells only when hypothermia was protective. We further showed that TREM2 positive cells are present as early as a day after MCAO, but peak at about 7 days. Both hypothermic paradigms led to decreases in TREM2 positive cells at 3 and 14 days, but when these counts were normalized to the numbers of microglia/macrophages, we found that

protective hypothermia, but not non-protective hypothermia or normothermia, actually increased the proportion of TREM2 positive inflammatory cells. Interestingly, while HSP60 has been suggested as an endogenous ligand for TREM2 (Stefano, Racchetti, Bianco, Passini, Gupta, Panina Bordignon and Meldolesi, 2009), we failed to see any changes in HSP60 expression following MCAO or hypothermia.

It is now well known that the inflammatory response contributes significantly to injury after ischemia.(Barone and Feuerstein, 1999, Yenari, et al., 1998) And we and others have shown that intra-ischemic and post-ischemic hypothermia can be a neuroprotective partially by inhibiting the acute inflammatory response.(Deng, et al., 2003, Han, et al., 2002, Kim, et al., 2011, Maier, et al., 1998, Toyoda, et al., 1996) Nevertheless, the mechanisms underlying this protective effect are still not fully elucidated.

Consistent reports from numerous laboratories have shown that cooling is remarkably neuroprotective when applied especially during ischemia.(van der Worp, et al., 2010, Yenari and Hemmen, 2010) However, any mechanistic observations reported as a result of cooling have been criticized because hypothermia is thought to affect multiple pathological processes, that no one mechanism could explain the robust beneficial effect. In spite of its robust neuroprotective effect, there are still conditions

where hypothermia fails to protect. In this study, we established that a relatively brief period of cooling delayed by an hour actually fails to protect in a model of permanent MCAO. Thus, we could use these paradigms to isolate specific factors that may lead to protection, rather than factors that may simply be epiphenomenon of cooling.

While delayed cooling would have obvious clinical implications, the purpose of this study was not to test whether delayed cooling is protective or not. In fact, prior studies from other labs have shown that delayed cooling, provided the cooling is maintained for durations of 24 h or more, lead to durable and robust protection (Colbourne, et al., 2000, Yanamoto, et al., 2001). Rather, we hoped to develop a platform from which potential pathomechanisms could be compared. Here, we showed that while a non-specific inflammatory response (microglial/macrophage activation) is likely an epiphenomenon of cooling itself, one neuroprotective benefit of cooling might be the relative upregulation of TREM2 on microglia/macrophages.

Microglia are the resident myeloid-derived cells in the central nervous system that provide constant surveillance of the brain and spinal cord.(El Khoury, et al., 1998, Kreutzberg, 1996, Thomas, 1992) Studies in brain ischemia models indicate that microglia participate in the progression of ischemic injury, and are activated in response to brain ischemia and many other stressors in the brain as early as 6 hours after MCAO,

and generate a variety of damaging substances.(Deng, Han, Cheng, Sun and Yenari, 2003) Although many studies have shown that microglia exacerbate ischemic injury(Chou, et al., 2004, Giulian, et al., 1993, Huang, et al., 2010, Lehnardt, et al., 2003, Yenari, et al., 2006), there is also evidence that some aspects of the inflammatory response are important for tissue repair, including phagocytosis of cellular debris, remodeling of the extracellular matrix, and the release of cytokines and trophic factors.(Kriz, 2006, Watanabe, et al., 2000, Zhao, et al., 2006) A challenge will be to find way to selectively suppress the deleterious effects of microglial activation after stroke without compromising neurovascular repair and remodeling.

Loss-of-function mutations in either TREM2 cause Nasu-Hakola disease, a rare and fatal neurodegenerative disease also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy.(Satoh, et al., 2011) Further, recent studies now implicate TREM2 variants as a risk factor for Alzheimer's disease. (Guerreiro, et al., 2013, Jonsson, et al., 2013) The mechanisms of neurodegeneration in these disorders are still unknown, but one hypothesis is that lack of either TREM2 impairs the clearance of apoptotic neurons or beta-amyloid deposition by microglia, leading to the accumulation of necrotic debris and toxic build up of Alzheimer's related plaques. Previous work identified the microglia as the brain cells expressing the highest

levels of TREM2,(Schmid, et al., 2002, Sessa, Podini, Mariani, Meroni, Spreafico, Sinigaglia, Colonna, Panina and Meldolesi, 2004) and has been experimentally shown that TREM2 mediates microglial phagocytosis of apoptotic neurons. (Hsieh, Koike, Spusta, Niemi, Yenari, Nakamura and Seaman, 2009, Neumann, et al., 2009, Neumann and Takahashi, 2007) In vitro, TREM2 was shown to promote phagocytosis of apoptotic neurons without up-regulation of antigen presentation molecules, tumor necrosis factor- α transcripts or the release of reactive oxygen species.(Takahashi, Rochford and Neumann, 2005) Conversely, loss of TREM2 impairs phagocytosis and promotes inflammation. In an in vivo model of experimental autoimmune encephalomyelitis, blockade of TREM2 using a monoclonal antibody led to the exacerbation of immune responses with increased demyelination and worsened neurological function.(Piccio, et al., 2007) Very little has been studied about TREM2 in acute neurological insults. However, Sieber et al.(Sieber, et al., 2013) recently documented the temporal profile of TREM2 mRNA expression in a stroke model. They found that TREM2 mRNA peaked 7 d after MCAO and persisted at 28 d, observations in line with our immunohistochemical data presented here. They also documented decreased mRNA levels in several pro-inflammatory cytokines and cytokines and decreased microglial activation in TREM2 knockout mice. However, they failed to see

any effect on ischemic lesion size. Since behavioral testing was not carried out, it remains to be seen whether TREM2 plays any role in ultimate neurological outcome. Existing data would predict that TREM2 augmentation might facilitate brain repair, and Takahashi and colleagues showed that intravenous application of TREM2-rich stem cells to facilitate brain repair in experimental autoimmune encephalomyelitis.(Takahashi, et al., 2007)

In this report we found that TREM2 is upregulated on myeloid cells of the ischemic brain at about day 7 and persists. Previous work identified the microglia as the major brain cell expressing the highest levels of TREM2, with up to 57% of cortical microglia expressing TREM2.(Schmid, Sautkulis, Danielson, Cooper, Hasel, Hilbush, Sutcliffe and Carson, 2002) We should note that we do not attempt to identify the precise myeloid cell on which TREM2 was observed. We used two different commonly used markers for all myeloid cells (IB4) and phagocytes (CD68). Since circulating monocytes/macrophages are known to enter the ischemic brain, our tools do not allow us to differentiate these cells from resident brain microglia. However, we do not feel this limitation detracts from the novel observations regarding TREM2 in brain ischemia and therapeutic hypothermia.

A limitation in TREM biology is the difficulty in identifying its ligand,

particularly its endogenous ligand in the brain.(Hsieh, Koike, Spusta, Niemi, Yenari, Nakamura and Seaman, 2009, Klesney-Tait, et al., 2006, Stefano, Racchetti, Bianco, Passini, Gupta, Panina Bordignon and Meldolesi, 2009, Turnbull, et al., 2006) Initial work using a fusion protein consisting of the TREM2 molecule covalently bound to a Fc receptor identified anionic ligands belonging to various microbial moieties, but curiously, TREM2 also bound brain cells.(Daws, Sullam, Niemi, Chen, Tchao and Seaman, 2003) By using the TREM2 fusion protein plus a reporter cell line, we were previously able to show that TREM2's ligand was upregulated on and activated by apoptotic neurons, and microglial phagocytosis involved direct recognition of TREM2.(Hsieh, Koike, Spusta, Niemi, Yenari, Nakamura and Seaman, 2009) One study suggested that one potential ligand might be HSP60.(Stefano, Racchetti, Bianco, Passini, Gupta, Panina Bordignon and Meldolesi, 2009) Stimulation of HSP60 stimulated phagocytic activity of TREM2-expressing microglia, but not TREM2-deficient microglia.(Stefano, Racchetti, Bianco, Passini, Gupta, Panina Bordignon and Meldolesi, 2009) However, in the current study, we were failed to see any changes in HSP60 expression due to hypothermia. Further investigations are clearly needed to determine the ligand for TREM2 in brain ischemia. However, this is beyond the scope of the present report.

In sum, while many inflammatory responses are damaging to the ischemic brain, others may be necessary for recovery and repair. Our data here suggest that while cooling, whether neuroprotective or not, suppresses microglial and macrophage activation, and this is consistent with our prior observations (Han, Qiao, Karabiyikoglu, Giffard and Yenari, 2002). However, it is only in the setting of therapeutic cooling that there is a proportionate increase in TREM2 expression which suggests that TREM2 may confer beneficial effects in the post stroke period. Strategies to augment TREM2 might be predicted to improve stroke outcome. However, blockade of TREM2 by using gene knockout or by using specific agonist or antagonists should be considered to in the setting of protective hypothermia as defined in this model would be necessary to elucidate more precise mechanisms of TREM2.

References

- Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: New opportunities for novel therapeutics. *J Cereb Blood Flow Metab* 1999;19:819-834
- Borlongan CV, Cahill DW, Sanberg PR. Locomotor and passive avoidance deficits following occlusion of the middle cerebral artery. *Physiol Behav* 1995;58:909-917
- Busto R, Globus MY, Dietrich WD, Martinez E, Valdes I, Ginsberg MD. Effect of mild hypothermia on ischemia-induced release of neurotransmitters and free fatty acids in rat brain. *Stroke* 1989;20:904-910
- Chou WH, Choi DS, Zhang H, Mu D, McMahon T, Kharazia VN, Lowell CA, Ferriero DM, Messing RO. Neutrophil protein kinase cdelta as a mediator of stroke-reperfusion injury. *J Clin Invest*

2004;114:49-56

- Colbourne F, Corbett D, Zhao Z, Yang J, Buchan AM. Prolonged but delayed postischemic hypothermia: A long-term outcome study in the rat middle cerebral artery occlusion model. *J Cereb Blood Flow Metab* 2000;20:1702-1708
- Daws MR, Lanier LL, Seaman WE, Ryan JC. Cloning and characterization of a novel mouse myeloid dap12-associated receptor family. *Eur J Immunol* 2001;31:783-791
- Daws MR, Sullam PM, Niemi EC, Chen TT, Tchao NK, Seaman WE. Pattern recognition by trem-2: Binding of anionic ligands. *J Immunol* 2003;171:594-599
- Deng H, Han HS, Cheng D, Sun GH, Yenari MA. Mild hypothermia inhibits inflammation after experimental stroke and brain inflammation. *Stroke* 2003;34:2495-2501
- El Khoury J, Hickman SE, Thomas CA, Loike JD, Silverstein SC. Microglia, scavenger receptors, and the pathogenesis of alzheimer's disease. *Neurobiol Aging* 1998;19:S81-84
- Feuerstein GZ, Wang X, Barone FC. Inflammatory gene expression in cerebral ischemia and trauma. Potential new therapeutic targets. *Ann N Y Acad Sci* 1997;825:179-193
- Giulian D, Corpuz M, Chapman S, Mansouri M, Robertson C. Reactive mononuclear phagocytes release neurotoxins after ischemic and traumatic injury to the central nervous system. *J Neurosci Res* 1993;36:681-693
- Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, Cruchaga C, Sassi C, Kauwe JS, Younkin S, Hazrati L, Collinge J, Pocock J, Lashley T, Williams J, Lambert JC, Amouyel P, Goate A, Rademakers R, Morgan K, Powell J, St George-Hyslop P, Singleton A, Hardy J. Trem2 variants in alzheimer's disease. *N Engl J Med* 2013;368:117-127
- Han HS, Qiao Y, Karabiyikoglu M, Giffard RG, Yenari MA. Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. *J Neurosci* 2002;22:3921-3928
- Hsieh CL, Koike M, Spusta SC, Niemi EC, Yenari M, Nakamura MC, Seaman WE. A role for trem2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem* 2009;109:1144-1156
- Huang WC, Qiao Y, Xu L, Kacimi R, Sun X, Giffard RG, Yenari MA. Direct protection of cultured neurons from ischemia-like injury by minocycline. *Anat Cell Biol* 2010;43:325-331
- Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, Bjornsson S, Huttenlocher J, Levey AI, Lah JJ, Rujescu D, Hampel H, Giegling I, Andreassen OA, Engedal K, Ulstein I, Djurovic S, Ibrahim-Verbaas C, Hofman A, Ikram MA, van Duijn CM, Thorsteinsdottir U, Kong A, Stefansson K. Variant of trem2 associated with the risk of alzheimer's disease. *N Engl J Med* 2013;368:107-116
- Kawabori M, Kuroda S, Ito M, Shichinohe H, Houkin K, Kuge Y, Tamaki N. Timing and cell dose determine therapeutic effects of bone marrow stromal cell transplantation in rat model of

cerebral infarct. *Neuropathology* 2012

- Kawabori M, Kuroda S, Sugiyama T, Ito M, Shichinohe H, Houkin K, Kuge Y, Tamaki N. Intracerebral, but not intravenous, transplantation of bone marrow stromal cells enhances functional recovery in rat cerebral infarct: An optical imaging study. *Neuropathology* 2012;32:217-226
- Kim JY, Kim N, Yenari MA, Chang W. Mild hypothermia suppresses calcium-sensing receptor (casr) induction following forebrain ischemia while increasing gaba-b receptor 1 (gaba-b-r1) expression. *Transl Stroke Res* 2011;2:195-201
- Klesney-Tait J, Turnbull IR, Colonna M. The trem receptor family and signal integration. *Nat Immunol* 2006;7:1266-1273
- Kreutzberg GW. Microglia: A sensor for pathological events in the cns. *Trends Neurosci* 1996;19:312-318
- Kriz J. Inflammation in ischemic brain injury: Timing is important. *Crit Rev Neurobiol* 2006;18:145-157
- Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. Activation of innate immunity in the cns triggers neurodegeneration through a toll-like receptor 4-dependent pathway. *Proc Natl Acad Sci U S A* 2003;100:8514-8519
- Maier CM, Ahern K, Cheng ML, Lee JE, Yenari MA, Steinberg GK. Optimal depth and duration of mild hypothermia in a focal model of transient cerebral ischemia: Effects on neurologic outcome, infarct size, apoptosis, and inflammation. *Stroke* 1998;29:2171-2180
- Neumann H, Kotter MR, Franklin RJ. Debris clearance by microglia: An essential link between degeneration and regeneration. *Brain* 2009;132:288-295
- Neumann H, Takahashi K. Essential role of the microglial triggering receptor expressed on myeloid cells-2 (trem2) for central nervous tissue immune homeostasis. *J Neuroimmunol* 2007;184:92-99
- Piccio L, Buonsanti C, Mariani M, Cella M, Gilfillan S, Cross AH, Colonna M, Panina-Bordignon P. Blockade of trem-2 exacerbates experimental autoimmune encephalomyelitis. *Eur J Immunol* 2007;37:1290-1301
- Satoh J, Tabunoki H, Ishida T, Yagishita S, Jinnai K, Futamura N, Kobayashi M, Toyoshima I, Yoshioka T, Enomoto K, Arai N, Saito Y, Arima K. Phosphorylated syk expression is enhanced in nasu-hakola disease brains. *Neuropathology* 2011;32:149-157
- Schmid CD, Sautkulis LN, Danielson PE, Cooper J, Hasel KW, Hilbush BS, Sutcliffe JG, Carson MJ. Heterogeneous expression of the triggering receptor expressed on myeloid cells-2 on adult murine microglia. *J Neurochem* 2002;83:1309-1320
- Sessa G, Podini P, Mariani M, Meroni A, Spreafico R, Sinigaglia F, Colonna M, Panina P, Meldolesi J. Distribution and signaling of trem2/dap12, the receptor system mutated in human polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy dementia. *Eur J Neurosci* 2004;20:2617-2628
- Sieber MW, Jaenisch N, Brehm M, Guenther M, Linnartz-Gerlach B, Neumann H, Witte OW, Frahm C. Attenuated inflammatory response in triggering receptor expressed on myeloid cells 2 (trem2)

- knock-out mice following stroke. *PLoS One* 2013;8:e52982
- Stefano L, Racchetti G, Bianco F, Passini N, Gupta RS, Panina Bordignon P, Meldolesi J. The surface-exposed chaperone, hsp60, is an agonist of the microglial trem2 receptor. *J Neurochem* 2009;110:284-294
- Takahashi K, Prinz M, Stagi M, Chechneva O, Neumann H. Trem2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med* 2007;4:e124
- Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 2005;201:647-657
- Tang XN, Cairns B, Cairns N, Yenari MA. Apocynin improves outcome in experimental stroke with a narrow dose range. *Neuroscience* 2008;154:556-562
- Tang XN, Wang Q, Koike MA, Cheng D, Goris ML, Blankenberg FG, Yenari MA. Monitoring the protective effects of minocycline treatment with radiolabeled annexin v in an experimental model of focal cerebral ischemia. *J Nucl Med* 2007;48:1822-1828
- Tang XN, Zheng Z, Giffard RG, Yenari MA. Significance of marrow-derived nicotinamide adenine dinucleotide phosphate oxidase in experimental ischemic stroke. *Ann Neurol* 2011;70:606-615
- Thomas WE. Brain macrophages: Evaluation of microglia and their functions. *Brain Res Brain Res Rev* 1992;17:61-74
- Toyoda T, Suzuki S, Kassell NF, Lee KS. Intraischemic hypothermia attenuates neutrophil infiltration in the rat neocortex after focal ischemia-reperfusion injury. *Neurosurgery* 1996;39:1200-1205
- Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L, Hernandez M, Colonna M. Cutting edge: Trem-2 attenuates macrophage activation. *J Immunol* 2006;177:3520-3524
- van der Worp HB, Macleod MR, Kollmar R. Therapeutic hypothermia for acute ischemic stroke: Ready to start large randomized trials? *J Cereb Blood Flow Metab* 2010;30:1079-1093
- Watanabe H, Abe H, Takeuchi S, Tanaka R. Protective effect of microglial conditioning medium on neuronal damage induced by glutamate. *Neurosci Lett* 2000;289:53-56
- Yanamoto H, Nagata I, Niitsu Y, Zhang Z, Xue JH, Sakai N, Kikuchi H. Prolonged mild hypothermia therapy protects the brain against permanent focal ischemia. *Stroke* 2001;32:232-239
- Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. *Nat Rev Neurosci* 2012;13:267-278
- Yenari MA, Hemmen TM. Therapeutic hypothermia for brain ischemia: Where have we come and where do we go? *Stroke* 2010;41:S72-74
- Yenari MA, Kunis D, Sun GH, Onley D, Watson L, Turner S, Whitaker S, Steinberg GK. Hu23f2g, an antibody recognizing the leukocyte cd11/cd18 integrin, reduces injury in a rabbit model of transient focal cerebral ischemia. *Exp Neurol* 1998;153:223-233
- Yenari MA, Onley D, Hedehus M, deCrespigny A, Sun GH, Moseley ME, Steinberg GK. Diffusion- and

perfusion-weighted magnetic resonance imaging of focal cerebral ischemia and cortical spreading depression under conditions of mild hypothermia. *Brain Res* 2000;885:208-219

Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia potentiate damage to blood-brain barrier constituents: Improvement by minocycline in vivo and in vitro. *Stroke* 2006;37:1087-1093

Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, Wang X, Lo EH. Role of matrix metalloproteinases in delayed cortical responses after stroke. *Nat Med* 2006;12:441-445

Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA. Anti-inflammatory effects of the 70 kda heat shock protein in experimental stroke. *J Cereb Blood Flow Metab* 2008;28:53-63

Figure Legends

Figure 1.

Schematic of the experimental paradigms. Mice were randomly allocated in to 3 groups. In the normothermia group (NT), body temperature was maintained between 36.5-37.5 °C during the course of the experiment. In the delayed hypothermia group (dHT), hypothermia (29.5-30.5 °C) was induced one hour after the ischemic insult (distal MCA occlusion; dMCAO), and maintained for 2 hours. In the early hypothermia group (eHT), cooling began at the time of dMCAO and continued for 2 hours followed by rewarming to the normal temperature. The duration of the anesthesia was maintained the same for all three groups. Dotted lines show the period of cooling.

Figure 2.

Early hypothermia showed significantly less neurological deficit compared to

normothermia and delayed hypothermia (A: Modified Bederson Score, B: Elevated Body Swing Test). ($\star p < 0.05$ vs normothermia, $\star\star p < 0.01$ vs normothermia, $\star P < 0.05$ vs delayed hypothermia, $\star\star P < 0.01$ vs delayed hypothermia)

Figure 3.

Early, but not delayed hypothermia is protective. Images of representative cresyl violet stained brain sections from the normothermia (A; NT), delayed hypothermia (B; dHT), and early hypothermia (C; eHT) groups are shown at 7 days post MCAO. Only the early hypothermia group showed significant reduction of the infarction by about 30% compared to NT and dHT (D). ($\star p < 0.05$ vs normothermia and delayed hypothermia)

Figure 4.

Hypothermia decreased numbers of microglia and macrophages post MCAO.

Representative micrographs of isolectin B4 (IB4) microglia/macrophages 7 days post MCAO (A; normothermia, B; delayed normothermia, C; early hypothermia). Both the delayed hypothermia (B; dHT) and early hypothermia (C; eHT) showed significant reduction of the activated microglia at Day 3 (D), while only the eHT group showed

marked reduction of them at Day 7 compared to the other groups. (* $p < 0.01$) Bar = 40 μm

Figure 5.

Early, but not delayed hypothermia decreased numbers of macrophages.

Representative photographs of CD68 positive phagocytes at Day 7 (A; normothermia, B; delayed normothermia, C; early hypothermia). The early hypothermia group showed marked reduction at Day 3 and at Day 7 compared to the other two groups. (* $p < 0.01$)

Bar = 40 μm

Figure 6.

Hypothermia decreased overall TREM2 expression, but increased the proportion

of TREM2 positive macrophages. Representative photographs of TREM2 positive cells at Days 7 (A; normothermia, B; delayed normothermia, C; early hypothermia) and 14 (D; normothermia, E; delayed normothermia, F; early hypothermia). G: TREM2 expression amongst the normothermia group showed little to no staining at day 1, but markedly increased by day 3, peaking at day 7, and with a few positive cells remaining by 30 day. Hypothermia, whether early or delayed, decreased numbers of TREM2

positive cells at day 3 and 14, but little difference was noticed at Day 7 and 30. H: TREM2 counts normalized to total IB4 counts suggest that the proportion of TREM2 microglia/macrophages is higher in the early hypothermia group compared to the other 2 groups at Day 7. (* $p < 0.01$) Bar = 40 μm

Figure 7.

Early, but not delayed hypothermia increased the proportion of TREM2 positive macrophages. Representative micrographs of double labeled TREM2 and CD68 positive phagocytes. A brain from the early hypothermia group at Day 7 shows that TREM2 is expressed in CD68 positive cells at the infarct border (red; TREM2, green; CD68, blue; DAPI) (A). The proportion of the TREM2 positive CD68 positive phagocytes was significantly increased in the early hypothermia group (B). High power views of the staining is shown in C-F. TREM2 (D) is highly co-localized with CD68 positive cells (C). DAPI (E). Merged image (F) (* $p < 0.01$) Bar = 80 μm

Figure 8.

Immunostaining for HSP 60, the proposed ligand for TREM2 is unaffected by hypothermia. DAB staining for HSP 60 revealed that ischemic insult up-regulates its

expression (A; Sham, B; ischemic penumbra at day 7). However, cooling paradigm did not changed the numbers of HSP 60 positive at any time point (C). Brains were double labeled for HSP60 (red, E, I) plus a neuron (MAP2, D) or astrocyte (GFAP, H) marker, and counterstained with DAPI (F, J). HSP 60 was present in both neurons (G) and astrocytes (K). Stains from day 7 are shown.

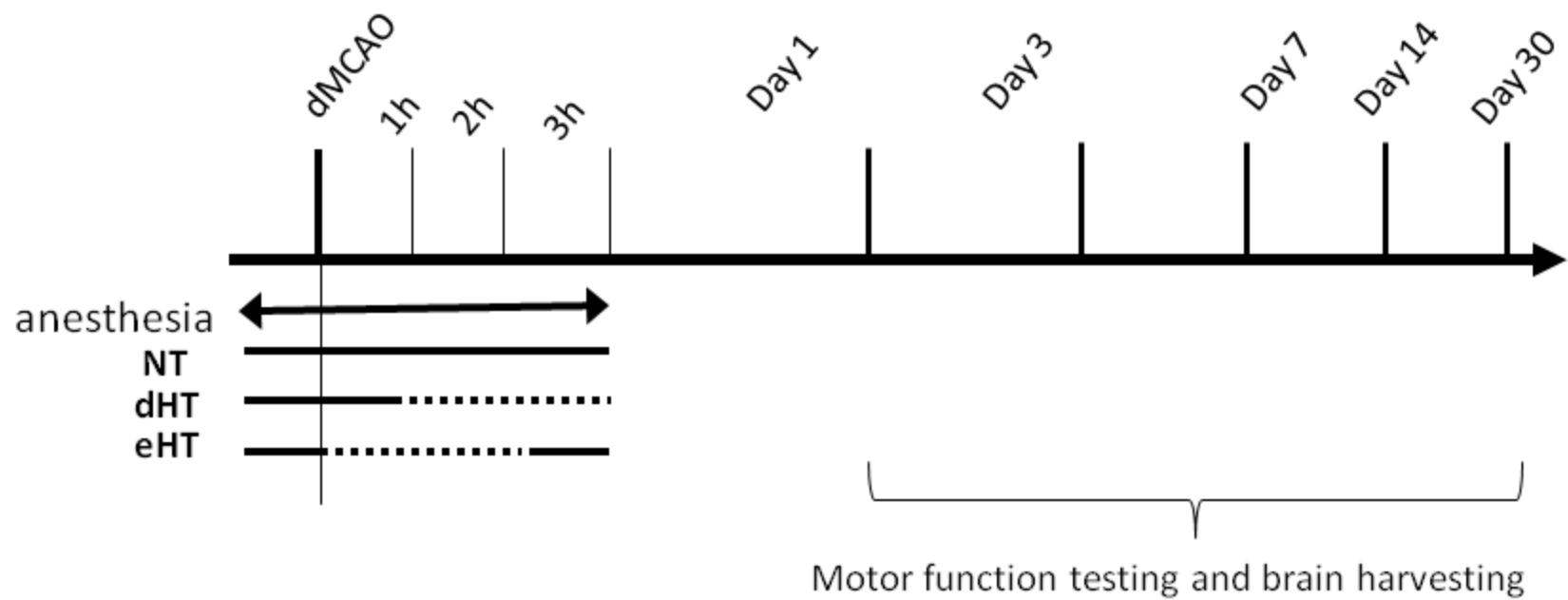


Fig.1

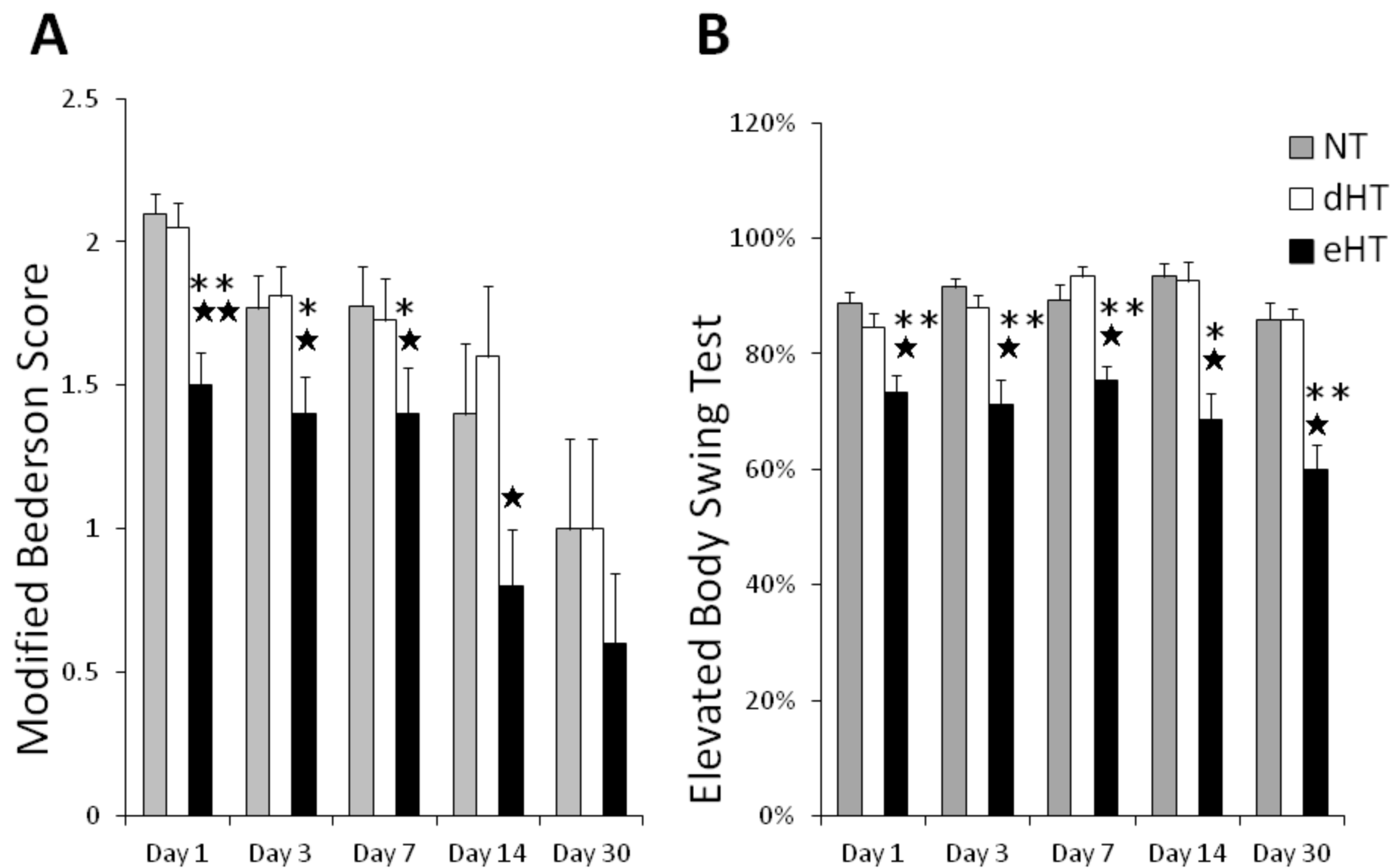


Fig.2

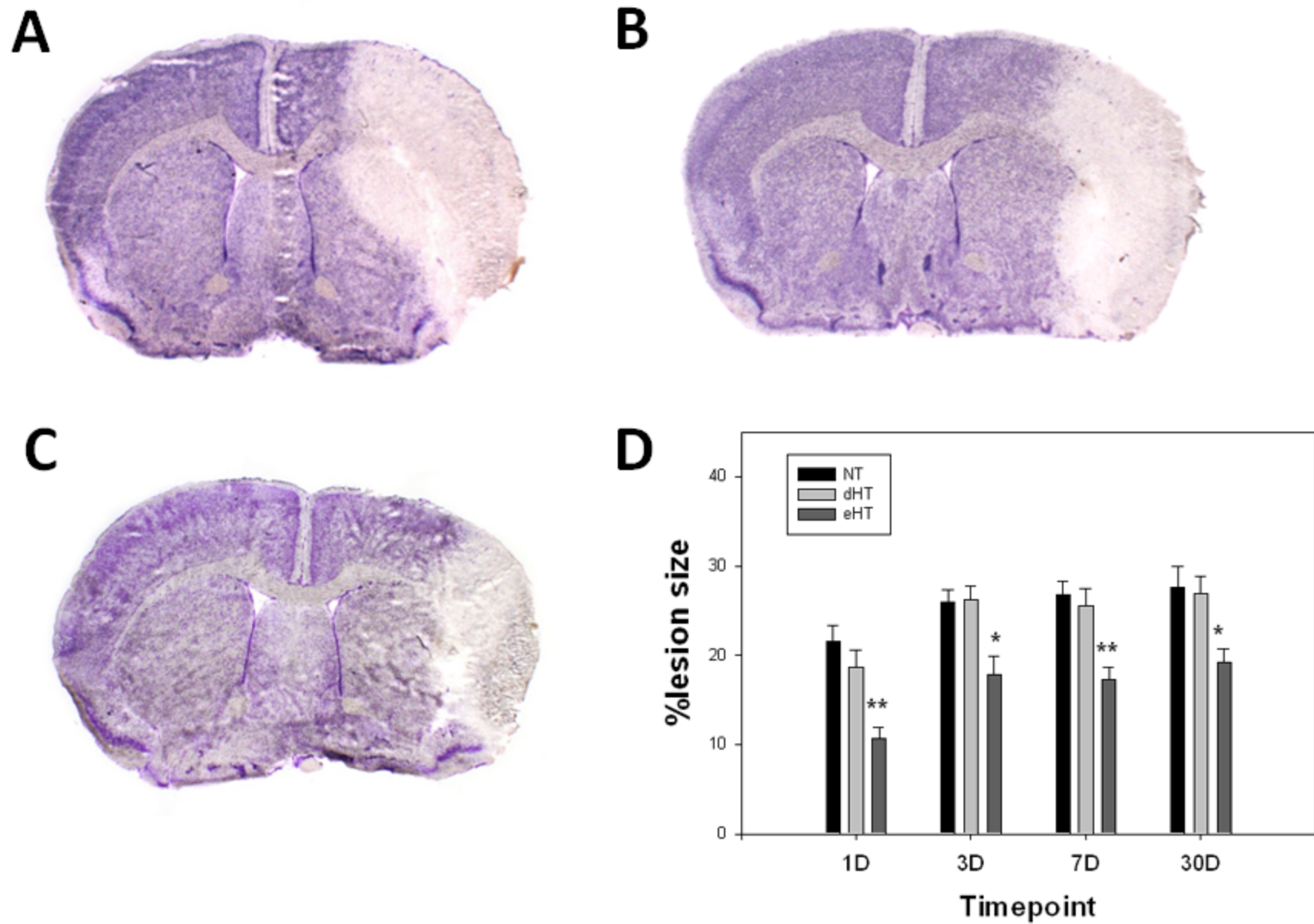


Fig.3

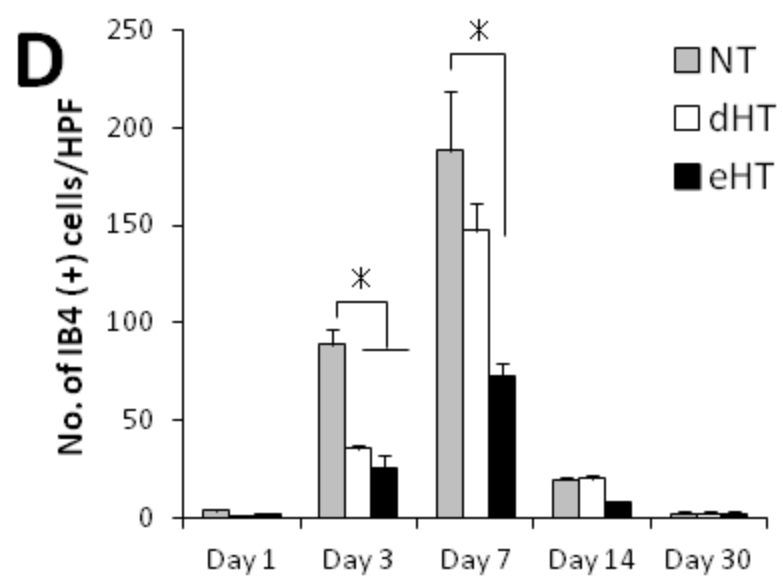
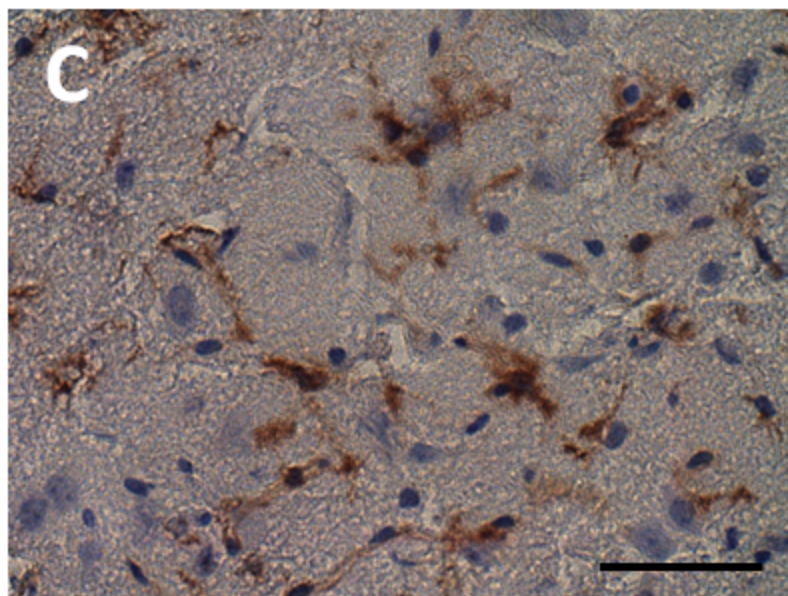
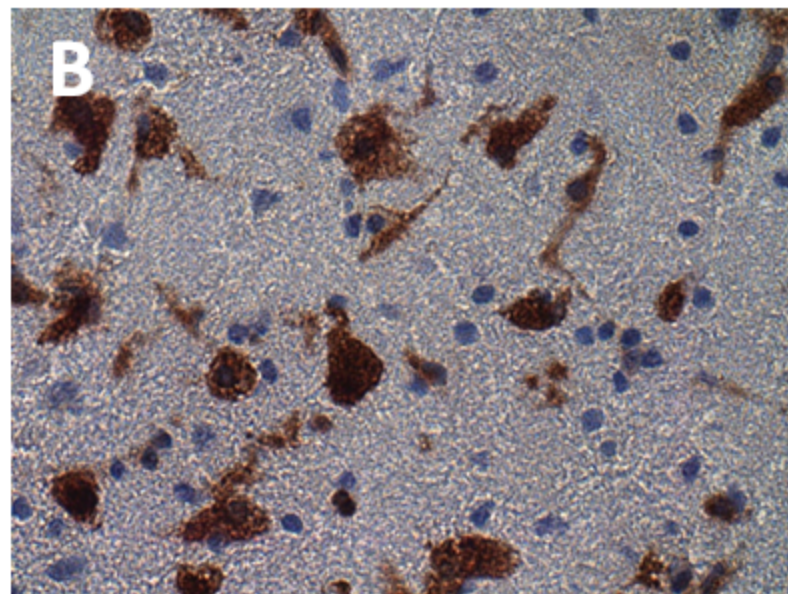
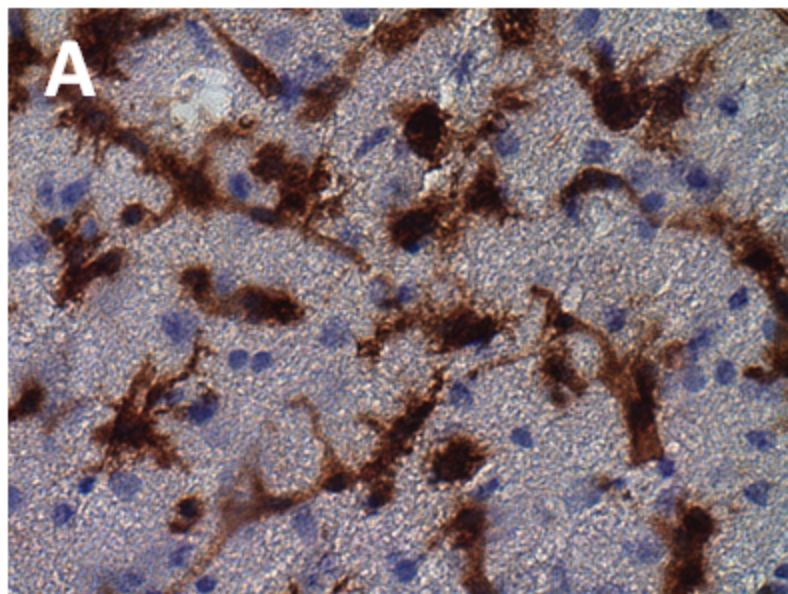


Fig.4

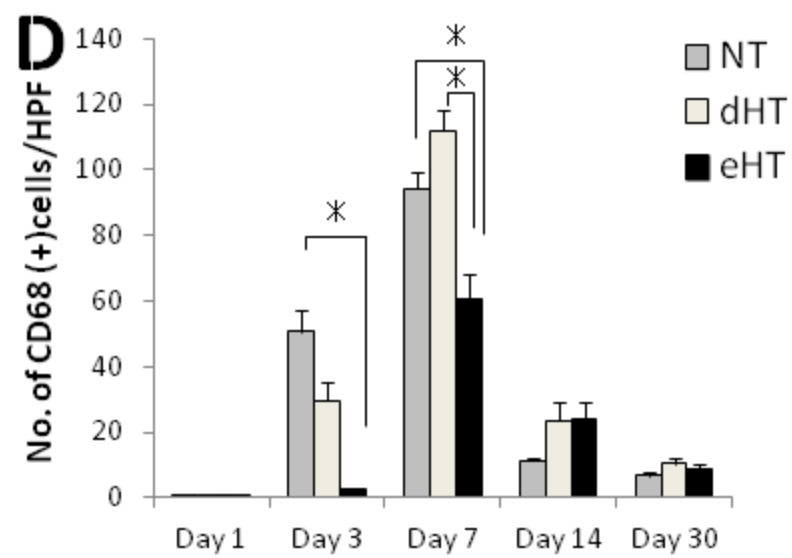
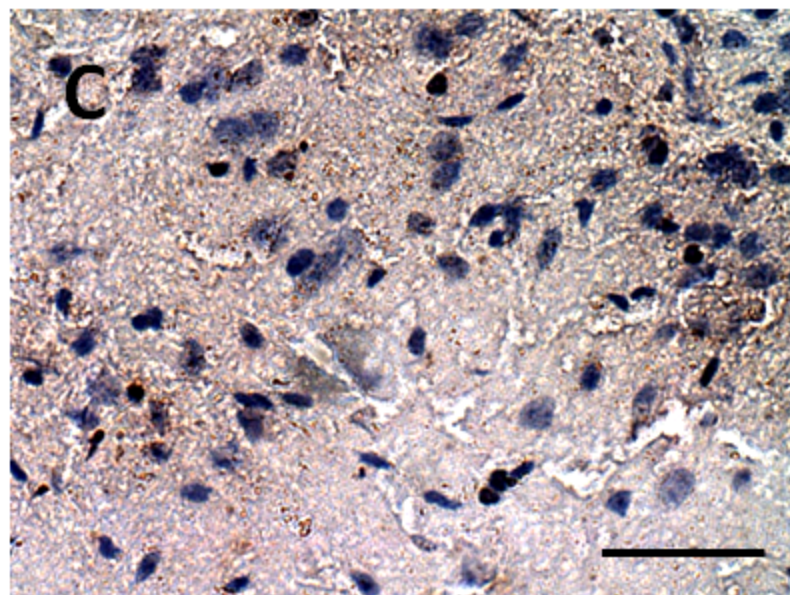
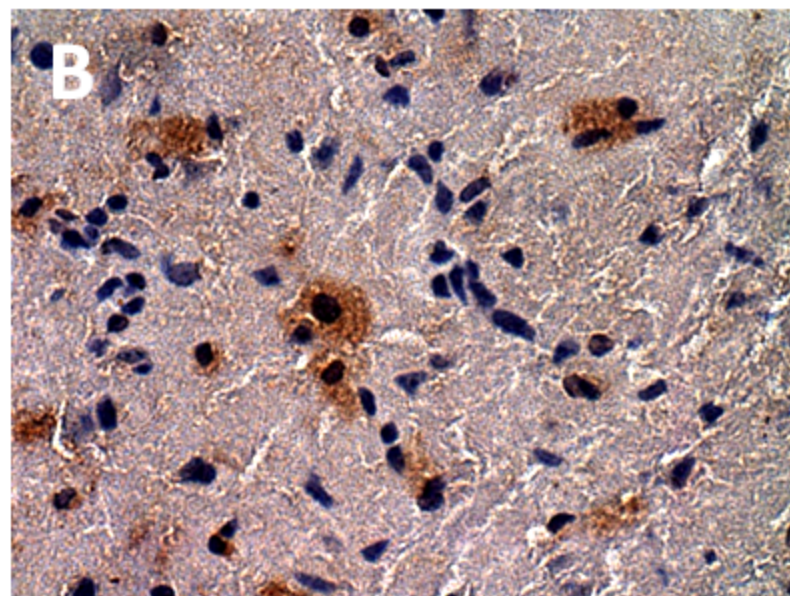
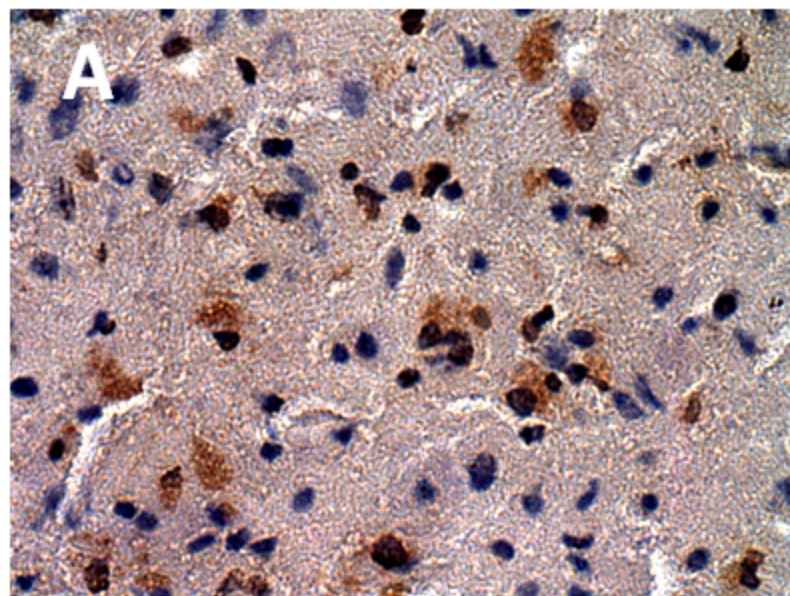


Fig.5

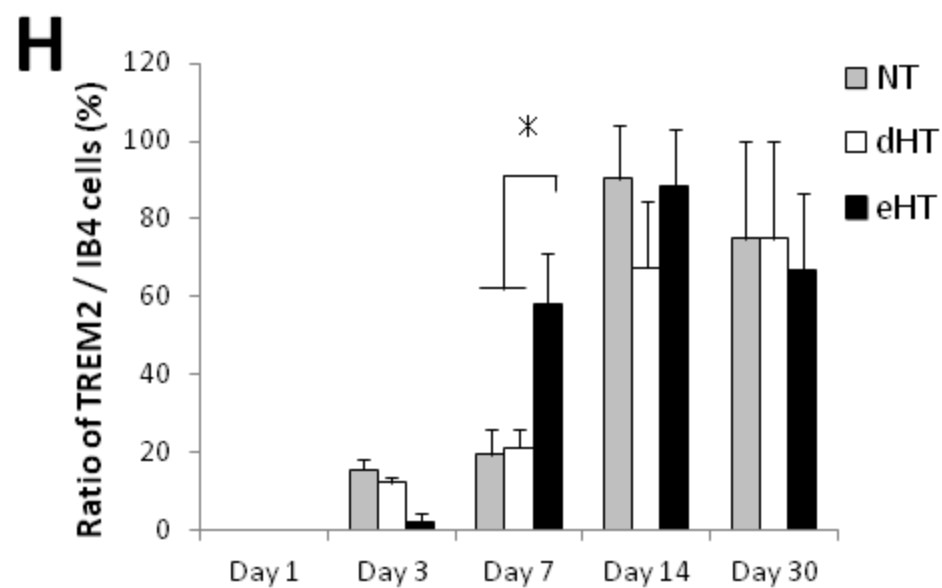
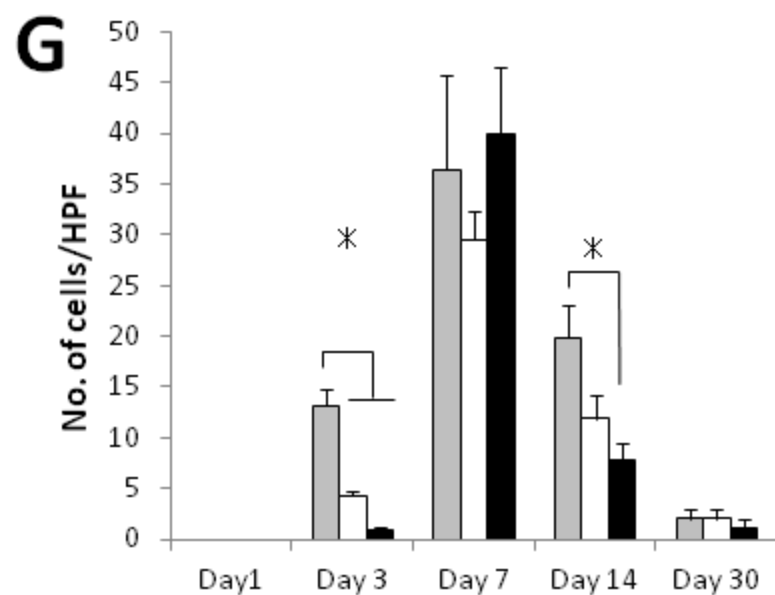
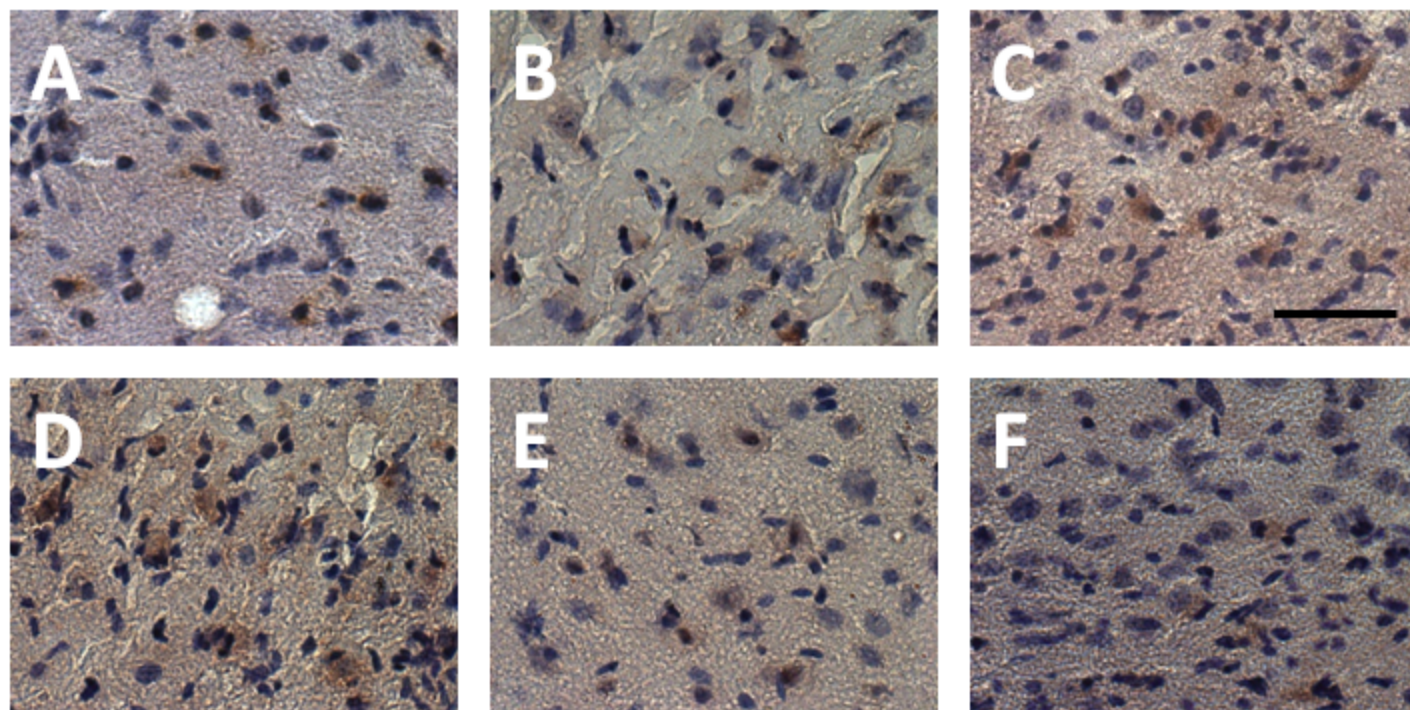


Fig.6

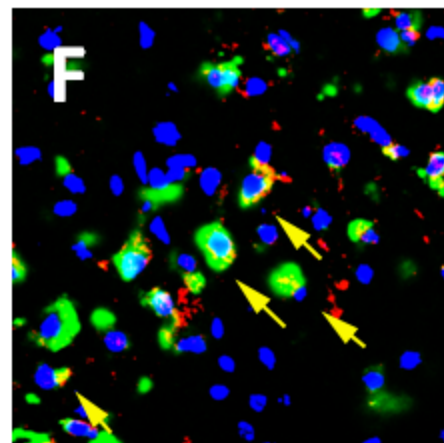
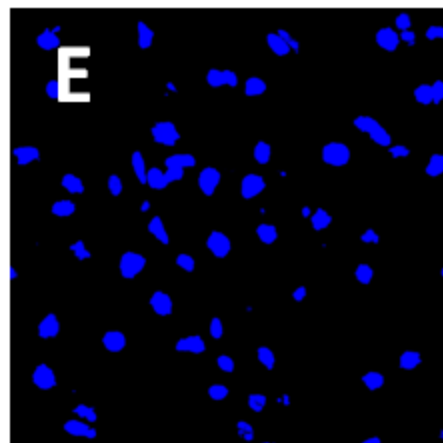
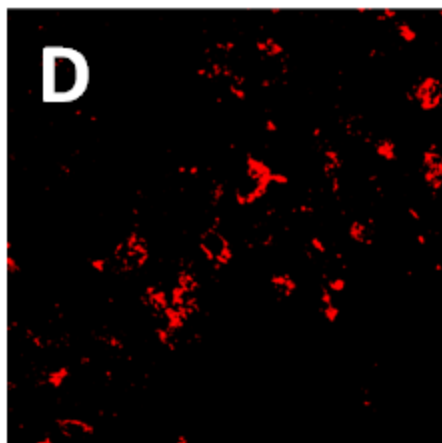
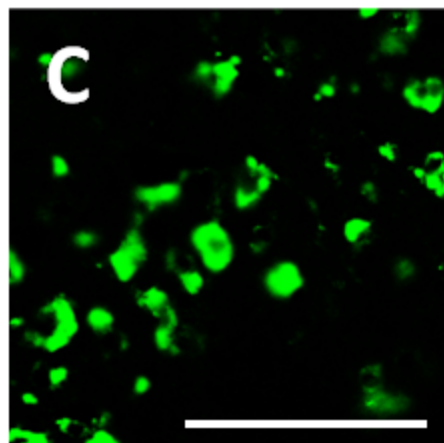
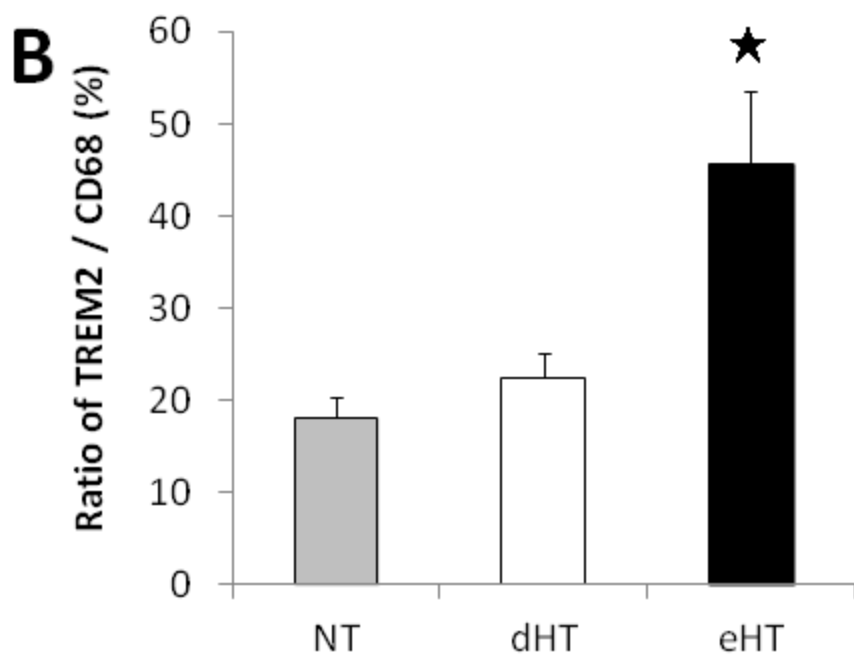
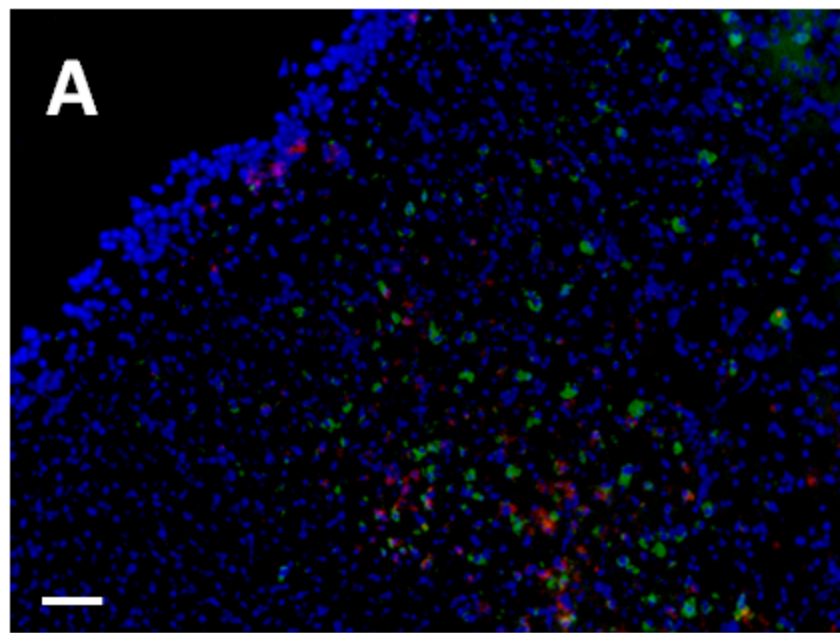


Fig.7

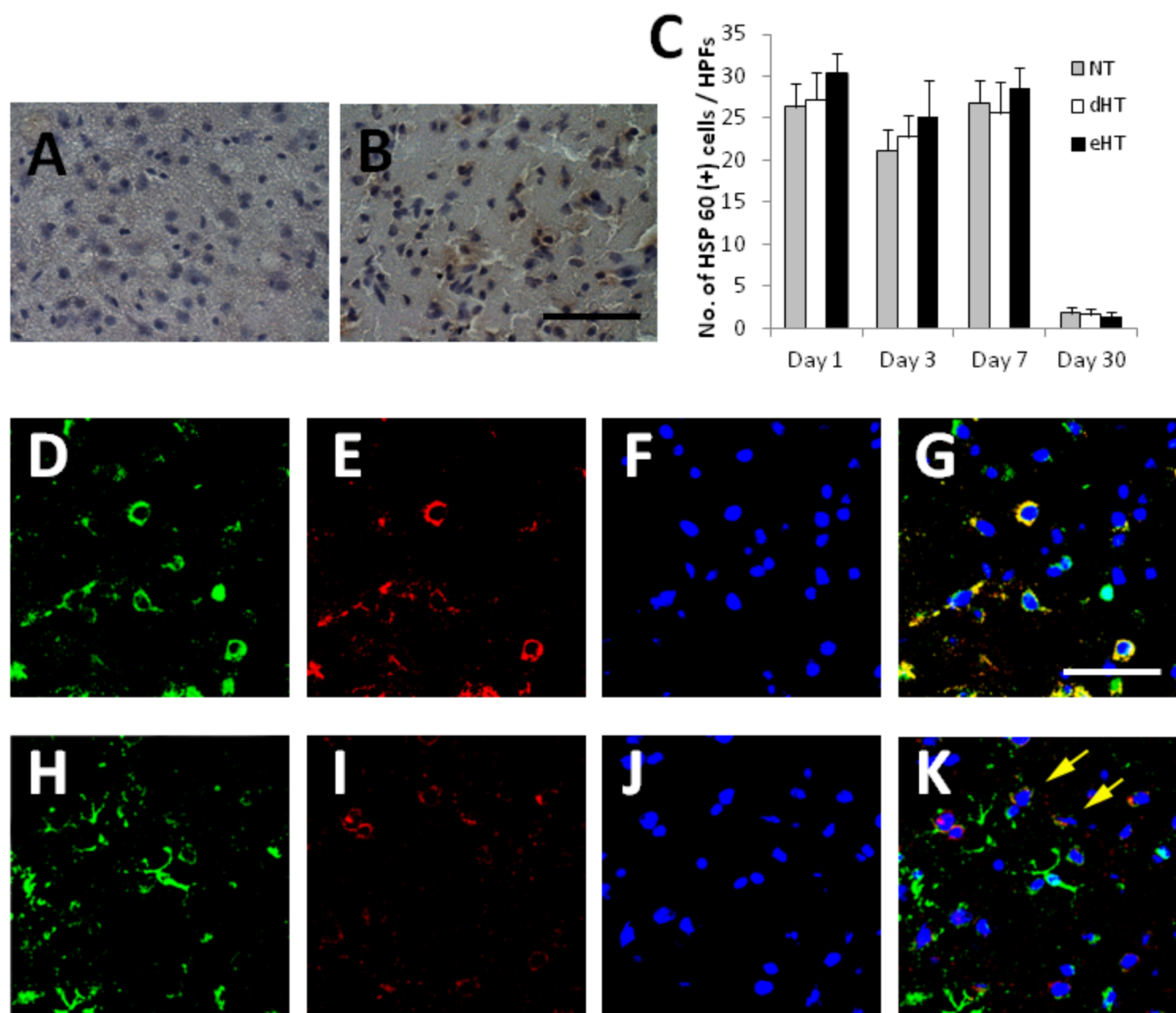


Fig.8