Aggravated brain injury after neonatal hypoxic ischemia in microglia depleted male mice

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Abstract

Background: Neuroinflammation plays important roles in neonatal hypoxic–ischemic encephalopathy (HIE). Microglia are largely responsible for the injury-induced inflammatory response, but they also play beneficial roles in both normal and disease states. Nevertheless, the effects of microglial depletion in neonatal HIE remain unclear.

Methods: Tamoxifen was administered to Cx3cr1 CreER/+ Rosa26 DTA/+ (microglia-depleted model) and Cx3cr1 CreER/+ Rosa26 DTA/- (control) mice at P8 and P9 in order to assess the effect of microglial depletion. The density of microglia was quantified using Iba-1 staining and the proportion of resident microglia after the hypoxic–ischemic (HI) insult was also analyzed using flow cytometric analysis. The HI insult employed the Rice-Vannucci procedure at P10. Infarct size and apoptotic cells were analyzed at P13. Cytokine analysis was performed by quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) at P13.

Results: Tamoxifen administration induced over 99% microglial depletion at P10 in DTA + mice. Microglial depletion over 97% persisted at P13 following the HI insult. Male DTA + mice exhibited significantly larger infarct volumes than did male DTA - mice, but there was no significant difference in females. The density of TUNEL + cells in DTA + mice was significantly higher than that in DTA - mice in the caudoputamen, cerebral cortex, and thalamus in males. Females showed significantly greater numbers of TUNEL + cells in the hippocampus and thalamus in DTA + mice compared to DTA - mice. ELISA revealed that IL-10 and TGF- β levels were significantly lower in both male and female DTA + mice compared to DTA - mice, both under normal conditions and, more pronounced, after HI.

Conclusion: We established a model of microglial depletion that aggravated neuronal damage and apoptosis after the HI insult, an effect most predominant in males.

Background

Neonatal hypoxic–ischemic encephalopathy (HIE) is a major cause of neonatal death and long-term disability, including mental retardation, visual motor or visual perceptive dysfunction, increased hyperactivity, cerebral palsy, and epilepsy, worldwide. Hypoxic–ischemic (HI) insults are known to induce inflammatory reactions within hours in rodent models, and microglia play an important role in this etiology [1–5].

As resident cells in the brain, microglia are the first responders to ischemia and are engaged in intimate cross-talk with other intrinsic brain cells and infiltrating leukocytes from the periphery that enter the brain through the compromised blood–brain barrier [6–8]. In response to brain ischemia, microglia promptly initiate reactive oxygen species generation, antigen presentation, phagocytosis, and the production of inflammatory mediators, including interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , IL-6, and matrix metalloproteinases [8–10]. Conversely, microglia also have an anti-inflammatory role by producing factors such as IL-4, IL-10 and transforming growth factor- β (TGF- β) during the resolution phase of brain

inflammation and on return back to homeostatic surveillance [10, 11]. The role of microglia as active players in ischemic brain injury in the adult brain has been extensively studied [12–17]. However, the precise influence of microglia on brain inflammation and their contribution to ischemic brain injury has not been adequately studied in neonatal brain. The response to HIE is largely different between the adult and neonatal brain [7, 18, 19]. Also the microglial response is dependent on developmental status [7, 20, 21].

The specific role of microglia can be investigated using models of microglial depletion [22–32]. Most studies in microglial depletion use pharmacological strategies with colony-stimulating factor 1 receptor (CSF1R), such as PLX33397 or PLX5622[33]. However, this procedure requires at least two weeks to deplete microglia. Thus, other methods are needed in order to study the role of microglia depletion in neonatal HIE. We have recently demonstrated competitive repopulation of the CNS following depletion of microglia and development of fatal demyelinating disease by using acute depletion model, which is a microglial-depletion Cx3cr1^{CreER}Rosa26^{DTA} model [23, 34]. This genetic microglial depletion model is suitable for neonatal experiments. Furthermore, we also found no disruption of the blood–brain barrier (BBB) after microglial depletion in this model [34].

Neonatal HIE is known to show sexual dimorphism. According to one study of 1,864,766 infants born in California, males have a 1.27-fold higher risk (95% CI, 1.2–1.4) of HIE [35]. Rodent models show no difference between sexes in infarct size on day 1 after HI, although male neonates exhibit significantly larger infarct sizes 3 days after HI [36]. Likewise, there are sex differences in microglial numbers, morphology, migration, and phagocytic activity [37–39].

We aimed to establish a model to study the effects of microglial depletion in experimental neonatal HIE, including infarct size, apoptotic cells, and cytokine response. In addition, we aimed to study sex differences in the effects of microglial depletion after HI.

Materials And Methods

Animals

This study was approved by the ethics committee at Karolinska Institutet, Stockholms norra djurförsöksetiska nämnd (approval number: N94/15 and N126/16) and carried out in accordance with the relevant guidelines and regulations (Swedish Animal Welfare Act 1988:543) and the ARRIVE guidelines for animal experiments. We obtained Cx3cr1^{EYFP-CreER} mice, which express a Cre-ERT2 fusion protein and enhanced yellow fluorescent protein (EYFP), and Rosa26^{DTA+/-} mice, which carry a loxP-flanked stop cassette associated with an attenuated diphtheria toxin, originally from Jackson Laboratory, as previously reported [34]. Crossbreeding by these two strains generated Cx3cr1^{CreER-EYFP+/Rosa26^{DTA+/-}} mice (DTA+ mice; microglia-depleted mice) and Cx3cr1^{CreER-EYFP+/Rosa26^{DTA-/-}} mice (DTA- mice; control mice) (Fig. 1a). All mice were housed in a humidity-controlled room with a 12-h light–dark cycle and ad libitum access to food and water.

Tamoxifen administration

Tamoxifen was purchased from Sigma-Aldrich (Cat. #T5648) and dissolved in corn oil (8 mg tamoxifen/1 ml corn oil) at 70 °C. It was administered at P8 and P9 via intraperitoneal injection (50 mg/kg).

Induction of hypoxic–ischemic encephalopathy

Induction of HIE was performed without knowledge of the genotype. Unilateral HI was induced at P10 using the modified Rice-Vannucci model [40, 41]. After sedation with isoflurane (4% for induction and 2% for maintenance), and local anesthesia with Marcaine (Astra Zeneca), the left common carotid artery was isolated and occluded by 8-Watt electronic coagulation (Additional file 1; Figure S1). The skin incision was sutured with 6 – 0 silk surgical thread and infiltrated with additional local anesthesia. All mice were subjected to ischemic surgery within less than 5 min. Pups were returned to the dam for 1 h and then placed in a hypoxic chamber (BioSpherix, US) with 10% O₂ in 90% N₂ for 1 h. In the sham group, the carotid artery was isolated but not cauterized. For injury evaluation, 40 pups were subjected to the HI insult. For cytokine analysis, 64 of 105 pups were randomly selected to be subjected to the HI insult; therefore, the sham group had 41 pups.

Immunohistochemistry

Mice were anesthetized by injection of 50 mg/kg pentobarbital (APL, Sweden) and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) at P10, P13, P17, and P24 for a repopulation study and at P13 after HI for evaluation of infarction size. Removed brains were fixed in 4% PFA for 24 h. Fixed brains were dehydrated and embedded in a paraffin block and sectioned coronally at 5 µm by a sliding microtome. Six levels of each brain were collected based on previous studies [18, 42, 43]. The first level was obtained corresponding to the bregma 1.3 mm in the adult mouse brain, and every 100th section was collected and deparaffinized. After deparaffinization, antigen retrieval was performed in 10 mM citric buffer (pH 6.0) for 10 min. After blocking, sections were exposed to primary antibodies in a humidity chamber box overnight at 4 °C. The primary antibodies were those against rabbit Iba-1 from Wako (#1919741) diluted 1:1000, mouse microtubule-associated protein-2 (MAP2) from Sigma (#M4403) diluted 1:1000 and rabbit anti glial fibrillary acidic protein (GFAP) from Millipore diluted 1:500 (AB5804). After washing, biotin-conjugated donkey anti-rabbit secondary antibody (Jackson Immuno Research) diluted 1:1000 was applied for Iba-1. For MAP-2, VECTOR M.O.M.[™] Immunodetection Kit was used. After blocking endogenous peroxidase using 0.3% H₂O₂, sections were visualized by VECTASTAIN Elite ABC-Peroxidase Kit and 3,3'-diaminobenzidine (DAB). For GFAP, biotinylated donkey anti rabbit secondary antibody was applied for 1 h before incubation with VECTASTAIN® Elite ABC-Peroxidase Kit. For immunofluorescence analyses, the following secondary

antibodies were used: donkey anti-rabbit IgG Alexa Fluor 555 (1:1000). Coverslips were mounted onto slides using antifade reagent ProLong Gold (Molecular probes/Life technologies).

Iba-1 cell counting

Sections were observed using Zeiss Axio Imager M2 (Carl Zeiss, Göttingen, Germany). The number of microglia was measured by counting Iba-1-positive cells in the whole hemisphere. The total number of Iba-1⁺ cells was calculated using the fractionator option in Stereo Investigator software (MicroBrightField, Colchester, Vt., USA). Briefly, each section was divided equally into approximately 200 squares (400 × 400 µm, sampling grid), and the number of Iba-1⁺ cells with a complete cell body was counted in each 100 × 100 µm red-green square (counting frame) within the sampling grid using a 20x air objective. The software provided the estimated total number of cells and the area in the whole coronal section. Slides from DTA⁺ mice at P10, P13, and P17 showed few unevenly distributed microglial cells, hence all Iba-1⁺ cells were counted directly without the use of the fractionator. Based on these data, the density of Iba-1⁺ cells was calculated.

Fluorescence Activated Cell Sorting (FACS)

Mice were sacrificed by intraperitoneal injection of 50 mg/kg pentobarbital (APL, Sweden) and perfused transcardially with PBS. After 5 minutes of perfusion, brains were extracted, the olfactory bulbs and the cerebellum were removed, and the right and left hemispheres separated and processed individually. The tissue was then minced with a scalpel and digested for 30 min at 37 °C with Hank's Balanced Salt Solution (HBSS) containing 0.02% DNase 1 (Sigma-Aldrich) and 0.005% collagenase (Sigma-Aldrich). The reaction was stopped with cold HBSS containing 0.01% ethylenediaminetetraacetic acid (EDTA) and the samples were strained and centrifuged at 4 °C (300 g, 10 min). The pellet was resuspended in 38% percoll solution and centrifuged at 4 °C (800 g, 10 min). After removing the supernatant carefully, the pellet was rinsed with PBS and transferred to a V-bottom plate for staining. The samples were incubated for 20 min at 4 °C with PBS containing CD11b (1:100, M1-70, BioLegend), CD45 (1:1000, 30-F11, BioLegend), and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen), and then rinsed before analysis. Cell numbers were acquired using Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza software (Beckman Coulter).

Injury evaluation

Tissue loss was assessed by determining the MAP-2-stained area at P13 as described previously.[18, 42] First, the MAP-2-positive area was measured in each hemisphere separately by using Stereo Investigator software (MicroBrightField, USA). Second, the difference in areas was calculated by subtracting the staining area in the contralateral hemisphere minus that in the ipsilateral hemisphere. Next, total tissue loss volume was calculated according to the Cavalieri principle using the following formula: $V = \Sigma A \cdot P \cdot T$

(where V is the total volume, ΣA is the sum of the areas measured, P is the inverse of the sampling fraction, and T is the section thickness). This evaluation was performed without knowledge of the genotype.

TUNEL and NeuN double staining

After deparaffinization, antigen retrieval was performed in 10 mM citric buffer (pH 6.0) for 15 min. Sections were incubated with 5% normal donkey serum and 0.3% Triton X-100 in PBS for 30 min at room temperature, and then with rabbit monoclonal anti-NeuN antibody (1:100, MABN140, Millipore Corpo, CA, USA) at 4 °C overnight. After washing out the primary antibody, donkey anti-rabbit Alexa Flour 488 (1:300, A-212206, Thermo Fisher Scientific) was applied for 2 hours at room temperature. Sections were rinsed with PBS, and the terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) (Click-iT Plus TUNEL Assay Kit, C10618, Thermo Fisher Scientific Europe BV) assay was performed according to the manufacturer's protocol. Stereological analysis was performed using Stereo Investigator software (MicroBrightField, USA). The number of TUNEL⁺ cells was counted under 200x magnification [44, 45]. First, the contour of each region (striatum, hippocampus, cortex, and thalamus) was traced using a 5x air objective. After setting the counting frame size to 150 × 150 μm and grid size to 150 × 150 μm, the number of TUNEL-positive cells was counted with a 20x air objective. Cell counting was performed by an investigator blinded to genotyping. Fluorescent images were obtained using a LSM700 laser scanning confocal microscope (Axio-observer Z1; Carl Zeiss microscopy, Germany), and analyzed using the software ZEN 2.3 (blue edition; Zeiss).

Genotyping

Genotyping was performed after sacrifice by using a piece of tissue, which was placed in 75 μl of extraction buffer containing 25 mM sodium hydroxide and 0.2 mM EDTA in distilled water and incubated at 98°C for 60 min. Next, 75 μl of 40 mM trishydroxymethylaminomethane-hydrochloric acid (pH 5.0) was added and centrifuged at 6000 rpm for 3 minutes. The supernatant was collected for genotyping. For the polymerase chain reaction (PCR), 2 μl of cDNA was mixed into 0.25 μl of MyTaq™ HS DNA Polymerase, 10 μl of 5x MyTaq™ Reaction Buffer (Bioline meridian, London, UK), 3 μl of each of the corresponding primers (0.5 μM), and 34.75 μl of distilled water (total 50 μl). Each primer sequence is shown in additional file 2 (Table S1). The Cre allele was amplified using the following cycle conditions: 95 °C for 30 s, 61.5 °C for 30 s, and 72 °C for 60 s. The cycle was repeated 30 times. For DTA allele, the cycle was 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 60 s. The cycle was repeated 35 times. PCR products were separated on 1% agarose gel and stained with GelRed® Nucleic Acid Gel Stain (Biotium, Inc. Fremont, CA, USA).

Quantitative polymerase chain reaction (PCR)

Total RNA was isolated from the ipsilateral hemisphere after HI by using the RNeasy Kit (QIAGEN) and was measured using a Nano Drop (Thermo Scientific). The nucleotide ratio (A260:A280) of all samples were within the range 1.9–2.1. A total of 1 µg of extracted RNA was underwent reverse transcription into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Quantitative PCR (qPCR) was performed using the Step-One-Plus Real Time PCR machine (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) and primers described in additional file 3 (Table S2). The set-up condition was initial denaturation step at 95 °C for 5 min, denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s, and elongation for 30 s. The total number of cycles was 40. The cycle time values were normalized to β -actin of the same sample. The mRNA expression levels were calculated using the delta-delta CT method as fold changes in comparison with DTA⁻ sham samples at P13, and all samples were examined in triplicate. These analyses were performed using StepOne software program (version 2.3, Applied Biosystems).

Quantitative enzyme-linked immunosorbent assay

Ipsilateral hemispheres were collected from pups at P13 after HI at P10. Tissue was homogenized in N-PER™ Neuronal Protein Extraction Reagent (Thermo Fisher) with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich) using pellet pestles, blue polypropylene (Sigma-Aldrich) on ice. After 1-h agitation at 4 °C, lysates were centrifuged 14000 rpm for 10 min. The protein concentration of the supernatant was measured by Pierce BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's protocol. Aliquots of lysates were stored at -80 °C until further analysis. After all samples were prepared, the IL-10 and TGF- β contents in the lysates were measured using Mouse IL-10 Quantikine ELISA Kit (R&D system) and Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (R&D system). For TGF- β assessments, samples were treated with 1N HCL to activate the latent TGF- β before measurement using ELISA kit. The optical density was determined at 450 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Germany) within 15 min after the reactions were stopped. All measurements were performed in duplicate.

Statistical analysis

Values are presented as mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism ver.7 (GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA was used for comparison of multiple samples followed by Sidak test to determine the statistical significance. Whenever normal distribution was not found, statistical significance was analyzed using nonparametric tests (Dunn's multiple comparison test). Statistical significance was defined at $p < 0.05$ in all cases.

Results

Microglial depletion and repopulation using tamoxifen in $Cx3cr1^{CreER}Rosa26^{DTA}$ neonatal mice

Iba-1⁺ cells were counted at P10, P13, P17, and P24 after tamoxifen administration at P8 and P9 to evaluate the depletion and repopulation of microglia (Fig. 1b). Over 99% of the microglia were eliminated in both sexes at P10 (striatum and hippocampus level) with no significant difference between males and females. At P13, male DTA⁺ mice had 1% microglia at the level of striatum and 2% at the hippocampus level in comparison with DTA⁻ mice. Likewise, female DTA⁺ mice had 1% microglia at both levels in comparison with DTA⁻ mice. However, microglia had rapidly repopulated by P17. DTA⁺ mice of both sexes showed microglial repopulation of up to approximately 60% at the striatum level and 70% at the hippocampus level, although these ratios varied between groups. At P24, microglia of both sexes had completely repopulated at both the striatum and hippocampus levels (Fig. 1c-e).

Microglial depletion persisted after the HI insult

To determine the depletion status after HI, we performed FACS analyses using surface markers at P13. We defined resident microglia as CD11b⁺CD45⁺EYFP⁺ cells and examined their ratio over the total number of live cells (Fig. 2a). In both males and females, the differences between DTA⁻ and DTA⁺ mice at P13 were similar to those seen at P10 in both the ipsilateral and contralateral sides of the brain after HI and in the sham group (Fig. 2b, c). Therefore, we concluded that microglia were still depleted 3 days after HI and 4 days after the second and last tamoxifen injection.

Microglial depletion aggravated the infarction size following HI in male mice

To study the effect of microglia in neonatal brain injury, we performed HI at P10 after microglial depletion. Mice were sacrificed at P13 and genotyped (Fig. 3a). All DTA groups displayed halted weight gain during tamoxifen administration compared to the non-treated groups (Fig. 3b), and there was no mortality at P13. Neuronal damage was evaluated by MAP-2 staining (Fig. 3c). There was a significant difference in tissue loss between male DTA⁻ and male DTA⁺ mice (Fig. 3d). Conversely, there was no significant difference between female DTA⁻ and female DTA⁺ mice (Fig. 3d). GFAP staining showed higher gliosis in DTA⁺ mice than in DTA⁻ mice (Additional file 4; Figure S2).

Microglial depletion aggravated apoptosis of neurons following HI

TUNEL and NeuN staining were performed in sections parallel to those used for MAP-2 evaluation at P13 to assess the effects of microglia on neuronal TUNEL-related apoptosis. At both the striatum and hippocampus levels, the area of the TUNEL⁺ clusters matched the areas where NeuN staining (Fig. 4a, Additional file 5; Figure S3) and MAP2 staining (Fig. 3c) were lacking. High magnification demonstrated that TUNEL⁺ cells co-localized with NeuN⁺ cells (Fig. 4C). Among male mice, the density of TUNEL⁺ cells was significantly greater in DTA⁺ mice compared to that in DTA⁻ mice in the caudoputamen, cerebral cortex, and thalamus (Fig. 4b, c, Additional file 5; Figure S3). In females, the density of TUNEL⁺ cells was significantly greater in DTA⁺ mice compared to that in DTA⁻ mice in the hippocampus and thalamus (Additional file 5; Figure S3), but not in the caudoputamen or cerebral cortex.

Expression of inflammation-related genes at P13 following HI at P10

To investigate if microglial depletion affects pro- and anti-inflammatory cytokine expression at P13 following HI at P10, we performed quantitative PCR using homogenates of the ipsilateral hemisphere. In males, the expressions of IL-10 and TGF- β genes were significantly suppressed in the DTA⁺ HI group compared to those in the DTA⁻ HI group (Fig. 5A). There were no differences in IL-1 β , TNF- α , iNOS, or IL-4 expressions (Fig. 5A). In females, only TGF- β was differently expressed between the DTA⁻ HI and DTA⁺ HI groups (Fig. 5B).

Microglial depletion attenuated the protein levels of IL-10 and TGF- β

Based on the results of quantitative PCR, the protein levels of IL-10 and TGF- β were measured by ELISA in the ipsilateral hemispheres at P13 following HI at P10. Male mice there showed higher expression levels of IL-10 in the DTA⁻ than in the DTA⁺ group, where it was undetectable (Fig. 6A). There were significant differences in TGF- β levels between the DTA⁻ and DTA⁺ groups among both sham and HI mice (Fig. 6A). Female mice showed significant differences in IL-10 and TGF- β expressions between the DTA⁻ and DTA⁺ groups among both sham and HI mice (Fig. 6B).

Discussion

Herein, we report a model of microglial depletion early in postnatal life that aggravated neuronal damage after HI injury. Microglial depletion reduced the levels of the anti-inflammatory IL-10 and TGF- β both under normal conditions and, even more pronounced, after HI. There was a sex-specific sensitivity in that males were more affected than females.

To our knowledge, this is the first report on the effect of microglia in neonatal HIE using a neonatal genetic microglial-depletion model. Several studies have reported microglial-depletion models in adult stroke, Alzheimer's disease, and retinal disease [26, 28, 30, 33, 46, 47]. In order to study the role of microglia early in life, a model for acute microglial depletion was needed. Intracerebral administration of clodronate encapsulated into liposomes (Clod-lip) has been shown to deplete microglia immediately. However, this method causes unselective cell damage, including damage to blood vessels, and depletion is incomplete [22, 33]. Cx3cr1^{CreER}Rosa26^{DTR} mice were also reported as a microglial acute depletion model through tamoxifen treatment followed by diphtheria toxin administration in adult mice [31, 48]. To avoid multiple injections in pups, the Cx3cr1^{CreER}Rosa26^{DTA} strain was selected for this study.

Firstly, we confirmed the depletion and repopulation of microglia in our neonatal model. The efficiency of resident microglial depletion was over 99% compared to that in DTA⁻ mice in P10. This depletion status remained until at least P13. We identified clusters of repopulating microglia at P17 (Additional file 6; Figure S4), in agreement with the findings in adult models of microglial depletion [48]. These clusters were few and asymmetrically distributed throughout the brain. This asymmetrical distribution might cause considerable variation of the results if the analysis is performed during repopulation; therefore, we evaluated brain damage at P13. Next, we confirmed that these depletion conditions remained even after HI. We identified resident microglia by EYFP using FACS and recorded a high grade of depletion (97%) after four days (P13), covering the time period of the HI insult and recovery.

Our data demonstrated that microglial depletion leads to aggravation of neuronal damage. This is in concordance with previous results of a neonatal depletion model using Clod-lip, which demonstrated that lack of microglia increased the severity and volume of brain injury using middle cerebral artery occlusion (MCAO) [49]. An adult depletion model also reported that depletion of microglia enhances the size of ischemic brain injury at 24 h after MCAO [26]. Our data also indicated a sex difference in the effect of microglial depletion after HIE. MAP2 evaluation of brain injury only revealed significant differences after microglia depletion in males. TUNEL staining showed sex-specific effects in different regions. Sex differences in brain inflammation and microglial effects have recently attracted attention [37–39]. For example, male microglia have higher migrational capacity than do female microglia, which might be related to the protective effect of microglia depletion [39]. Another possibility is that sex differences in our model could be attributed to the estrogen effect of tamoxifen. Estrogen also has neuroprotective effects in the ischemic brain, and these effects are stronger in females through a more effective upregulation of estrogen receptors [50–52].

Previous studies of microglial depletion in adult stroke models reported that the levels of inflammatory mediators were significantly increased in injured tissues at 24 h following MCAO [26]. In our study, analyses at 72 h after the injury showed no significant differences in the levels of several pro-inflammatory cytokines. The result may be attributed to our experimental design of the evaluation for pro-inflammatory cytokines at the end of sub-acute phase [3, 53]. Our findings demonstrated that anti-inflammatory cytokines were differentially affected in DTA⁻ and DTA⁺ mice. IL-10 upregulation was suppressed in microglia-depleted mice and TGF- β was downregulated in microglia-depleted mice three

days after the HI insult. It has been demonstrated that microglia are major sources of TGF- β after stroke [54]. To confirm these quantitative PCR results at the protein level, we performed ELISA for IL-10 and TGF- β and observed a significantly decreased concentration of both cytokines in the DTA⁺ group, even in the sham group. We propose that microglial depletion aggravated neuronal death due to a lack of anti-inflammatory cytokines such as IL-10 and TGF- β . In line with our results. Jin WN demonstrated that microglial depletion increased ischemia-induced astrocytic inflammatory response and neural injury [26]. Therefore, studying astrocytic responses is also important to understand the impact of microglia depletion in neonatal hypoxic-ischemic mice.

Our findings focused on resident microglia including the classic pro-inflammatory type microglia (M1-like) and alternative neuroprotective type microglia (M2-like). Infiltrating monocytes/macrophages also have polarization after HI insult and influence on neuronal cell death [55]. Although resident microglia contribute to the earlier and more pronounced inflammatory reaction in neonatal brain rather than blood-derived macrophage, further studies are needed to understand how microglial depletion affect polarization of infiltrated monocytes/macrophages [7].

Conclusions

We established a model showing over 99% microglial depletion in neonatal mice. The current study suggests that resident microglia have a neuroprotective role early after neonatal hypoxic ischemia in males and that the protective pathway involves anti-inflammatory cytokines.

Abbreviations

HIE: hypoxic–ischemic encephalopathy; IL-1 β : interleukin; TNF- α : tumor necrosis factor; TGF- β : tumor necrosis factor; BBB: blood–brain barrier; EYFP: enhanced yellow fluorescent protein; PFA: paraformaldehyde; DAB: 3,3'-diaminobenzidine; FACS: Fluorescence Activated Cell Sorting; EDTA: ethylenediaminetetraacetic acid; TUNEL: transferase-dUTP nick end labeling; PCR: Polymerase Chain Reaction; ELISA: enzyme-linked immunosorbent assay; Clod-lip: clodronate encapsulated into liposomes

Declarations

Acknowledgments

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Author contribution

Shunichiro Tsuji designed and conducted the experiments, analyzed the data and drafted the manuscript, Elena Di Martino designed and conducted the experiments, Takeo Mukai designed and conducted the

experiments, Shoko Tsuji conducted the experiments, Takashi Murakami interpreted the data and edited the manuscript, Robert A. Harris conceived the experiments and edited the manuscript, Klas Blomgren conceived and designed the experiments, interpreted the data, and edited the manuscript, Ulrika Åden conceived and designed the experiments, interpreted the data, wrote and edited the manuscript, and acquired funding for the study. All authors approved the manuscript before submission.

Ethics approval

This study was approved by the ethics committee at Karolinska Institutet, Stockholms norra djurförsöksetiska nämnd (Approval number; N94/15 and N126/16) and carried out in accordance with relevant guideline and regulations (Swedish Animal Welfare Act 1988:543) and the ARRIVE guidelines for animal experiments.

Availability of data and materials

All data used in this manuscript are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplemental Information

Supplemental figure 1

Occlusion procedure by electronic coagulation to left common carotid artery. **a** Separation from vagus nerve. **b** Grasp the artery by bipolar electronic micro forceps. **c** Coagulation by bipolar electronic micro forceps. **d** Occlusion by electronic coagulation.

Supplemental figure 2

Representative slides of GFAP staining. **a** Low magnification in male and female DTA⁻ mice and DTA⁺ mice. Upper row shows the striatum level, and lower row shows the hippocampus level. Scale bar indicates 500 μ m. The square indicates the higher magnification. **b** High magnification of GFAP staining. Scale bar indicates 100 μ m.

Supplemental figure 3

TUNEL and NeuN staining at P13 following HI insult at P10. **a** Representative image of co-immunofluorescent staining at the hippocampus level. Scale bar indicates 500 μm . **b** Quantification of TUNEL⁺ cells/ mm^2 in the caudoputamen and cerebral cortex. (DTA⁻ male, n = 12; DTA⁺ male, n = 10; DTA⁻ female, n = 6; DTA⁺ female, n = 12) *P < 0.05, **P < 0.01, ***P < 0.001; Dunn's multiple comparison test. Bars depict mean $\pm$ SEM.

Supplemental figure 4

Uneven repopulation at P17 following tamoxifen administration at P8 and P9. **a** Iba-1 staining cells could be identified in whole brain equally in DTA⁻ mice. **b** In DTA⁺ mice, Iba-1 staining cells were detected partially. Furthermore, distribution was uneven. Scale bar indicates 500 μm .

Figures

Figure 1

Microglial depletion and repopulation following tamoxifen (TAM) administration. **a** Scheme of genetic design. **b** Experimental plan of the repopulation study. **c** Representative slides of Iba-1 staining for microglia in the striatum level at each time point. Scale bar indicates 200 μm in the upper column and 50 μm in the lower column. The square in the upper column indicates the higher magnification in the lower

column. d The density of Iba-1+ cells in the striatum level and hippocampus level in male mice. e The density of Iba-1+ cells in the hippocampus level in female mice.

Figure 2

The microglial depletion persisted at P13 following HI at P10. a Gating strategy for FACS analysis. After elimination of dead cells, resident microglial cells were defined by CD11b+CD45+EYFP+ status. b Quantification of resident microglial cells in each group. (Male; DTA- HI group n = 7, DTA+ HI group n = 5, DTA- sham group n = 3, DTA+ sham group n = 3, Female; DTA- HI group n = 8, DTA+ HI group n = 3, DTA- sham group n = 4, DTA+ sham group n = 3) **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way ANOVA followed by Sidak test. Bars reflect mean $\pm$ SEM.

Figure 3

Evaluation of infarct size by MAP2 staining at P13 following HI insult at P10. a Experimental design. b The ratio of body weight change in this experiment. c Representative slides of MAP2 staining in male and female DTA- mice and DTA+ mice. Upper row shows the striatum level, and lower row shows the hippocampus level. Scale bar indicates 500 μ m. d Quantification of infarct volume in DTA- mice and DTA+ mice of each gender (DTA- male, n = 12; DTA+ male, n = 10; DTA- female, n = 6; DTA+ female, n = 12; no tamoxifen male, n = 6; no tamoxifen female n = 9) *P < 0.05; Dunn's multiple comparison test.

Figure 4

TUNEL and NeuN staining at P13 following HI insult at P10. a Representative image of co-immunofluorescent staining at the striatum level. Scale bar indicates 500 μ m. b High magnification of the TUNEL+ area at the striatum level in each gender and genotype in the square of figure 1. Scale bar indicates 20 μ m. c Quantification of TUNEL+ cells/mm² in the caudoputamen and cerebral cortex. (DTA- male, n = 12; DTA+ male, n = 10; DTA- female, n = 6; DTA+ female, n = 12) *P < 0.05, **P < 0.01, ***P < 0.001; In the caudoputamen, one-way ANOVA followed by Sidak multiple comparison test. In cerebral cortex, Dunn's multiple comparison test. Bars depict mean $\pm$ SEM.

Figure 5

Expression of pro- and anti-inflammatory genes at P13 following HI insult at P10. Relative expression of pro-inflammatory genes (IL-1 β , TNF- α , iNOS) and anti-inflammatory genes (IL-4, IL-10, TGF- β) was measured by quantitative PCR at P13 in male a (DTA- sham, n = 3; DTA+ sham, n = 4; DTA- HI, n = 6; DTA+ HI, n = 9), and in female b (DTA- sham, n = 3; DTA+ sham, n = 3; DTA- HI, n = 6; DTA+ HI, n = 6). All values were normalized to β -Actin. The expression levels of target mRNAs were calculated in comparison with

DTA- sham. Values are presented as mean $\pm$ SEM. *P < 0.05, **P < 0.01; Regarding TNF- α , iNOS, IL-4, TGF- β in male and IL-1 β , iNOS, IL-4, TGF- β in female, one-way ANOVA followed by Sidak multiple comparison test. Regarding IL- β , IL-10 in male and TNF- α , IL-10 in female, Dunn's multiple comparison test.

Figure 6

Quantification of IL-10 and TGF- β protein levels at P13 following HI insult at P10 by ELISA in male a (DTA- sham, n = 7; DTA+ sham, n = 10; DTA- HI, n = 9; DTA+ HI, n = 9), and in female b (DTA- sham, n = 4; DTA+ sham, n = 7; DTA- HI, n = 9; DTA+ HI, n = 10) *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; Dunn's multiple comparison test. Bars depict mean $\pm$ SEM.

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