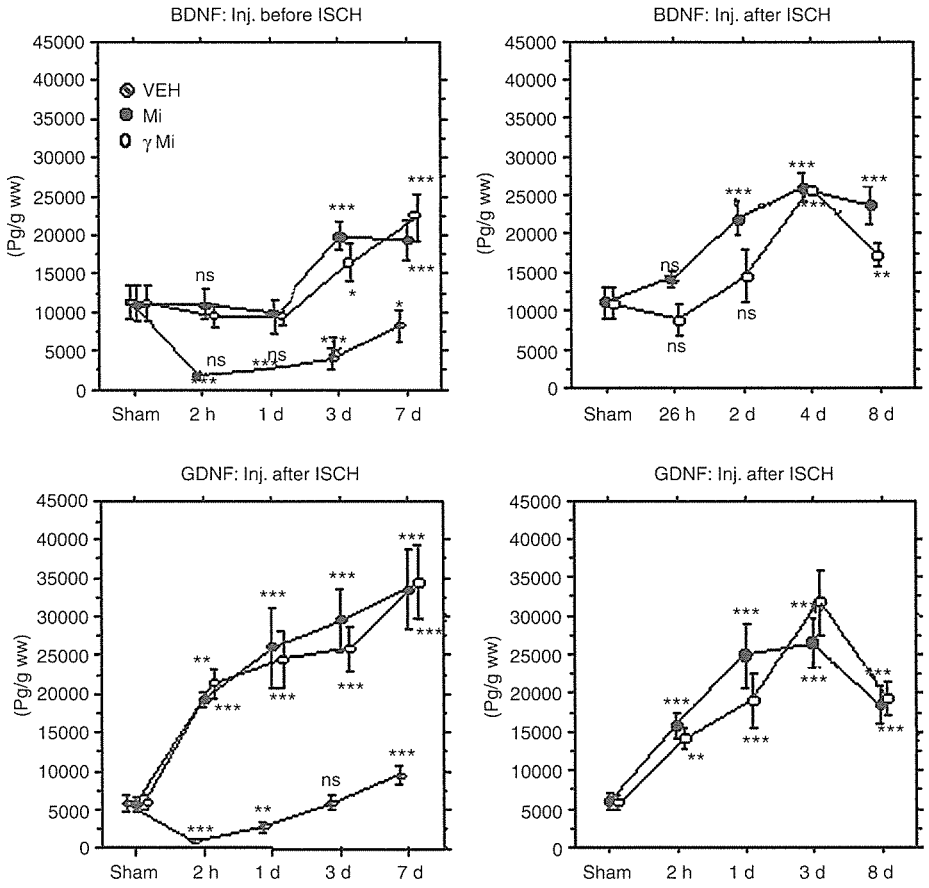

Figure 5 The effects of exogenous microglia on the time course of hippocampal BDNF and GDNF expression after transient global ischemia. Graphs indicate the BDNF and GDNF expression level in the hippocampus detected by enzyme-linked immunosorbent assay. Results are expressed as means of picograms of BDNF or GDNF normalized to gram of wet tissue weight (pg/g ww). As a control, we used SHAM/VEH hippocampus, which was dissected 6 h after the treatment. In SHAM/VEH hippocampus, BDNF and GDNF expression was observed. In ISCH/VEH-PRE animals, however, the levels were less than 20% of the baseline level (SHAM/VEH-PRE level) 2 h after ischemia. Injection of microglia prevented the decrease of both BDNF and GDNF and then induced an increase in both factors above baseline levels. In ISCH/Mi-PRE hippocampus, the neurotrophin levels were measured 2 h, 1 day, 3 days, and 7 days after ischemia. In ISCH/Mi-POST hippocampus, neurotrophin levels were measured 26 h, 2 days, 4 days, and 8 days after ischemia. Vertical bars represent the mean $\pm$ s.d. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from SHAM control with Bonferroni *post hoc* test. Abbreviations are as in Figure 2. Hatched circles (VEH) indicate values of BDNF or GDNF expression level in the ischemia-treated hippocampus injected with culture medium, black circles (Mi) indicate those injected with nonstimulated microglia, and open circles (γMi) indicate those injected with IFNγ-stimulated microglia.

rather than microglia or astrocytes, were the main source of the later high expression of BDNF and GDNF in the hippocampus observed 7 days after ischemia. Injections of microglia into SHAM animals did not significantly increase BDNF or GDNF expression in the brain, although a large number of PKH26-labeled microglia enter the brain from the circulation (Imai *et al*, 1997; Sawada *et al*, 1998). Astrocytes expressing these neurotrophic factors were not detected in ischemic hippocampus injected with microglia. The maintenance of BDNF and/or GDNF expression in injured neurons might be one mechanism of neuroprotection in the ischemic hippocampus.

Intraventricular administration of a Sendai virus vector carrying GDNF or nerve growth factor increases the expression of these neurotrophic factors in the hippocampus and prevents delayed neuronal death induced by transient global ischemia in gerbils, even when administered 6 h after ischemia (Shirakura *et al*, 2004). The time course for increased GDNF expression was similar to that in the present experiment, except that it took less time for exogenous microglia to express GDNF than it took for the Sendai virus vector. This might be one reason why we observed neuroprotection even when the microglia were injected 24 h after ischemia. Intrahippocampal administration of BDNF in adult rats improves performance in a spatial memory task (Cirulli *et al*, 2004). Administration of GDNF and BDNF to the site of ischemia might be one of the mechanisms underlying microglial neurotrophic effects on ischemic CA1 neurons.

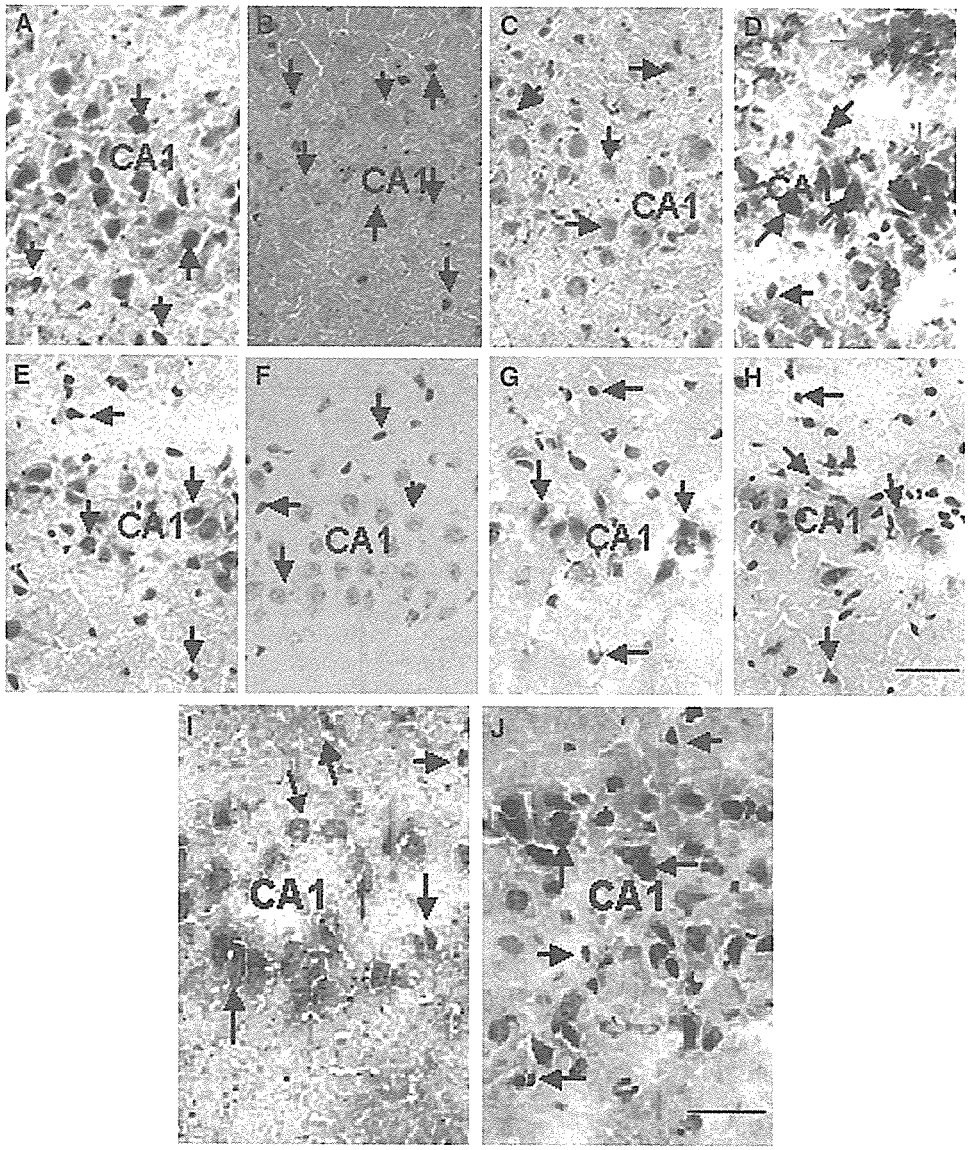

Figure 6 Immunohistochemical staining for BDNF and GDNF after transient global ischemia. (**A**–**H**) Time course of BDNF and GDNF expression in the hippocampus of SHAM and ischemia-treated animals injected with culture medium. (**A**) Most of the CA1 neurons expressed BDNF in the SHAM hippocampus. (**B**) Two hours after ischemia, there was little, if any, BDNF expression in CA1 neurons. BDNF-expressing endogenous microglia were observed around the CA1 field. (**C**) Three days after ischemia, most of the CA1 neurons weakly expressed BDNF. (**D**) Seven days after ischemia, a large number of damaged CA1 neurons and reactive astrocytes expressed BDNF. (**E**) Most of the CA1 neurons expressed GDNF in the SHAM hippocampus. (**F**) Two hours after ischemia, there was little, if any, GDNF expression in CA1 neurons, whereas there were a few GDNF-expressing endogenous microglia. (**G**) Three days after ischemia, most of the CA1 neurons expressed GDNF. (**H**) Seven days after ischemia, damaged CA1 neurons expressed GDNF and many GDNF-expressing exogenous microglia were scattered around them. (**I** and **J**) BDNF and GDNF expression, respectively, in the hippocampus 2 h after ischemia. Nonstimulated microglia were injected 24 h before ischemia treatment. Injection of microglia prevented the loss of both BDNF and GDNF expression in the hippocampus immediately after ischemia. Red arrows: CA1 neurons; black arrows: microglia; green arrows: reactive astrocytes. Scale bars = 5 μm.

Lee *et al* (2002) reported that BDNF mRNA increases 3 h after ischemia and returns to sham-operated level in the CA1 pyramidal neurons 1 day after ischemia, whereas BDNF protein decreases 3 h after ischemia and recovers to some extent at 7 days after ischemia. This expression disparity between neurotrophin mRNA and protein was reported to be because of a translational and/or posttranslational dysfunction of protein synthesis, release, or transport immediately after cerebral injury (Lee *et al*, 2002). In our study, the recovery of BDNF and GDNF expression was observed in degenerated neurons. The expression of neurotrophic factors might not enhance the survival of

ischemic pyramidal neurons, however, because most degenerating cells do not express BDNF or GDNF receptors after ischemic insult (Ferrer *et al*, 1998; Wang *et al*, 2004). Although pretreatment of microglia with IFNγ significantly enhanced microglial neuroprotective effects on ischemic CA1 pyramidal neurons, it did not increase hippocampal BDNF or GDNF expression, compared with nontreated microglia. Nevertheless, pretreatment with IFNγ increased the number of microglia migrating to the ischemic hippocampus. It is possible that microglia pretreated with IFNγ induce expression of neurotrophic factor receptors in ischemic pyramidal neurons, thus providing a neuroprotective effect. We are now investigating the mechanism.

Cytokines secreted by microglia might also affect ischemic CA1 neurons. We did not examine cytokine activity in this study because neurons and microglia interact with each other and produce complicated effects *in vivo*. For example, activated microglia release factors capable of generating oxidative damage, such as superoxide anions (O_2^-) and nitric oxide (Saud *et al*, 2005), whereas transient global ischemia increases transforming growth factor-β1 expression in CA1 pyramidal neurons (Zhu *et al*, 2000), which could eliminate microglial O_2^- and nitric oxide production (Herrera-Molina and von Bernhardi, 2005).

Microglial activation, after neuronal injury, appears to serve a neuroprotective function. A strong inflammatory response, however, could induce microglia to be hyperactive, which allows them to escape endogenous control and become toxic to neurons (Polazzi and Contenstabile, 2002). Interleukin-1β secreted by microglia activates p38 mitogen-associated protein kinase and cyclic adenosine monophosphate-response element binding protein to suppress long-term potentiation, which is thought to be an important underlying mechanism of learning and memory (Srinivasan *et al*, 2004). In our preliminary data, however, systemic injection of exogenous microglia in rabbits subjected to transient global ischemia prevented the ischemia-induced suppression of long-term potentiation (unpublished data). Microglia also produce interleukin-10 (Wu *et al*, 2005; Broderick *et al*, 2000), which reverses lipopolysaccharide-induced increases in signaling in the hippocampus (Lynch *et al*, 2004). Exogenous microglia introduced to the site of ischemia might reverse ischemia-induced disruption of endogenous control by secreting antiinflammatory cytokines such as interleukin-10 (Wu *et al*, 2005; Ooboshi *et al*, 2005; Broderick *et al*, 2000). Further studies are necessary to verify the mechanism of microglial neuroprotective effects on ischemic CA1 neurons.

It is also possible that injection of nonautologous microglia induced an immune reaction that is responsible for the present findings. We believe that this is unlikely because there is no evidence in the literature for a nonspecific immune response to be neuroprotective and/or reverse behavioral deficits. This will be examined in the future.

In conclusion, peripherally injected exogenous microglia exhibit a specific affinity for ischemic brain lesions and protect against neuronal damage in our ischemia model, suggesting that one role of microglia is to protect damaged neurons after transient global ischemic insult. To continue the exploration of the uses of exogenous microglia, we are currently isolating microglia from bone marrow (Ono *et al*, 1999; Tanaka *et al*, 2003). In the future, the administration of microglia might be a potential tool for cell or gene therapy in the treatment of brain disease.

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References

Barger SW, Harmon AD (1997) Microglial activation by Alzheimer amyloid precursor protein and modulation by apolipoprotein E. *Nature* 388:878–81

Beyer M, Gimsa U, Eyuponglu IY, Hailer NP, Nitsch R (2000) Phagocytosis of neuronal or glial debris by microglial cells: upregulation of MHC class II expression and multinuclear giant cell formation *in vitro*. *Glia* 31:262–6

Broderick C, Duncan L, Taylor N, Dick AD (2000) IFN-gamma and LPS-mediated IL-10-dependent suppression of retinal microglial activation. *Invest Ophthalmol Vis Sci* 41:2613–22

Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK (1992) Activated microglia mediate neuronal injury via nitric oxide mechanism. *J Immunol* 149:2736–41

Cirulli F, Berry A, Chiarotti F, Alleva E (2004) Intrahippocampal administration of BDNF in adult rats affects short-term behavioral plasticity in the Morris water maze and performance in the elevated plus-maze. *Hippocampus* 14:802–7

Diemel LT, Copelman CA, Cuzner ML (1998) Macrophages in CNS remyelination: friend or foe? *Neurochem Res* 23:341–7

Ebadi M, Bashir RM, Heidrick MK, Hamada FM, Refaey HE *et al* (1997) Neurotrophins and their receptors in nerve injury and repair. *Neurochem Int* 30:347–74

Ferrer I, Lopez E, Pozas E, Ballabriga J, Marti E (1998) Multiple neurotrophic signals converge in surviving CA1 neurons of the gerbil hippocampus following transient forebrain ischemia. *J Comp Neurol* 394:416–30

Gehrmann J, Matsumoto Y, Kreutzberg GW (1995) Microglia: intrinsic immunoeffector cell of the brain. *Brain Res Rev* 20:269–87

Giulian D, Baker TJ, Shin LC, Lachman LB (1986) Interleukin-1 of the central nervous system is produced by ameboid microglia. *J Exp Med* 164:594–604

Giulian D, Yu J, Li X, Tom D, Li J, Wendt E, Lin SN, Schwarcz R, Noonan C (1996) Study of receptor-mediated neurotoxins released by HIV-1-infected mononuclear phagocytes found in human brain. *J Neurosci* 16:3139–53

Hamanoue M, Takemoto N, Matsumoto KT, Nakamura T, Nakajima K, Kohsaka S (1996) Neurotrophic effect of hepatocyte growth factor on central nervous system neurons *in vitro*. *J Neurosci Res* 43:554–64

Herrera-Molina R, von Bernhardi R (2005) Neurotransforming growth factor-beta 1 produced by hippocampal cells modulates microglial reactivity in culture. *Neurobiol Dis* 19:229–36

Horan PK, Slezak SE (1989) Stable cell membrane labeling. *Nature* 340:167–8

Hortega D (1932) Microglia. In: *Cytology and cellular pathology of the nervous system* (Penfield W, ed), New York: Hoeber, 481–534

Imai F, Sawada M, Suzuki H, Kiya N, Hayakawa M, Nagatsu T, Marunouchi TK (1997) Migration activity of microglia and macrophages into rat brain. *Neurosci Lett* 237:49–52

Imai F, Sawada M, Suzuki H, Zlokovic BV, Kojima J, Kuno S *et al* (1999) Exogenous microglia enter the brain and migrate into ischaemic hippocampal lesions. *Neurosci Lett* 272:127–30

Ishihara S, Sawada M, Chang L, Kim JM, Brightman M (1993) Brain vessels near autografts are sites for entry of isogenic macrophages into brain. *Exp Neurol* 124:219–30

Kataoka Y, Cui Y, Yamada H, Utsunomiya K, Niiya H, Yanase H (2000) Neovascularization with blood–brain barrier breakdown in delayed neuronal death. *Biochem Biophys Res Commun* 273:637–41

Kokaia Z, Nawa H, Uchino H, Elmer E, Kokaia M, Carnahan J *et al* (1996) Regional brain-derived neurotrophic factor mRNA and protein levels following transient forebrain ischemia in the rat. *Brain Res Mol Brain Res* 38:139–44

Lawson LJ, Perry VH, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* 39:151–70

Lee TH, Kato H, Chen ST, Kogure K, Itoyama Y (2002) Expression disparity of brain-derived neurotrophic factor immunoreactivity and mRNA in ischemic hippocampal neurons. *NeuroReport* 13:2271–5

Lees GJ (1993) The possible contribution of microglia and macrophages to delayed neuronal death after ischemia. *J Neurol Sci* 114:119–22

Ling EA, Wong WC (1993) The origin and nature of ramified and ameboid microglia: a historical review and current concepts. *Glia* 7:9–18

Lynch AM, Walsh C, Delaney A, Nolan Y, Campbell VA *et al* (2004) Lipopolysaccharide-induced increase in signaling in hippocampus is abrogated by IL-10—a role for IL-1 beta? *J Neurochem* 88:635–46

Mallat M, Houlgatte R, Brachet P, Ptochiantz A (1989) Lipopolysaccharide-stimulated rat brain macrophages release NGF *in vitro*. *Dev Biol* 133:309–11

Marmigere F, Givalois L, Rage F, Arancibia S, Tapia-Arancibia L (2003) Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus* 13:646–55

Miyazaki H, Nagashima K, Okuma Y, Nomura Y (2001) Expression of glial cell line-derived neurotrophic factor induced by transient forebrain ischemia in rat. *Brain Res* 922:165–72

Nanri M, Yamamoto J, Miyake H, Watanabe H (1998) Protective effect of GTS-21, a novel nicotinic receptor agonist, on delayed neuronal death induced by ischaemia in gerbils. *Jpn J Pharmacol* 76:23–9

Neumann J, Gunzer M, Gutzeit HO, Ulrich O, Reymann KG *et al* (2006) Microglia provide neuroprotection after ischemia. *FASEB J* 20:714–6

Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K *et al* (1995) Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischaemia is apoptosis. *J Neurosci* 15:1001–11

Ono K, Takii T, Onozaki K, Ikawa M, Okabe M, Sawada M (1999) Migration of exogenous immature hematopoietic cells into adult mouse brain parenchyma under GFP-expressing bone marrow chimera. *Biochem Biophys Res Commun* 262:610–4

Ooboshi H, Ibayashi S, Shitita T, Kumai Y, Takada J *et al* (2005) Postischemic gene transfer of interleukin-10 protects against both focal and global brain ischemia. *Circulation* 111:913–9

Pinteaux E, Rothwell NJ, Boutin H (2006) Neuroprotective actions of endogenous interleukin-1 receptor antagonist (IL-ra) are mediated by glia. *Glia* 53:551–6

Polazzi E, Contenstabile A (2002) Reciprocal interaction between microglia and neurons: from survival to neuropathology. *Rev Neurosci* 13:221–42

Saud K, Herrera-Molina R, Von Bernhardi R (2005) Pro- and anti-inflammatory cytokines regulate the ERK pathway: implication of the timing for the activation of microglial cells. *Neurotox Res* 8:277–87

Sawada M, Kondo N, Suzumura A, Marunouchi T (1989) Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Res* 491:394–7

Sawada M, Imai F, Suzuki H, Hayakawa M, Kanno T, Nagatsu T (1998) Brain-specific gene expression by immortalized microglia cell-mediated gene transfer in the mammalian brain. *FEBS Lett* 433:37–40

Sawada M, Suzumura A, Marunouchi T (1992) Down regulation of CD4 expression in cultured microglia by immunosuppressants and lipopolysaccharides. *Biochem Biophys Res Commun* 189:869–76

Sawada M, Suzumura A, Marunouchi T (1995) Cytokine network in the central nervous system and its roles in growth and differentiation of glia and neuronal cells. *Int J Dev Neurosci* 13:253–64

Sawada M, Suzumura A, Yamamoto H, Marunouchi T (1990) Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C. *Brain Res* 509:119–24

Shimojo M, Nakajima K, Takei N, Hamanoue M, Kohsaka S (1991) Production of basic fibroblast growth factor in cultured brain microglia. *Neurosci Lett* 123:229–31

Shirakura M, Inoue M, Fujikawa S, Washizawa K, Komabe S, Maeda M *et al* (2004) Postischemic administration of Sendai virus vector carrying neurotrophic factor genes prevents delayed neuronal death in gerbils. *Gene Therapy* 11:784–90

Silverman AJ, Sutherland AK, Wilhelm M, Silver R (2000) Mast cells migrate from blood to brain. *J Neurosci* 20:401–8

Srinivasan D, Yen JH, Joseph DJ, Friedman W (2004) Cell type-specific interleukin 1β signaling in the CNS. *J Neurosci* 24:6482–8

Streit WJ (1990) An improved stain method for rat microglia cells using the lectin from *Griffonia simplifolia* (GSAI-B4). *J Histochem Cytochem* 38:1683–6

Streit WJ (1993) Microglial–neuronal interactions. *J Chem Neuroanat* 6:261–6

Suzuki H, Imai F, Kanno T, Sawada M (2001) Preservation of neurotrophin expression in microglia that migrate into the gerbil's brain across the blood–brain barrier. *Neurosci Lett* 312:95–8

Suzumura A, Bhat S, Eccleston PA, Lisak RP, Silverberg DH (1984) The isolation and long-term culture of oligodendrocytes from new-born mouse brain. *Brain Res* 324:379–83

Suzumura A, Sawada M, Yamamoto H, Marunouchi T (1993) Transforming growth factor-beta suppresses activation and proliferation of microglia *in vitro*. *J Immunol* 15:2150–8

Tanaka R, Komine-Kobayashi M, Mochizuki H, Yamada M, Furuya T *et al* (2003) Migration of enhanced green fluorescent protein expressing bone marrow-derived microglia/macrophage into the mouse brain following permanent focal ischemia. *Neuroscience* 117:531–9

Tone O, Miler JC, Bell JM, Rapoport SI (1987) Regional cerebral palmitate incorporation following transient bilateral carotid occlusion in awake gerbils. *Stroke* 18:1120–7

Wang W, Redecker C, Bidmon HJ, Witte OW (2004) Delayed neuronal death and damage of GDNF family receptors in CA1 following focal cerebral ischemia. *Brain Res* 1023:92–101

Wu Z, Zhang J, Nakanishi H (2005) Leptomeningeal cells activate microglia and astrocytes to induce IL-10 production by releasing pro-inflammatory cytokines during systemic inflammation. *J Immunol* 167:90–8

Zhu Y, Roth-Eichhorn S, Braun N, Culmsee C, Rami A, Krieglstei J (2000) The expression of transforming growth factor-beta 1 (TGF-beta 1) in hippocampal neurons: temporary upregulated protein level after transient forebrain ischemia in the rat. *Brain Res* 866:286–98